Analysing components of the Torso-activation module: Do they pattern the end-terminal regions of two Hymenopteran insect species, *Apis mellifera* and *Nasonia vitripennis*?

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This project sought to determine if components of the Torso-activation module pattern the embryonic anterior and posterior ends of the honey bee, *Apis mellifera*, and the parasitoid wasp, *Nasonia vitripennis* via a patterning process termed terminal patterning. The Torso-activation module is the collective term for the set of proteins Trunk, Torso, prothoracicotrophic hormone (PTTH) and Torso-like (Duncan *et al.*, 2013). Throughout arthropod evolution, the Torso-activation module’s evolutionary history is complex, with different components of it being implemented for a variety of developmental functions among different species (Duncan *et al.*, 2013). The overarching aim of this project was to narrow down when, in the evolutionary history of arthropods, the Torso activation module was adapted to function in end-terminal patterning. An additional aim was to determine the functional role of torso-like in *A. mellifera*. As this insect species does not express the other components of the Torso-activation module, it is hoped that this research may help to reveal the individual function of torso-like, outside of interacting with the Torso-activation module.

The Torso-activation module’s role in terminal patterning appears to have a limited phylogenetic distribution and has only been found in those species that express trunk. To date, it has only been confirmed in the red flour beetle, *Tribolium castaneum*, and some species of Diptera. It is currently hypothesized that the Torso-activation module has only recently been adapted for the role of terminal patterning, with its older role being that of initiating larval moulting (Duncan *et al.*, 2014).

It is worth noting that the genomes of both *N. vitripennis* and *A. mellifera* do not contain the full Torso-activation module as the genome *N. vitripennis* does not contain trunk, and *A. mellifera* expresses only torso-like. Regardless, if components of the Torso-activation module were found to pattern the end-terminals of *N. vitripennis*, it would have provided evidence that
this adaptation of it evolved more basally in the radiation of the holometabolous insects than what is currently hypothesised (Duncan et al., 2014). Furthermore, it would have been the first known example of the Torso-activation module patterning the end-terminals of an insect that does not express trunk (Duncan et al., 2014).

It’s unsurprising then that completion of parental RNAi (pRNAi) experiments in N. vitripennis here suggest that neither PTTH nor torso function to pattern the end-terminal regions in this species. Here, the cuticle structures of larvae collected from N. vitripennis females injected with torso and PTTH dsRNA appeared wildtype. However, these results will have to be confirmed via RT-qPCR.

Additionally, these functional pRNAi experiments would benefit from completion of in situ hybridization to visualize where, and at what developmental stage, the transcripts of PTTH and torso localize in N. vitripennis. By determining whether these transcripts localize to the embryo poles, it may be possible to assess if they play a role in patterning the end-terminal regions of this species. Here, several attempts were made to perform in situ hybridization of these two genes. Unfortunately, for a variety of reasons, these attempts were unsuccessful and yielded no informative results.

As for A. mellifera, pRNAi experiments performed here suggest that torso-like, functioning independently from the Torso-activation module, may play a role in patterning the anterior terminal end of this species. Here, larvae collected from A. mellifera queens injected with torso-like dsRNA were completely missing the mandible, maxilla, labium and all thoracic segments. This is exciting because it adds yet another example to the ever-growing list of torso-like’s functional roles between different insect species. However, as there are no other known experiments in which pRNAi has been reported on this species, these results will have to be repeated and confirmed via RT-qPCR.
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LIST OF ABBREVIATIONS

~ - approximately

°C - degrees Celsius

*A.m – Apis mellifera*

AP- alkaline phosphatase

BCIP- 5-bromo-chloro-3-indoyl phosphate

BLAST- basic local alignment search tool

bp- base pairs

BSA- bovine serum albumin

cDNA- complementary DNA

dH$_2$O- distilled H$_2$O

DIG- digoxigenin

*D.m- Drosophila melanogaster*

DNA- deoxyribonucleic acid
DNAse- deoxyribonuclease

dsRNA- double-stranded Ribonucleic acid

_E. coli_ Escherichia coli

EDTA- ethylenediaminetetraacetic acid

g- force of gravity on earth (~ 9.81 m.s\(^{-2}\))

kPA- kilopascal (kg.m\(^{-1}\).s\(^{-2}\))

IPTG- isopropyl β-D-1-thiogalactopyranoside

L- litre

LB- lysogeny broth

m- milli

M- moles of solute per litre of solution

mRNA- messenger ribonucleic acid

n- nano

NBT- p-nitro blue tetrazolium

_N. v-_ Nasonia vitripennis

PBS- phosphate buffered saline

PCR- polymerase chain reaction

pRNAi- parental RNA interference

PTw- 0.1% (v/v) Tween diluted in PBS PBS

PBTw- 0.1% Bovine serum albumin (BSA) (v/v) diluted in PTw
PTx- Triton-X in PBS

RNAs- ribonuclease

RNAi- ribonucleic acid interference

RPM- revolutions per minute

RT-qPCR- quantitative reverse transcription polymerase chain reaction

s- second

SDS- sodium dodecyl sulphate

ssDNA- salmon sperm deoxyribonucleic acid

ssRNA- single-stranded ribonucleic acid

µ- micro

U- units of enzyme activity

UV- ultraviolet

v/v- volume per volume

w/v- weight per volume
1.1 EVOLUTIONARY DEVELOPMENT AND INSECT ANTERIOR-POSTERIOR PATTERNING

1.1.1 Introduction

Evolutionary development is a union of two broad fields of biology: evolution and development. Developmental biology aims to discover the processes by which complex organisms develop from a single cell. The evolutionary component of evolutionary development is concerned with discovering the ancestral relationships between these developmental processes thereby answering how they evolved between species. This project focuses on comparing the development of the terminal ends (the furthermost anterior and posterior ends) in three insect species of interest, *Drosophila melanogaster*, *Apis mellifera* and *Nasonia vitripennis*.

Note, the following sections focus only on those insects that are long germ as *D. melanogaster*, *A. mellifera* and *N. vitripennis* all fall under this category. Long germ is a term given to insects whose embryos pattern all segments of the embryo simultaneously, as opposed to short germ insects, which generate segments sequentially from a posterior region in the embryo (Krause, 1939).

1.1.2 Anterior-posterior patterning

Despite vast differences in morphology, the insect body plan can be roughly generalized as follows: from anterior to posterior it consists of a head region made of 6–7 segments, a thorax of three segments, and an abdomen of 8–11 segments. Producing these segments relies on creating an anterior to posterior axis of positional information along the embryo. Individual
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cells exposed to this positional information are then properly specified into their developmental fate. (Sander, 1965).

In Drosophila, the foundations of the anterior-posterior axis are laid out at the syncytial stage of embryogenesis, a stage defined by the having all nuclei within a single cytoplasm. The lack of cellular membranes between the nuclei at the syncytial stage allows for the diffusion of genetic material throughout the embryo (Sander, 1975). The anterior-posterior axis is specified when the follicle cells surrounding the oocyte deposit mRNA that is localised in different regions of the egg. Once translated, these maternally deposited mRNAs create a gradient of high protein concentration at the localised mRNA site, to low protein concentration at those regions furthest from the away from these sites. The mRNAs which are deposited from the mother to the oocyte in this regard are called maternal effect genes, and the protein concentration gradients they produce are what give the first positional clues to the embryonic cells (Nusslein-Volhard and Wieschaus, 1980; Schupbach and Wieschaus, 1986).

Once the concentration gradients of maternal effect genes are established, they are later used to set off a cascade of regulatory transcription factors (termed the segmentation genes) that act in a sequential fashion to subdivide the embryo into increasingly smaller domains (Nusslein-Volhard and Wieschaus, 1980). A schematic diagram illustrating an overview anterior-posterior axis formation in Drosophila melanogaster can be seen in Figure 1.1. Whilst there is detailed knowledge of the processes by which maternal effect and segmentation genes pattern the embryos of Drosophila, biologists are still in the early stages of understanding their specific roles in other insects, bar a few non-drosophilid species (Rosenberg et al., 2009). As Drosophila have a highly derived form of embryogenesis, the study the of embryonic patterning in other insect species is necessary to have a broader phylogenetic understanding of insect development. (Rosenberg et al., 2014).
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Figure 1.1 An overview of anterior-posterior axis formation in *Drosophila melanogaster*. Embryos are orientated anterior to the left, ventral side down. (a) Transcripts of the maternal effect genes *bicoid* and *nanos* are loaded into the anterior and posterior ends of the embryo respectively. The morphogenic gradients of *bicoid* and *nanos* define where the maternal effect genes caudal and hunchback are expressed. This establishes a gradient of high *hunchback* and *bicoid* expression at the embryo's anterior, decreasing in concentration towards the posterior of the embryo; and a gradient of high *nanos* and *caudal* expression in the embryo's posterior, decreasing in concentration towards the anterior of the embryo. The morphogenic gradients of the maternal effect genes regulate the expression of the Gap genes which define large domains of the embryo (b). The gap genes then activate the pair-rule genes which divide the embryo into 15 segments (c). Pair rule genes then activate the segment polarity genes which specify the anterior and posterior borders of the segments established by the pair-rule genes (d).
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1.1.2.1 A summary of maternal effect genes in *Drosophila melanogaster*

As previously stated, maternal effect genes are those genes whose mRNAs are loaded into the oocyte from the surrounding follicle cells. Regarding the initiation of the anterior-posterior axis there are four key maternal effect genes worth noting: *bicoid* and *hunchback* which function to specify the anterior end of the Drosophila embryo (Driever and Nusslein-Volhard, 1989); and *nanos* (Lehmann and Nusslein-Volhard, 1991) and *caudal* (Wu and Lengyel, 1988) which specify the posterior end of the embryo. Whilst *bicoid* and *nanos* mRNAs are loaded into the anterior and posterior ends of the embryo respectively, *hunchback* and *caudal* are distributed evenly throughout the embryo (Driever and Nusslein-Volhard, 1988; Driever and Nusslein-Volhard, 1989; Tautz, 1988; Wreden et al., 1997). Together the maternal effect genes govern the expression domains of gap genes, a class of segmentation genes involved in specifying broad regions, or contiguous segments of the embryo (Nusslein-Volhard and Wieschaus, 1980; Schupbach and Wieschaus, 1986).

Bicoid achieves anterior specification through the activation of *hunchback* (Driever and Nusslein-Volhard, 1989; Struhl et al., 1989). Once *hunchback* is activated it cooperates with Bicoid to activate the anterior gap genes *buttonhead*, *empty spiracles* and *orthodenticle* (Simpson-Brose et al., 1994; Reinitz et al., 1995). Additionally, Bicoid inhibits the translation of *caudal* (Dubnau and Struhl, 1996).

*Caudal's* translation, which is now confined to the posterior region of the embryo, activates posterior gap genes, *knirps* and *giant* (Schulz and Tautz, 1995). Nanos governs posterior specification by directly inhibiting the translation of *hunchback*, thereby confining its expression to the anterior of the embryo (Wreden et al., 1997).

In summary, interactions between these four genes create a gradient of high *hunchback* and *bicoid* expression at the embryo's anterior, decreasing in concentration towards the
posterior of the embryo; and a gradient of high *nanos* and *caudal* expression in the embryo's posterior, decreasing in concentration towards the anterior of the embryo. These concentration gradients relay positional information to the embryo and regulate the expression of the gap genes.

There is one third class of maternal effect genes, those that are restricted to the terminal ends of the embryo. These are *torso*, *torso-like* and *trunk* and are the focus of this research. These genes will be discussed in greater detail in later sections.

1.1.2.2 Segmentation genes (gap, pair rule, and segment polarity genes) in *Drosophila melanogaster*

1.1.2.2.1 Gap genes

Gap genes function to specify broad regions of the embryo. The initial expression boundaries of the gap genes are first defined by the anterior-posterior gradients of maternal effect genes. Later these boundaries are reinforced by repressive interactions between the gap genes themselves (Gaul and Jackle, 1990). The genes *orthodenticle*, *empty spiracles* and *buttonhead* are activated by Bicoid and pattern the head (Simpson-Brose *et al.*, 1994; Reinitz *et al.*, 1995). The gap genes *tailless* and *huckebein* pattern the terminal ends and are activated downstream of *torso* (Furriols and Casanova, 2003; Jiménez *et al.*, 2000). Four gap genes, *krüppel*, *knirps*, *hunchback* and *giant* have partially overlapping domains in the trunk of the embryo (Nusslein-Volhard and Wieschaus, 1980). This overlap of gap gene domains produces different combinations of gap gene concentrations in each cell (Gaul and Jackle, 1990). These differing concentrations of gap genes are now used as positional information to regulate the expression of the pair rule genes (Nusslein-Volhard and Wieschaus, 1980).
1.1.2.2 Pair rule and segment polarity genes

The expression pattern of the pair rule genes is a series of vertical bands along the anterior-posterior axis of the embryo. Adjacent vertical bands of nuclei express each pair rule gene in an “on/off” fashion. This creates the appearance of stripes and divides the embryo into 15 subunits along the anterior-posterior axis of the embryo (Hafen et al., 1984). The primary pair rule genes are expressed first, and their expression is predominantly regulated by different concentrations of gap genes. Once expression of the primary pair rule genes is established, they act to allow or repress expression of the secondary pair rule genes (Ingham and Gergen, 1988; Arias et al., 1988).

Whilst the bands of the pair rule genes do overlap to a degree, each vertical band of nuclei contains a unique combination of pair rule gene expression, thus giving them a unique anterior-posterior identity (Pankratz et al., 1990). The domains of the pair rule genes function to regulate the final tier of the segmentation genes, the segment polarity genes.

Segment polarity genes act to reinforce the boundaries produced by pair rule genes by specifying the anterior and posterior regions within each band of nuclei. The two main segment polarity genes are wingless and engrailed. Wingless is expressed in the anterior border of each band, whilst engrailed is expressed in the entire posterior of the band (Bhanot et al., 1996; Siegfried et al., 1994).

1.2 THE TORSO-ACTIVATION MODULE AND CANONICAL END-TERMINAL PATTERNING IN DROSOPHILA

1.2.1 The Torso-activation module and its role in terminal end patterning
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In *Drosophila melanogaster*, the process of terminal end patterning is governed by activation of the maternal effect gene *torso*, a receptor tyrosine kinase. Despite the receptor’s ubiquitous expression throughout the embryo’s cellular surface, it is only activated at the embryonic poles. Whilst the exact mechanism for Torso activation is not known, it is accepted that it depends the ligand Trunk, also ubiquitously expressed, (Casali and Casanova, 2001) and localized expression of *torso-like* in the end-terminals (Mineo *et al.*, 2015). Together the proteins, Trunk, Torso-like, Torso and PTTH (a peptide related to Trunk) are referred to as the Torso-activation module.

1.2.1.1 Evidence that Torso-like activates Torso indirectly

Early evidence of Torso-like’s involvement in defining the terminal regions were a lack of anterior acron and posterior telson derivatives (together, these are the terminal features of the embryo) in *torso-like* null mutant embryos (Strecker *et al.*, 1988). Furthermore, ectopic *torso-like* expression during oogenesis causes central portions of the early embryo to develop terminal structures (Savant-Bhonsale and Montell, 1993). Additionally, *torso’s* localized activation appears to require the genes *nasrat, polehole* and *closca*, which have known roles in anchoring Torso-like to the terminal ends of the eggshell (Casanova and Struhl, 1989). Later experiments revealed that ectopic expression of *torso-like* in *trunk* mutant Drosophila appeared to have an absence of Torso activation. (Furriols *et al.*, 1998). These experiments, coupled with evidence that a cleaved form of Trunk can activate Torso alone (Casali and Casanova, 2001), indicates that Torso-like acts indirectly via Trunk to activate Torso, and is not Torso’s ligand.
1.2.1.2 Evidence that Trunk is the ligand for Torso

Perhaps the clearest evidence that Trunk is the ligand for Torso came from a study performed by Casali and Casanova (2001) which reported that a fragment containing the carboxy-terminal 108 amino acids of the Trunk protein retains Trunk activity and is sufficient to activate Torso signalling. This fragment of Trunk activates the Torso pathway even in a torso-like null mutant background. Furthermore, this fragment alone can bypass requirements of other genes, such as nasrat, closca and pole hole, in the activation of the Torso Receptor, indicating a cleaved, active form of Trunk acts as the ligand. (Casali and Casanova, 2001).

1.2.1.3 Torso, once activated, turns on expression of huckebein and tailless, via the MAP-Kinase/ERK signalling cascade

After activation, Torso triggers phosphorylation and activation of the MAP-Kinase (originally termed ERK) pathway. The mechanisms of the Torso-activated MAP-Kinase signalling pathway are as follows. Signal transduction begins with the activation of small membrane proteins such as GTPases Ras (or Rap) by Torso (Kolch, 2000). Upon activation Ras then forms a large signalling complex which activates Raf which in turn activates MEK by phosphorylation. From here MEK recognizes and activates specific MAP-Kinases. Activated MAP-Kinases can phosphorylate over 80 substrates in the cytoplasm and the nucleus, and generally alter gene transcription by directly or indirectly targeting transcriptional factors (Orton et al., 2005). Torso’s activation of the MAP-Kinase pathway ultimately determines end-terminal patterning by reducing Capicua (Cic) levels, a repressor which controls many aspects of Drosophila development (Grimm et al., 2012). Reduction in Cic derepresses expression of transcription factors huckebein and tailless, whose functions are described below, at both poles of the embryo (Furriols and Casanova, 2003; Jiménez et al.,
The derepression of *huckebein* and *tailless* from activation of the MAP-kinase signalling pathway can be seen in Figure 1.2.

**Figure 1.2: Generalised diagram illustrating Torso’s activation of the MAP-kinase signalling cascade.** Signal transduction begins with the activation of small membrane proteins such as GTPases Ras (also termed Rap) by Torso (Kolch, 2000). Upon activation Ras then forms a large signaling complex which activates Raf which in turn activates MEK by phosphorylation. From here MEK recognizes and activates specific MAP-Kinases. Activated MAP-Kinases can phosphorylate over 80 substrates in the cytoplasm and the nucleus and generally alter gene transcription by directly or indirectly targeting transcriptional factors (Orton *et al.*, 2005). Torso’s activation of the MAP-Kinase pathway ultimately determines end-terminal patterning by reducing Capicua (Cic) levels, a repressor which controls many aspects of Drosophila development (Grimm *et al.*, 2012). Reduction in Cic derepresses expression of transcription factors *huckebein* and *tailless*.

### 1.2.1.3.1 Huckebein

In Drosophila, *huckebein* functions as a terminal gap gene, preventing segmentation by suppressing the activity of the central gap gene, *giant*. Additionally, Huckebein limits the
expansion of the ventral furrow to the central portion of the embryo by repressing the genes *twist* and *snails*. The ventral furrow is an invagination along the ventral most region of the gastrulating embryo, which internalizes mesodermal precursor cells. *Huckebein* null mutant embryos display an expansion of the ventral furrow, and a decrease in the number of endodermal primordia, which are located at the polls of the embryo. (Bronner and Jackle, 1991).

1.2.1.3.2 Tailless

In *Drosophila*, Tailless develops the acron and the telson (terminal features of the embryo) by functioning as a constitutive repressor of central gap genes *giant*, *knirps* and *krüppel* (Moran and Jimenez, 2006). Tailless’s repressor function involves interaction with the protein brakeless, a co-repressor protein. (Haecker et al., 2007) This repression of genes regionalizes the early embryo by repressing abdominal development and promoting terminal fate (Pignoni et al., 1990).

1.3 EVOLUTION OF THE TORSO-ACTIVATION MODULE

1.3.1 Introduction

This section aims to introduce the complexity of the Torso-activation module’s evolution. It discusses both its conservation and diversification in roles throughout the holometabolous (undergoes a pupal stage of development) and hemimetabolous (does not undergo a pupal stage of development) insects. Furthermore, this section illustrates how, aside from its role in terminal patterning, the Torso-activation module is implemented in another broad context: it has a role in initiating larval moulting and metamorphosis. Finally, this section
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aims to give an indication of how dynamic the Torso-activation module is, as it has a variety of functional roles in species who have lost various components of it altogether.

1.3.2 The Torso-activation module and its role in determining larval moulting, metamorphosis.

A key concept of the Torso-activation module’s evolution is that it has some redundancy and does not need all the components of it to be implemented. More specifically, trunk is homologous to the neuropeptide hormone PTTH, and sequence similarity between them reveals both proteins belong to the cysteine knot growth factor superfamily (Duncan et al., 2013). Both Trunk and PTTH are virtually interchangeable ligands for Torso, as illustrated by research that revealed ectopic expression of PTTH in the Drosophila embryo partially rescues trunk mutants (Rewitz et al., 2013).

Additionally, PTTH replaces the function of Trunk as the ligand of Torso in the prothoracic gland. Here, the Torso-activation module is implemented in a different developmental context from terminal end patterning: initiating metamorphosis, the process by which insects develop from larval to pupal, or pupal to adult, forms. PTTH, released as a response to key environmental and nutritional cues, stimulates the prothoracic gland by activating the Torso receptor (McBrayer et al., 2007). This induces a MAP-kinase signalling cascade, which initiates synthesis and release of the steroid hormone ecdysone. This in turn, initiates larval moulting and metamorphosis (Gilbert et al., 2002). Additionally, there is some diversification between genera as to which gland is innervated in this process. Whilst in Drosophila, these neurons directly innervate the prothoracic gland, in the genus Bombyx (moth) these neurons innervate the corpus allatum (Mizoguchi et al., 1990).
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In addition to PTTH and torso, torso-like is also expressed in the prothoracic gland of Drosophila and has a role on developmental timing. However, whilst metamorphosis is delayed in torso-like null mutants, in torso:torso-like double mutants this delay in larval moulting was greatly enhanced. This indicated that loss of torso-like is additive rather than epistatic of the loss of torso in this context and that Torso-like is therefore not affecting timing of metamorphosis through the direct activation of Torso (Johnson, et al., 2013) Since this discovery, a study done by Henstridge et al., (2018) has shown that Torso-like influences larval moulting through upregulation of insulin signalling. Despite insulin signalling being one of the many nutritional cues that regulate production of ecdysone (Columbani et al., 200+5), this still confirmed that Torso-like influences developmental timing outside of direct interaction with the Torso-activation module.

1.3.3 The conservation and role of the torso-activation module outside of Drosophila

In arthropods, there is a large discrepancy in patterns of conservation between the different components of the Torso-activation module. Illustrating this is comparing trunk’s conservation with PTTH. PTTH, and its role in initiating larval moulting, is detected widely in the genomes of both holometabolous and hemimetabolous insects (Duncan et al., 2013). However, the conservation of trunk is a rarer occurrence as trunk is only found in some holometabolous insects, but not in hemimetabolous insects. More specifically, in the holometabolous insects, trunk is only found in Diptera, the order containing Drosophila and the genus Tribolium. As the Torso-activation module’s role in terminal patterning has only been confirmed in Tribolium and Drosophila there is a potential that Torso-activation module-determined terminal patterning may only be found in those species that express trunk (Duncan et al., 2013). This observation has led some to propose that the Torso-activation
module has only recently been adapted to a role in terminal patterning, with this adaptation arising in a common ancestor of Drosophila and Tribolium (Duncan et al., 2014). A phylogram displaying the evolution of the genes of the Torso-activation module can be seen in Figure 1.3.

![Figure 1.3: The evolution of the Torso-activation module.](image)

**Figure 1.3: The evolution of the Torso-activation module.** (a) Schematic phylogram, reproduced with permission from Duncan et al., (2014) showing the origins and losses of different components of the Torso-activation module. (b) Schematic phylogram, reproduced with permission from Duncan et al., (2014) representing the evolution of functionary roles of the Torso-activation module. Note, the proposed evolution of the Torso-activation module’s role in terminal patterning is in a common ancestor of Tribolium and Drosophila (Duncan et al., 2014).

For this project it is worth noting that the genomes of *Apis mellifera* and *Nasonia vitripennis* do not contain orthologues of *trunk*. (Duncan et al., 2013). Interestingly though, a target of the terminal patterning pathway, *tailless*, is expressed anteriorly and posteriorly in both these species. This appears to indicate that terminal patterning is occurring in both species.
but does not require the activation of Torso (Lynch et al., 2006b; Wilson and Dearden, 2009; Duncan et al., 2014).

However, as outlined previously, both PTTH and Trunk can act as ligands for Torso. As PTTH is expressed in *N. vitripennis*, it cannot yet be determined that the Torso-activation module is not involved in terminal patterning in this species. If the Torso-activation module is found to play a role in terminal patterning of *N. vitripennis* it would indicate that this adaptation of the Torso-activation module evolved earlier than expected. Indeed, rather than the prospective evolution of this adaptation occurring in a common ancestor of only Tribolium and Drosophila, it would have occurred in a common ancestor of Tribolium, Drosophila and Nasonia. Furthermore, it would be the first known example of the Torso-activation module patterning the end-terminals of an insect that does not express trunk.

*Torso-like* is present in both *N. vitripennis* and *A. mellifera* and has been found in the ovaries of both species (Duncan et al., 2013). Interestingly, *PTTH, trunk* and *torso* are missing in *A. mellifera*. This is of special interest for two reasons. Firstly, as its genome does not contain *PTTH, trunk* or *torso*, it raises questions regarding how exactly this species facilitates larval moulting and metamorphosis. Secondly, if *torso-like* is found to have a role in terminal patterning in *A. mellifera*, it will provide supporting evidence that *torso-like* can pattern embryonic terminal regions independently of the Torso-activation module.

It is worth reiterating that this won’t be the first instance of *torso-like* showing independence from the Torso-activation module. One example is the maternal torso-like RNAi knockdown in *Oncopeltus fasciatus* (commonly named the milkweed bug). Knockdown of *torso-like* in this species disrupts a key invagination process, whereby the blastoderm cannot fully embed into the yolk (Weisbrod et al., 2013). Additionally, RNAi of torso-like in *Oncopeltus fasciatus* appears to have an essential role in defining the posterior growth zone,
the region of the embryo from which segments are generated in a sequential fashion in developing short germ insects (Weisbrod et al., 2013). This role of torso-like functions outside of the Torso-activation module as torso is not expressed in this species. Because of these findings it is hypothesized that torso-like was the original coordinator of the posterior growth zone and Torso signalling was later co-opted for this function. Then, in the evolutionary transition from short germ to long germ insects this posterior patterning process was recruited to pattern both terminal regions of the embryo. (Weisbrod et al., 2013).

Even in Drosophila there are examples of Torso-like displaying functional independence from the Torso-activation module. Specifically, this refers to, aside from the previously discussed role in insulin regulation, Torso-like’s role in invaginating the ventral furrow. The ventral furrow functions to bring mesodermal and endodermal precursor cells into the interior of the embryo and is a key developmental process. Correct formation of the ventral furrow requires precisely timed apical constriction of invaginating cells, resulting in them adopting a ‘wedge-like shape’ (Johnson et al., 2017). In a recent study by Johnson et al. (2017), they show Torso-like is essential for coordinated apical constriction of cells along the edge of the ventral furrow, likely through the upregulation of the gene fog. Loss of maternal torso-like leads to a ‘cuticular hole’ phenotype resulting from incomplete ventral furrow formation (Johnson et al., 2017).

However, despite the variation in roles of torso-like, its specific biochemical activity appears to be conserved. Experiments in which aphid and honeybee torso-like genes were ectopically expressed in the Drosophila ovary showed no phenotypic difference to the ectopic ovarian expression of Drosophila torso-like (Duncan et al., 2013).

1.3.3.1 Noggin-like proteins
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Interestingly, there is some evidence to suggest that the Torso-activation module is activated by another ligand, in contexts where orthologues of either trunk or PTTH are missing in an insect species’ genome. This theory arose from evidence that both PTTH and trunk are related to noggin, a regulator of BMP signalling (Smith et al., 1993). Bayesian phylogenetic analysis has identified a class of Noggin-like molecules, which are more homologous to noggin than to trunk or PTTH, in crustacean and hemimetabolous insect genomes only (Duncan et al., 2013). Cladistic analysis indicates that PTTH and trunk arose from the duplication and divergence of a noggin-like gene. Despite the ancestral relationships between these three ligands however, there is no further evidence to suggest that these Noggin-like proteins act as Torso ligands (similar to Trunk and PTTH), thus forming part of the Torso-activation module, or as BMP regulators (similar to Noggin). Additionally, torso has currently been found only in the genomes of species where homologues either PTTH or trunk have been found, suggesting that none of these Noggin-like proteins replace Trunk or PTTH as the ligands of Torso (Duncan et al., 2013). Noteworthy for this project, these noggin-like genes have not been found in the genome of the holometabolous insects N. vitripennis and A. mellifera.

1.3.3.2 Summary

The genes and functions of the Torso-activation module are not detected equally throughout the arthropods. Whereas PTTH and its role in metamorphosis is detected widely in the holometabolous and hemimetabolous insects, trunk and its role in end-terminal patterning has only been found in some Diptera and Tribolium castaneum. This has led some to hypothesize that the Torso-activation module’s older purpose was initiating larval moulting and that its implementation in terminal patterning is a relatively recent adaptation (Duncan et al., 2014). How this occurred is not fully understood but importantly for this master’s project
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it explains why the presence of genes of the Torso-activation module does not guarantee that they are implemented in terminal patterning. This is an important consideration for the species *N. vitripennis* and *A. mellifera*, which each contain some, but not all, components of the Torso-activation module.

If the Torso-activation module is found to play a role in terminal patterning of *N. vitripennis* it would indicate that this adaptation of the Torso-activation module evolved earlier than expected. Indeed, rather than the prospective evolution of this adaptation occurring in a common ancestor of only Tribolium and Drosophila, it would have occurred in a common ancestor of Tribolium, Drosophila and Nasonia. Furthermore, it would be the first known example of the Torso-activation module patterning the end end-terminals of an insect that does not express *trunk*.

As for *A. mellifera*, whose genome does not contain *PTTH, trunk* or *torso*, it raises questions regarding how exactly this species facilitates larval moulting and metamorphosis. Secondly, if *torso-like* is found to have a role in terminal patterning in *A. mellifera*, it will provide supporting evidence that *torso-like* can pattern embryonic terminal regions independently of the Torso-activation module.

Furthermore, as illustrated by *torso-like*’s role in *Oncopeltus fasciatus*, these genes may have entirely different roles outside terminal patterning and initiating larval moulting, which remain to be discovered.

1.4 HOW TORSO-LIKE AND TRUNK INTERACT TO ACTIVATE TORSO

1.4.1 Introduction
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This section aims to introduce a hotly debated topic: the exact mechanism of how Torso-like and Trunk interact to activate Torso signalling in Drosophila. It addresses the relevant features, and then the methods of interaction that these features suggest, of both Trunk and Torso-like. It will also highlight prominent research that studies the possible modes of action that Torso-like and Trunk use to activate Torso. It is important to note that there is no conclusive evidence that determines the exact mechanism of Torso-like’s restriction of Torso’s activation to the embryonic poles, and that this is still the topic of some controversy. An equally important consideration is that *Nasonia vitripennis* and *Drosophila melanogaster* may have different mechanisms by which they activate Torso. Hence, it is yet to be determined whether the research highlighted in this section also applies to *Nasonia vitripennis*.

1.4.2 Notable features of *torso*-like and *trunk*

1.4.2.1 Trunk has similarity to Spatzle

Trunk encodes a terminal cysteine-knot region and the protein is most similar to the *noggin*-like branch of the cysteine-knot superfamily (Duncan *et al*., 2013; Groppe *et al*., 2002). More precisely it resembles *spatzle*, another member of the cysteine-knot superfamily. Spatzle undergoes cleavage to become an active ligand and is the growth factor that binds the Toll receptor, which initiates dorso-ventral patterning in Drosophila (Hu *et al*., 2004). In this system, the Spatzle ligand is generated through a proteolytic cascade (Reeves and Stathopoulos, 2009). Given that Trunk has similarities with Spatzle, it led to research which indicated that a cleaved form of the Trunk protein acts as a signal for the Torso receptor, and the proposal that a restricted activation of the Torso receptor is defined by the spatial control of the proteolytic processing of Trunk (Casali and Casanova, 2001).
1.4.2.2 Torso-like is member of the MACPF protein superfamily

Bioinformatic studies reveal that *torso-like* encodes a membrane attack complex/perforin (MACPF) domain (Ponting, 1999). There is a large variety of functions in proteins belonging to this superfamily including: roles in vertebrate immunity, development of the embryo, and neural-cell migration. The MACPF domain has structural similarity with pore-forming cholesterol-dependent cytolysins (CDCs) from Gram-positive bacteria and they are, therefore, collectively referred to as the MACPF/CDC superfamily (Rosado *et al*., 2007). This has provided important insight in the mechanism of their function and suggests that lytic MACPF proteins may use a CDC-like mechanism to form pores and disrupt cell membranes (Gilbert *et al*., 2013; Rosado *et al*., 2007). This has important implications for Torso-like as it may activate Torso via some unknown pore-forming mechanism.

1.4.3 Theories on how Torso-like and Trunk interact to activate Torso

1.4.3.1 Theory 1: Trunk is secreted then cleaved, independently of Torso-like at the embryonic termini, allowing Trunk to interact with Torso

Similarities of *trunk* to *spatzle*, which imply that Trunk may be cleaved to become active, as well as evidence that the active form of Trunk diffuses through the perivitelline space (the space between the vitelline membrane (eggshell) and the Drosophila embryo) led some to postulate the following theory: That Trunk is secreted intact into the perivitelline fluid layer and is locally activated by proteolytic cleavage, allowing it to activate the Torso receptor (Casanova *et al*., 1995). It was further hypothesized that this process was mediated by Torso-like which may direct an unidentified protease to cleave and activate Trunk only at the embryonic poles (Casanova *et al*., 1995). An image depicting this model of Torso activation
can be seen in Figure 1.4. This idea was bolstered by studies indicating that torso-like’s restricted expression in the end-terminals appears to be critical for localized Torso activation. However, more recent studies indicate that the process of cleaving and activating Trunk is likely independent of Torso-like as it was shown that no change in the cleavage pattern was observed in torso-like null mutants (Henstridge et al., 2014). Additionally, it has been discovered that Trunk cleavage is dependent on the Furin proteases Fur1 and Fur2, and this process likely occurs intracellularly, within the Drosophila embryo, before being excreted into the perivitelline space. Therefore, this process would likely be independent of Torso-like which is localized to the vitelline membrane (VM), surrounding the Drosophila embryo. (Johnson et al., 2015)

A variation of this theory is that Trunk is secreted in an active form into the perivitelline layer and, independently of Torso-like, binds and activates Torso which has localized to the poles of the embryo (Sprenger and Nusslein-Volhard, 1992). However, this hypothesis seems to contradict previously reported data that shows Torso-like plays an essential role in Torso’s activation. Additionally, this theory relies on a localized distribution of the Torso receptor at the embryo poles. As Torso is detected ubiquitously on the embryonic cell surface it is unlikely this is the mechanism of Torso’s localized activation.

1.4.3.2. Theory 2: Torso-like allows localized secretion of Trunk into the embryonic poles of the perivitelline layer which causes restricted activation of Torso

Evidence that Torso-like is critical for localized Torso activation, and that Trunk cleavage is independent of Torso-like, has led to the development of a second prevailing theory: Cleavage of Trunk occurs prior to Trunk secretion, and Torso-like which is localized at the terminal ends of the VM, interacts with the plasma membrane to regulate localized secretion of Trunk from the terminal regions of the embryo, allowing Trunk to interact with Torso and...
trigger signalling. An image depicting this model of Torso activation can be seen in Figure 1.4. Supporting this theory is evidence that torso-like is member of the MACPF family, therefore Torso-like likely involves some element of membrane interaction such as the formation of membrane pores. Torso-like is anchored to the VM, which is not a phospholipid membrane, and is therefore unlikely targeted by the MACPF complex (Duncan et al., 2013). The only membrane close to the VM is the plasma membrane of the embryo. As this is the membrane in which Trunk translocates through, it is not implausible to consider that Torso-like facilitates this transport via pore-formation (Alessandro et al., 2015). Furthermore, recently there has been an additional study which provides evidence in support of the hypothesis that Torso-like facilitates the tranlocation of Trunk into the perivitelline membrane. In this study, an experiment using a fluorescently tagged Trunk protein has shown that accumulation of Trunk into the poles of the perivitelline space is dramatically reduced in torso-like null mutant embryos (Johnson et al., 2015).
1.4.3.3 Theory 3: Torso-like and Trunk act synergistically to activate Torso.

Recently, a third theory has been put forth regarding Torso’s restricted activation to the poles of the embryo: Trunk and Torso-like act synergistically to activate Torso. Upon using a cell-culture base system that expressed high levels of Torso, Amarnath et al. (2017) discovered that expression of Torso-like or Trunk alone in this system was sufficient to activate Torso. Additionally, when Torso concentrations were reduced in this system to resemble more closely those concentrations found in the Drosophila embryo, neither Torso-like nor Trunk alone could activate Torso. Instead, at this concentration of torso expression, Torso activation was only detected upon exposing the cells to both trunk and torso-like. This suggests that Torso’s
localized activation in the poles of the embryo may be facilitated, at least in part, by some synergistic action between Trunk and Torso-like. This synergistic action would hypothetically be restricted to the embryo poles, because this is the only location where there is a colocalization of Trunk and Torso-like. It is unclear how this synergistic activity would be facilitated. Furthermore, these experiments seem to contradict evidence that a cleaved form Trunk can activate Torso even in torso-like null mutant embryos (Casali and Casanova, 2001).

1.5 NASONIA VITRIPENNIS AS A MODEL ORGANISM

1.5.1 Overview on *N. vitripennis*

Nasonia, originally called *Mormoniella*, is a genus name given to four closely related species of wasp: *Nasonia longicornis*, *Nasonia oneida*, *Nasonia vitripennis*, and *Nasonia giraulti* (Darling and Werren, 1990). The species most commonly experimented on is *N. vitripennis* and is often referred to as the “lab rat” of parasitoid wasps. The term ‘emerging model organism’ is somewhat unfairly given to *N. vitripennis* as it has been the subject of genetic analysis for over 65 years (Whiting, 1950). However, *N. vitripennis* as a system for genetic research is seeing an increase in popularity as of late, most-likely as a result of its “rapidly expanding genetic toolbox” (Lynch, 2015).

Importantly for this project, the genomes of *N. vitripennis* and two closely related species, *N. giraulti* and *N. longicornis* are accessible on NasoniaBase a publicly available bioinformatics repository for the different Nasonia species (http://hymenopteragenome.org/nasonia). Additionally, a method of pRNAi has already been published for *N. vitripennis*, as well as techniques for visualizing localization of mRNA transcripts within embryos by *in situ* hybridization (Lynch and Desplan, 2006).
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*N. vitripennis* is also proving itself as an exceptionally useful tool to study the evolutionary development of insects. This is illustrated, for example, by comparing developmental characteristics between *N. vitripennis* and the more commonly studied insect model organisms, *T. castaneum* and *D. melanogaster*. Phylogeny studies indicate that the Hymenoptera (the order of insects containing bees, wasps, sawflies and ants) diverged at the base of the holometabolous insect radiation (Savard et al., 2006). Coleoptera (the order of insects containing beetles such as *T. castaneum*) and Diptera (the order of insects containing flies such as *D. melanogaster*) in comparison, diverged a short time after the Hymenoptera (Lynch et al., 2012). Whilst *T. castaneum* is short germ, *N. vitripennis* and *D. melanogaster* are long germ, likely as result of convergent evolution (Peter Dearden, personal communication). In their review Lynch et al. (2012) state that characteristics shared by *D. melanogaster* and *N. vitripennis* may represent strategies that have convergently evolved to facilitate long germ embryogenesis, whereas characteristics shared between *D. melanogaster* and *T. castaneum* may represent traits that arose in the holometabolous insects that diverged from the Hymenoptera.

More general features *N. vitripennis* are described herein. Firstly, *N. vitripennis* is a parasitoid of fly pupae, meaning that a *N. vitripennis* larva use fly pupae as hosts, both as a food source and as an environment in which to complete larval and pupal development. After reaching adulthood the wasps are then free living. To parasitize their host, female *N. vitripennis* inject venom into the host pupae, causing arrest of host pupal development, then lays eggs on the surface of the host pupa (Whiting, 1967). *N. vitripennis* are extremely easy to raise as they have a short generation time of 14 days at 25 °C or 10 days at 28 °C. Additionally, refrigerating *N. vitripennis* results in a larval diapause. Wasps can be refrigerated for up to two months with no appreciable effect on fecundity or health (Lynch and Desplan, 2006). This allows further adjustment to schedules as projects can resume when it is convenient
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(Saunders, 1965). Their storage is also easy as they are around 2mm in size, allowing many to be kept in an average size test tube or culture tube. For food, *N. vitripennis* is a generalist that parasitizes blow flies, flesh flies, house flies and others. Finally, when transferring *N. vitripennis* adults between storage containers, anaesthetization of adults is unnecessary as they have a low tendency for flight (Werrin and Loehlin, 2009).

1.6 APIS MELLIFERA AS A MODEL ORGANISM

1.6.1 Overview on *Apis mellifera*

*Apis mellifera*, more commonly known as the honeybee, has been distributed globally because of their commensal relationship with humans (Whitfield et al., 2006). It is estimated that *A. mellifera* pollinates US $215 billion worth of crops and pasture world-wide. As a result of this critical service *A. mellifera* plays in sustaining our ecosystems and agriculture, the importance of this species cannot be understated (Smith et al., 2013). Unfortunately, a number of factors, such as the spread of pests, pathogens and application of neonicotinoid insecticides are driving a global decline in *A. mellifera* populations. Therefore, understanding how we can mitigate or eliminate causes of the global *A. mellifera* decline is becoming a task of increasing importance (Smith et al., 2013). Putting this in context with this master’s project, it may therefore prove beneficial to gain a deeper understanding of the developmental genetics of *A. mellifera*.

Beekeeping as a practice has spread worldwide and one outcome of this is that acquiring *A. mellifera*, and the equipment to raise them is often an inexpensive and easy process. Additionally, information on raising them is readily available as there is a wealth of online beekeeping forums detailing the processes of all stages of beekeeping. Regarding genetics experiments, protocol for RNA interference (RNAi) and sectioned *in situ* hybridisation of the
honey bee has already been developed as well (Dearden \textit{et al.}, 2009). Additionally, the honeybee genome is well annotated and assembled. The latest genome assembly, Amel 4.5, has a contig N50 of 46 kb and a scaffold N50 of 997 kb (Elsik \textit{et al.}, 2014). Morphology of the embryonic stages of \textit{A. mellifera} has been carefully documented (DuPraw, 1967; Nelson, 1915). Additionally, scanning electron microscope images to stage embryos have also been published (Fleig & Sander, 1988) providing an invaluable tool for analysing the RNAi experiments in this project.

1.7 AIMS

The overall aim of this project is to determine if components of the Torso-activation module pattern the end-terminals of the two hymenopteran species \textit{A. mellifera} and \textit{N. vitripennis}.

Additionally, this project aims to narrow-down when, in the evolutionary history of insects, the Torso-activation module was adapted to its role in end-terminal patterning. If the genes \textit{PTTH} and \textit{torso} are found to pattern the end-terminal regions of \textit{N. vitripennis} it will provide evidence that this functional adaptation of the Torso-activation module evolved more basally in the radiation of the holometabolous insects than what is currently hypothesised (Duncan \textit{et al.}, 2014).

Lastly, this project aims to determine the function of \textit{torso-like} in \textit{A. mellifera}. Analysis of the functional role of \textit{torso-like} in \textit{A. mellifera} will determine if \textit{torso-like} can pattern the end-terminals of this species independently from the rest of the Torso-activation module.

To determine the functional roles of these genes pRNAi will be performed on \textit{A. mellifera} and \textit{N. vitripennis}. The pRNAi experiments performed here will reduce the
maternally donated transcript levels of the genes of the Torso-activation module in these two species. This is achieved by injecting a solution of dsRNA that is complementary to the target gene, into an adult female insect. This injection of dsRNA provokes an endogenous response in the organism which then reduces the expression of the target transcript. After performing pRNAi on _A. mellifera_ and _N. vitripennis_ females, their larvae will be examined to see if they display a terminal-patterning phenotype. If they do, it will suggest that the Torso-activation module patterns the end-terminal regions in these species.

To localize the expression domains of _PTTH_ and _torso_ in the embryos of _N. vitripennis_, Digoxigenin (DIG)-labelled _in situ_ hybridisation will be performed on these two genes.. DIG-labelled probes are antisense ssRNA transcripts complementary to the transcript of interest, which are synthesised with DIG-labelled uracil nucleobases. The uracil nucleobases in the DIG-labelled probes act as an antigen for anti-DIG antibodies which are conjugated to alkaline phosphatase. The alkaline phosphatase, which is now localised to those domains of the embryo which express _PTTH_ and _torso_, catalyses a colourgenic reaction when washed with nitro-blue-tetrazolium-chloride (NBT) and 5-Bromo-Chromo-3-indolyl phosphate (BCIP).
CHAPTER TWO: MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 DNA Plasmids

pBluescript II SK (+)  \textit{Genscript}

pBluescript SK (+)  \textit{Genscript}

LITMUS 38i  \textit{Genscript}

2.1.2 Purification of nucleic Acids

Phenol:chloroform:isoamyl alcohol (25:24:1)  \textit{Invitrogen}

TRIzol® reagent  \textit{Invitrogen}

2.1.3 Restriction endonuclease Reactions

2.1.3.1 Restriction enzymes

\textit{EcoRI}  \textit{Roche}

\textit{HindIII}  \textit{Roche}

\textit{PstI}  \textit{Roche}

\textit{SphI}  \textit{Roche}
2.1.3.2 Buffers
H \quad Roche

M \quad Roche

2.1.4 Kits used
PureLink™ HiPure Plasmid Midiprep Kit \quad Thermo Fisher Scientific

Megascript RNAi Kit from Invitrogen \quad Thermo Fisher Scientific

2.1.5 E. coli bacteria strains
HT115 \quad propagated in the lab

XL1- blue MRF \quad propagated in the lab

2.1.6 Insect species

Nasonia vitripennis \quad Dr David Wheeler, Massey University

Lucilia sericata \quad Dr David Wheeler, Massey University

Apis mellifera \quad Otto Hyink

2.1.7 in vitro transcription

10x Transcription buffer \quad Roche

10 x Dig Labelling mix \quad Roche
T3 RNA polymerase \( \textit{Roche} \)

T7 RNA polymerase \( \textit{Roche} \)

Dnase I \( \textit{Invitrogen} \)

RnaseOUT \( \textit{Invitrogen} \)

### 2.1.8 \textit{in situ} hybridization

Anti-digoxigenin-alkaline Phosphatase (AP) antibody \( \textit{Roche} \)

75 mg/mL nitro-blue-tetrazolium-chloride in dimethylformamide (NBT) \( \textit{Roche} \)

50 mg/mL 5-Bromo-Chromo-3-indolyl phosphate (BCIP) \( \textit{Roche} \)

Bovine Serum Albumin (BSA) \( \textit{Invitrogen} \)

Tween-20 \( \textit{Nuplex Industries} \)

### 2.1.9 Injection apparatus

Needle puller \( \textit{Narashige} \)

Micro injector \( \textit{Harvard Apparatus} \)

100 µL syringe \( \textit{Hamilton} \)

27 gauge needle \( \textit{Hamilton} \)
Chapter Two: Materials and Methods

2.1.10 Miscellaneous

Queen cages  
*EZI Queen Systems*

Incubator  
*Black Chick*

*EC 250-90 electrophoresis power supply*  
*Thermo Electron Corporation*

Bx61 Compound microscope  
*Olympus*

2.2 METHODS

2.2.1 Maintenance of *Nasonia vitripennis* stocks

Wild-type *Nasonia vitripennis* were supplied by Dr David Wheeler, Massey University, in *Lucillia sericata* pupae (commonly referred to as the green bottle fly). After the adults emerged they were divided into 20 30 mL scintillation vials containing 20 adults and supplied with 40 *L. sericata* pupae to parasitize. Using a Pasteur pipette, three drops of 50:50 (v/v) honey and water were applied to the inside of the vials for food and hydration of adults. The vials were then placed in a 25°C incubator for 17 days: three days to allow adults to parasitize the fly pupae; 14 days for the wasps to complete larval and pupal development. After the adults emerged, the above protocol was repeated to maintain stocks.

2.2.2 Plasmid preparation

The gene *orthodenticle-1* was as chosen as a positive control for *N. vitripennis* and A.
mellifera pRNAi experiments, as in both of these species it has a well characterized terminal-end mutant phenotype (Lynch et al., 2006a; Wilson and Dearden, 2009). For a negative control, enhanced green fluorescent protein, eGFP, was chosen as it is not endogenously expressed in N. vitripennis or A. mellifera. DNA sequences of eGFP, and N. vitripennis orthologs of PTTH, torso and orthodenticle-1 were accessed on the sequence database Genbank (https://www.ncbi.nlm.nih.gov/genbank/). The sequences of A. mellifera orthologs of orthodenticle-1 and torso-like were accessed on Genbank too. These sequences were then used to order cDNA clones, which came preinserted into plasmid vectors from the biotechnology company, Genscript. The gene inserts, their corresponding plasmid vectors, species of origin and cloning sites can be seen in Table 2.1. The plasmids used can be seen in the appendix (Figures A.1, A.2, and A.3). Sequences of the cDNA inserts can be seen in the appendix (Figures A.4, A.5, A.6, A.7, A.8, and A.9).

<table>
<thead>
<tr>
<th>Species</th>
<th>orthodenticle-1</th>
<th>Torso</th>
<th>PTTH</th>
<th>orthodenticle-1</th>
<th>torso-like</th>
<th>eGFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. vitripennis</td>
<td>N. vitripennis</td>
<td>N.</td>
<td>A. mellifera</td>
<td>A. mellifera</td>
<td>A. Victoria</td>
<td></td>
</tr>
<tr>
<td>Cloning site</td>
<td>EcoRI-HindIII</td>
<td>EcoRI-XbaI</td>
<td>EcoRI-XbaI</td>
<td>EcoRI-EcoRI</td>
<td>BamHI-EcoRI</td>
<td>EcoRV</td>
</tr>
<tr>
<td>Plasmid vector</td>
<td>pBlueScript SK (+)</td>
<td>pBlueScript</td>
<td>pBlueScript</td>
<td>Litmus 38i</td>
<td>Litmus 38i</td>
<td>Litmus 38i</td>
</tr>
</tbody>
</table>
2.2.3 Bacterial transformations and culturing

2.2.3.1 Growth media

Bacterial cultures were grown using Lysogeny broth (LB), or 2xYT as media. Dry bacterial nutrients were prepared in dH₂O and sterilized by autoclaving using a ‘media’ setting. Agar plates were prepared by adding 1.5% (w/v) agar to media prior to autoclaving it. If media was LB with agar, it was left to cool to ~50 °C before adding 100 µg/mL of ampicillin. If media was 2x YT with agar it was left to cool to ~50 °C before adding 100 µg/mL of ampicillin and 12.5 µg/ml of tetracycline. After antibiotics were added, media was poured into plates aseptically and cooled to room temperature to set.

*Lysogeny Broth (LB)*: 1% (w/v) tryptone from casein, 0.5% (w/v) yeast extract, 1% (w/v) NaCl.

*2xYT*: 6.25% (w/v) Tryptone from casein, 1% (w/v) yeast extract, 1% (w/v) NaCl.
Adjusted pH to 7.0 with 5N NaOH.

2.2.3.2 Transformation of *E. coli* bacteria

Twenty µL aliquots of calcium competent *E. coli* strains XL1-blue MRF, or HT115, were transformed by the addition of 1 µL of plasmid solution (section 2.2.2). The cells were then incubated on ice for 20 minutes and mixed by gently flicking the tubes every 5 minutes during this incubation step. The cells were then heat-shocked at 42 °C for one minute, and re-incubated on ice for a further minute. Nine hundred µL of LB media was then added aseptically to the cells which were then shaken at 300 RPM whilst incubating at 37 °C for one hour. After which, the cells were then pelleted by centrifuging at 2000 x g for 1 minute and ~800 µL of the supernatant was removed. The cells were then resuspended in the remaining supernatant.
2.2.3.3 Culturing transformed *E. coli* strain XL1-blue MRF, for *in vitro* synthesis of dsRNA or DIG-labelled probes

Twenty mL of XL1-blue MRF *E. coli* in suspension from section 2.2.3.2 were spread over LB + Ampicillin (100 µg/mL) agar plates. The agar plates were then left to incubate at 37 °C for 16 hours. Single colonies of *E. coli* were then used to inoculate 50 mL of LB + Ampicillin (100 µg/mL) culture. Inoculated LB media was then shaken at 300 RPM for 16 hours at 37 °C. Plasmids cloned by growth of transformed *E. coli* cultures were then isolated and purified with a midiprep kit (section 2.2.4).

2.2.3.4 Culturing transformed *E. coli* strain HT115 for *in vivo* synthesis of dsRNA

Twenty mL of HT115 *E. coli* in suspension, prepared from section 2.2.3.2 were spread over 2xYT + Ampicillin (100 µg/mL) + tetracycline (12 µg/mL) agar plates. The agar plates were then left to incubate at 37 °C for 16 hours. Single colonies of *E. coli* were then used to inoculate 5 mL of LB containing Ampicillin (100 µg/mL) and tetracycline (12.5 µg/µL). Inoculated LB media was then shaken at 300 RPM for 16 hours at 37 °C. The bacterial starter culture was diluted 100-fold with 2xYT media containing ampicillin (100 µg/mL) and tetracycline (12.5 µg/mL). One mL of the diluted culture was then used to inoculate 50 mL of 2xYT medium. The 2xYT media was incubated at 37 °C until the OD600 (optical density at 600nm) reached 0.4, as measured by a spectrophotometer. Once the bacterial cultures reached an OD600 of 0.4, they were used for synthesis of dsRNA by *in vivo* bacterial expression (2.2.8).

2.2.4 Midiprep procedure to isolate and purify plasmid DNA

Midiprep purifications of plasmid DNA were performed on cultures prepared in section
2.2.3.3 using the PureLink™ HiPure Plasmid Midiprep kit by Thermofisher Scientific. The manufacturer’s protocol was followed barring the following alterations. After precipitation, lysate was centrifuged at 4,000 x g for 15 minutes instead of 12,000 x g for 10 minutes. Additionally, once DNA was eluted from the binding column, the 8.5 mL eluate was not centrifuged in an ultracentrifuge tube, but divided into 6 microcentrifuge tubes and centrifuged for 99 minutes at 12,000 g. After centrifuging, 20 µL of TE buffer was then added to each microcentrifuge tube to resuspend the DNA pellets. Purity and concentration of the plasmid DNA was then determined using a nanodrop spectrophotometer. Only DNA concentrations of ≥700 ng/µL were used for further steps.

2.2.5 Restriction endonuclease reactions

Purified plasmid solutions prepared from section 2.2.4 were linearized by performing restriction endonuclease digests, using cut sites on opposite sides of the gene insert. Buffers and restriction endonucleases used can be seen in Table 2.2. Additional reactions were performed using both restriction endonuclease enzymes in a single digest. The presence of two bands after running on the completed reaction on an electrophoresis gel indicates that the restriction reaction was successful. Restriction endonuclease reactions were set up as follows:

5 µL of buffer (either H or M from Roche)

2 µL of endonuclease enzyme (4 µL if performing a double digest)

Plasmid DNA to give 10µg of DNA

Brought to a total volume of 50 µL with dH₂O

After reagents were added together the reaction was incubated at 37 °C in a PCR machine for
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4 hours. Note: later the restriction endonuclease reactions were run at 37 °C in a PCR machine for 5 hours. 5 µL of the reaction solution was then run on an electrophoresis gel (2.2.12) to determine if the digest was successful.

### Table 2.2 Gene inserts and the corresponding buffers and restriction enzymes used for restriction endonuclease reactions.

<table>
<thead>
<tr>
<th>cDNA insert</th>
<th>Restriction enzymes used</th>
<th>Buffer used</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. mellifera orthodenticle-1</em></td>
<td>Sphi and HindIII</td>
<td>M from Roche</td>
</tr>
<tr>
<td><em>A. mellifera torso-like</em></td>
<td>EcoRI and PstI</td>
<td>H from Roche</td>
</tr>
<tr>
<td><em>N. vitripennis orthodenticle-1</em></td>
<td>XbaI and EcoRI</td>
<td>H from Roche</td>
</tr>
<tr>
<td><em>N. vitripennis torso</em></td>
<td>XbaI and EcoRI</td>
<td>H from Roche</td>
</tr>
<tr>
<td><em>N. vitripennis PTTH</em></td>
<td>XbaI and EcoRI</td>
<td>H from Roche</td>
</tr>
<tr>
<td><em>eGFP</em></td>
<td>XbaI and HindIII</td>
<td>H from Roche</td>
</tr>
</tbody>
</table>

### 2.2.6 Purification of Nucleic acids

#### 2.2.6.1 Phenol/chloroform extraction of nucleic acids

Two hundred µL of phenol:chloroform:isoamyl alcohol (50% (v/v) phenol, 48% (v/v) chloroform, 2% (v/v) isoamyl alcohol) was added to either 200 µL of a solution of linearized DNA, RNA, or dsRNA prepared in sections mentioned elsewhere. The solution was vortexed for ~15 seconds prior to being centrifuged at 17000 x g for 5 minutes. After centrifugation, 175 µL of the upper aqueous phase was transferred to a new microcentrifuge tube whilst taking care not to transfer any of the lower organic phase. Two hundred µL of chloroform was added to the aqueous solution, which was then vortexed for ~15 seconds, before being centrifuged at
17000 x g for 5 minutes. Then, ~150 µL of the upper layer was transferred to a new microcentrifuge tube. Nucleic acids in solution were precipitated, and purified further, by ethanol precipitation (section 2.2.6.2).

2.2.6.2 Ethanol precipitation

A 0.1 volume of 3M sodium acetate (pH 5.2) was added to the nucleic acid solution and vortexed for ~15 seconds. Then, 2.5 volumes of ice cold 96% lab grade ethanol was added to the solution, prior to mixing it by inversion, and storing it at -20 °C for 48 hours.

If the desired precipitate was DNA then it was centrifuged at 17000 x g for 99 minutes after which the supernatant was removed. The DNA pellet was then washed with 200 µL of 70% ribonuclease (RNAse) free ethanol and centrifuged at 17000 x g for 5 minutes. The ethanol was removed, and the pellet was air-dried at room temperature for 10 mins before being resuspended in 20 µL RNAse free dH20 and stored at -20 °C.

If the desired precipitate was dsRNA/RNA then it was centrifuged at 17000 x g for 30 minutes, after which the supernatant was removed. The RNA pellet was then washed with 200 µL of 70% RNAse free ethanol and centrifuged at 17000 x g for 5 minutes. The ethanol was then replaced with 200 µL of fresh 70% RNAse free ethanol and centrifuged again at 17000 x g for 5 minutes. Ethanol was then removed, and the pellet was resuspended in 10 µL of RNAse free dH20 and stored at -80 °C. Purity and concentration of the resulting DNA/RNA/dsRNA solution was quantified by nanodrop spectrophotometry.

2.2.7 dsRNA synthesis using the Megascript RNAi Kit from Invitrogen by Thermo Fisher Scientific
Purified linearized DNA produced (sections 2.2.4, 2.2.5, 2.2.6.1, 2.2.6.2) was used to synthesize dsRNA with the Megascript RNAi kit from Invitrogen by Thermo Fisher Scientific. All reagents/protocols used in the following steps are supplied with the kit. The kit’s protocols are summarized as follows. An in vitro transcription reaction was performed to generate antisense and sense transcripts of the gene insert contained within the linearized plasmid. T7 and T3 polymerases used in the transcription reaction were paired with plasmids linearized by restriction endonucleases according to Table 2.3.

**Table 2.3: cDNA inserts, the restriction enzymes used to linearize them, and the corresponding RNA polymerases used in in vitro transcription reactions.**

<table>
<thead>
<tr>
<th>cDNA insert</th>
<th>Restriction endonuclease</th>
<th>Polymerase</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. m orthodenticle-1</em></td>
<td><em>SphI</em></td>
<td>T7 (sense)</td>
</tr>
<tr>
<td><em>A. m orthodenticle-1</em></td>
<td><em>HindIII</em></td>
<td>T7 (antisense)</td>
</tr>
<tr>
<td><em>A. m torso-like</em></td>
<td><em>EcoRI</em></td>
<td>T7 (sense)</td>
</tr>
<tr>
<td><em>A. m torso-like</em></td>
<td><em>PstI</em></td>
<td>T7 (antisense)</td>
</tr>
<tr>
<td><em>N. v torso</em></td>
<td><em>XbaI</em></td>
<td>T7 (sense)</td>
</tr>
<tr>
<td><em>N. v PTTH</em></td>
<td><em>EcoRI</em></td>
<td>T7 (sense)</td>
</tr>
<tr>
<td><em>N. v PTTH</em></td>
<td><em>XbaI</em></td>
<td>T7 (antisense)</td>
</tr>
<tr>
<td><em>N. v orthodenticle-1</em></td>
<td><em>XbaI</em></td>
<td>T7 (sense)</td>
</tr>
<tr>
<td><em>N. v orthodenticle-1</em></td>
<td><em>EcoRI</em></td>
<td>T7 (antisense)</td>
</tr>
<tr>
<td><em>eGFP</em></td>
<td><em>EcoRI</em></td>
<td>T7 (sense)</td>
</tr>
<tr>
<td><em>eGFP</em></td>
<td><em>HindIII</em></td>
<td>T7 (antisense)</td>
</tr>
</tbody>
</table>
Endonuclease restriction sites were paired with polymerase binding sites located on the opposite side of the cDNA insert. This ensured polymerases dissociated from the plasmid after they complete transcript synthesis of the plasmid insert. A schematic diagram representing the method by which RNA polymerases were paired with restriction endonuclease cut sites can be seen in Figure 2.1.

![Diagram of DNA Plasmid with restriction cut sites and polymerases](image)

**Figure 2.1: A representation of the pairing strategy between RNA polymerases and Linearized plasmids in *in vitro* transcription reactions.** Plasmids that are linearized by restriction enzymes that cut at cut site 2, are paired with the T7 polymerases in *in vitro* transcription reactions; Plasmids that are linearized by restriction enzymes that cut at cut site 1 are paired with T3 polymerases in *in vitro* transcription reactions.

Next the solutions of sense and antisense transcripts were pooled and left at room temperature to cool for four hours. This annealed the two ssRNA transcripts producing dsRNA. Then DNA and RNA were digested by adding ribonucleases and nucleases to the solution and allowing it to incubate. Lastly, the dsRNA is purified by centrifuging the solution through a filter column supplied by the kit. Note: this purification step, in which the dsRNA is centrifuged through a filter column, was later substituted with the phenol/chloroform extraction, and ethanol precipitation methods of nucleic acid purification (sections 2.2.6.1 and 2.2.6.2 respectively).
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Purity and concentration of the resulting dsRNA solution is then quantified by nanodrop spectrophotometry, before storing at -80 °C until use in pRNAi experiments (sections 2.2.10, 2.2.11). Note: In performing later attempts at dsRNA synthesis, all the kit protocol reactions were scaled up by a factor of four, to increase dsRNA yield.

2.2.8 Synthesis of dsRNA by in vivo bacterial expression

Bacterial cultures of *E. coli* strain HT115 cells prepared in section 2.2.3.4 were induced to express dsRNA by the addition of 0.4 mM of IPTG. The bacterial culture was further incubated at 37 °C for 4 hours. The bacterial cells were pelleted by centrifugation at 6000 x g for 5 min at 4 °C. To lyse cells, the pellet was resuspended in 2.5 mL of 0.1% Sodium dodecyl sulfate (SDS) prior to boiling for 2 minutes in a water bath. Then, 50 µg of RNase A in a total volume of 3.25 mL of buffer was added to the cells and incubated at 37 °C for 5 min to remove any endogenously expressed RNA of the bacterial host. The remaining dsRNA was purified with TRIzol® reagent (section 2.2.8.1).

*Buffer*: (10 mM Tris-Cl pH 7.5, and 5 mM EDTA, 300 mM sodium acetate.)

2.2.8.1 Purification of dsRNA with TRIzol® reagent.

To the 3.25 mL samples of cells in solution prepared from section 2.2.8, 7.5 mL of TRIzol® reagent was added prior to pipetting the solution up and down several times to homogenize it. Then, 3.75 mL of isopropanol was added to the aqueous phase before incubating it at room temperature for 10 minutes. To pellet the dsRNA, the aqueous solution was centrifuged for 10 minutes at 12,000 x g at 4 °C, after which the supernatant was discarded. The pellet was then washed with 10 mL of RNase-free 70% ethanol and centrifuged for 5
minutes at 7,500 x g at 4 ℃. The ethanol was removed, and the pellet was resuspended in 50µL of RNAaese-free water. Purity and concentration of the dsRNA in solution was measured by nanodrop spectrophotometry.

2.2.9 *N. vitripennis* in situ hybridization with DIG-labelled probes

2.2.9.1 Synthesis of DIG-labelled probes

The following methods were used to synthesize sense and antisense digoxigenin (DIG)-labelled probes of *N. vitripennis* PTTH, torso and orthodenticle-1. Twenty µg of plasmid DNA prepared by Midiprep procedure (section 2.2.4) was linearized in two separate reactions (section 2.2.5). To determine if endonuclease digestion was complete, 5 µL of the total reaction volume was analyzed on an electrophoresis gel (2.2.12). Linearized plasmid DNA was brought to a total volume of 200 µL by adding dH₂O then performing a phenol/chloroform extraction (section 2.2.6.1). DNA in solution was then ethanol precipitated (section 2.2.6.2). The DNA pellet was then resuspended in 30 µL of RNAse free dH₂O. Concentration and purity of DNA in solution was analyzed by nanodrop spectrophotometry.

An *in vitro* transcription reaction was set up by adding the following reagents to 28 uL of plasmid DNA:

- 4 uL of 1x transcription buffer
- 4 uL of 1x DIG RNA labelling mix
- 160 U RNAse inhibitor
- 40 U of T7 or T3 polymerase (refer to Table 2.3)

1 x transcription buffer: 40 mM Tris-HCl (pH 8.0), 8 mM MgCl₂, 50mM NaCl, 2mM
spermidine, in dH\textsubscript{2}O

The transcription reaction was incubated for 4 hours at 37 °C. To digest the DNA template, 1 µL of DNAse was added to the reaction and then incubated at 37 °C for 15 minutes. DIG-labelled RNA probes were then ethanol precipitated (section 2.2.6.2) and 1 µL of the probe solution was added to 9 µL of dH\textsubscript{2}O. Of the diluted probe solution, 1 µL was analyzed using a nanodrop spectrophotometer, and the remainder was analyzed using gel electrophoresis (2.2.12) to assess RNA concentration and integrity. 50 µL of hybridization buffer was added to the remaining DIG-labelled RNA prior to storing it at -80 °C.

2.2.9.2 Preparing \textit{N. vitripennis} embryos for \textit{in situ} hybridization.

To collect embryos from a range of early developmental stages, adult \textit{N. vitripennis} were supplied with \textit{Lucillia sericata} pupae and allowed to lay on hosts for 24 hours. The adult \textit{N. vitripennis} and \textit{Lucillia sericata} pupae were incubated at 28 °C for the duration of the lay period. After the lay period, embryos were collected from \textit{Lucillia sericata} larvae using an 18.5 gauge needle and placed in a scintillation vial, containing 5 mL of heptane and 1.5 mL of phosphate buffered saline (PBS). Once embryos were collected, 500 µL of 37% formaldehyde solution was added to the scintillation vial and shaken vigorously on a platform shaker for 25 minutes. After fixing, embryos were then transferred onto a rectangular (\textasciitilde 2 \times 4 cm) piece of \textit{Whatman} paper and allowed to dry until there was no liquid standing on the paper. Then, a petri dish was lined with a piece of double-sided sticky tape. Embryos were then transferred to the petri dish by pressing the \textit{Whatman} paper, embryo side up to the sticky tape. Embryos were then covered with 0.1% (v/v) Triton-X 100 in PBS. Chorions and vitelline membranes were then removed using a using a 28.5 gauge needle under a dissecting microscope. This technique required disrupting the outer membranes on one end of the embryo, then pushing the embryo out of the membranes from the opposite side of the embryo. After removing the chorion and
vitelline membranes, embryos were transferred to a microcentrifuge tube using a 200 µL pipette. Embryos were dehydrated with four changes of methanol of consecutively increasing concentrations: 25%, 50%, 75% and 100%. Embryos were then stored at -20 °C until use for in situ hybridization (2.2.9.3)

*Phosphate buffered saline (PBS):* 137 mM NaCl, 2.7mM KCl, 10mM Na₂HPO₄ in dH₂O.

**2.2.9.3 In situ hybridization of *N. vitripennis* embryos**

Embryos prepared from section 2.2.9.2 were rehydrated with four changes of PTw. PTw was removed and replaced with 0.1% (v/v) proteinase K in PTw. Initially, the embryos were immersed in the solution of proteinase K for 5 minutes. Later optimization of this protocol however immersed embryos in the proteinase K solution for 7 minutes. PTw with proteinase K was washed off the embryos via three changes of PTw. The PTw was then removed and replaced with 10% (v/v) of 40% formaldehyde in PTw and incubated at room temperature for 10 minutes. The embryos were then rinsed six times in PTw, before replacing PTw with 1 mL of hybridization buffer. Embryos were incubated in a hybridization buffer for 2 hours at 52 °C. Most of the hybridization buffer was then removed, leaving ~100 µL, and 1 µL of DIG-labelled RNA probe (section 2.2.9.1) was added to the solution. Embryos were left to incubate in the hybridization buffer and probe overnight at 52 °C.

After incubating, the hybridization buffer/probe solution was replaced with Drosophila wash and incubated at 52 °C for 5 minutes. This wash/incubation step was repeated for the following lengths of time: 5, 10, 15, 30 minutes and 1 hour. After which, embryos were left overnight at 52 °C in Drosophila wash.

After completion of the wash steps, the embryos were rinsed three times in PTw at
room temperature. The PTw was removed, replaced with PBTw and incubated for 30 minutes at room temperature. The PBTw was then replaced with 1:1000 (v/v) anti-DIG-AP antibody in PBTw and incubated at room temperature for 90 minutes. Embryos were rinsed three times in PTw, PTw was replaced and embryos were incubated in a series of PTw washes for the following lengths of time: 5, 10, 15, 30 minutes and 1 hour. Embryos were washed with two incubations in AP buffer for five minutes. AP buffer was removed and replaced with 4.5 µL of NBT and 3.5 µL of X-phos diluted in 1 mL of AP buffer. Embryos in labelling solution were transferred to microtiter plates and monitored under a microscope until completion of labelling. The labelling solution was removed by washing embryos 3 times in PTw. PTw was removed and replaced with 50% (v/v) methanol in PBS and incubated at room temperature for five minutes. The methanol/PBS solution was replaced with 100% methanol to remove secondary labelling from the embryos. Following de-staining, embryos were rehydrated in 50% (v/v) methanol in PBS for 5 minutes, prior to being washed 3 times in PBS. PBS was removed and replaced with 50% (v/v) glycerol in dH2O until embryos settled. Once the embryos had settled, the 50% glycerol was replaced with 70% (v/v) glycerol in dH2O. The embryos were then mounted onto glass slides and imaged with an *Olympus BX61* compound microscope.

*PTw*: 0.1% Tween diluted in PBS

*PBTw*: 0.1% Bovine serum albumin (BSA) (v/v) diluted in PTw

*Hybridization buffer*: 50% deionized formamide, 4x Standard Saline Citrate, 1x Denhardt’s solution, 250 µg/mL tRNA, 250 µg/mL boiled ssDNA, 50µg/mL heparin, 0.1% Tween-20, 5% dextran sulfate

*Alkaline Phosphatase (AP) buffer*: 50mM MgCl$_2$, 100mM NaCl, 100mM Tris-HCl (pH 9.5), 0.2% (v/v) Tween-20, in dH$_2$O
2.2.10 N. vitripennis pRNAi experiments

2.2.10.1 Needle production

The injection apparatus for the pRNAi procedure includes a needle drawn from borosilicate glass capillary tubing. Tubing dimensions are an outer diameter of 1.00mm and an inner diameter of 0.58mm. Needles are made using the Narashige needle puller which uses weight to pull the needle whilst applying heat to the glass capillary. The Narashige settings used were the number No.2 heater setting, set to 65% maximum heat. Needle tips were then sharpened by applying the ends to a fine rotating sander.

2.2.10.2 Injection of dsRNA into yellow stage N. vitripennis.

N. vitripennis wasps were injected at ‘yellow stage’, the pupal stage that is most responsive to RNAi, according to protocol outlined by Lynch and Desplan (2006). Using a 28.5 gauge needle, Yellow stage N. vitripennis were collected from L. sericata pupae 7 days after adult females had laid embryos. Females were adhered ventral side up along a glass slide using non-toxic stick glue from Crayola. Once needles were made (section 2.2.10.1), 4 µL of dsRNA of either N. v orthodenticle-1, N. v torso, N. v PTTH or eGFP (prepared in section 2.2.7) was loaded into them. Then, they were attached to a Harvard Apparatus microinjector. The microinjector uses pressurized nitrogen gas to pump liquids out of attached needles. The initial microinjector settings were an injection pressure of 115 kPa and an inject time of 210 ms, although these were adjusted depending on viscosity of the dsRNA solutions. Wasps were injected into lateral areas of the abdomen, and caution was taken to avoid injecting the ventral midline. Using the above injection apparatus there is no way to determine the exact volume of dsRNA solution injected into an individual wasp. Therefore, the criteria for a successful
injection were wasps whose abdomens had visibly swelled upon injection. The 4 µl of dsRNA loaded into needles was enough to inject ~40 wasps, after which needles were reloaded. The glass slide with wasps adhered to it was placed in a 50 mL falcon tube and capped with cotton to allow airflow. Wasps were then kept in a 25 °C incubator and allowed to develop into adulthood.

2.2.10.3 Analyzing N. vitripennis pRNAi phenotypes

Once emerged, adult female N. vitripennis which had been injected with dsRNA (section 2.2.10.2) were then supplied with fresh fly pupae to parasitize. The embryos were then collected and placed on slides. The slides were placed on damp paper towels and sealed within petri dishes to keep embryos humid. The petri dishes were stored at 28 °C in an incubator until embryos developed signs of segmentation. Once embryos developed into larvae which displayed movement and segmentation features, they were covered with a 20 µL solution of 10% (v/v) ethanol in lactic acid. The application of lactic acid and ethanol solution here is to dissolve internal features of the larvae allowing for better visualization of the cuticle structures. Larvae are then covered with a coverslip, incubated at 65 °C overnight, and imaged with an Olympus BX61 compound microscope.

2.2.11 pRNAi experiments in A. mellifera

A. mellifera queens were supplied by a local beekeeper, Otto Hyink. Queens were inserted into clear plastic tubing with an inner diameter of 8mm and an outer diameter of 10mm. Halfway down the length of the plastic tubing was the designated injection site, which bees were then confined to by stuffing cotton wool down both ends of plastic tubing. Encircling the injection site were six holes 1mm in diameter which were drilled through the sides of the tube. Once queen movement was restricted, a 27 gauge (outer diameter: 0.4128mm) Hamilton
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needle, attached to a 100 µL Hamilton syringe containing dsRNA solution, was inserted through one of the six holes encircling the injecting site and used to inject the queens’ abdomens with dsRNA solutions (section 2.2.7) of eGFP, or A. mellifera orthologs of orthodenticle-1 or torso-like. At first, queens were injected with 5 µL of dsRNA solution, although this volume was increased to 10µL and then 15µL to elicit stronger phenotypic responses from these experiments. Once injected, queens were returned to their hives and encased in Api Ezi Queen cages inserted onto a fresh (yet to have brood laid in it) hive frame. Api Ezi Queen cages restrict movement of the queens, ensuring that they only lay onto the area of frame that is encased within the cage. Additionally, as the frame is fresh, only those embryos which were laid after the queens were injected will be found contained within the Ezi Queen cages. After injecting them, queens were left in Ezi Queen cages for 24 hours before removing the frames and collecting the embryos. The embryos were then stored at for 48 hours at 28 °C, relative humidity (RH) 96% in a Black Chick incubator. Embryos were then placed on a glass slide, immersed in halocarbon oil, and imaged with an Olympus Bx61 compound microscope.

2.2.12 Gel electrophoresis

Agarose gels were made from 1% agarose (w/v) in 1x SB buffer. One µL of 6x loading dye was added to nucleic acids and samples were brought to a final volume of 10 µL with RNAse free dH₂O, prior to loading samples into gel lanes. Gels were run for 20 minutes at 180 volts provided by a Thermo EC 250-90 electrophoresis power supply. Ethidium bromide in SB buffer intercalates with nucleic acids and fluoresces under UV exposure. The Bio-Rad gel doc system was used to photograph this fluorescence, and Quantity One software was used for gel image analysis. All samples were run parallel to Invitrogen 1Kb + size markers, to approximate the length of fragments on the gel.
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20 x SB buffer: 0.8% (w/v) NaOH was diluted in dH$_2$O. pH of SB was adjusted to 8.0 with boric acid prior to adding ethidium bromide to a final concentration of 50mg/mL.

1 x SB buffer was prepared by diluting 20 x SB in dH$_2$O.

6 x Loading dye: 0.25% (v/v) bromophenol blue sigma, 0.25% (v/v) xylene cyanol, Sigma, and 30% glycerol (v/v) diluted in dH$_2$O.
CHAPTER THREE: RESULTS

3.1 FUNCTIONAL STUDIES

3.1.1 Introduction

To determine if components of the Torso-activation module pattern the end-terminal regions of A. mellifera and N. vitripennis, the maternal expression of these genes were 'knocked down' (reduced), and the resulting embryo phenotypes were analysed.

These genes (torso and PTTH in N. vitripennis; torso-like in A. mellifera) were knocked down by performing pRNAi. This is a method of RNAi by which the expression of the target gene is reduced in an adult female insect, thereby reducing the capacity of the female to donate transcripts of this gene to her progeny. The function of the maternally-donated gene is then determined upon analysing the phenotypes of the adult female's progeny.

In these experiments, pRNAi was performed by injecting adult females with dsRNA that is complementary to the target gene. This injection of dsRNA provokes an endogenous response in the organism which is summarised in Figure 3.1.
3.1.2 Synthesis of dsRNA by \textit{in vitro} transcription

\textbf{Figure 3.1:} Diagram illustrating the pathway by which dsRNA initiates RNAi in the host organism. Gene silencing is engaged when the eukaryotic enzyme, Dicer, recognizes and processes dsRNA ~20bp in length. These siRNAs are then unwound to form singles strands and one strand, termed the guide strand, is incorporated into the protein Argonaute. Argonaute is one protein belonging to RISC (RNA-induced silencing complex), and it is this complex which enforces RNAi. RNAi occurs when the guide strand basepairs with the target mRNA. Binding via sequence complementarity of the guide RNA and target mRNA induces Argonaute-mediated cleavage of the mRNA, effectively silencing the gene of interest (Hannon, 2002).
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All plasmids were transformed into *E. coli* strain XL1-blue MRF, cultured, and midprepped successfully. Plasmid midprep concentrations of all plasmids can be seen in Table 3.1. Note: These concentrations were high enough to provide the 5 µg of DNA required for the 50 µL restriction enzyme endonuclease reactions. The resulting plasmid solutions were then linearized by performing restriction endonuclease digest reactions, which upon running them on an electrophoresis gels, appeared to be successful (Figures 3.2, 3.3, 3.4 and 3.5). The gel images show that all but one of the double digests appeared as two bands, and all single digests appear as one band. Furthermore, all bands were the correct size, approximating the size of the plasmid, insert, or the combined size of the plasmid and insert where appropriate (gene insert and plasmid vector sizes can be seen in Table 3.2). The one exception was the *N. vitripennis* gene *orthodenticle-1*, which upon digesting it in double digest endonuclease reaction appeared as degraded DNA at the bottom of the gel. It’s unclear what caused this degradation. However, repeating the double digest of *orthodenticle-1* under the same reaction conditions appeared successful however, as indicated by Figure 3.6.

**Table 3.1 Plasmid insert concentrations following midprep isolation**

<table>
<thead>
<tr>
<th>Plasmid insert</th>
<th>Concentration (ng/µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. m orthodenticle-1</em></td>
<td>1947.52</td>
</tr>
<tr>
<td><em>A. m torso-like</em></td>
<td>1382.56</td>
</tr>
<tr>
<td><em>eGFP</em></td>
<td>1520.14</td>
</tr>
<tr>
<td><em>N. v torso</em></td>
<td>1653.13</td>
</tr>
<tr>
<td><em>N. v PTTH</em></td>
<td>1930.50</td>
</tr>
<tr>
<td><em>N. v orthodenticle-1</em></td>
<td>1172.32</td>
</tr>
</tbody>
</table>
Table 3.2: Gene insert and plasmid vector lengths in base pairs

<table>
<thead>
<tr>
<th>Gene</th>
<th>Length in base pairs (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. m orthodenticle-1</em></td>
<td>1400</td>
</tr>
<tr>
<td><em>A. m torso-like</em></td>
<td>1800</td>
</tr>
<tr>
<td><em>eGFP</em></td>
<td>726</td>
</tr>
<tr>
<td><em>N. v torso</em></td>
<td>1876</td>
</tr>
<tr>
<td><em>N. v PTTH</em></td>
<td>751</td>
</tr>
<tr>
<td><em>N. v orthodenticle-1</em></td>
<td>1138</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Length in base pairs (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBlueScript II SK (+)</td>
<td>2961</td>
</tr>
<tr>
<td>pBlueScript SK (+)</td>
<td>2958</td>
</tr>
<tr>
<td>LITMUS 38i</td>
<td>2814</td>
</tr>
</tbody>
</table>

Figure 3.2: Agarose gel electrophoresis of restriction digests (*N. vitripennis* genes and *eGFP*). Lane 1: 1kb+ ladder (sizes in base pairs on the left of the panel). Lane 2: double digest of PTTH. Lane 3: double digest of orthodenticle-1. Lane 4: double digest of PTTH. Lane 5: double digest of torso. Lane 6: EcoRI digest of PTTH (antisense). Lane 7: XbaI digest of PTTH (sense). Lane 8: EcoRI digest of orthodenticle-1. .
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Figure 3.3: Agarose gel electrophoresis of restriction digests (*N. vitripennis* genes and eGFP) (continued).
Lane 1: 1kb + ladder (sizes in base pairs on the left of the panel). Lane 2: *Xba*I digest of *orthodentine*-1 (sense). Lane 3: *EcoRI* digest of *torso* (antisense). Lane 4: *Xba*I digest of *torso* (sense). Lane 5: *HindIII* digest of eGFP (antisense). Lane 6: *EcoRI* digest of eGFP (sense).

Figure 3.4: Agarose gel electrophoresis of restriction digest (*A. mellifera* orthodentine-1).
Lane 1: 1kb + ladder (sizes in base pairs on the left of the panel). Lane 2: Double digest of *A. m* orthodentine-1. Lane 3: *SphI* digest of *A. m* orthodentine-1 (sense). Lane 4: *HindIII* digest of *A. m* orthodentine-1 (antisense).

Figure 3.5: Agarose gel electrophoresis of restriction digest (*A. m* torso-like).
Lane 1: 1kb + ladder (sizes in base pairs on the left of the panel). Lane 2: *EcoRI* digest of *A. m* torso-like (sense). Lane 3: *PstI* digest of *A. m* torso-like (antisense).

Figure 3.6: Agarose gel electrophoresis of repeat restriction digest (double digest of *N. v* orthodentine-1).
Lane 1: 1kb + ladder (sizes in base pairs on the left of the panel). Lane 2: Double digest of *N. v* orthodentine-1.
Linearized plasmid DNA was then phenol/chloroform extracted and ethanol precipitated. Purified DNA concentrations can be seen in Table 3.3. These concentrations were high enough to provide the 1 µg of DNA required for \textit{in vitro} transcription reactions. The purified solutions of plasmid DNA were then used to synthesise and purify dsRNA using the \textit{Megascript RNAi kit} from \textit{Invitrogen} by \textit{Thermo Fisher Scientific}.

\begin{table}[h]
\centering
\caption{Linearized plasmid concentrations following ethanol precipitation}
\begin{tabular}{|l|c|}
\hline
Gene (antisense) & Concentration \\
\hline
\textit{A. m orthodenticle-1} & 704.23 ng/µL \\
\textit{A. m torso-like} & 711.60 ng/µL \\
eGFP & 484.56 ng/µL \\
\textit{N. v torso} & 669.18 ng/µL \\
\textit{N. v PTTH} & 747.32 ng/µL \\
\textit{N. v orthodenticle-1} & 588.30 ng/µL \\
\hline
\end{tabular}
\end{table}

As it was expected that protocol of dsRNA synthesis would require some optimisation to be performed successfully, the initial attempts at dsRNA synthesis were performed only on the \textit{N. vitripennis} genes \textit{PTTH}, \textit{torso} and \textit{orthodenticle-1}. These \textit{in vitro} transcription reactions, and subsequent purification steps, yielded concentrations of dsRNA were below that of what is required for pRNAi experiments. In published protocol, the concentration of dsRNA recommended for pRNAi on \textit{N. vitripennis} is \(\sim 1 \mu g/\mu L\) (Lynch and Desplan, 2006). To date, experiments performing pRNAi on \textit{A. mellifera} have not been published, but typical \textit{A. mellifera} RNAi experiments use a dsRNA concentration range of 0.5-10 \(\mu g/\mu L\) (Amdam \textit{et al}.,
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2003; Antonio et al., 2008; Maleszka et al., 2007; Schlüns and Crozier, 2007). The dsRNA concentrations of the N. vitripennis gene PTTH, torso, and orthodenticle-1 were 289.53 ng/µL, 58.04 ng/µL, and 347.30 ng/µL respectively. Furthermore, analysing the integrity of dsRNA solutions by running them on electrophoresis gel revealed bright bands sized <100bp (Figure 3.7)

![Figure 3.7: Agarose gel electrophoresis of dsRNA of N. v genes PTTH, torso, orthodenticle-1.](image)

Lane 1: 1kb + ladder (sizes in base pairs on the left of the panel). Lane 2: dsRNA of N. v PTTH. Lane 3: dsRNA of N. v torso. Lane 4: dsRNA of N. v orthodenticle-1.

The presence of these bands indicates that an abundance of single nucleotides, or very short molecules of ssRNA/dsRNA/DNA, are present in the final solution. The absence of dsRNA bands approximating size of the plasmid inserts coupled with the appearance of these short bright bands indicates two things. Firstly, it means that dsRNA was either degraded during some stage of the protocol, or it was never correctly synthesised in the in vitro transcription reaction. Secondly it indicates there is a failure to correctly purify short molecules of ssRNA/dsRNA/DNA and single nucleotides out from the solution.

To increase the purity, integrity and yield of dsRNA solutions the following adjustments were then made to the following protocols before performing a repeat attempt at dsRNA synthesis. The steps used here to refine the protocol were first tested on the N. vitripennis gene torso before applying the protocol to other genes. Firstly, dH₂O used in any protocol from performing a midiprep procedure onwards was substituted with dH₂O that had been treated with diethylpyrocarbonate (DEPC). This ensured all purified solutions of linearized DNA were ribonuclease (proteins which catalyse the degradation of RNA) free. It
was hoped therefore, that upon using these solutions of DNA for in vitro transcription reactions, any RNA transcripts synthesised in the reaction would not be degraded by ribonucleases. Additionally, when repeating restriction endonuclease reactions, they were run for five hours instead of four to ensure all plasmids were completely digested. The protocol supplied by the manufacturers of the Megascript RNAi kit states that undigested plasmid can inhibit disassociation of the RNA polymerases in in vitro transcription reaction. This results in a lower yield of ssRNA transcripts from the reaction and therefore less dsRNA is produced when the ssRNA transcripts are annealed. Upon repeating the restriction digest reactions and running them on an electrophoresis gel, it showed that all the double digests appeared as two bands, and all single digests appeared as one band. Once again, all bands were the correct size, approximating the size of the plasmid, insert, or the combined size of the plasmid and insert. This linearized DNA was then phenol/chloroform extracted. During this procedure extra care was taken to ensure none of the lower organic phase contaminated the upper aqueous phase when pipetting it into a new Eppendorf tube, to avoid potential chloroform contamination of the precipitated DNA. Chloroform contamination was avoided in this manner because chloroform is known to denature proteins (Asakura et al., 1978). Therefore, it was considered that chloroform contamination could denature RNA polymerase, thereby inhibiting the in vitro transcription reactions. Additionally, when ethanol precipitating the solution, DNA was only resuspended once all the ethanol had evaporated, as ethanol can also denature proteins (Asakura et al., 1978).

The resulting solution of linearized N. vitripennis torso plasmid DNA in solution was then used to synthesise and purify dsRNA using the Megascript RNAi kit. Upon analysing the solution of N. vitripennis torso dsRNA by nanodrop spectrophotometry the concentration was 273.20 ng/μL. Once again, this is lower than 1μg/μL, the concentration recommended for pRNAi experiments in N. vitripennis. However, the electrophoresis gel image (Figure 3.8)
suggests that the integrity of the dsRNA solution had improved considerably. The gel image shows a bright band slightly larger than 1650bp, which approximates the size of *N. vitripennis torso*, which is 1876 bp in length.

Additionally, the appearance of bands larger than the expected size do not indicate that the dsRNA is of poor quality or integrity. According to the protocol supplied by the manufacturers of the *Megascript RNAi kit*, these larger bands are the result of aggregates of multiple RNA strands. The protocol further states that these RNA aggregates can induce RNAi as effectively as dsRNA of the expected size. However, there was one additional band that appeared in both lanes that is unlikely to induce RNAi. The bands seen on the gel that appear 1000 bp in length, which are approximately half the size of the *N. vitripennis torso* insert, are likely the result of undegraded ssRNA according to the manufacturers protocol.

Because these changes in protocol vastly improved the integrity of the dsRNA they were used in all following repeat attempts at dsRNA synthesis.

Next, to increase the yield of dsRNA, a repeat attempt at dsRNA synthesis was performed in which all reactions/purification steps performed with the *Megascript RNAi kit* were scaled up by a factor of four. The resulting concentrations of the dsRNA solutions of *A. m orthodenticle-1* and *A. m torso-like* were 3,272.04 ng/µL and 3,814.60 ng/µL respectively.
These concentrations are therefore within the typically used concentration range of 0.5-10µg/µL. Furthermore, upon running the dsRNA solutions on an electrophoresis gel it displayed two clear bands, with no accumulation of degraded dsRNA/ssRNA/DNA at the bottom of the gel (Figure 3.9). Interestingly, the size of both bands from both genes were greater than 4000bp, which is larger than the 1370bp and 1800bp sized bands expected for A. m orthodenticle-1 and A. m torso-like respectively. However, as these bands are most-likely the result of multi-strand RNA aggregates, these dsRNA solutions were used for pRNAi experiments in A. mellifera.

Because dsRNA production of the A. mellifera homologues of orthodenticle-1 and torso-like was successful these methods were repeated for eGFP and the N. vitripennis homologues of PTTH, torso and orthodenticle-1.

Upon running the resulting dsRNA solution of eGFP on a gel, the dsRNA appeared to be of acceptable integrity (Figure 3.10). Whilst there was the presence of a band of small nucleotides at the bottom of the gel, this was faint compared to the bright band approximating the expected size of eGFP dsRNA (720 bp). Therefore, upon analysing the concentration of nucleotides in this solution by nanodrop spectrophotometry, most of the nucleotide concentration will be attributable to eGFP dsRNA. The concentration of dsRNA was 1704.05
ng/µL, and therefore it was considered sufficient for pRNAi experiments in *N. vitripennis* and *A. mellifera*.

Figure 3.10: Agarose gel electrophoresis of dsRNA of *eGFP*.
Lane 1: 1kb + ladder (sizes in base pairs on the left of the panel). Lane 2: *eGFP* dsRNA.

Surprisingly, the dsRNA of the *N. vitripennis* genes yielded here appeared degraded upon running the solutions on an electrophoresis gel (Figure 3.11). It is unclear why the dsRNA synthesised of the *N. v.*, but not *A. m.*, genes was degraded. It was considered that a possible cause of dsRNA degradation was by use of the filter cartridges supplied in the *Megascript RNAi kit*. When purifying dsRNA solutions with this kit, the dsRNA is drawn through the filter cartridges by centrifugation. Therefore, it was considered that the process of applying the dsRNA solutions onto these filter cartridges may subject the dsRNA to oxidation damage, as dsRNA may briefly be removed from the eluate, when moving through the cartridge.

Figure 3.11: Agarose gel electrophoresis of dsRNA of *N. v.* genes *PTTH*, *torso*, *orthodenticle-1*.
Lane 1: dsRNA of *N. v* orthodenticle-1. lane 2: 1 kb + ladder (sizes in base pairs on the left of the panel). Lane 3: dsRNA of *N. v* torso. Lane 4: dsRNA of *N. v* *PTTH*. 
To avoid any possible dsRNA degradation caused by using filter cartridges, the modified protocol of dsRNA synthesis was repeated albeit with a different method of purifying the dsRNA solution. This time, upon attempting to purify the dsRNA in solution, the dsRNA was phenol/chloroform extracted and then ethanol precipitated. The precipitated dsRNA was then resuspended in 100 µL of RNAse-free dH2O. The resulting solutions of dsRNA were then analysed by running them on an electrophoresis gel (Figure 3.12). Whilst the bands of the gel appeared smeared, their lengths are within the range 1000-12000bp, indicating that partial, but not complete degradation of RNA aggregates has occurred. Because dsRNA molecule lengths as low as 20bp can induce RNAi (Scott et al., 2013), these dsRNA solutions were of good enough integrity to use in pRNAi experiments. Upon analysing the concentrations by nanodrop spectrophotometry the dsRNA concentrations of the N. vitripennis homologues of PTTH, torso and orthodenticle-1 were 538.90 ng/µL, 331.73 ng/µL and 344.05 ng/µL respectively. As these concentrations of dsRNA were below the 1µg/µL recommended for pRNAi experiments they were concentrated by the methods of phenol/chloroform extraction and ethanol precipitation.

![Figure 3.12: Agarose gel electrophoresis of dsRNA of N. v genes PTTH, torso, orthodenticle-1 (eluted in 100µL of dH2O).](image)

Lane 1: 1kb + ladder (sizes in base pairs on the left of the panel). Lane 2: dsRNA of N. v PTTH. Lane 3: dsRNA of N. v torso. Lane 4: dsRNA of N. v orthodenticle-1.

This time, after ethanol precipitating the dsRNA solutions, all pellets were resuspended in 20µL of RNAase-free dH2O. Upon running the resulting solutions on an electrophoresis gel
it appeared that the integrity of the dsRNA remained the same (Figure 3.13). Furthermore, the concentrations of the dsRNA solutions were successfully increased to 876.36 ng/µL, 1104.18 ng/µL and 1431.68 ng/µL for the *N. vitripennis* genes torso, orthodenticle-1, and *PTTH* respectively. Whilst the dsRNA solution of *N. vitripennis* torso was below the 1 µg/µL recommended for pRNAi in *N. vitripennis*, it was considered to be of close enough concentration to use it in pRNAi experiments regardless.

![Figure 3.13: Agarose gel electrophoresis of dsRNA of *N. v* genes *PTTH*, torso, orthodenticle-1 (eluted in 20µL of dH2O).](image)

Lane 1: 1kb + ladder (sizes in base pairs on the left of the panel). Lane 2: dsRNA of *N. v* orthodenticle-1. Lane 3: dsRNA of *N. v* torso. Lane 4: dsRNA of *N. v* PTTH.

### 3.1.3 Synthesis of dsRNA by *in vivo* bacterial expression

Discussed further in other sections, several unsuccessful attempts at synthesizing dsRNA by *in vitro* transcription were performed, before considering alternative methods of dsRNA synthesis. Here, an attempt was made at using a method of dsRNA production described first by Ongvarrisoponea *et al.* (2007). This method uses *in vivo* bacterial expression to synthesise sense and antisense transcripts of plasmid inserts, which anneal to form dsRNA.

Transformations of *E. coli* strain HT115, were first performed with Litmus38i plasmids containing cDNA inserts of either eGFP, *A. mellifera* orthodenticle-1 or *A. mellifera* torso-like. HT115 cells have T7 RNA polymerase under an IPTG inducible promotor. Therefore,
Litmus38i plasmids were chosen to test the efficacy of *in vivo* dsRNA synthesis in HT115 cultures as they contain two T7 polymerase binding sites flanking the plasmid insert site. Gene inserts in pBlue Script (I and II) SK (+) vectors were not used for these methods as they required T3 polymerase, which is not expressed in HT115 cells, to synthesise the antisense transcripts of the plasmid insert. If the initial attempts to synthesise dsRNA via the methods described by Ongvarrasoponea *et al.* (2007) were successful, then they were to be repeated on inserts contained in the plasmids pBlue Script (I and II) SK (+), after subcloning them into Litmus38i plasmids.

Transformations of *E. coli* strain HT115, with both Litmus38i plasmid inserts appeared successful. This was indicated by the appearance of bacterial colonies on agar plates supplemented with ampicillin and tetracycline antibiotics. The *E. coli* strain HT115 contains resistance to tetracycline only. Therefore, growth of HT115 cells on ampicillin-supplemented agar plates is indicative that this *E. coli* strain was successfully transformed with Litmus38i plasmids, which contain ampicillin resistance.

Further culturing of transformed HT115 cells appeared successful, as all 50 mL cultures of 2xYT media inoculated with HT115 cells reached an OD600 of 0.4 after ~4hours of incubation.

However, despite indications that transformed HT115 cells were successfully cultured, dsRNA synthesized from *in vivo* bacterial production was of low yield upon purifying it with TRIzol® reagent. Upon analysing the integrity of the dsRNA by running the solutions on an electrophoresis gel, no bands could be detected upon imaging the gels. This is likely because very low concentrations of nucleic acids are undetectable on an electrophoresis gel. Concentrations of the dsRNA solutions were 26.03ng/μL, 4.56ng/μL, 19.89ng/μL for *A. m orthodenticle-1*, *A. m torso-like*, and eGFP respectively.
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The method of producing dsRNA by *in vivo* bacterial production was repeated for both Litmus38i plasmid inserts. Upon repeating this method of dsRNA by *in vivo* bacterial production all steps were performed as before bar one exception: This time, HT115 cells were not boiled to lyse them as this was considered to be a possible cause for the dsRNA degradation. Boiling may result in dsRNA degradation because the boiling process separates the ssRNA strands that form the dsRNA. If the dsRNA in solution was being unannealed to form ssRNA, then it would be degraded after the addition of RNaseA, a ribonuclease enzyme which is added with the intention of degrading the ssRNA of the bacterial host. Therefore, Instead of lysing cells by boiling them, 7.5 mL TRIzol® reagent was added directly to the 3.25 mL samples and the solution was incubated for 10 minutes. According the manufacturer’s protocol, TRIzol® reagent can be added directly to cells in suspension as it contains cell lysis properties. No dsRNA pellet appeared after performing the TRIzol® RNA extraction however, and upon analysing the solutions by nanodrop spectrophotometry the solutions contained a dsRNA concentration ~0ng/µL. This seemed to indicate that TRIzol® itself could not lyse the cells as effectively as boiling them. Therefore, these methods were repeated with the following modifications. Firstly, the cells in suspension were only boiled for one minute to lyse them. It was hoped that by decreasing the boiling time, separation of the ssRNA strands that form the dsRNA would occur. The second modification was to introduce a dsRNA annealing step. After boiling the cells in suspension they were left at room temperature for four hours to allow the sense and antisense transcripts of the Litmus38i plasmid inserts to reanneal, before continuing with the protocol. However, upon repeating these methods with the above modifications the resulting dsRNA in solution was of low integrity and yield (concentrations were 23.45ng/µL, 12.80ng/µL, 33.01ng/µL for *A. m orthodenticle-1*, *A. m torso-like*, and eGFP respectively). Upon analysing the integrity of the dsRNA by running the solutions on an electrophoresis gel, no bands could be detected upon imaging the gels.
3.1.4 pRNAi experiments in *A. mellifera*

In the initial pRNAi experiments performed on *A. mellifera*, 5 µL of a dsRNA solution of either *eGFP*, *A. m orthodenticle-1*, or *A. m torso-like*, was injected into three separate queens. The queens came from three large hives, similar in size, with fresh brood laid in the hives’ frames. This indicated that queens were healthy and had laid embryos within the previous few days prior to injecting. However, after performing injections of dsRNA, only the queen that was injected with a dsRNA solution of *eGFP* laid embryos. As expected, embryos laid by the *eGFP*-injected queen appeared wildtype upon developing to Du Praw stage 9 (46.9 ± 2 hours after egg laying) (Du Praw, 1967). After four days however, all three queens, resumed laying embryos. After resumption of queen laying, embryos from all three queens, including the positive control *A. m orthodenticle-1*, appeared wildtype. This indicated that either expression of these genes were not knocked down successfully, or that gene expression had been knocked down but gene expression had returned to normal levels by the time queens had resumed laying.

These experiments were repeated one week after performing the initial injections. Here, the same queens were injected 5 µL of the same solutions of dsRNA that they had been injected with previously. Again, only the queen that was injected with the negative control, *eGFP*, laid embryos. Once again, upon developing to Du Praw stage 9, these appeared wildtype. Likewise, with the previous attempt at pRNAi in *A. mellifera*, all three queens resumed laying wildtype embryos three days after performing the dsRNA injections. It is unclear why only the queen bee that was injected with a dsRNA solution of *eGFP* laid embryos initially. It was considered that a possible contributing factor causing only the *eGFP*-injected queens to lay is that these experiments were performed in late February (nearing the end of Summer in Dunedin, New Zealand). Queen bees typically display a sharp decline in brood laying as a response to shorter
photo periods (Avitabile, 1978). Therefore, these experiments were repeated later that year in September, when queen bees displayed an increased level of brood laying.

Three different queens, from that of the previous set of pRNAi experiments, were chosen for the continuation of experiments in September. Likewise, with queens used previously, the new queens came from three large hives, similar in size, with fresh brood laid in the hives’ frames. 5µL of a dsRNA solution of either eGFP, A. m orthodenticle-1, or A. m torso-like, was injected into each queen. In contrast to the queens injected previously, all three of the new queens laid plenty of embryos (~300 each) 24 hours post-injection. Upon reaching Du Praw stage 9, the phenotypes of embryos from the queens injected with eGFP and torso-like appeared wildtype; embryos from the queen injected with A. m orthodenticle-1 displayed a similar but weaker phenotype to RNAi studies previously performed on this gene (Wilson and Dearden, 2011). More specifically they displayed a reduction in size of the mandible, maxilla, and labium (together these are anterior features of the developing bee) whilst Wilson and Dearden (2011) have reported A. m orthodenticle-1 RNAi phenotypes in which developing embryos are missing the mandible, maxilla, labium, thoracic segments and some abdominal segments. Despite this, because the embryos from queen injected with A. m orthodenticle-1 were displaying a similar phenotype to that which has been reported previously, it was an indication that this positive pRNAi control gene had been knocked down successfully. Images of embryo phenotypes can be seen in Figure 3.14. Table 3.4 shows, from each gene group, how many bees appeared wildtype and how many displayed a reduction in anterior features, from this set of injections.

Table 3.4: Number of A. mellifera embryos appearing wildtype or displaying a reduction in anterior features after performing pRNAi (5µL injections)
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<table>
<thead>
<tr>
<th>Gene</th>
<th>Total number of embryos</th>
<th>Number of embryos appearing wildtype</th>
<th>Number of embryos missing anterior features</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. m Orthodenticle-1</td>
<td>292</td>
<td>234</td>
<td>58</td>
</tr>
<tr>
<td>A. m torso-like</td>
<td>305</td>
<td>305</td>
<td>0</td>
</tr>
<tr>
<td>eGFP</td>
<td>318</td>
<td>318</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 3.14: A. mellifera larval images following pRNAi knockdown of A. m orthodenticle-1, A. m torso-like, and eGFP (5 μL injection experiments).

All larvae are orientated ventral side down, anterior on the left. (A) In A. mellifera the pRNAi of eGFP phenotype appears wildtype. (B) In A. mellifera the pRNAi of orthodenticle-1 phenotype displays a reduction of anterior segments. (C) In A. mellifera the pRNAi of torso-like phenotype appears wildtype. Features are labelled, mn, mandible; mx, maxilla; lb, labium; T, thoracic limb buds; A, abdominal buds; tl, telson.
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The strength of phenotypic change produced from RNAi is often dependant on the dose of dsRNA administered to an organism (Scott et al., 2013). Therefore, to enhance the phenotypic response seen in Figure 3.14, the volume of injected dsRNA was increased from 5µL to 10µL and the experiment was repeated. Note that the same queens used for the previous set of injections were used here as well. Here, upon reaching Du Praw stage 9, phenotypes of embryos from the queen injected with eGFP appeared wildtype. Upon reaching Du Praw stage 9, embryos from the queen injected with A. m orthodenticle-1 produced a stronger phenotypic response with severely affected embryos completely missing the mandible, maxilla, and labium. Surprisingly, embryos from the queen injected with A. m torso-like dsRNA were completely missing the mandible, maxilla, and labium and all thoracic segments. Images of embryo phenotypes can be seen in Figure 3.15. Table 3.5 shows, from each gene group, how many bees appeared wildtype and how many displayed a reduction in anterior features, from this set of injections.
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Table 3.5: Number of *A. mellifera* embryos appearing wildtype or displaying a reduction in anterior features after performing pRNAi (10 µL injections)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Total number of embryos</th>
<th>Number of embryos appearing WildType</th>
<th>Number of embryos missing anterior features</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. m orthodenticle-I</em></td>
<td>346</td>
<td>252</td>
<td>94</td>
</tr>
<tr>
<td><em>A. m torso-like</em></td>
<td>275</td>
<td>226</td>
<td>47</td>
</tr>
<tr>
<td><em>eGFP</em></td>
<td>310</td>
<td>310</td>
<td>0</td>
</tr>
</tbody>
</table>
In order to confirm that *A. mellifera* queens injected with a dsRNA solution of *torso-like* exhibit a loss of anterior features, an attempt was made to repeat this injection experiment. Additionally, to determine whether the phenotypic responses exhibited in the previous injection experiment could be enhanced, the injection dose of dsRNA solutions were increased from 10µL to 15µL. Unfortunately, because of a displayed lack of fine motor skills by the experimenter, the *torso-like* dsRNA solution was irretrievably injected outside of the *A. mellifera* queen, and absorbed into the cotton that encased her. As this was the last of the dsRNA solution of *torso-like* this set of injections was discontinued and the resulting embryo phenotypes were not analysed. An attempt was made to resynthesise more dsRNA of all three genes, but this was not achieved successfully before the end of summer. Correspondence with Otto Hyink, the supplier of the queen bees confirmed that the queens had stopped laying embryos.

### 3.1.5 pRNAi experiments in *Nasonia vitripennis*

For each of the four *N. vitripennis* pRNAi gene targets, *N. v PTTH, N. v orthodenticle-1, N. v torso or eGFP*, 200 ‘yellow’ stage wasps were injected with solutions of dsRNA. After injections were performed, wasps displayed a high mortalit rate, as only ~20% of wasps survived to adulthood from each of the four sets of injections. Despite this, three days after emerging, the surviving wasps displayed a high rate of parasitization on the fly hosts, which allowed plenty of embryos to be collected. After allowing the embryos to develop cuticle structures (~24 hours after laying) the embryos collected from wasps injected with the negative control, *eGFP*, and the *N. vitripennis* homologues of *torso* or *PTTH* appeared wildtype. The
embryos from wasps injected with the positive control, *orthodenticle-1*, appeared to be missing anterior and posterior segments, mouthparts and the spiracle (external respiratory opening) associated with the second thoracic denticle belt. The pRNAi *orthodenticle-1* phenotype displayed here is the same phenotype previously reported for pRNAi of *orthodenticle-1* in *N. vitripennis* (Lynch et al., 2006a). Therefore, this is an indication that the positive control, *orthodenticle-1*, was successfully knocked down in *N. vitripennis*. Images of the embryos collected from this set of pRNAi experiments can be seen in Figure 3.16. Table 3.6 shows, from each gene group, how many wasps appeared wildtype and how many displayed a reduction in anterior/posterior features, from this set of injections. Unfortunately, because this pRNAi experiment required the entire use of the *N. vitripennis* dsRNA solutions that were made in earlier steps, a repeat pRNAi experiment in *N. vitripennis* was unable to be performed.
Figure 3.16: *N. vitripennis* larval images following pRNAi knockdown of *N. v. othodonticle*-I, *N. v. torso*, *N. v. PTTH* and eGFP.

All larvae are orientated anterior side on the left. **(A)** The pRNAi of *eGFP* phenotype appears wildtype (spiracles are covered by trapped particles in slides). **(B)** The pRNAi of *othodonticle*-I phenotype displays a loss of anterior/poster features and segments. **(C)** The pRNAi of *torso* phenotype appears wildtype (note: extra spiracles can be seen because embryos are orientated ventral side towards the viewer). **(D)** The pRNAi of *PTTH* appears wildtype. Yellow arrows indicate developing mouthparts. Green arrows indicate spiracle associated with second thoracic denticle belt. The red arrows indicate spiracles associated with abdominal spiracles. The blue arrows indicate denticle belts.
Table 3.6: Number of \textit{N. vitripennis} embryos appearing wildtype or displaying a reduction in terminal features after performing pRNAi

<table>
<thead>
<tr>
<th>Gene</th>
<th>Total number of embryos</th>
<th>Number of embryos appearing WildType</th>
<th>Number of embryos missing terminal features</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{N. v torso}</td>
<td>35</td>
<td>35</td>
<td>0</td>
</tr>
<tr>
<td>\textit{N. v PTTH}</td>
<td>41</td>
<td>41</td>
<td>0</td>
</tr>
<tr>
<td>\textit{N. v orthodenticle-1}</td>
<td>38</td>
<td>26</td>
<td>12</td>
</tr>
<tr>
<td>\textit{eGFP}</td>
<td>28</td>
<td>28</td>
<td>0</td>
</tr>
</tbody>
</table>

3.2 EXPRESSION STUDIES

3.2.1 Introduction

Expression studies determine where a gene is expressed, thereby allowing a researcher to gain insight into what tissues the gene of interest may develop/have functional roles in. Here, several attempts were made to localise the expression domains of \textit{PTTH} and \textit{torso} in \textit{N. vitripennis}. This was done by performing \textit{in situ} hybridisation on \textit{N. vitripennis} embryos with Digoxigenin-labelled (DIG) probes. DIG-labelled probes are antisense ssRNA transcripts complementary to the transcript of interest, which are synthesised with DIG-labelled uracil nucleobases. The process by which the expression domain of a gene is labelled with DIG-labelled probe is summarised in Figure 3.17.
3.2.2 Synthesis of DIG-labelled Probes

The pBluesScript II SK (+) plasmids containing gene inserts of *N. vitripennis* orthologues of *orthodenticle-1*, *torso*, and *PTTH* were transformed into *E. coli* XL1-blue MRF, cultured, and midiprepped successfully. These plasmid midiprep concentrations were high enough to provide the 5 µg of DNA required for the 50 µL restriction enzyme endonuclease reaction (Table 3.1). The resulting plasmid solutions were then linearized by performing restriction endonuclease digest reactions, which upon running them on an electrophoresis gel, showed that all double digests appeared as two bands, and all single digests appear as one band (Table 3.2, 3.3). Furthermore, all bands were the correct size, approximating the size of the plasmid, insert, or the combined size of the plasmid and insert where appropriate. Linearized plasmid DNA was then phenol chloroform extracted and ethanol precipitated. The resulting DNA concentrations were high enough to provide the 1µg of DNA template required for the
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\textit{in vitro} transcription reaction (Table 3.3). Note: this purified, linearized plasmid DNA was the same as that used for the initial attempts at synthesising dsRNA (section 3.1.2). Purified plasmid DNA was then used in DIG-labelled transcription reactions. The concentration of the majority of the DIG-labelled probes were within a concentration range of 150-400 ng/µL, which is the lab protocol’s recommended concentration range for samples of probe solution that have been diluted 1:10 in water. The probes that were not within this concentration range were the sense transcripts of \textit{N. vitripennis} \textit{PTTH}, and \textit{torso}. The sense transcripts of genes act as a negative control as they cannot hybridize to the endogenously expressed transcripts of the organism being studied. Because the DIG-labelled sense transcript of \textit{N. v orthodenticle-1}, was the only DIG-labelled transcript within the recommended concentration range of 150-400 ng/µL, it was used as the negative control. The concentrations of the probes can be seen in Table 3.7. Analysis of the electrophoresis gels reveal bright clear bands approximating the expected size of the transcripts. Note: because transcripts are ssRNA they should appear as half the size of the cDNA inserts (the cDNA inserts of \textit{N. v torso}, \textit{N. v PTTH} and \textit{N. v orthodenticle-1} are 1800, 751 and 1400 bp in length respectively). Additional bands can be seen above the expected sizes but this is most-likely caused by the transcripts forming mult-strand aggregates and running slower on the gel. Interestingly, upon running the solution on an electrophoresis gel, \textit{N. v PTTH}, displayed no bands at all. The integrity of the probes can be seen in Figures 3.18 and 3.19.

![Figure 3.18: Agarose gel electrophoresis of DIG-labelled probes (\textit{N. vitripennis} genes PTTH and orthodenticle-1).](image)

Lane 1: Antisense probe of \textit{N. v PTTH}. Lane 2: Sense probe of \textit{N. v PTTH} (note: probe was not synthesised correctly). Lane 3: 1kb + ladder with sizes in base pairs to the left of the panel. Lane 4: Sense probe of \textit{N. v orthodenticle-1}. Lane 5: Antisense probe of \textit{N. v orthodenticle-1}. 

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Table 3.7: DIG-labelled probe concentrations of *N. vitripennis* genes *torso*, *PTTH* and *orthodenticle-1*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense probe concentration (ng/µL)</th>
<th>Antisense probe concentration (ng/µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>N. v torso</em></td>
<td>71.60</td>
<td>111.99</td>
</tr>
<tr>
<td><em>N. v PTTH</em></td>
<td>0</td>
<td>275.30</td>
</tr>
<tr>
<td><em>N. v orthodenticle-1</em></td>
<td>221.76</td>
<td>264.00</td>
</tr>
</tbody>
</table>

3.2.3 *In situ* hybridisation experiments.

Attempts were made to perform *in situ* hybridisation experiments in an effort to localise where, and at what developmental stage *torso* and *PTTH* are transcribed in *N. vitripennis* embryos. *N. vitripennis* embryos were collected at the cellular blastoderm stage (4-6 hours after egg laying) to perform an *in situ* hybridisation on the positive control, *N. v orthodenticle-1*, as at this developmental stage it has a clear expression pattern at the poles of the embryo (Lynch *et al.*, 2006a). As it has yet to be determined when in the development of *N. vitripennis* *PTTH*
and torso are expressed, N. vitripennis embryos were collected from a range (between 0-24 hours) of developmental timepoints when performing in situ hybridisation on these two genes.

Initial in situ hybridisation experiments performed indicated that embryos were only staining in those areas that were damaged in the process of chorion removal. This is evident when examining embryos that were labelled with a sense transcript (negative control) probe solution of N. v orthodenticle-1 (Figure 3.20). This is further demonstrated when examining embryos labelled with the antisense transcript (positive control) of N. v orthodenticle-1, as they showed no labelling, in the poles of the embryo or otherwise, unless they had displayed signs of damage (Figure 3.20). The process of chorion removal as described by Lynch and Desplan (2006) requires the experimenter to possess a certain amount of fine motor skills, as it involves the use of a needle to remove the chorion without piercing the embryo. Therefore, many practice attempts were made to remove the chorions of the embryos before performing repeat attempts of the in situ hybridization protocol. Furthermore, in all subsequent attempts at performing the in situ hybridisation protocol, embryos were only labelled if they received no damage from chorion removal. This was to ensure that any labelling observed as a result of performing in situ hybridisation was not a result of probes being trapped in damaged areas of the embryo, but of the probes binding to RNA transcripts.
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Because performing in situ hybridization on embryos with the positive control showed no signs of labelling, the protocol was repeated bar one modification: embryos were immersed in a 0.1% (v/v) of proteinase K for 10 minutes as opposed to the original time of 5 minutes. A summary of why the embryos were immersing in proteinase K for a longer period is as follows. Formaldehyde used to fix the embryo forms protein cross-links that may encase endogenous RNA transcripts of the N. vitripennis specimen, thereby producing a weak labelling signal. Proteinase K is used to break the protein cross-links and allow the in situ probes to access the endogenous RNA transcripts. However, for most in situ hybridization experiments, the use of proteinase K to break protein cross-links requires some optimisation, as tissue can often become over digested by proteinase K and lose its structural integrity (Nuovo, 2013). Unfortunately, when submerging the embryos in 0.1% proteinase K for 10 minutes all the embryos completely disintegrated, most-likely as result of over digesting the tissue.

Figure 3.20: N. vitripennis embryos following in situ hybridisation stains with DIG-labelled RNA probes. (A) Embryos stained with sense RNA probe of N. v orthodenticle-1 show probe trapping in areas of embryo damage (indicated by arrows). (B) Embryos stained with antisense RNA probe of N. v orthodenticle show no significant localisation of staining.
Therefore, the submersion time of embryos in 0.1% proteinase K was reduced to 7 minutes and the protocol for *in situ* hybridization was repeated, with one further modification: to increase the hybridization signal, the concentration of probe that embryos were immersed in was increased from 0.1% to 0.2% (v/v). Immersing the embryos in 0.1% proteinase K for this reduced length of time still resulted in over digestion. Because of this, most embryos had completely disintegrated, including all of the embryos that were collected with the intention of labelling them with the negative control. After completion of the protocol, all of the embryos displayed staining over their entire surface. It’s unclear what caused this uniform stain, but a possibility is that it was caused by excessive probe signal. Moreover, it is not an indication that *torso, PTTH* and *orthodenticle-1* (which localises to the embryo poles), are expressed uniformly over the entire embryo. Images of *in situ* hybridization of these embryos can be seen in Figure 3.21.
Figure 3.21: Non-localised staining in *N. vitripennis* embryos following a repeat attempt at performing an *in situ* hybridisation stain with DIG-labelled RNA probes (antisense *N. v probes PTTH, orthodenticle-I, and torso*.

All larvae are orientated ventral side down, anterior side on the left (A) DIG-labelled stain of *N. v PTTH* (B) DIG-labelled stain of *N. v orthodenticle-I*. (C) DIG-labelled stain of *N. v torso*. 
The aim of this project was to determine if components of the Torso-activation module pattern the end-terminals of the two hymenopteran species *Apis mellifera* and *Nasonia vitripennis* embryos. If the genes *PTTH* and *torso* were found to pattern the end-terminals of *N. vitripennis* it would have provided evidence that this function of the Torso-activation module evolved more basally in the radiation of the holometabolous insects than is currently hypothesised (Duncan *et al.*, 2014). Analysis of the functional role of *torso-like* in *A. mellifera* would determine if *torso-like* can pattern the end-terminals of this species independently from the rest of the Torso-activation module. To determine the functional roles of these genes pRNAi was performed on *A. mellifera* and *N. vitripennis*. Additionally, in *N. vitripennis* several attempts were made to perform DIG-labelled *in situ* hybridisation on ovaries for *PTTH* and *torso*.

4.1 FUNCTIONAL STUDIES

4.1.1 pRNAi in *A. mellifera*.

As previously mentioned, there have been no published reports of pRNAi in *A. mellifera*. This presented an opportunity to test this method of RNAi in this species. Currently, researchers have a limited capacity to predict the efficiency of a mode of RNAi delivery in a species until it is empirically tested. It has been well established that successful RNAi performance varies between species, stages of development, modes of delivery and genes targeted. Why this variance occurs between species is yet to be fully understood, however it is accepted that it is a result of a large number of biological variations between species, tissues
and developmental stages. Key variables to note are the presence of core RNAi machinery, cellular uptake of dsRNA and propagation of the RNAi signal (Scott et al., 2013).

This uncertainty, regarding whether pRNAi could be performed successfully in A. mellifera necessitated the use of controls in these experiments. Here, eGFP was used as a negative control as it is not expressed in the A. mellifera genome. Upon analysing larvae from A. mellifera queens injected with a dsRNA solution of eGFP they appeared wildtype. This suggests that any phenotypic responses observed in these experiments were the result of knocking down expression of the genes orthodenticle-1 or torso-like, and not a result of trauma from performing the injections, or as physiological response from the non-specific engagement of the RNAi machinery (Scott et al., 2013).

The positive control used in this pRNAi experiment was A. mellifera orthodenticle-1. A previous study has reported that performing RNAi on A. m orthodenticle-1 results in a loss of head, thoracic and anterior abdominal segments (Wilson and Dearden, 2011). The phenotype produced from the pRNAi of orthodenticle-1 here, produced a weaker but similar phenotype, as the embryos displayed a loss of head segments only. Therefore, these results suggest that the pRNAi of orthodenticle-1, the positive control, was likely successful. Furthermore, this suggests that the phenotype observed from the pRNAi of torso-like is a result of reducing transcript levels of maternally expressed torso-like. The phenotypic results from the pRNAi experiments performed here indicate that torso-like functions to pattern a large part of the A. mellifera embryo. This is interesting for two reasons: Firstly, it appears to pattern a larger domain than what would be expected if it patterned only the anterior terminal end of this species; secondly, it contradicts a previous hypothesis stating that torso-like is most-likely not donated to the A. mellifera embryo and therefore does not play a role in anterior-posterior patterning (Duncan et al., 2013).
The successful knockdown of *A. m orthodenticle-1* and *A. m torso-like* strongly indicates that the solutions of dsRNA used in this experiment were of high enough concentration and integrity to knockdown the expression of these two genes. In all species thus far examined, the strength of the pRNAi response is correlated with the dose of the dsRNA administered to the organism (Scott *et al.*, 2013). As a protocol for pRNAi in *A. mellifera* has not been published there was no accurate way to predict how much dsRNA needed to be administered to the queen bees to elicit phenotypic responses from pRNAi. Because of this, it was decided that any concentration between 0.5-10 µg/µL (Amdam *et al.*, 2003; Antonio *et al.*, 2008; Maleszka *et al.*, 2007; Schlüns and Crozier, 2007), the range that is typically used for other forms of RNAi in *A. mellifera*, could potentially knockdown gene expression via pRNAi in this species. Here, the synthesis of solutions of dsRNA within this concentration range was achieved for all three genes (*A. m torso-like*, *A. m orthodenticle-1*, *eGFP*). Additionally, the dsRNA solutions of *A. m orthodenticle-1* and *A. m torso-like* appeared to be of high integrity. Whilst running the *A. m* genes on a gel revealed that their bands were all over 4000bp in size (the expected size for *A. m orthodenticle-1* and *A. m torso-like* were 1400bp and 1800bp respectively) this is most-likely the result of multi-stranded dsRNA aggregates. This is because RNAse and DNAse (ribonuclease and nuclease enzymes respectively) were added to the solutions of dsRNA. The application of these enzymes to the dsRNA solutions would degrade any DNA and RNA. Any degraded DNA/RNA that wasn’t subsequently purified from the solutions would appear as a band sized <100bp in length, and not as large as the minimum band size on the gel, which is 4000bp in length. Unfortunately, the concentration of the dsRNA solution of *eGFP* was approximately half that of *A. m orthodenticle-1* and *A. m torso-like* (concentrations were 1704.05 ng/µL, 3,272.04 ng/µL and 3,814.60 ng/µL for *eGFP*, *A. m orthodenticle-1* and *A. m torso-like* respectively). Furthermore, upon running the dsRNA solution on a gel it indicated that the solution was contaminated with very short molecules of
nucleotides. Regardless of whether this contamination is degraded dsRNA, DNA or RNA it is unlikely to trigger an RNAi response because the minimum required length of dsRNA to induce RNAi is 20bp (Bolognesi et al., 2012). The effective dsRNA concentration, therefore, is even lower, as upon analysing this solution with a nanodrop spectrophotometer, some of the absorbance reading will be attributable to this contaminating band. Therefore, this solution of *eGFP* dsRNA may not be an adequate negative control, as there is a chance that administering a higher concentration of *eGFP* dsRNA solution to the queen bees may elicit a phenotypic response in the embryos that they lay. If this were to happen then it would indicate that nonspecific engagement of the RNAi machinery is eliciting a phenotypic response in the *A. mellifera* embryos collected from these experiments (Scott et al., 2013). Note: it would not indicate that the phenotype is caused by trauma induced from the injection of the queen, as this would be independent of dsRNA concentration. Furthermore, if this phenotype were the same one observed for the pRNAi of *A. m torso-like* and *A. m orthodenticle-1* then it would indicate that the phenotypes observed from these experiments were only artefacts of administering a solution of dsRNA itself, and not a result of successful pRNAi of these genes. It is unlikely, therefore, that the phenotypes observed in these experiments are only experimental artefacts. It’s unlikely that an experimental artefact would produce the same phenotypic response as that achieved by Wilson and Dearden (2011) when performing RNAi on *A. m orthodenticle-1*.

The pRNAi experiments performed here should be repeated to determine whether the knockdown phenotypes of *A. m orthodenticle-1* and *A.m torso-like* achieved here are replicable. Furthermore, upon replicating these results the reduction of the target gene transcript levels should be measured by quantitative reverse transcription polymerase chain reaction (RT-qPCR). By comparing abundance of *torso-like* and *orthodenticle-1* transcripts in those embryos from the treatment group compared to controls it will allow measurement of exactly how much the transcripts of these two genes were reduced.
4.1.2 pRNAi in *Nasonia vitripennis*

For this aim, there was already a published protocol detailing how to perform pRNAi on *Nasonia vitripennis* (Lynch and Desplan, 2006). Fortunately, this eliminates some uncertainty regarding the validity of the results obtained from these experiments. Because this protocol has been proven to work, and was followed exactly, it is likely that the expression of *N. v PTTH* and *N. v torso* was successfully reduced, despite the apparent absence of a phenotypic response in these embryos. Further supporting this is that pRNAi of *N. vitripennis orthodenticle-1*, the positive control, was successful. Previously, Lynch *et al.* (2006a) have shown that pRNAi of *N. v orthodenticle-1* results in embryos that lack mouth and thoracic segments in the anterior, and lack abdominal segments in the posterior. As the results obtained from pRNAi of this gene replicated this phenotype, the pRNAi of *N. v orthodenticle-1* was considered to be successful.

The successful phenotype of *N. v orthodenticle-1* was obtained by performing pRNAi with a dsRNA solution of similar integrity and concentration as that used for pRNAi of *N. v torso* and *N. v PTTH*. The concentration of *N. v PTTH* dsRNA was even higher than that used for the successful pRNAi of *N. v orthodenticle-1* (dsRNA solutions of *N. v orthodenticle-1* and *N. v PTTH* were 1104.18ng/µL and 1431.68 ng/µL respectively). Therefore, it is likely that the expression of *N. v PTTH* was reduced in this experiment. Furthermore, because no end-terminal phenotype was observed from pRNAi of *N. v PTTH*, it is likely that *PTTH* is not involved in terminal patterning in this species. To date, the only known ligands of Torso are *PTTH* and *Trunk*. Because the *N. vitripennis* genome does not contain *trunk*, the lack of an end-terminal phenotype in embryos after pRNAi of *PTTH* indicates that the Torso-activation module does not pattern the end-terminal regions of this species. However, this alone does not conclusively
prove that the Torso-activation module does not pattern the end-terminal regions of *N. vitripennis*. This is because of the slim possibility that, in this species at least, *torso* is activated by a different and unidentified ligand to pattern the end-terminal regions. Therefore, it is unfortunate that the concentration of *N. v torso* dsRNA was 876.36 ng/µL, which is below the concentration of 1µg/µL recommended for pRNAi in *Nasonia vitripennis* (Lynch and Desplan, 2006). Because the concentration of *torso* dsRNA was below that recommended in the protocol there is a possibility that the expression of *torso* was not reduced sufficiently to elicit a detectable end-terminal phenotype. However, because the concentration of *torso* dsRNA ~87% of the recommended concentration, it is likely that this difference in concentration is negligible. This means that by using this concentration of *N. v torso* dsRNA an end-terminal phenotype should appear, albeit a little weaker, should *N. v torso* function to pattern the end-terminals of *N. vitripennis*. Alternatively, it may be that there is some mismatch between the sequences of the dsRNA solutions used here and the sequences of the endogenous RNA transcripts of the target genes. If this is true, then the suppressive effect of the dsRNA solutions on the target genes’ expression would have been reduced. The sequences used for dsRNA synthesis of the genes *N. v torso* and *N. v PTTH* were accessed via the sequence database *Genbank*, which displays only the consensus sequences of genes. Sequence mismatch between the dsRNA and endogenous RNA transcripts could have arisen from sequence variability of the genes *torso* and *PTTH* within the *N. vritripennis* species. This uncertainty regarding the reduction of the *N. v torso* and *N. v PTTH* transcripts necessitates that these experiments performed here should be repeated, and the reduction of transcript levels should be measured by RT-qPCR.

4.1.3 Synthesis of dsRNA by *in vivo* bacterial expression
Several attempts were made at using a method of dsRNA production described first by Ongvarrasoponea et al. (2007). This method uses *in vivo* bacterial expression to synthesise sense and antisense transcripts of plasmid inserts, which anneal to form dsRNA. It is not clear why the attempts at performing this method of dsRNA synthesis were not successful.

One possible reason why the dsRNA was not correctly synthesised was because the ssRNA inserts were not synthesised exactly as described by Ongvarrasoponea et al. (2007). In their protocol, the plasmid inserts they use contain the sense transcript of the gene of interest, followed by 200 bp of sequence from *eGFP*, followed by the antisense transcript of the gene. Therefore, in one transcription run, a single RNA polymerase generates the sense and antisense transcripts of the gene of interest, with an *eGFP* sequence connecting them. By connecting the sense and antisense transcripts the *eGFP* spacer sequence facilitates the annealing of the two ssRNA transcripts by keeping them in close proximity to each other. This forms a dsRNA sequence of the gene of interest with an *eGFP* hairpin loop at one end. Later, after the addition of RNAase to the solution of dsRNA, this hairpin loop which is ssRNA, is degraded.

However, the plasmid inserts used in this project differed in the way they generated sense and antisense transcripts. Here the plasmid inserts were sense cDNA sequences of a gene of interest, flanked by two T7 RNA polymerase promotors. Therefore, two transcription runs (one transcription run from each promotor) were required to generate the sense and antisense and sense transcript of the gene. Because the sense and antisense transcripts generated here were not connected by a sequence of *eGFP*, this may have resulted in inefficient annealing of the ssRNA transcripts, and therefore less generation of dsRNA. Aside from this difference in plasmid insert design, the protocol described by Ongvarrasoponea et al. (2007) was followed exactly. Therefore, it is likely that these unsuccessful attempts at dsRNA synthesis were caused by using the different plasmid design.
4.2 EXPRESSION STUDIES

4.2.1 In situ hybridisation detection of PTTH and torso RNA in N. vitripennis

The attempts performed here to localise the expression domains of PTTH and torso in N. vitripennis by performing in situ hybridisation were unsuccessful. These unsuccessful attempts were caused by several issues with the protocol. One of the initial issues was probe trapping caused by the damage the embryos receive in the process of removing the chorion and vitelline membranes. This meant that upon observing the embryos after completing the protocol, they appeared to be stained wherever the embryos had received damage. Fortunately, later embryos did not display signs of either probe-trapping or tissue damage. This suggests that this issue was resolved as a result of practicing the removal of the chorion and vitelline embryos without damaging them. Later attempts at performing in situ hybridisation suggest that the embryos were being over-digested with proteinase K. This was apparent when upon incubating the embryos in proteinase K for ten minutes they completely disintegrated. Proteinase K is used in the in situ hybridisation protocol to break down protein crosslinks that may mask the mRNA antigen. However, for most in situ hybridization experiments, the use of proteinase K to break protein cross-links requires some optimisation, as tissue can often become over-digested by proteinase K and lose its structural integrity (Nuovo, 2013). An attempt was made to optimize the proteinase K incubation step by lowering the time that the embryos were submerged in proteinase K from 10 to 7 minutes. There were two indications that this shorter incubation time was still too long: Firstly, the majority of the embryos still disintegrated upon incubating them in proteinase K; secondly, upon completing the in situ hybridization protocol, the remaining embryos that didn’t disintegrate displayed a uniform labelling all over their surface. This uniform labelling could be the result of probe-trapping occurring in areas in which...
proteinase K has damaged the embryo tissue. Alternatively, the excessive probe signal apparent in the embryos could be the result of incubating the embryos in a DIG-labelled probe solution that is too concentrated. Therefore, this protocol requires further optimisation, such as incubating the embryos in proteinase K for a shorter period of time and a lower concentration of probe solution.

4.3 CONCLUDING REMARKS AND FUTURE RESEARCH

The phenotypic results obtained from the pRNAi experiments performed here suggest that the components of the Torso-activation module do not pattern the terminal ends of *A. mellifera* and *N. vitripennis*. Overall these results support the hypothesis put forth by Duncan *et al.* (2014), that in the evolutionary history of the Torso-activation module it has only recently been adapted to terminal end patterning, with its older role being that of initiating metamorphosis and larval moulting. Furthermore, they support the findings from Wilson and Dearden (2009). Here this research could detect no labelling of activated ERK, the MAPkinase that is activated in response to Torso signalling, at the poles of the *A. mellifera* embryo. They also support a personal observation from Jeremy Lynch, that activated ERK labelling could not be detected in the poles of the *N. vitripennis* embryo (Lynch *et al.*, 2012)

There are two reasons to suggest that whilst *torso-like* appears to play a role in patterning the anterior of *A. mellifera* this is not an indication that it has a functional role in patterning the terminal ends of this species. Firstly, knocking down a gene responsible for patterning the terminal-ends of a species would produce a disruption to the formation of both ends of the embryo. Secondly, the phenotype observed from pRNAi of *A. mellifera torso-like* was a loss of a large proportion of the anterior features of the embryo. This indicates that *torso-
like plays a role in patterning the anterior of this species, but this patterning process is not confined to the anterior terminal end of the embryo.

Regarding end-terminal patterning, the results from these experiments seem to raise more questions than they answer. Firstly, if not through activation of the Torso-activation module, how do these two species pattern their end-terminal regions? It could be that some species of insects do not employ an entirely separate class of genes to pattern their end-terminal regions. This could mean that in these species the maternal effect genes responsible for patterning the central regions of the embryo also pattern the end-terminal regions. This is plausible when considering the function of Orthodenticle-1 in A. mellifera and N. vitripennis. In these two species Orthodenticle-1 appears to replace the role of Bicoid, one of the four key maternal effect genes expressed in D. melanogaster and other Diptera. Here, in A. mellifera and N. vitripennis, Orthodenticle-1 appears to function like Bicoid, as it activates gap genes whose expression domains are in the central regions of the embryo. However, unlike Bicoid’s role in D. melanogaster, Orthodenticle-1 is also responsible for activating tailless, a target of the terminal patterning pathway, in both these (Lynch et al., 2006b; Wilson and Dearden, 2009). This raises the possibility that having an entire separate class of genes to pattern the end-terminal regions is a highly derived form of development. Moreover, it could be that this strategy of using a separate class of genes to pattern the embryonic end-terminals is shared only between Tribolium castaneum and some species of Diptera.

There is also the possibility that the positional cues that differentiate the end-terminals of these species are structural or mechanical in nature. For example, the mechanism by which the end-terminal regions of A. mellifera and N. vitripennis are patterned could be analogous to a hypothesis put forth by Johnson et al. (2015). Here they considered that one possible cause of Trunk concentrating at the poles of the D. melanogaster embryo was because the perivitelline space in which it accumulates is larger at the embryonic poles. Therefore, it
follows that more space at the embryo poles allows for a greater amount of the ligand to accumulate in this region. Note that in this study, the researchers conclude that it is Torso-like, not the relatively large perivitelline space at the embryo poles, that mediates the accumulation of Trunk to the end-terminal regions of *D. melanogaster*. However, this does not suggest that the relative size of the perivitelline space can’t relay positional information to the embryo poles of *A. mellifera* and *N. vitripennis*. Furthermore, it must be emphasised that this is just one example of a potential way by which these two species could use physical information to define their end-terminal regions. Other examples of physical information that has been proven to different cells includes the following: mechanical forces, cell shape, properties of the extracellular matrix, and contacts between cells (Clause *et al.*, 2010).

The results obtained from this project also have broad implications for the evolutionary development of insects in general. Termed the hourglass model, there is a theory which proposes that throughout the Kingdom animalia, the middle stage of embryogenesis is highly conserved, whilst the later and earlier stages are more diverged (Duboule, 1994; Raff and Slack, 1996). After this theory was first proposed the concept was later extended to encapsulate not just the evolution of animal morphology but the evolution of genetic pathways too (Kalinka *et al.*, 2010). Therefore, according to the hourglass model, gene regulation should be highly conserved in the middle stage of embryogenesis, whilst later stages are more diverged. The results of the pRNAi studies performed here can be used as further support to this theory. Here it has been shown that in early embryogenesis the Torso-activation module does not pattern the end-terminal regions of *N. vitripennis* and *A. mellifera*. However, despite this, *tailless*, a target of the Torso-activation module in *D. melanogaster*, is expressed in the pole regions of both of these species during early to middle embryogenesis (Lynch *et al.*, 2006b; Wilson and Dearden, 2009).
As previously stated, the pRNAi experiments performed here should be repeated, and then subsequently analysed by performing RT-qPCR to determine that the transcripts of the target genes were successfully knocked down. This is especially true for the pRNAi experiments performed here on *N. vitripennis*. Because of the low number of embryos collected from this experiment, it is unlikely that the results obtained from this experiment are statistically significant.

To localise the transcript domains of *PTTH* and *torso* in *N. vitripennis*, the *in situ* hybridisation experiments performed here should be repeated and optimised.

Finally, RNAi studies should be performed on *N. v PTTH* and *N. v torso* to determine what their function is in this species. Torso-activation module’s role in larval moulting and metamorphosis is detected widely in the holometabolous and hemimetabolous insects. Therefore, RNAi of *PTTH* and *torso* should be performed on *N. vitripennis* larvae, prior to observing their larval moulting times compared to a negative control. This would determine whether or not these two genes regulate larval moulting in this species.
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Rosado, C. J., Buckle, A. M., Law, R. H., Butcher, R. E., Kan, W. T., Bird, C. H., ... &


APPENDIX

Figure A1: Plasmid map of pBluescript II SK (+). Image sourced from transOMIC technologies.

Figure A2: Plasmid map of pLITMUS 38i. Image sourced from New England Biolabs.
Figure A.3: Plasmid map of pBluescript SK (+). Images sourced from NovoPro

Figure A.4: coding sequence of A. mellifera orthodenticle-1. Sourced from Genscript.

1 atgacctgcc acacagttg aaagttcagc aagttcagc aagttcagc aagttcagc aagttcagc
61 ccacagttg aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc
121 ttcagcagct cagcttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc
181 cagcttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc
241 cagcttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc
301 cagcttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc
361 cagcttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc
421 cagcttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc
481 cagcttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc
541 cagcttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc
601 cagcttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc
661 cagcttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc
721 cagcttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc
781 cagcttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc
841 cagcttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc
901 cagcttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc
961 cagcttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc
1021 cagcttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc
1081 cagcttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc
1141 cagcttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc
1201 cagcttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc
1261 cagcttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc
1321 cagcttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc

1 gaaggcttag gattcaca aagttcagc aagttcagc aagttcagc aagttcagc aagttcagc
61 aatattttatttaga aatattttatttaga aatattttatttaga aatattttatttaga aatattttatttaga aatattttatttaga aatattttatttaga

110
Figure A.6: coding sequence of *A. mellifera* torso-like. Sourced from Genscript.
**Figure A.7: coding sequence of eGFP.** Note, coding sequence is highlighted in brown and is 726 bp. Sourced from Genscript.

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181 ctctcggctg cagcatgagg tgcgtctcta cgtgcggctg ctcgcgtcag ccggggttct
241 ggctgtcttg cgtgcggctg ctcgcgtcag ccggggttct
301 cgcgtagtgt gcagaatccta aatgcaggttc gcagaatccta aatgcaggttc gcagaatcct
361 cgacatctt acatgtcttc agtctctttc acattatcttc aataatcttc aataatcttc
421 ccaatggctg ccacccagatg ccacccagatg ccacccagatg ccacccagatg ccacccagatg
481 tgtatcttgc ccctctcgctgc ccctctcgctgc ccctctcgctgc ccctctcgctgc ccctctcgctgc
541 taatggctg ccctctcgctgc ccctctcgctgc ccctctcgctgc ccctctcgctgc ccctctcgctgc
601 tgtatcttgc ccctctcgctgc ccctctcgctgc ccctctcgctgc ccctctcgctgc ccctctcgctgc
661 tgtatcttgc ccctctcgctgc ccctctcgctgc ccctctcgctgc ccctctcgctgc ccctctcgctgc
721 tgtatcttgc ccctctcgctgc ccctctcgctgc ccctctcgctgc ccctctcgctgc ccctctcgctgc
```

**Figure A.8: coding sequence of Nasonia vitripennis torso.** Sourced from Genscript.

```
1 caaatgacgc gcagagcagg attcggggat caagggatga agctcattca gtggaccgtc
61 tcggaagagc gcagagcagg attcggggat caagggatga agctcattca gtggaccgtc
121 ctcgcggcag ctcgcggcag ctcgcggcag ctcgcggcag ctcgcggcag ctcgcggcag
181 gcagagcagg gcagagcagg gcagagcagg gcagagcagg gcagagcagg gcagagcagg
241 gcagagcagg gcagagcagg gcagagcagg gcagagcagg gcagagcagg gcagagcagg
301 gcagagcagg gcagagcagg gcagagcagg gcagagcagg gcagagcagg gcagagcagg
361 gcagagcagg gcagagcagg gcagagcagg gcagagcagg gcagagcagg gcagagcagg
421 gcagagcagg gcagagcagg gcagagcagg gcagagcagg gcagagcagg gcagagcagg
481 gcagagcagg gcagagcagg gcagagcagg gcagagcagg gcagagcagg gcagagcagg
541 gcagagcagg gcagagcagg gcagagcagg gcagagcagg gcagagcagg gcagagcagg
601 gcagagcagg gcagagcagg gcagagcagg gcagagcagg gcagagcagg gcagagcagg
661 gcagagcagg gcagagcagg gcagagcagg gcagagcagg gcagagcagg gcagagcagg
721 gcagagcagg gcagagcagg gcagagcagg gcagagcagg gcagagcagg gcagagcagg
781 gcagagcagg gcagagcagg gcagagcagg gcagagcagg gcagagcagg gcagagcagg
841 gcagagcagg gcagagcagg gcagagcagg gcagagcagg gcagagcagg gcagagcagg
901 gcagagcagg gcagagcagg gcagagcagg gcagagcagg gcagagcagg gcagagcagg
961 gcagagcagg gcagagcagg gcagagcagg gcagagcagg gcagagcagg gcagagcagg
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Figure A.9: coding sequence of *Nasonia vitripennis PTTH*. Sourced from Genscript.

```
1 atggcggccg ctggggcccc gaattcggtc gtcggtctgc agtaccatc a ccaggcggta
61 tcgcaccatc ac ccagactca tctgggctgc cctgggctgc ttctaacg tctgagct
121 cagactcatca ccagactctc agctctctgc attcggactc ctgcaccatc ttcgccctgc
181 ccagactctc cagactcatc tctgagct ccaggtgtta tcgcaccatc ttcgccctgc
241 tcgcaccatc ccagactctc agctctctgc attcggactc ctgcaccatc ttcgccctgc
301 tctgagct ccagactctc agctctctgc attcggactc ctgcaccatc ttcgccctgc
361 atgcgcggctc ccaggtgtta tcgcaccatc ttcgccctgc ttctaacg tctgagct
421 cagactcatca ccagactctc agctctctgc attcggactc ctgcaccatc ttcgccctgc
481 ccaggtgtta tcgcaccatc ttcgccctgc ttctaacg tctgagct ccaggtgtta
541 ccaggtgtta tcgcaccatc ttcgccctgc ttctaacg tctgagct ccaggtgtta
601 cagactcatca ccaggtgtta tcgcaccatc ttcgccctgc ttctaacg tctgagct ccaggtgtta
661 ccaggtgtta tcgcaccatc ttcgccctgc ttctaacg tctgagct ccaggtgtta
721 ccaggtgtta tcgcaccatc ttcgccctgc ttctaacg tctgagct ccaggtgtta
781 ccaggtgtta tcgcaccatc ttcgccctgc ttctaacg tctgagct ccaggtgtta
841 ccaggtgtta tcgcaccatc ttcgccctgc ttctaacg tctgagct ccaggtgtta
901 ccaggtgtta tcgcaccatc ttcgccctgc ttctaacg tctgagct ccaggtgtta
961 ccaggtgtta tcgcaccatc ttcgccctgc ttctaacg tctgagct ccaggtgtta
1021 ccaggtgtta tcgcaccatc ttcgccctgc ttctaacg tctgagct ccaggtgtta
1081 ccaggtgtta tcgcaccatc ttcgccctgc ttctaacg tctgagct ccaggtgtta
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