Gene expression analysis of *Thaumatotibia leucotreta* in response to the *Cryptophlebia leucotreta* granulovirus

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Abstract

Gene expression studies provide baseline information on the interactions of insects with their environment. Despite the importance of this information, limited gene expression data are available for most insect pests, including the family Tortricidae (Lepidoptera), which includes *Thaumatotibia leucotreta* (Meyr), an important agricultural pest in Africa. Because *T. leucotreta* can be controlled successfully by a granulovirus, this system is a good model for exploring insect-virus susceptibility. The main aim of this study was to investigate gene expression of *T. leucotreta* in responce to virus infection. However, before pursuing this aim, two objectives required completion. First, the most suitable RNA extraction method for insects needed to be determined and second, the most suitable reference genes for qPCR for Tortricidae pests needed to be identified. Once these objectives were accomplished, the response of *T. leucotreta* to its granulovirus was evaluated at different temperatures and points after infection.

Four RNA extraction methods, the RNeasy® Mini Kit, SV Total RNA isolation system, TRIzol® reagent, and a CTAB-based method, were compared using two beetle and two moth species, including *T. leucotreta*. The quality of extracted RNA was similar for all four species for all extraction methods. Based on several criteria, the best RNA extraction method was the SV Total RNA isolation system.

Six candidate reference genes were evaluated for qPCR using different tissue types of *T. leucotreta* and two other Tortricidae pests. Additionally, reference genes were evaluated for *T. leucotreta* with and without its granulovirus at different temperatures. Reference gene stability was found to be dependent on species and tissue type. Overall the most suitable combination of reference genes for *T. leucotreta* were α-actin, arginine kinase and elongation factor 1-α.

Gene expression of *T. leucotreta* in response to granulovirus infection at different temperatures and intervals after infection was evaluated by qPCR using 13 target genes associated with the infection process. Most genes were down-regulated after 24 and 48 h.p.i. However, after 72 h.p.i most genes were up-regulated. The same trend was observed at different temperatures, where most genes were down-regulated at 15°C and 25°C but up-regulated at 35°C. These results show that there is a dynamic gene expression response in *T. leucotreta* due to granulovirus infection under different conditions. Not only do these findings provide insight into the control of this tortricid pest, they also contribute further to our knowledge of insect-virus interactions.

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List of Abbreviations and symbols

α	Alpha
$	United States of America Dollar
%	Percentage
(Pty) Ltd	Private company, limited
°C	Degrees Celsius
µL	Microlitres
µM	Micromole
ACT	α-Actin
AGE	Agarose gel electrophoresis
AK	Arginine kinase
BLAST	Basic Local Alignment Search Tool
bp	Base pair
Bt	*Bacillus thuringiensis*
CD	Cell Death
cDNA	Circular DNA
COI	Cytochrome oxidase I
CP	Crossing point
CpGV	*Cydia pomonella* granulovirus
CrleGV	*Cryptophlebia leucotreta* granulovirus
Ct	Cyclic threshold
CTAB	Cetyltrimethylammonium bromide
CV	Coefficient of variation
DI	Dicer-2
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphates
EF1α	Elongation factor 1- α
ENO	Enolase
EppoNPV	*Epiphyas postvittana* nucleopolyhedrovirus
GEL	Gelsolin
h	Hours
h.p.i	Hours post infection
HSP	Heat shock protein

I5A	Initiation factor 5a
JHEH	Juvenile hormone epoxide hydrolase
M	Mole
MAD	Mitochondrial aldehyde dehydrogenase
min	Minutes
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
mM	Millimoles
mtDNA	Mitochondrial DNA
NAN	No amplification noted
NCBI	National Centre for Biotechnology Information
ng	Nanogram
nM	Nanomole
NTC	Non-template control
OBs	Occlusion bodies
PCR	Polymerase chain reaction
R	South African Rand
R^2	Correlation coefficient
r	Pearson correlation coefficient
RFLP	Restriction Fragment Length Polymorphisms
RIN	RNA integrity number
RNA	Ribosomal nucleic acid
rRNA	Ribosomal RNA
qPCR	Real-time-quantitative PCR
SD	Standard deviation
SE	Standard error
sec	seconds
siRNAs	Short interfering RNA
SNP	Single Nucleotide Polymorphism
Taq polymerase	*Thermus aquaticus* polymerase
™	Trade mark
TUB	α -Tubulin
USA	United States of America
V	Volts

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Outputs

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Brits D, Ridgeway JA, Timm AE (2015) Laboratory evaluation of temperature effects on the efficacy of *Cryptophlebia leucotreta* granulovirus (CrleGV-SA) on fourth-instar false codling moth larvae. African Entomol 23: 243-246

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Disclaimer

Any opinion, findings and conclusions or recommendations expressed in this material are those of the author and do not necessarily reflect the views of the National Research Foundation or Citrus Research International.

Declaration

The following thesis has not been submitted to any university other than Rhodes University, Grahamstown, South Africa. The work presented here is that of the author.

Dedication

I dedicate this thesis to Marc Ridgeway, who would have been the only one happier than me to have seen it done. Your love for me and belief in my potential will guide me forever.

Hope to always “be happy”.

1

General Introduction

Gene expression studies are an exciting new method of research that have allowed scientists to understand interactions at the most basic level. These technique do however need benchmark studies in order to produce reliable data with meaningful results [1]. One such area of gene expression research is focused on insect biopesticide interactions and in particular the genetic basis of resistance.

There are multiple pest species worldwide that are controlled using biopesticides and as more studies become available it is easier to incorporate previous methods and standards to advance future research. The results obtained from these gene expression studies may prove to be useful in controlling insect pests like *Thaumatotibia leucotreta*.

1.1 Thaumatotibia leucotreta

Thaumatotibia leucotreta (Meyrick, 1912) (Lepidoptera: Tortricidae) is a polyphagous insect indigenous to sub-Saharan Africa and surrounding islands [2–4]. Its larvae feed on at least 74 species of host plants, of which 24 are cultivated crops [2,5–8]. Control of this pest in orchards is challenging due to the large number of alternative hosts, particularly wild plants.

Thaumatotibia leucotreta populations have 5-6 generations per year, with individual females able to lay up to 456 eggs [2]. Eggs are laid individually on fruit of the host plant. After 6-12 days, larvae hatch and bore into the host plant within 24 hours. Larval development takes between 25 and 67 days, depending on the season [2]. Once larval development is completed to the final (5^{th}) instar [9], larvae exit the host plant and pupate in the first few centimetres of soil [10]. Depending on the season *T. leucotreta* adults will eclose 21-80 days after pupation [11] with the ability to fly more than 1 km [12]. With multiple host

plant species, prolific reproduction and a wide migration range, *T. leucotreta* is a major threat to crop production.

The first record of a pestilent infestation of *T. leucotreta* was in KwaZulu-Natal on citrus in 1901 [13].The insect is now directly responsible for an estimated annual loss of R100 million (about $10 million) to the southern African citrus industry [14]. The effect of *T. leucotreta* on the citrus industry is not restricted to crop losses, but includes the loss of potential export markets due to its phytosanitary status [15]. Strict regulations are imposed by importers to prevent accidental introductions of pests or diseases, and the bulk of citrus production in South Africa must meet these standards because it is intended for export. The European Union signed the Sanitary and Phytosanitary (SPS) Bill, which codified the requirements for imports. Lapses in meeting these requirements result in closure of these export markets, leading to the potential collapse of the citrus export industry [16]. Therefore, for the preservation of the South African citrus industry, it is crucial that *T. leucotreta* populations within orchards are controlled to an internationally acceptable level.

1.2 Thaumatotibia leucotreta pest control

Control measures currently used to combat *T. leucotreta* infestations require a dynamic systems approach to fully meet citrus export standards. Apart from restricting the biotic contamination of fruit, common export regulations include a maximum acceptable level of pesticide residues. This requirement restricts chemical spray regimes and therefore calls for the incorporation of additional control strategies, which include orchard sanitation [17], mating disruption [18], sterile insect releases [19], attract-and-kill technologies [6] and biopesticides [20]. Arguably the most successful of these additional controls has been the use of *Cryptophlebia leucotreta* granulovirus (CrleGV) as a biopesticide [17].

The CrleGV was first described in 1965 by Angelini *et al.* [21]. However, it was only in 2002 that it was proposed as a potential biopesticide against *T. leucotreta* [15]. The potential biopesticide use of CrleGV was promising as the closely related *Cydia pomonella* granulovirus (CpGV) had already been described and commercially deployed in European orchards as a control against European *C. pomonella* (Linnaeus, 1758) (Lepidoptera: Tortricidae) for more than two decades [22]. As *T. leucotreta* and *C. pomonella* are in the same tribe and have very similar life histories, it was believed that like CpGV, CrleGV would successfully control its target pest, *T. leucotreta*.

There are currently two registered CrleGV isolates commercially available for the control of *T. leucotreta* - Cryptogran™ [14,15] and Cryptex® [23]. The use of these isolates to manage *T. leucotreta* populations on citrus and other commercial crops [14,15,24] has provided a forward-thinking systems approach in the agricultural industry. Combining the application of CrleGV with other pre-harvest controls such as non-copper pesticide sprays [6], sterile insect releases, attract-and-kill techniques and mating disruption provides the dynamic systems approach required to control a pest of this severity.

New isolates of CrleGV have been identified and are continually tested against different *T. leucotreta* populations to better understand the infection processes and virus efficacy [25]. It has been shown that by rotating isolates of the same virus species, insects are less likely to develop resistance [22,26–28]. However, the tests used to study the isolates are labour-intensive, restricted to laboratory or small field trials, technically demanding and provide results only at the organism level. Determining the genetic markers in *T. leucotreta* linked to CrleGV infection could provide a higher throughput of tests and a more detailed understanding of the interactions of CrleGV and *T. leucotreta*.

1.3 Biopesticide resistance development

Biopesticides have often been lauded as superior to conventional insecticides. One underlying belief was that it was impossible for organisms to develop resistance to naturally occurring pathogens [29] since the two species are most likely to have co-evolved [30,31]. It was hypothesized that since biological control agents are genetically diverse, they are able to evolve when necessary to allow adaptation to the host, unlike chemical insecticides [32,33]. Thus, it was with some surprise that field resistance to *Bacillus thuringiensis* was reported in diamondback moth *Plutella xylostella* (Lepidoptera: Plutellidae) in the early 1990s [34,35]. This resistance development showed that it is important to evaluate the threat of resistance for all biopesticides.

Advances in research on CrleGV **are** permanently threatened by the development of host resistance. Resistance to *C. pomonella* granulovirus (CpGV) in *C. pomonella* from Germany [36], Italy, Switzerland, Netherlands, Austria [37,38] and the Czech Republic [39] and France [40] caused alarm, since the biopesticide had been used effectively for 20 years. This occurrence led to the study of a variety of matters concerning resistance to biopesticides, which included the interaction of the virus with its host at a variety of levels, including the genomic level.

The concerns about biopesticide resistance resonate in Africa due to the close relatedness of the viruses CpGV and CrleGV [41] and of the insects *C. pomonella* and *T. leucotreta* [42]. However, understanding the development of resistance in *C. pomonella* will aid the decisions made for control strategies for *T. leucotreta* populations. The possibility of resistance in *T. leucotreta* populations provides incentive for researchers to identify the mechanisms behind resistance before it occurs in the field. Research identifying genetic markers that are associated with resistance to CrleGV can be used to evaluate the rate of development of resistance within a population of *T. leucotreta*, with the eventual goal of delaying the advent of resistance.

1.4 Genomics of biopesticide resistance

Viruses and their insect hosts form close associations, often influencing each other in unexpected ways. Infection by a microbial pathogen involves a variety of interactions between the pathogen and its host at the cellular and molecular levels. To improve the action of a biopesticide, it is necessary that these mechanisms be understood. Knowledge of transcriptional processes in the genome offers the possibility of improved understanding of biopesticide transmission and of finding new ways to enhance their efficacies. Despite this, little is known of this infection process at the genomic level as only a small number of studies have focused on this interaction [43–46]. One such study identified the response of *Epiphyas postvittana* (Walker, 1863) (Lepidoptera: Tortricidae) to *Epiphyas postvittana* nucleopolyhedrovirus (EppoNPV) (Baculoviridae) [46]. Microarray analysis revealed 102 genes that were differentially expressed in virus-infected larvae compared to uninfected larvae. These genes were associated with functions such as apoptosis (programmed cell death), endocrine modulation and digestion. The study thus provided baseline data for indicating which viral genes are necessary for pathogenesis and how the insect is capable to defend itself against infection.

Literature reporting on the molecular mechanisms of resistance in insects is dominated by responses to either insecticides or *Bacillus thuringiensis* (Bt). Research concerning resistance of *C. pomonella* to CpGV at the molecular level is the most applicable when planning studies for *T. leucotreta*. However, research focused on *C. pomonella* and CpGV focuses on the mode of resistance inheritance [37,47], resistance genes [48,49], and viral enhancers [49] and there is no published literature about the quantification of gene expression that compares resistant to susceptible *C. pomonella* larvae, or other abiotic influencing factors.

1.5 Gene expression study tools

Genomic expression studies offer a uniquely powerful way to investigate genes involved in evolutionary processes by clarifying aspects such as the adaptive importance of gene expression, gene functions and their consequences [50–52]. As such, their use is becoming increasingly important for the study of interactions among different species, e.g. [53–57]. The availability of more genetic data coupled with high-throughput genetic tools has increased the amount of insect genetic research over the last 5 years. The three most commonly used tools to study gene expression are microarrays, transcriptome sequencing and real-time quantitative PCR (qPCR).

Microarrays have been suggested as the best method for high-throughput screening of multiple target genes [58,59]. This method requires a microarray construction whereby oligonucleotide probes specific for the known or predicted genes of the target species are used. The cDNA is fluorescently labelled and can therefore be used to quantify the amplification of the target gene. However, this method is expensive and prior knowledge of specific target genes is vital. Microarrays are essential for many projects that require reliable screening of multiple genes to identify only the genes of interest with expression.

Transcriptome sequencing provides a snapshot of gene expression that can be compared among insects species or conditions. Therefore, this method identifies all of the genes that were expressed at a particular time and can provide quantitative information about individual gene expression. This is the most detailed and accurate method for investigating the holistic genetic expression of an insects. However, this technique is labour-intensive and requires an understanding of bioinformatics and statistics to correctly standardize and represent the data. For comparative work using a transcriptome, it is also important to have a reference genome or transcriptome for gene identification. Gene studies using transcriptomics can provide extensive information about genetic interactions. However, until more reference genomes become available this method is restrictive for studying insect-virus interactions.

The third commonly used method for analysis of gene expression, real-time quantitative PCR (qPCR), is currently the gold standard tool for studying gene expression with lower-throughput [60,61]. It allows researchers to evaluate individual genes, providing the most accurate expression data relatively quickly and easily [62]. Primers for specific target genes are used and the protocol is the same as with standard PCR. The qPCR method provides more accurate assessment of gene expression compared to microarrays while being

cheaper and more easily analysed compared to transcriptomics. However, the reliability of this method is based on the assumption that all preliminary tests have been adequately performed and are acceptable. These preliminary assessments require RNA and cDNA of high quality, primers with acceptable amplification efficiency, no evidence of primer-dimers, and two or more reference 'house-keeping' genes that are stably-expressed across all potential samples. To obtain reliable, reproducible data from qPCR, these three key requirements must be first assessed.

1.6 Thesis aims and objectives

The main aim of this thesis was to determine the change in gene expression of *T. leucotreta* when infected with CrleGV, at different time intervals and at different temperatures. For this aim to be met, three objectives needed to be completed.

Objective 1: Evaluation of RNA extraction methods

The quality of RNA has been shown to have an effect on gene expression studies as it provides the measure of gene expression. A number of factors can influence RNA quality, including the extraction method and the tissue type. A number of different RNA extraction methods are available and by referring to up-to-date literature or manufacturers' recommendations, the right methods can be selected for most tissue types. A thorough comparison of RNA extraction methods using insect tissue, however, have not been evaluated in over 22 years [63] and a number of new or improved extraction methods have since become available. As insect species are diverse and have different tissue compositions, determining how this might affect the quality of RNA extracted could be meaningful. This evaluation would allow the most robust methods to be selected for future insect studies requiring RNA. Therefore, an evaluation of candidate RNA extraction methods was performed. The four most common methods evaluated were a cetyl trimethylammonium bromide (CTAB)-based method, a TRIzol reagent method, the SV total RNA isolation kit and the RNeasy isolation kit. To evaluate each method the whole bodies of larvae of four different insect species were used: *Thaumatotibia leucotreta*, *Plutella xylostella* (Linnaeus, 1758) (Lepidoptera: Plutellidae), *Thanatophilus micans* (Fabricius 1794) (Coleoptera, Silphidae), and *Tenebrio molitor* (Linnaeus 1758) (Coleoptera: Tenebrionidae). Using multiple species for comparison provides an additional assessment of whether species-specific tissues affect RNA quality and therefore indicates the optimal method to be used.

Objective 2: Reference gene selection

The most important criterion for a good gene expression study using qPCR is accurate normalization. Normalization is performed by identifying stable reference genes that will be used as a baseline to quantify the change in expression of specific target genes. Unstable (or absent) reference genes could result in false positive or false negative results. Accurate normalization requires evaluation of reference genes based on the specific conditions of the study. Little is known about the effect that different conditions have on reference gene stability and changes in gene expression. Reference gene selection is greatly influenced by species differences, with some studies incorporating multiple species [64]. No study has been done using three different closely related species, and this would therefore provide invaluable information towards understanding possible taxonomic trends regarding reference gene selection. Comparing different reference genes for different species using the same primers would not only provide a foundation for future gene expression studies, but would also help to determine the probability of using generalised target gene primers for qPCR. Therefore, our objective was to evaluate six reference genes (α-actin, arginine kinase, cytochrome c oxidase I, elongation factor 1-α, enolase, and α-tubulin) using three different tissue types from *T. leucotreta, C. peltastica* and *C. pomonella*. Furthermore, these genes were evaluated using *T. leucotreta* midgut tissue under different stress conditions to identify the three best-performing genes for normalization of the response of T. leucotreta to CrleGV infection, which is discussed below.

Objective 3: Target gene expression responses to *T. leucotreta* midgut CrleGV infection at different times and temperatures

Quantification of change in gene expression levels between virus-infected treatments and uninfected controls could identify the genetic responses of the host during the infection process. Using a range of target genes with established links to virus interactions could help reveal the host's virus defence processes and ultimately its susceptibility to the virus's infection pathways. Incorporating different conditions, such as time and temperature variation, allows for further insight into the effects that these conditions have on the infection process and what conditions are better suited for host defence or susceptibility. Therefore, the final objective of this study evaluated changes in the expression of 12 target genes: cell death, dicer-2, gelsolin, glutathione peroxidise, heat shock protein 70, heat shock protein 90, initiation factor 5a, juvenile hormone epoxide hydrolase, mitochondrial aldehyde

dehydrogenase, enolase, α-tubulin, and cytochrome oxidase I. The expression profiles of these genes were quantified for *T. leucotreta* larvae midgut infected with CrleGV and uninfected at 15°C, 25°C, 35°C after 48 h post-infection, as well as 24h and 72h post-infection at 25°C. These data identify which of these genes show the greatest expression differences during infection as well as infection under different conditions. This could therefore assist future management if links are identified between environmental conditions and virus infection.

2

Comparison of RNA isolation methods from insect larvae

2.1 Abstract

Isolating RNA from insects is becoming increasingly important in molecular entomology. Four methods, including three commercial kits (RNeasy® Mini Kit (Qiagen), SV Total RNA isolation system (Promega), TRIzol® reagent (Invitrogen)) and a CTAB-based method were compared regarding their ability to isolate RNA from whole-body larvae of *Thaumatotibia leucotreta, Thanatophilus micans, Plutella xylostella* and *Tenebrio molitor*. A differences were observed among the four methods regarding RNA quality but not quantity. However, RNA quality and quantity obtained was not dependent on the insect species. The agarose gel electrophoresis, 260/280 OD readings and RNA integrity number (RIN) showed that the CTAB-based method produced low quality RNA and the TRIzol® reagent produced partially degraded RNA whereas the RNeasy® Mini Kit and SV Total RNA isolation system produced RNA of consistently high quality. However, after reverse transcription to cDNA, RNA produced using all four extraction methods could be used to successfully amplify a 708 bp fragment of the cytochrome oxidase I gene. Of the four methods, the SV Total RNA isolation system showed the least amount of DNA contamination with the highest RIN and is thus recommended for stringent applications where high quality RNA is required. This is the first comparison of RNA isolation methods among different insect species and the first to compare RNA isolation methods in insects in the last 20 years.

Keywords: CTAB, RNA extraction, RNeasy® Mini Kit, SV Total RNA isolation system, TRIzol® reagent

2.2 Introduction

Isolating RNA from insects is becoming increasingly important as a growing number of molecular biology applications rely on using RNA rather than DNA [65–68]. Currently, limited research is available on RNA isolation methods from insects. One of the only published research extensively comparing RNA isolation methods in insects is by Noriega & Wells (1993), where three different RNA isolation protocols were compared. However, as molecular entomology has advanced substantially in the last 20 years, these methods may not be suitable for stringent RNA applications. In addition, isolation methods that have since been developed may produce RNA of a higher quality.

Different RNA isolation methods often produce RNA of varying quantity and quality, that may depend on the type of tissue used. Isolating RNA from tissue requires separation of the nucleotides from secondary metabolites such as polyphenolics, etheric oils, carbohydrates and lignins; which differ according to tissue composition [69]. It is therefore vital that the optimal RNA isolation method is selected according to the type of tissue used to provide RNA of a suitably high quality for the selected downstream application.

Currently, many insect gene expression studies have focussed on agriculturally important insects [46,70,71]. Thus, for this study three agriculturally important insect pests were chosen for comparison: the false codling moth *Thaumatotibia leucotreta* (Meyrick, 1913) (Lepidoptera: Tortricidae), diamondback moth *Plutella xylostella* (Linnaeus, 1767) (Lepidoptera: Plutellidae) and mealworm *Tenebrio molitor* (Linnaeus, 1758) (Coleoptera: Tenebrionidae), as they are particularly damaging to both the commercial and subsistence agriculture sectors in South Africa [72–74]. In addition, the forensically important insect *Thanatophilus micans* (Castelnau, 1840) (Coleoptera: Silphidae) [75] was chosen for comparison. Two different species each of Coleoptera and Lepidoptera were selected to provide a comparison between and within different holometabolous insect orders. Whole-body extractions were analysed as they cover all potential tissue types for use and are commonly used for gene expression studies [46].

A number of criteria determine a good RNA isolation method. The method needs to be highly efficient to limit the chance of RNA degradation [76], must provide high quality RNA that contains no contaminants, must produce RNA of a reasonable quantity and overall needs to be consistent and robust. In addition, the safety of the RNA isolation method should also be considered, since many RNA isolation methods include the use of toxic or hazardous

chemicals such as phenol, chlorophorm or 2-mercaptonoethanol. In general, a good RNA isolation method is characterised as being economically viable; where any costs of the method, including isolation time, monetary cost and health risk, are justified by the above-mentioned criteria. The aims of this study were thus to compare different RNA extraction methods from insect whole-body larvae and to evaluate the RNA produced based on the criteria described above. This is the first comparison of RNA isolation methods among different insect species and the first to compare RNA isolation methods in insects in the last 20 years.

2.3 Methods and Materials

2.3.1 Insect material

Late instar larvae of *T. leucotreta, P. xylostella*, *Te. molitor* and *Th. micans* were collected from established cultures at Rhodes University. Live individuals were flash-frozen in liquid nitrogen and homogenised using a sterile mortar and pestle. Homogenised tissue (30 mg) was then placed into a pre-frozen microcentrifuge tube. This was the standard sample preparation for all RNA isolation methods. Three replicates were included for *T. leucotreta, P. xylostella*, *Te. molitor* and *Th. micans* using each method TRIzol® reagent (Invitrogen, Carlsbad, USA), the SV Total RNA isolation system (Promega, Madison, Wisconsin, USA) and the RNeasy® Mini Kit (Qiagen, Hilden, Germany), resulting in a total of 48 independent RNA isolations.

2.3.2 RNA isolation

Four RNA isolation methods were compared in this study. Three of these methods are based on commercial kits: TRIzol® reagent, the SV Total RNA isolation system and the RNeasy® Mini Kit. The remaining RNA isolation method is a complex CTAB-based method [77]. Reagents for the CTAB-based method were purchased from the Merck group (Merck, South Africa).

The RNeasy® Mini Kit and SV Total RNA isolation system were followed according to manufacturers' specifications. The CTAB protocol was adjusted by including three ethanol washes instead of two for improved RNA precipitation. In addition, the pellet was suspended in 100 µL of H_2O instead of the recommended 30 µL before the optional cleanup to better dissolve the RNA pellet. The TRIzol® reagent protocol was followed according to the

manufacturer's specifications with the exception that only 30 mg of tissue was used instead of the suggested 50-100 mg to allow direct comparison with the other four RNA isolation methods used in this study. All RNA extracted was stored at -80°C before further analysis.

2.3.3 DNase digestion

To evaluate the necessity of additional DNase digestion steps, the samples were subdivided and 10 µl RNA from each method was digested using the RQ1 RNase-Free DNase kit (Promega, Madison, Wisconsin, USA) according to manufacturer's specifications to remove any remaining DNA from the RNA extractions. DNA contamination of RNA isolations before digestion with DNase enzyme was evaluated using PCR, along with positive and negative controls.

2.3.4 PCR

Any DNA contamination of the isolated RNA was evaluated by PCR which amplified the 708 bp fragment of the cytochrome oxidase I (COI) gene using the previously described primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-5') [78]. DNA contamination was assumed to be present if the PCR was successful. PCR tubes contained 12.5 µl of Master Mix (Promega, Madison, Wisconsin, USA), 1.5 µl of each primer (10 pmol), 1.5 µl $MgCl_2$ (25 mM), 7 µl H_2O and 1 µl RNA, resulting in a total volume of 25 µl. The conditions for each of 40 PCR cycles were as follows: denaturation at 95°C for 30 sec, annealing at 54°C for 1 min, and extension at 72°C for 1.5 min. The reaction was preceded with an initial denaturation step of 95°C for 5 min and ended with a final extension step of 72°C for 10 min. PCRs were checked for amplification using agarose gel electrophoresis.

2.3.5 cDNA conversion

The RNA of *T. leucotreta*, isolated using the four methods described above, was converted to cDNA using the Maxima H Minus Reverse Transcriptase kit with a random hexamer primer (5'-d (NNNNNN) -3') (Thermo Scientific, Wilmington, Delaware, USA). The sample was incubated at 25°C for 10 min followed by 30 min at 50°C. The reaction was terminated by heating the sample to 85°C for 5 min. cDNA was then tested in a PCR reaction

using the conditions described above to determine its applicability for basic downstream applications.

2.4 Results

2.4.1 RNA quality

Spectrophotometer readings for RNA isolations conducted using the CTAB isolation protocol were generally poor, with only 70% of the isolations showing acceptable 280/260 and 260/230 ratios. In addition, agarose gel electrophoresis of RNA extracted using the CTAB isolation protocol showed only faint smearing, with no well-defined bands. The average RIN for CTAB extractions was 1, with no peak visible for either the 18S or 28S fragments on the electropherogram (Figure 2). The quality of RNA using TRIzol® reagent was fairly inconsistent as only half of the reactions had acceptable 260/230 ratios with the rest below 1.3. The mean RIN values of samples extracted with TRIzol® reagent was 5.3 (SD = 0.14), and could thus be considered partially degraded. RNeasy® Mini Kit and SV Total RNA isolation system showed acceptable purity ratios, with a 280/260 mean value of 2.01 (SD = 0.15) and 230/260 ratio of 1.85 (SD = 0.37). The mean RIN for RNeasy® was 8.2 (SD = 0.57), which is considered intact RNA. Electropherogram graphs of RNA isolated using the RNeasy® mini kit showed the highest 28S peak out of the four methods, with a good 5S fragment (Figure 2) The mean RIN for SV total RNA extraction kit was 9.25 (SD = 0.21). These RNA extractions showed well defined peaks with limited contamination (Figure 2).

2.4.2 RNA quantity

According to spectrophotometer results, the mean RNA yield per extraction was 96319 ng (SD = 39463) for CTAB isolations, 52314 ng (SD = 2593) for the SV Total RNA isolation system, 40127 ng (SD = 14407) for RNeasy® Mini Kit isolations and 85079 ng (SD = 52154) for TRIzol® reagent isolations.

2.4.3 DNA contamination

RNA isolated using TRIzol® reagent, RNeasy® Mini Kit and the CTAB method showed high amounts of DNA contamination, resulting in PCR products of high concentration. Only RNA extracted using the SV Total RNA isolation system did not show high amounts of DNA contamination. Additional DNase digestions were performed for a

subsample of RNA extracted using each of the four methods. After DNase digestion, PCR amplification was unsuccessful for RNA extracted using each of the four methods, showing that detectable DNA contamination had been eliminated.

2.4.4 PCR application

Thaumatotibia leucotreta RNA was reverse-transcribed to cDNA and used to amplify a 708 bp fragment of the cytochrome oxidase I gene to compare the success of different RNA isolation methods for basic downstream applications. It was found that RNA isolated using all four methods included in this study could be used to produce well-defined amplification products (figure not shown).

2.5 Discussion

2.5.1 RNA quality

There was no clear difference among the four insect species regarding the quality of RNA extracted. However, there was a variation among different RNA isolation methods for both the 280/260 and 230/260 quality ratios and the Agilent Bioanalyser results. The SV Total RNA isolation system and RNeasy® mini kit extracted the highest quality RNA. RNA extracted using the TRIzol® reagent showed partially degraded RNA. The low 230/260 quality ratio could indicate a residue of phenol from the TRIzol® extraction. Although it is preferable for gene expression studies that the RNA is of a high quality, RIN above 5 have been noted as acceptable RNA for microarrays and gene expression studies as long as the product length is less than 400bp [79–81]. Based on the electropherogram graphs, it was assumed that high quality RNA had a 28S peak twice the height of the 18S peak [82]. This, however, is rarely achieved as the 28S RNA subunit degrades more rapidly than the 18S subunit [79]. The major cause of this denaturing is heating of the RNA during the extraction procedure, which may denature the 28S ribosomal subunit to produce two fragments similar in size to that of the 18S RNA subunit [83,84]. SV Total RNA isolation system and the TRIzol® reagent both had heating steps during the procedure, which may account for their lower 28S peaks (Figure 2). The complete lack of a 28S peak with TRIzol extractions is most likely a result of the 15 min heating process at 60°C during the isolation procedure. The CTAB method did not produce RNA of a high quality or quantity. However, this method is cost-effective, especially when large sample sizes are used, and can be used for simple

downstream applications such as basic PCR for phylogenetic studies. For example, phylogenetics analysis based on RNA may be performed using lower quality RNA, and as such may not require stringent RNA isolation methods [85–90].

2.5.2 RNA quantity

As spectrophotometer results may confuse contamination within the RNA sample with increased RNA concentration, only RNA samples with acceptable 280/260 and 230/260 ratio readings were considered. Thus, no CTAB RNA concentration readings were analysed. RNA extractions using TRIzol® reagent showed the highest concentrations of RNA. The RNeasy® Mini Kit and SV total RNA system extracted similar and consistent concentrations of RNA.

2.5.3 DNA contamination

That three of the methods showed high levels of DNA contamination demonstrated the importance for an additional DNase step following such RNA extractions. The exception was the SV Total RNA isolation system.

2.5.4 PCR application

PCR results have been found to not be generally affected by RNA integrity [91], which would explain why PCR was successful using low quality RNA produced by the CTAB-based protocol. However, the performance of more stringent techniques, such as microarray analysis, is affected by RNA integrity. Therefore, depending on the downstream applications, the choice of RNA isolation method would vary depending on the quantity and quality of RNA desired, the cost of the isolation method, and the time taken to perform isolations.

2.5.5 Additional considerations

Besides the quantity and quality of RNA isolated, an important consideration for selecting an appropriate RNA isolation method is cost. The commercial kits were significantly more expensive per isolation than the CTAB method. The CTAB method costs less than $0.2 per isolation if more than 50 reactions were performed, due to the initial start-up costs. The RNeasy® Mini Kit and SV Total RNA isolation system were the most

expensive at $7 and $6.2 per isolation respectively. The TRIzol® method, which cost $4.5 per isolation, was cheaper than these. However, isopropanol and ethanol were required but were not included in the kit, necessitating an additional cost of $20 for 1000 reactions. In addition to the RNA extraction costs, a further DNase step was considered with a cost of $110 for 1000 reactions .

A further factor to consider when selecting an RNA extraction method is the time taken to perform the isolation. There was an inverse relationship between cost and isolation time for each RNA isolation method included in this study. The RNeasy® Mini and SV Total RNA isolation system both averaged bench and total times of 40 min and the CTAB protocol required a bench time of 2.5 hours.

The safety of the isolation procedure for the user is also an important consideration. The CTAB and TRIzol isolation protocols rely on the use of chloroform and phenol respectively, which are both hazardous to users, thus making the RNeasy® Mini Kit and SV Total RNA isolation system the safest isolation methods of the four that were tested.

2.6 Conclusions

All four isolation methods evaluated produced RNA of a similar quantity and quality regardless of insect species analysed, indicating that these results may be applicable to larvae from a range of insect species. RNA isolated using the RNeasy® Mini Kit was found to be of high quality and always of a consistent concentration, with the method also being the most robust and safe, since no harmful chemicals were used. The RNeasy® Mini Kit does not require any heating steps, allowing for the quantity and quality of the RNA to be determined without any interference due to denaturation of the 28S ribosomal subunit.

Even though TRIzol® reagent has been most commonly used for gene expression analysis in insects [46,58,71,92,93], which requires very high quality of RNA [79,91,94], it was found that this method was not consistent and produced a high quantity but lower quality of RNA when compared to RNA isolated using the RNeasy® Mini Kit and SV Total RNA isolation system. RNA isolations performed using the SV total RNA system were of the highest quality and of consistently high quantity. In addition, RNA isolated using this method did not require an additional DNase digestion step, thus saving time and money. The SV total RNA system is thus recommended for any downstream applications requiring a consistently good quality and quantity of RNA. The SV total RNA system will be the RNA extraction kit used for this studies reference gene selection and gene expression analysis.

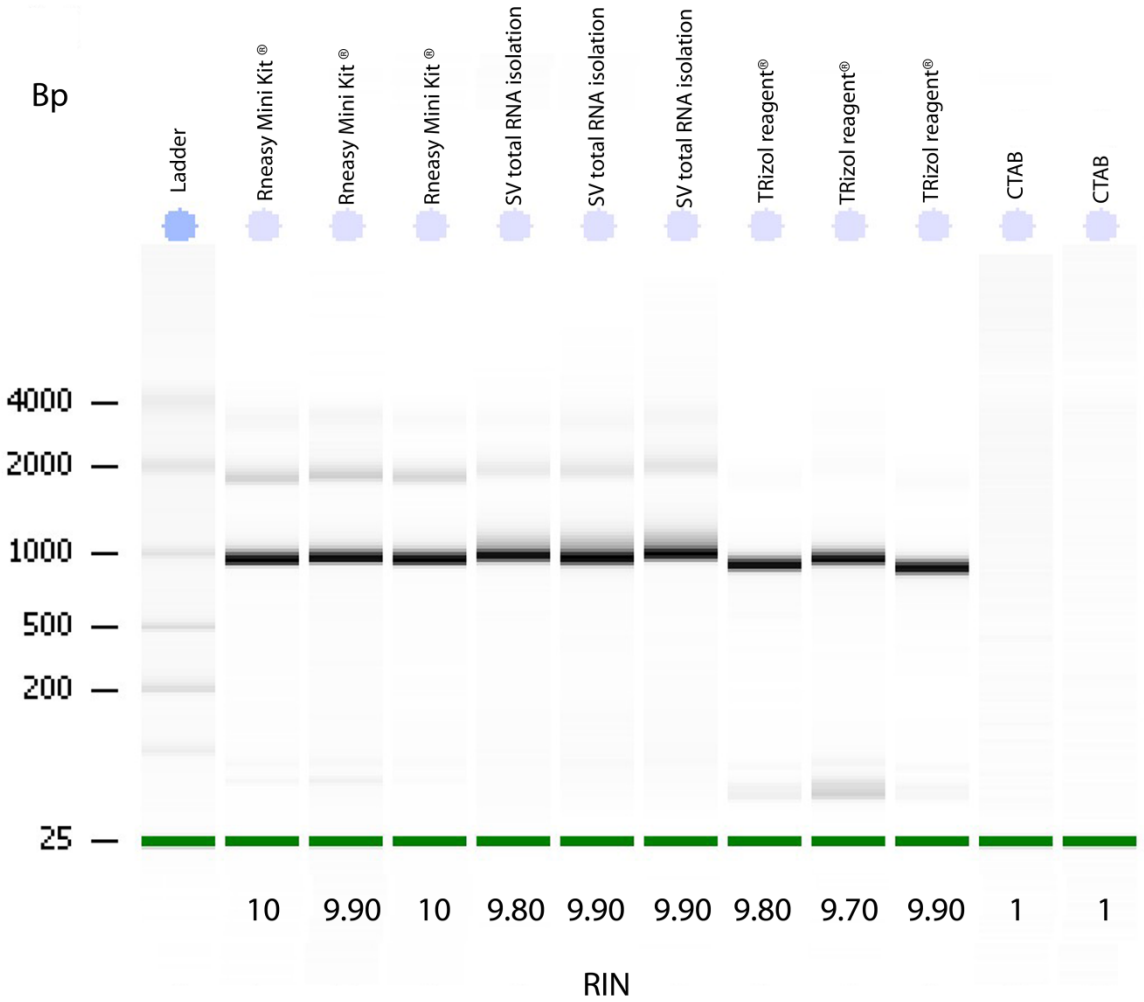

Figure 1. Pseudo-gel image produced using an Agilent 2100 Bioanalyser, showing the results of RNA extracted from *T. leucotreta* using the RNeasy® Mini Kit, a CTAB-based protocol, TRIzol® reagent and the SV total RNA kit. The 18S RNA subunit is visible at 2000nt, the 28S large subunit at 3800nt and a combination of 5S, 5.8S and tRNAs at about 180 nt. RNA integrity was established based on the RNA integrity number (RIN).

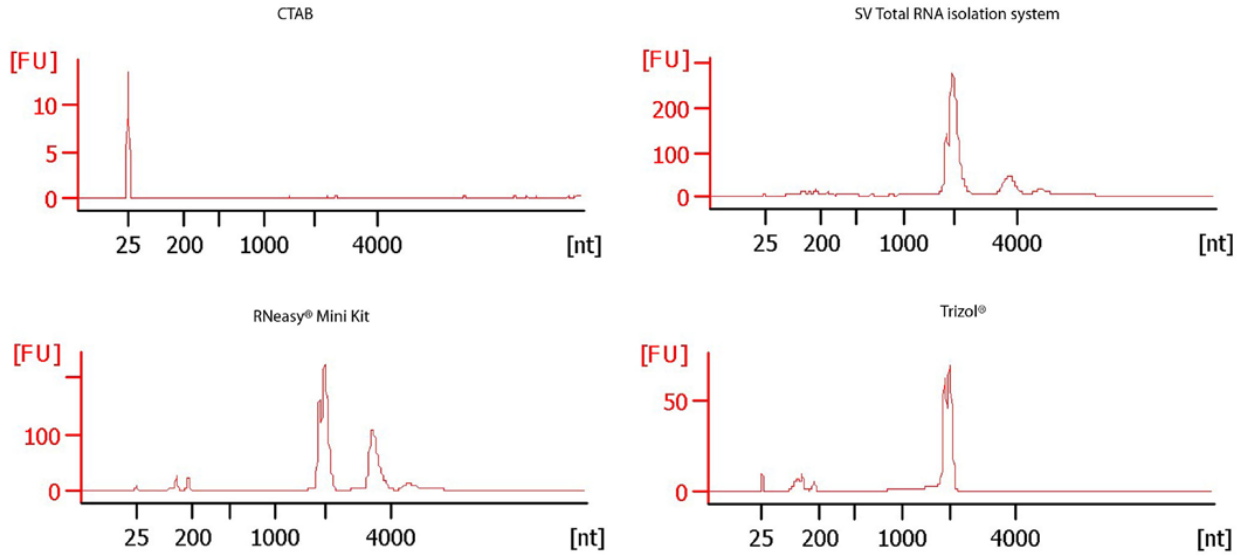

Figure 2. Agilent 2100 Bioanalyser electropherogram graphs showing RNA extracted using a CTAB-based protocol, the SV Total RNA System, the RNeasy® Mini Kit and TRIzol® reagent. Markers can be seen at 25 nt, the 18S RNA subunit at 2000 nt, the 28S large subunit at 3800 nt and a combination of 5S, 5.8S and tRNAs at about180 nt.

3

Reference gene selection for quantitative real-time PCR normalization in larvae of three species of Grapholitini (Lepidoptera: Tortricidae)

3.1 Abstract

Despite the agricultural importance of species in the Grapholitini (Lepidoptera: Tortricidae), and the value of gene expression analysis for improved population management, few gene expression studies based on real-time quantitative PCR (qPCR) have been conducted for this tribe. Part of the reason for this is that suitable reference genes, which are fundamental for accurate normalization of qPCR studies, have not been identified for the tribe. Thus, the expressional stability of six potential reference genes (ACT, AK, COI, EF1, ENO and TUB) was assessed in three different tissues (whole body, midgut and cuticle) of *Cryptophlebia peltastica* (Meyr), *Cydia pomonella* (L.) and *Thaumatotibia leucotreta* (Meyr). Additionally, these reference genes were tested using *T. leucotreta* at different temperatures (15°C, 25°C and 35°C) with and without baculovirus infection. Suitable reference genes were identified for the whole body and midgut tissue of all three species, and for cuticle tissue of *C. pomonella* and *T. leucotreta*. When *T. leucotreta* was infected with the virus at all temperature conditions, ACT, AK and EF1 were found to be the most suitable reference genes for experimental normalization. In general, for all tissue types, species and stress conditions, AK and EF1 were the best-performing reference genes. However, even though the three species analysed were closely related and within the same tribe, each species required varying gene combinations for suitable normalization. This study provides the first reference gene evaluation for the Tortricidae, and paves the way for future qPCR analysis in Tortricidae.

3.2 Introduction

Insect gene expression studies have proliferated considerably during the last five years. The major influencing factor for this trend is the availability of genomic information paralleled with high-throughput, robust and accurate gene expression analysis tools such as real-time quantitative PCR (qPCR) [95,96]. In the agricultural sector, these gene expression studies can provide data crucial to improving production. An example is using gene expression to identify and quantify key genes involved with virus interactions, that can then be used to predict the success of biological control agents or improve current control measures [96]. As the use of biopesticides is increasing, genetic clues for improving the efficacy of these control measures will not only benefit production but also provide a more competitive and reliable alternative to chemical controls.

Although qPCR is the current gold standard for gene expression analysis, it has been recognised as having high variability if the recommended steps are not taken for proper normalization [1,97]. As with all basic experimental processes, results of the identified treatments are dependent on the stability of the controls. The importance of qPCR normalization has been reviewed several times [1,96,98]. However, as insect gene expression studies using qPCR are still relatively recent, the importance of accurate normalization bears repetition. Although a number of potential reference genes have been identified, no universal reference genes for insects have been identified yet as there are many different factors that influence gene expression and the subsequent stability of reference genes [64,96,99]. It is therefore crucial to assess reference genes for the same experimental conditions under which the target genes will be evaluated. Factors known to influence gene stability at the species level are temperature [62], virus infection [95], pesticide exposure [96], tissue type [100], geographic group [98], age [60], sex [101] and diet [102]. For normalization of qPCR, reference genes should provide consistent expression across all conditions. The qPCR process would be simplified if the same genes and primers could be used for different species. However, information about taxonomic trends for reference gene selection is limited. In a study examining reference genes in Lepidoptera, no genes were found that could be used universally [64]. Reference gene evaluation at lower taxonomic levels might still reveal trends across closely related species.

This study analysed species within the tribe Grapholitini (Lepidoptera: Tortricidae), which contains some of the world's most destructive horticultural pests. Three pest species of major economic importance were included in the analysis - *Cryptophlebia peltastica* (Meyr), *Thaumatotibia leucotreta* (Meyr) and *Cydia pomonella* (L.). Currently both *T. leucotreta* and *C. pomonella* are controlled using commercially-produced granulovirus formulations and research is under way for the formulation of a virus of *C. peltastica*. In the last decade *C. pomonella* has developed widespread resistance to the granulovirus used in its control [36,37,103]. This led to concerns that *T. leucotreta* and *C. peltastica* may also develop resistance to granuloviruses, due to the close relatedness of the three insect species and of their granuloviruses [41,42]. Thus, evaluating a foundation for future gene expression studies of Grapholitini is necessary.

The aim of this study was to evaluate candidate reference genes for species within the Grapholitini. Reference gene evaluation was performed for *C. peltastica, T. leucotreta* and *C. pomonella* to provide a comparison for reference gene selection among species that are closely related, which might provide an indication of possible trends at fine taxonomic levels. For each species, the whole bodies, midgut and cuticle tissue were compared to examine their effect on reference gene selection [71,104]. The three tissue types chosen for reference gene evaluation have advantages for a variety of future gene expression studies. In addition, the influence of biological stress on reference genes was examined by exposing *T. leucotreta* to a granulovirus used for its control at different temperatures. Temperature has been observed to have a major influence on the efficacy of viruses to control their hosts' popuations [105,106]. Results provided by this study will be fundamental for further qPCR studies on gene expression in *T. leucotreta*, *C. peltastica* and *C. pomonella* and the potential improvement for their control.

3.3 Materials and Methods

3.3.1 Insect material

The three species used in this study - *C. peltastica, T. leucotreta and C. pomonella* - were obtained from established cultures at River Bioscience (Pty) Ltd, Port Elizabeth. The larvae were received as 1st instars with their species-specific artificial diet formulation. Individual larvae were placed in separate Petri dishes with a 5 mm x 20 mm x 20 mm diet cube and reared at a temperature of 25°C (+-3°C) 50-70% relative humidity and a 12h photoperiod. The diet was rehydrated, using type 2 distilled water, after 48 h. As stable gene

expression is highly dependent on the age of the animal [107,108], only 4th-instars, selected based on head capsule size in accordance with Dyar's rule [109], were used in analyses to ensure that results could be meaningfully compared. The 4th-instars were examined as they were large enough to dissect and measure virus uptake accurately while still being immature enough to not pupate prematurely under stressful conditions.

3.3.2 Insect comparison

Three different tissue compositions – midgut, cuticle and whole body – were used for each of the three species to test the effect of tissue composition on reference gene stability. Dissections of the midgut and cuticle tissue were performed in insect Ringer's solution [110] and dissected tissue was immediately placed in either Qiagen RNAlater™ RNA stabilization reagent (Qiagen, Germantown, USA) or flash-frozen in liquid nitrogen, then stored in a −80°C freezer until RNA extraction. Three biological replicates consisting of a pool of eight individual insects per replicate were used for each tissue type for each species.

3.3.3 Biological treatment

Further biological treatments were performed using *T. leucotreta* as this species will be used in downstream qPCR experiments. All larvae were in their 4th-instar after the termination of each experiment. The required instar for the start of each treatment was determined using preliminary study data.

The *Cryptophlebia leucotreta* granulovirus (CrleGV-SA) [111] was purified as described elsewhere [15,112] and diluted to a concentration of 10^7 OBs/ml. This concentration was required to ensure a lethal dosage [113]. Droplet feeding treatments were done using previously published methods [114]. Upon reaching the desired instar, the diet cube was removed from the individual Petri dishes for 12 hours. Thereafter, a droplet of either distilled H_2O and dark blue food colouring mix (for controls) or diluted CrleGV-SA and dark blue food colouring (for treatments) was presented to the larvae. The food colouring provided a visible confirmation of consumption. Larvae were manipulated in a dimly-lit room (+- 3 lumens) with a number 2 paint brush to minimize their stress. Larvae were weighed on a Mettler Toledo XPE 205 analytical balance before and after droplet consumption. For each experiment, eight infected and eight control 4th-instar *T. leucotreta* larvae were selected. After droplet feeding, the diet cube was placed back in the Petri dish, which was sealed using

a rubber band. Experiments examining the effects of biological stress on reference gene selection in *T. leucotreta* were conducted at 15°C, 25°C and 35°C for 48 hours and 25°C for 24 and 72 hours, using CrleGV-infected and uninfected larvae. All experiments were repeated in triplicate, resulting in 15 independent treatments.

3.3.4 RNA extraction and cDNA convertion

RNA extractions were performed using the SV Total RNA Isolation System (Promega, Madison, WI, USA), as it is known to produce RNA of a good quality and quantity [115]. All tissue was homogenised with liquid nitrogen using a sterile mortar and pestle following the manufacturer's protocol.

RNA sample quality was tested using the NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, USA). For further confirmation of RNA quality and lack of DNA contamination, a random sample was tested with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA). The RNA was converted to cDNA using the Maxima H Minus Reverse Transcriptase kit with a random hexamer primer (5'-d(NNNNNN)-3') (Thermo Scientific, Wilmington, USA). cDNA concentration was determined using the NanoDrop 2000 spectrophotometer and then diluted to 1000ng/µl.

3.3.5 Confirmation of virus infection

To verify that control samples were uninfected and that treated samples were infected with CrleGV-SA, a PCR targeting the granulin gene was performed as described elsewhere [25]. The PCR products were electrophoresed on a 1% agarose gel for 30 minutes at 80V. Random infected samples were further verified for infection by Sanger sequencing conducted by Macrogen using the same primers used in the PCR.

3.3.6 Selection of candidate reference genes and primer design

Six genes were selected for analysis - α-Actin 5C (ACT), Elongation factor 1-α (EF1), α-Tubulin (TUB), Arginine kinase (AK), Cytochrome oxidase subunit 1 (COI) and Enolase (ENO) (Table 1). Primers amplifying the ACT, EF1, TUB genes were obtained from previous studies (Table 1) [46,116,117]. Primers amplifying the AK, COI, and ENO (Table 1) genes were designed using conserved regions of multiple Tortricidae sequences. These primers were designed using Primer3 0.4.0 software [118] (http://bioinfo.ut.ee/primer3-

0.4.0/primer3/). The parameters set for primer design required a melting temperature between 60-63°C, GC content of 50-60%, amplified region length 60-120 bp, max 3' self-complimentarity of 1, max poly-X = 3, primer length between 18 and 20 bp, primer paired toward 3'end and a max Tm difference of 10. A ten-times dilution series was performed (1000ng/µl, 100 ng/µl, 10 ng/µl and 1 ng/µl) using a pool of eight individual *C. peltastica, T. leucotreta and C. pomonella* whole body cDNA with three technical replicates. The CFX Connect ™ (BIO-RAD, Hercules, USA) program provided analysis of amplification efficiencies and melt curve data. For primers to be identified as suitable, it is required a melt curve with a single peak to verify the absence of primer-dimers, amplification efficiencies between 90-110% and an R-squared value above 0.95.

All qPCR experiments were conducted using the CFX Connect ™ machine and iTaq™ Universal SYBR® Green Supermix (BIO-RAD, Hercules, USA). The qPCR thermal cycling profile had a denaturing step of 50°C (2 mins), 95°C (10 mins) followed by 40 cycles of 95°C (15 sec) and 60°C (60 sec) with a melting curve analysis. This thermal cycling profile was set according to a previous study [116]. Species and tissue comparisons used a final reaction volume of 25 µl consisting of 1 µl of each primer (400nM), 10 µl Supermix, 0.25 µl cDNA (250ng/µl) and 12.75 µl H_2O. The *T. leucotreta* stress qPCR reactions comprised of a final volume of 10 µl, consisting of 1 µl cDNA (500ng/ µl), 5µl Supermix, 0.25µl of each primer (100nM) and 3.5 µl H_2O. Three technical replicates were used for each sample and H_2O replaced template for the non-template controls.

3.3.7 Analytical software

The three most widely accepted programs for reference gene evaluation, Bestkeeper [119], geNorm (Biogazelle qBase+) [120] and Normfinder [121], were used to analyse gene expression stability. Bestkeeper provides Pearson's correlation coefficient (r), statistical power (1-β) and the standard error (SE) for each gene. As Bestkeeper is highly sensitive to co-regulation and this effect is seen in the r-value and power value results, these values carried little weight in relation to recommendations for reference genes in this study. Therefore, the Bestkeeper values will be used as a guide and will not be considered if the geNorm and Normfinder results are in opposition to the Bestkeeper output. The geNorm analysis program is based on a similar algorithm to Bestkeeper but it provides a stability value (M) and a coefficient of variation (CV) of the normalized genes selected. The program also suggests the optimal gene pairing of up to six genes with a calculation of pair-wise

variation (V). To avoid misleading results due to co-regulation, genes were excluded from the analysis in a step-wise manner to determine the actual values for the selected reference genes. Normfinder identifies stability values and standard deviation (SD) of genes analysed. The program also identifies the best gene pair by using inter- and intragroup variation. However, if the sample is only based on one grouping variable, the program can only provide one best performing reference gene. By taking into account the strengths and limitations of all three programs, we provided a selection of the most suitable reference genes for all of the different conditions analysed.

3.4 Results

3.4.1 Sample validation

This study conforms with the Minimum Information for Publication of Quantitative Real-Time PCR guidelines [1]. The average RIN of the RNA sub-sampled was 9.87. The mean purity values measured using the NanoDrop 2000 spectrometer were 2.18 ± 0.03 (280/260) and 2.17 ± 0.14 (230/280) (Table S1). All larvae in this study ingested an average of 1.18 ml (± 0.5 ml) of droplet mix. For analysis of the response of *T. leucotreta* to its granulovirus, control treatments tested negative for CrleGV-SA while all treatment samples tested positive. Treated samples that were sequenced showed a 99% match with *C. leucotreta* granulovirus isolates (CrleGV-SA).

3.4.2 Primer optimization and amplification

For all three species, the ACT, AK, EF1 and TUB genes were found to have an expression level below the required value (Cq < 35) with amplification efficiencies between 90-110% and no evidence of primer-dimers; Correlation coefficients were all above 0.95. Primers for COI and ENO satisfied the conditions above for *C. pomonella* and *T. leucotreta.* However, COI for *C. peltastica* samples showed poor amplification efficiency and ENO did not amplify. Therefore, the COI and ENO genes were excluded from all *C. peltastica* analyses.

3.4.3 Expression levels

The expression levels of the six genes were all between 12 and 35 Cq (Fig. 3), calculated as a mean of biological and technical replicates of each sample. High Cq values represent low expression levels. The COI gene was the most highly expressed gene in all *T. leucotreta* samples with a mean CP value of 15.44 (± 2.32) and showed the lowest expression in the *C. pomonella* samples with a mean expression of 34.1 (± 0.144) (Figure 3). ACT had the highest variability of expression in the *C. peltastica* whole body samples with an SD of ± 2.99 (Figure 1). The SDs of the CP values for *T. leucotreta* for the different tissue types for the same genes was on average four times higher than the SDs of the other two species. In addition, expression levels for the same genes in the different tissues of *T. leucotreta* were more similar than in *C. peltastica* and *C. pomonella*.

3.4.5 Pair-wise variation

For the majority of species and tissue comparisons, geNorm identified the optimal number of reference genes as two, although *C. pomonella* midgut samples required four genes for accurate normalisation. The required number of reference genes varied with *T. leucotreta* under stressful conditions. At 15°C and 25°C for both virus-infected and uninfected samples, only two genes were required for normalization. Two genes were also required for normalization of uninfected samples at 35°C. However, when uninfected and infected samples were combined in the analysis at 35°C, four genes were required for normalization. Analysis including all three temperatures, with virus-infected and control samples required three genes for accurate normalization.

3.4.6 Analysis of gene expression stability

3.4.6.1 Species comparison

For *C. peltastica* samples, the Bestkeeper, Normfinder and geNorm programs all identified AK and TUB as the most stable reference genes and the best combination for analysing cDNA generated from whole body RNA extractions (Table 2). ACT was rejected by all three programs as it had high values for SD (±2.99), SE (±1.52) and power (7.63), and poor stability (3.02). In the *C. pomonella* whole body sample, AK and EF1 were the most stable and best gene combination by all three programs (Table 2). Normfinder ranked genes from most stable to least stable as AK, EF1, TUB, COI, ENO and ACT. In the *T. leucotreta*

whole body sample, Normfinder and geNorm found ACT and COI to be the overall best performing gene and best combination of genes for normalization (Table 2). However, Bestkeeper showed that although both genes had a suitable SD, their power and r values were unsatisfactory. According to Bestkeeper, AK and ENO were the best genes for normalization of this sample.

3.4.6.2 Tissue type comparison

geNorm and Bestkeeper identified only two genes that were stable enough to use as reference genes for analyses of midgut tissue for the three species – EF1 and TUB. Although Normfinder also identified EF1 and TUB as the most stable genes, in a separate analysis, these had a combined stability of 1.46, well above the proposed cut-off value of 1.00.

When midgut tissue was analysed separately for each species, all three programs identified suitable reference genes. For the *C. peltastica* midgut samples, geNorm identified AK and TUB as the best gene combination for normalization (Table 3). Normfinder also showed that AK and TUB were suitable reference genes but selected EF1 as the best overall performing gene. However, Bestkeeper found AK to have a power of 0.16 above the threshold and to have the highest SD. The remaining genes were all found to be acceptable by Bestkeeper.

For *C. pomonella* midgut tissue samples, geNorm required AK, EF1, ENO and TUB to be used in combination for accurate normalization (Table 3). Normfinder found AK to be the overall best-performing gene with only ACT being rejected due to a stability value of 1.31. These results were consistent with those of Bestkeeper, as ACT and COI showed power values above the recommended threshold of 2.

The best gene combination found by geNorm for *T. leucotreta* midgut tissue samples was AK and EF1 (Table 3). Although both these genes were found to be acceptable reference genes by Normfinder, TUB was selected as the overall best-performing gene. Bestkeeper found both EF1 and TUB to have power values above the recommended threshold, but AK was accepted as a potential reference gene.

Analysis of the cuticle tissue gene expression values in all three species showed that none of the three programs found genes suitable to be used as reference genes for normalization. Therefore, cuticle tissue samples of the individual species were analysed separately.

For *C. peltastica* cuticle tissue samples, Bestkeeper found that all genes had acceptable power and SD values (Table 4). TUB was the only gene with a positive r-value and was chosen by Normfinder as the overall best-performing gene. However, it was still not considered to be stable. Thus, Normfinder and geNorm found no genes suitable as reference genes in this sample type.

For *C. pomonella* cuticle tissue samples, geNorm identified ACT and EF1 as the best-suited gene pair for normalization (Table 4). In contrast, Normfinder rejected ACT as it had a stability value of 1.038. Normfinder found AK to be the best-performing gene and ranked the genes from most to least stable as AK, COI, EF1, ENO, TUB and ACT. Bestkeeper found that all genes satisfied the SD and power thresholds, but Bestkeeper suggested that only TUB and COI had acceptable r-values of 0.934 and 0.885 respectively.

For *T. leucotreta* cuticle samples, all three programs selected COI and EF1 as the most stable genes/gene combination (Table 4). Bestkeeper rejected TUB and AK as potential genes due to their SD being above 1.

3.4.6.3 Biotic stress of *Thaumatotibia leucotreta*

When all stress conditions (15°C, 25°C and 35°C with virus-infected and uninfected midgut tissue over 24-, 48- and 72-hour periods) for *T. leucotreta* were analysed in combination geNorm selected ACT, AK and EF1 as suitable genes for normalization (Table 5). Normfinder identified AK and EF1 as the best gene pair with ACT as the third best-performing gene. Bestkeeper found ACT and EF1 to be suitable reference genes, although AK exceeded the power value threshold by 0.19.

The geNorm analysis found that the AK and COI were the best combination of genes for normalizing the expression of the sample from *T. leucotreta* exposed to low temperature stress (15°C) for 48 hours (Table 5). Normfinder selected AK as the overall best-performing gene followed by ACT, EF1, COI and TUB. Normfinder rejected ENO as a possible reference gene due to its stability value above 1. Bestkeeper identified only EF1 and TUB as possible reference genes as the remaining genes had power values above 2. When the *T. leucotreta* virus-infected samples kept at low temperature were analysed with low temperature controls, geNorm selected ENO and EF1 as the best gene combination for normalization. Normfinder also selected EF1, although combined with AK. ENO was rejected by Normfinder as a potential reference gene. Bestkeeper also rejected ENO and AK but found all other genes acceptable.

With *T. leucotreta* exposed to high temperature (35°C) stress, only two genes, ACT and AK, were required by geNorm for accurate normalization (Table 5). AK was also selected by Normfinder as the best performing gene. Normfinder ranked the genes from most stable to least stable as AK, EF1, TUB, and ACT with COI and ENO being excluded as possible reference genes. ENO was also rejected by Bestkeeper as it showed a SD above 1. COI was the only gene that satisfied the Bestkeeper power value cut-off of 2, but it was also the only gene with a negative r-value (-0.796). Combining the high temperature virus-infected samples with the high temperature control samples reduced overall gene stabilities. geNorm required ACT, AK, EF1, and TUB in conjunction to satisfy normalization of the samples. Normfinder paired EF1 and TUB as the best gene combination and rejected ENO as a potential reference gene. Bestkeeper rejected ENO and TUB as potential reference genes but all other genes satisfied the requirements of this software (Table 5).

geNorm analysis of *T. leucotreta* virus-infected samples at 25°C for 24, 48 and 72 h.p.i,, selected ACT, AK, EF1 and TUB genes for normalization. Although the genes selected had individual M values above the 0.5 threshold, when combined they provided suitable stability. ACT and EF1 were selected as the best paired genes by Normfinder with the remaining genes still satisfying reference gene stability criteria. AK and TUB were rejected by Bestkeeper as they had power values above 2. However, all genes had low SD. Combining the *T. leucotreta* virus-infected and uninfected samples at 25°C for 24, 48 and 72 hours post-infection improved the overall gene stability. AK and ACT were selected by geNorm as the best gene combination whereas Normfinder selected AK and EF1. Bestkeeper found AK and TUB to have power values above 2.

3.5 Discussion

To our knowledge this is the first study evaluating potential reference genes for insects within the same tribe as well as an insect under biopesticide stress at different temperatures. This is also the first study comparing reference gene selection for species of Tortricidae and their different tissue types.

Although there have been dramatic improvements in the availability of insect genetic information, it is still limited for many non-model species, and sequences are often not available for important genes for these species [46,60]. It would be advantageous if the same primers could be used to evaluate related species. To determine whether a single primer pair could be used for multiple species in qPCR analysis, we used multiple species for reference

gene amplification. The six primer sets for ACT, AK, COI, EF1, ENO and TUB genes were found to be suitable for *C. pomonella* and *T. leucotreta* while the ACT, AK, EF1 and TUB genes were suitable for *C. peltastica*. These primers will also have a high likelihood of being suitable for other closely-related species for further comparative studies where sequence information is not available.

Ideal reference genes are known to differ among species. Previous studies have compared reference gene selection using two species within genus *Bombus* (Hymenoptera: Apidae) [100] and four species of lepidopteran from different families [64]. As expected more reference genes were found in common for species within the same genus than those from different families. However, the reference genes selected for these species were not identical and therefore further information is needed for the understanding of closley related species and the effect on reference gene selection. Here, we investigated whether the same reference genes could be used for species from the same tribe. No reference genes were stable enough to be used for all three species together or in pairs. COI expression levels differed dramatically between *C. pomonella* and *T. leucotreta* under the same conditions, with Cq differences of up to 21 Cq. These results were similar to those found a study that showed that GAPDH differed between lepidopteron families by up to 14 Cq [64]. Therefore, if taxonomic trends for reference gene selection exist for Tortricidae, it will be at a lower taxonomic level than tribe. This pattern of sample differences in reference gene selection was also found in the tissue comparisons.

Different tissue types were shown to have a major influence on gene stability. This was seen when the tissue samples from all three species were combined in the analysis. This poor gene stability using multiple tissue types is consistent with previous studies [61,62,98,122]. Thus, we assessed reference genes for different tissues separately [98,107]. None of the genes investigated in this study were suitable for *C. peltastica* cuticle tissue normalization and thus other genes should be evaluated for that purpose. However, *C. peltastica* whole body and midgut tissue samples at 25°C could be accurately normalized using AK and TUB as there was good consensus amongst the three analysis programs. All three tissue types included for *C. pomonella* incorporated EF1 as a reference gene, which has previously been found to be stable in different tissue types [100]. AK and EF1 are recommended reference genes for *C. pomonella* whole body extractions and AK, EF1, ENO and TUB are recommended for *C. pomonella* midgut tissue. There was a little consensus among the analysis programs for an additional, suitable gene for cuticle analysis of *C. pomonella*. Therefore, it is suggested that genes not included in this study are evaluated for

potential combination with EF1 for normalisation of *C. pomonella* cuticle tissue. For *T. leucotreta*, whole body tissue required ACT and COI and cuticle tissue required COI and EF1 for normalization. Midgut tissue analysis for *T. leucotreta* found TUB to be a stable gene but as accurate normalization requires genes to be both stable and well paired, the best gene combination was found to be AK and EF1.

Major differences were seen in the same tissue type under different stress conditions in *T. leucotreta*, confirming that tissue type is not the only factor to consider in reference gene selection (Table 2). When *T. leucotreta* midgut tissue samples of uninfected larvae were exposed to 35°C for 48 hours, the analysis showed that only two genes were required for normalization. However, when virus-infected samples at 35°C were also included in the analysis, four genes were required for normalization. This is further evidence to highlight the importance of testing reference genes under the specific conditions of the experiment. Stability could be affected in unexpected ways. At low temperatures AK was shown to be the most stable gene, paired with COI. This result confirms a previous finding [98] that AK performs well at low temperatures. Although EF1 has been found to be a stable gene in insects at high temperatures [61,62,123], our results showed that ACT and AK were the best combination of genes even though EF1 was still suitable for normalization. However, when virus-infected samples were assessed at high temperature EF1 was required for normalization, in combination with ACT, AK and TUB. When all temperature and virus-stress conditions were combined ACT, AK and EF1 was found to provide the best normalization for *T. leucotreta* midgut tissue. Only two studies on reference gene evaluation have been performed using insect viruses. These studies found stable expression of ACT [95] and EF1[122] during the virus infection. In the light of these results, and those produced in this study, it is recommended that ACT, AK and EF1 are used for normalisation of virus-infected *T. leucotreta* larvae at different temperatures.

In summary, this study identified potential reference genes for a number of different tissue types for three species of Tortricidae, which provides a foundation for future Tortricidae reference gene selection and gene expression research. We showed that the same primers can be used to successfully amplify gene regions from a number of species when assessing reference stability, and we identified stable reference genes for use in the following chapters gene *T. leucotreta* gene expression study focusing on the change in expression of CrleGV-SA-infected midgut tissue at different temperatures and times.

Table 1. Primers used for reference gene selection for normalisation of qPCR analysis of three species of Tortricidae

Gene	Primer sequence (5'-3')	Amplicon length (bp)	Reference species	Amplification efficiency % (R^2)		
				C. peltastica	*C. pomonella*	*T. leucotreta*
ACT	F: AAT TAC CAT TGG TAA CGA GCG ATT R: TGC TTC CAT ACC CAG GAA TGA	73	*Drosophila melanogaster* [116]	107.25 (0.965)	101.05 (0.987)	104.31 (0.996)
AK	F: CTA AGG AAA CCC AGC AGC AG R: GGC AGT CAC CAA CCT CTT GT	188	Tortricidae	101.25 (0.985)	106.9 (0.968)	97.85 (0.985)
CO1	F: CCG GGA TCT TTA ATT GGT GA R: CAT CCT GGT CCT GCT CCA TT	171	Tortricidae	2453.55 (0.955)	104 (0.993)	99.6 (0.994)
EF1	F: ACG TCT ACA AAA TCG GCG GT R: GAT GGT GGC AGG TGC GAA TA	61	*Epiphyas postvittana* [46]	107.2 (0.999)	105 (0.985)	101.85 (0.995)
ENO	F: ACT TGG TGC TAA CGC CAT TC R: GCC AAG TCA GCC AAG TGT TT	59	Tortricidae	No amplification	102.5 (0.959)	97.4 (0.987)
TUB	F: ACC CGC GTA TCC ACT TCC C R: AAC TCG CCC TCC TCC ATA CC	Not available	*Plutella xylostella* [117]	94 (0.939)	96 (0.985)	97.8 (0.986)

Table 2. Analysis of candidate reference genes for normalisation of *C. peltastica*, *C. pomonella* and *T. leucotreta* whole body tissue. Values in bold indicate best performing genes for Normfinder and geNorm analyses. Due to possible misleading results resulting from co-regulation, only the best gene combinations are shown for geNorm analysis. No suitable genes were identified for *C. peltastica* cuticle by geNorm analysis

	Bestkeeper analysis			**Normfinder analysis**		**geNorm analysis**	
	Coefficient of Correlation (r)	Power-x (> 2)	Standard Deviation (> 1)	Stability value (> 1)	Standard error (> 1)	M (> 0.5)	CV (> 0.3)
***C. peltastica* whole body**							
ACT	0.985	7.630	2.987	3.016	1.519		
AK	0.999	1.394	0.474	**0.386**	**0.306**	**0.208**	**0.021**
EF1	1.000	1.684	0.733	0.725	0.235		
TUB	0.890	1.123	0.182	0.693	0.234	**0.289**	**0.133**
***C. pomonella* whole body**							
ACT	0.256	1.042	0.064	0.414	0.222		
AK	0.996	1.284	0.302	**0.026**	**0.362**	**0.041**	**0.014**
COI	0.657	2.522	0.159	0.297	0.174		
EF1	0.971	1.943	0.293	0.026	0.362	**0.041**	**0.014**
ENO	0.895	1.865	0.459	0.344	0.192		
TUB	0.903	2.021	0.330	0.221	0.147		
***T. leucotreta* whole body**							
ACT	0.972	2.407	0.358	**0.095**	**0.157**	**0.118**	**0.040**
AK	0.981	1.084	0.057	0.222	0.123		
COI	-0.746	2.666	0.379	0.210	0.122	**0.118**	**0.041**
EF1	0.539	2.595	0.391	0.214	0.122		
ENO	0.956	0.496	0.340	0.726	0.238		
TUB	0.978	4.502	0.611	0.564	0.193		

Table 3. Analysis of candidate reference genes for normalisation of *C. peltastica*, *Cy. pomonella* and *T. leucotreta* midgut tissue. Values in bold indicate best performing genes for Normfinder and geNorm analyses. Due to possible misleading results resulting from co-regulation, only the best gene combinations are shown for geNorm analysis.

	Bestkeeper analysis			**Normfinder analysis**		**geNorm analysis**	
	Coefficient of Correlation (r)	Power-x (> 2)	Standard Deviation (> 1)	Stability value (> 1)	Standard error (> 1)	M (> 0.5)	CV (> 0.3)
***C. peltastica* midgut**							
ACT	0.976	1.639	0.420	0.416	0.171		
AK	0.893	2.160	0.697	0.590	0.204	**0.108**	**0.037**
EF1	0.626	1.386	0.429	**0.126**	**0.237**		
TUB	0.768	1.706	0.580	0.330	0.162	**0.108**	**0.037**
***C. pomonella* midgut**							
ACT	0.972	4.373	1.184	1.308	0.477		
AK	0.988	1.806	0.505	**0.114**	**0.503**	**0.439**	**0.040**
CO1	0.996	3.067	0.950	0.910	0.363		
EF1	0.829	1.742	0.514	0.114	0.503	0.662	0.307
ENO	0.824	2.030	0.698	0.629	0.266	0.721	0.318
TUB	0.869	1.297	0.235	0.218	0.316	**0.550**	**0.200**
***T. leucotreta* midgut**							
ACT	0.252	1.290	0.590	0.795	0.261		
AK	0.981	1.910	0.420	0.165	0.232	**0.152**	**0.054**
CO1	0.887	1.520	0.290	0.254	0.193		
EF1	1.000	2.210	0.500	0.533	0.208	**0.152**	**0.052**
ENO	0.678	2.650	0.780	1.362	0.402		
TUB	0.936	2.860	0.640	**0.163**	**0.234**		

Table 4. Analysis of candidate reference genes for normalisation of *C. peltastica*, *Cy. pomonella* and *T. leucotreta* cuticle tissue. Values in bold indicate best performing genes for Normfinder and geNorm analyses. Due to possible misleading results resulting from co-regulation, only the best gene combinations are shown for geNorm analysis.

	Bestkeeper analysis			**Normfinder analysis**		**geNorm analysis**	
	Coefficient of Correlation (r)	**Power-x (> 2)**	**Standard Deviation (> 1)**	**Stability value (> 1)**	**Standard error (> 1)**	**M (>0.5)**	**CV (>0.3)**
***C. peltastica* cuticle**							
ACT	-0.787	0.797	0.422	1.321	1.323		
AK	-0.532	0.855	0.399	1.495	1.345		
EF1	-0.775	0.647	0.822	2.087	1.489		
TUB	0.999	1.304	0.360	**0.461**	**1.788**		
***C. pomonella* cuticle**							
ACT	-0.787	0.797	0.422	1.038	0.538	**0.256**	**0.088**
AK	0.335	1.233	0.501	**0.158**	**0.305**		
CO1	0.885	1.293	0.249	0.470	0.303		
EF1	-0.262	0.903	0.324	0.483	0.308	**0.256**	**0.088**
ENO	-0.581	0.948	0.071	0.553	0.332		
TUB	0.934	1.667	0.461	0.566	0.337		
***T. leucotreta* cuticle**							
ACT	0.982	1.692	0.775	0.368	0.215		
AK	0.999	2.124	1.098	0.099	0.160		
CO1	0.998	1.878	0.920	0.026	0.399	0.015	0.005
EF1	0.994	1.859	0.908	0.096	0.162	0.015	0.005
ENO	0.994	1.789	0.838	0.189	0.157		
TUB	0.977	2.779	1.462	0.903	0.455		

Table 5 Analysis of candidate reference genes for qPCR normalisation of *T. leucotreta* under different biological conditions, including CrleGV infection at three different temperatures. At 25°C, uninfected midgut samples are as described in Table 4.

	Bestkeeper analysis			**Normfinder analysis**		**geNorm analysis**	
	Coefficient of Correlation (r)	**Power-x (> 2)**	**Standard Deviation (> 1)**	**Stability value (> 1)**	**Standard error (> 1)**	**M (>0.5)**	**CV (>0.3)**
15°C uninfected							
ACT	1.000	11.490	0.280	0.133	0.371		
AK	0.989	3.880	0.150	0.087	0.507	0.047	0.016
CO1	0.522	3.440	0.280	0.373	0.294	0.047	0.016
EF1	-0.370	0.720	0.100	0.155	0.339		
ENO	0.220	4.940	0.850	1.220	0.623		
TUB	-0.055	0.140	0.420	0.772	0.428		
15°C infected + uninfected							
ACT	0.376	1.320	0.200	0.296			
AK	0.936	2.300	0.300	0.095	0.134		
CO1	0.795	1.960	0.300	0.246			
EF1	0.517	1.300	0.160	0.257	0.134	0.046	0.161
ENO	0.537	5.200	1.060	1.064		0.046	0.161
TUB	0.189	1.460	0.620	0.924			
35°C uninfected							
ACT	0.868	4.050	0.860	0.775	0.448	0.103	0.036
AK	0.991	2.930	0.610	0.080	0.797	0.103	0.036
CO1	-0.796	0.730	0.200	1.000	0.539		
EF1	0.948	2.530	0.550	0.080	0.797		
ENO	0.897	5.710	1.010	1.073	0.570		
TUB	0.997	1.270	0.130	0.421	0.342		
35°C infected + uninfected							
ACT	0.641	1.890	0.770	0.404		0.442	0.165
AK	0.707	1.890	0.610	0.369		0.549	0.276
CO1	0.712	1.810	0.700	0.443			
EF1	0.580	1.340	0.380	0.122	0.121	0.459	0.163

	Bestkeeper analysis			Normfinder analysis		geNorm analysis	
	Coefficient of Correlation (r)	**Power-x (> 2)**	**Standard Deviation (> 1)**	**Stability value (> 1)**	**Standard error (> 1)**	**M (>0.5)**	**CV (>0.3)**
ENO	0.624	2.940	1.320	0.937			
TUB	0.932	2.230	0.760	0.187	0.121	0.104	0.036
25°C infected							
ACT	0.767	1.590	0.280	0.056	0.080	0.695	0.253
AK	0.931	3.060	0.530	0.223		0.725	0.337
CO1	0.470	1.490	0.370	0.310			
EF1	0.610	1.490	0.310	0.140	0.080	0.671	0.314
ENO	0.505	1.720	0.520	0.360			
TUB	0.889	3.030	0.490	0.238		0.664	0.282
25°C infected + uninfected							
ACT	0.625	1.900	0.520	0.347		0.309	0.106
AK	0.844	2.550	0.520	0.365	0.184	0.309	0.106
CO1	0.544	1.520	0.380	0.409			
EF1	0.659	1.630	0.360	0.294	0.184		
ENO	0.333	1.480	0.630	0.554			
TUB	0.922	3.250	0.590	0.338			
15°C, 25°C, 35°C infected + uninfected							
ACT	0.613	1.820	0.510	0.420		0.413	0.144
AK	0.788	2.190	0.490	0.377	0.309	0.553	0.270
CO1	0.609	1.730	0.480	0.657			
EF1	0.622	1.490	0.330	0.439	0.309	0.401	0.144
ENO	0.444	2.050	0.900	0.825			
TUB	0.798	2.620	0.650	0.591			

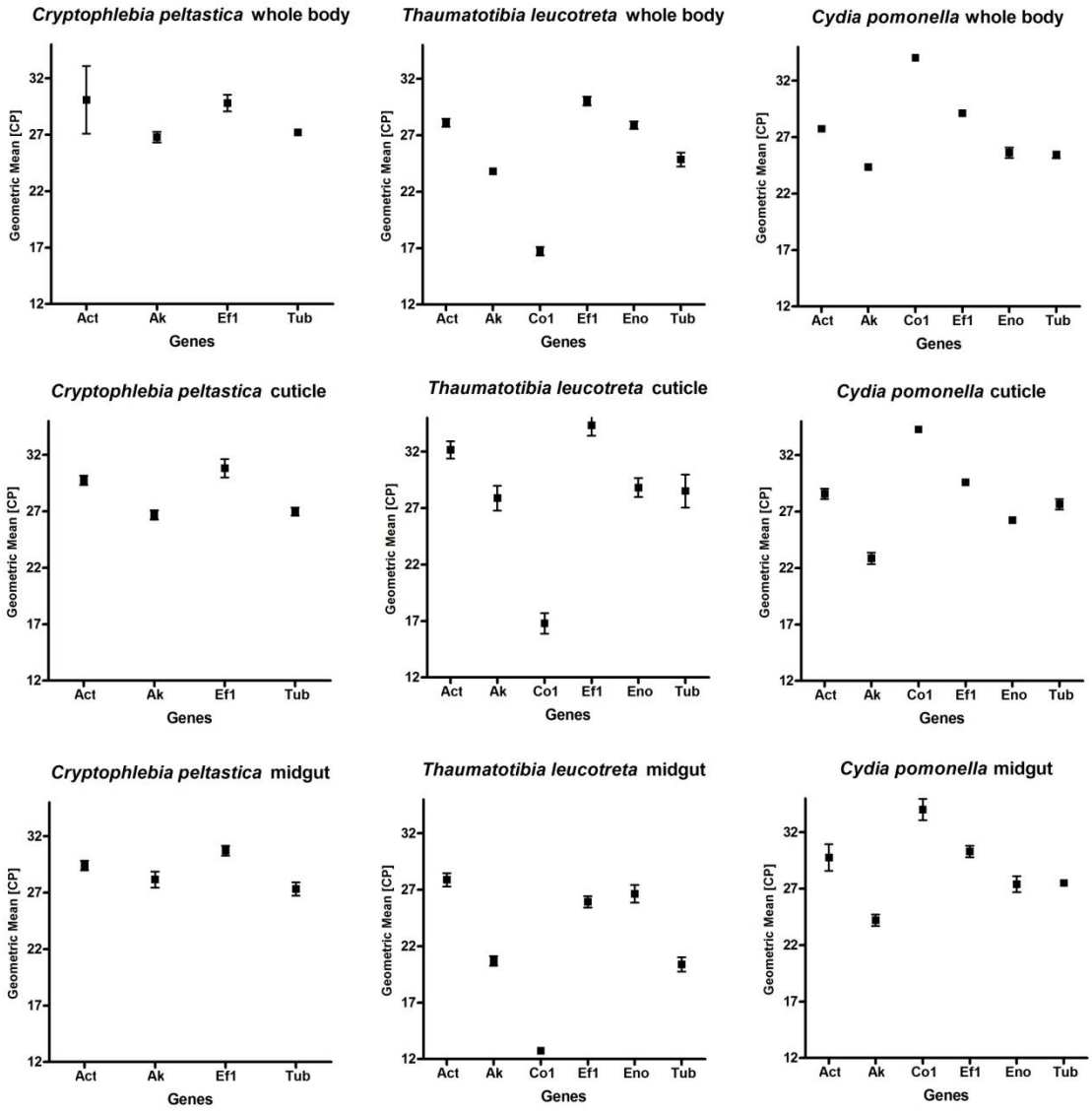

Figure 3. Expression levels for candidate reference genes for three different tissue types and species of Tortricidae. Expression levels of reference genes are expressed in cyclic-threshold (Cp). Boxes indicate Cp means and whiskers standard deviations from the means.

4

Thaumatotibia leucotreta gene expression in response to *Cryptophlebia leucotreta* granulovirus infection under different abiotic conditions

4.1 Abstract

Gene expression analysis is an important tool to improve pest management, particularly with resistance management. The *Cryptophlebia leucotreta* granulovirus (CrleGV) is currently used to control *Thaumatotibia leucotreta*, although resistance development is becoming a concern. Thus, the expression of 13 known virus-associated *T. leucotreta* genes were tested in response to *Cryptophlebia leucotreta* granulovirus (CrleGV) at 24h (early stage), 48h (mid-stage) and 72h (late stage) after infection at 25°C. In addition, changes in expression as a result of CrleGV infection were examined at three different non-lethal temperatures: 15°C, 25°C and 35°C. During the early stages of infection and at 15°C, genes were not highly expressed, and the early and mid-stages of infection showed an overall trend of down-regulation. However, a major shift was observed at late infection stages and at 35°C, when most genes were significantly up-regulated. At 35°C there was a decrease in *T. leucotreta* defence response genes at. This study provides the first insights into the interaction of *T. leucotreta* and its baculovirus at a genomic level. The study also offers important information for increasing the understanding of baculovirus infection processes in Lepidoptera.

4.2 Introduction

The agricultural industry is constantly pressured by new legislation to reduce chemical control measures and implement integrated pest management (IPM) strategies for insect pest control as public knowledge and subsequent public demand for 'more natural' produce increases [17,124]. Integrated pest management strategies incorporate numerous control measures for decreasing pest damage, which includes the use of baculoviruses as biopesticides. Baculoviruses have been accepted as a viable control method for commercial monocultures worldwide, and their production, availability and use is increasing in agricultural systems. Currently, over 50 baculovirus products are available globally for controlling pest species [17]. The family Baculoviridae can be divided into two genera: *Nucleopolyhedroviruses* (NPV) and *Granuloviruses* (GV). Compared to NPV infections, GV are species-specific [125] and currently known only to infect Lepidoptera.

In Africa the most common baculoviruses used as biopesticides are those used against *Cydia pomonella* and *Thaumatotibia leucotreta.* Research is underway to formulate isolates for other endemic pest species. The control of *C. pomonella* using the *Cydia pomonella* granulovirus is one of the most globally successful uses of baculoviruses. *Cydia pomonella* is an introduced pest in South Africa and is a major cause of deciduous fruit losses [126]. The closely related South African endemic pest species *T. leucotreta* is also responsible for large economic losses through direct damage and its phytosanitary status, which threatens export markets. *Cryptophlebia leucotreta* granulovirus, which is the first biopesticide to be commercially produced in Africa (under the product name Cryptogran), has provided good control of *T. leucotreta*. As *C. pomonella* and *T. leucotreta* and their biopesticide controls are so closely related, they provide good pest management models for comparison.

The core principle of IPM is that no individual control measure is sustainable, and this holds true for biopesticide use [17]. In recent years *C. pomonella* has been found to be less susceptible to CpGV. The close relationships between *C. pomonella* and *T. leucotreta* and their viral pathogens suggest that resistance might develop imminently in *T. leucotreta*. Resistance development in *C. pomonella* could be used as a model to avert or reduce the risk of resistance development in *T. leucotreta* in South Africa. Studying the interactions between *T. leucotreta* and CrleGV at a genetic level will allow for better overall IPM control, as the application and management of CrleGV will be based on detailed information about the interaction between the insect and its virus. This could also provide insight for virus recombinants that enhance infectivity [127], improve understanding of resistance

mechanisms [128] and identify selection pressures, such as isolate types or spray regimes, that can be implemented in population management strategies. Such gene expression information could also illuminate abiotic factors, such as temperature, that influence virus infectivity.

Extreme temperatures may inhibit viruses before they reduce larval growth [129]. The upper temperature limits of viruses vary from 29.4°C to 46°C, depending on the species [130–133]. Although CrleGV is still effective at 35°C [113], the upper temperature limits of this virus are unknown. Therefore, as temperature fluctuations can be dramatic under field conditions, it is important to have a detailed understanding of the influence of temperature on virus-insect interactions. This study evaluates the genetic response of *T. leucotreta* to CrleGV infection at different time points after infection and at different temperatures. Identifying key genes in this interaction and the timing of virus activation could allow virus efficacy to be improved. This is the first gene expression analysis of *T. leucotreta* and will provide a platform for future genetic research regarding this pest species and other tortricid pests.

4.3 Methods

4.3.1 Biological treatments and cDNA preparation

First instar *T. leucotreta* larvae were obtained from River BioScience (Port Elizabeth, South Africa) and stored at 25°C (+-3°C), 50-70% relative humidity and a 12h photoperiod.. As the different bioassays required larvae to be exposed to different temperatures for different time points, a range of instars were selected depending on the bioassays, which ensured that at termination of the bioassay only 4th instar larvae were examined. Each bioassay was individually replicated three times. The optimum temperature for *T. leucotreta* development is 25°C [134]. Therefore, this temperature was used to assess gene expression at three different time points after virus infection. Preliminary studies using PCR found that after 48 h.p.i. there was strong evidence of infection, and that after 96 h.p.i. RNA was degraded when extracted (Chapter 3). Therefore, 24 h.p.i., 48 h.p.i. and 72 h.p.i. were chosen for evaluation as they were equally separated by 24h, and tissue extractions still provided good quality RNA.

The effect of temperature on the CrleGV infection process was examined by comparing uninfected and infected individuals at 15°C, 25°C and 35°C at 48 h.p.i. As 25°C is the known optimal temperature for *T. leucotreta,* evaluating populations at 10°C above and 10°C below this optimal provided two additional sublethal temperatures towards the upper and lower

temperature extremes. All RNA extractions and reverse transcription of RNA were as described in Chapter 3.

4.3.2 qPCR analysis

4.3.2.1 Reference gene selection

The reference genes actin (ACT), arginine kinase (AK) and elongation factor 1-α (EF1) were selected for analysis (Chapter 3).

4.3.2.2 Target gene primers

A cDNA library for *T. leucotreta* was sequenced by Inqaba Biotechnical Industries (Pretoria, South Africa) on the Illumina sequencing platform using manufacturer's protocols (Illumina, San Diego, CA). As it is beyond the scope of this thesis, the transcriptome results will not be presented in this study. However, *g*ene sequences were selected from this transcriptome data to design primers for target genes of *T. leucotreta*. Genes known to be associated with virus infection in insects were selected for analysis if these sequences were identified from the *T. leucotreta* transcriptome.

All target gene primers were designed using Primer3Plus version 0.4.0 [118]. Parameters for primer design included a GC content of 50-60%, melting temperature between 60-63°C, max 3' self complimentarity of 1, amplicon length between 60-120 bp, max poly-x = 3, primer length between 18 bp and 20 bp, paired toward 3'end and a max Tm difference of 10°C between forward and reverse primers. Ten primers sets (Table 1) were designed for amplification of nine different target genes, cell Death (CD), dicer-2 (DI), gelsolin (GEL), glutathione peroxidise (GPO), heat shock protein 70 (HSP70), heat shock protein 90 (HSP90), initiation factor 5a (I5A), juvenile hormone epoxide hydrolase (JHEH), and mitochondrial aldehyde dehydrogenase (MAD). In addition, three target genes that are involved in metabolic pathways and initially evaluated as reference genes, enolase (ENO), α tubulin (TUB) and cytochrome oxidase I (COI), were also included in analyses as target genes as they showed differential expression under the experimental conditions (Chapter 3). Primer efficacy testing was performed using a ten-times dilution series (10 ng/µl, 100 ng/µl and 1000 ng/µl). Three technical replicates were included for each dilution. The cDNA used for this efficiency test was a pool of eight individual 4th instar *T. leucotreta* larvae. Primer amplification efficiencies, correlation coefficients and melt curve data were analysed using a CFX Connect ™ (Bio-Rad, CA, USA) program, with details described in Chapter 3.

4.3.2.3 *qPCR conditions*

Three technical replicates were performed for each bioassay. Reactions were performed in a final volume of 10 µl, which consisted of 0.25 µl of each primer at a concentration of 400 nM, *5* µl iTaq^{TM} Universal SYBR Green Supermix (Bio-Rad, CA, USA), 1 µl of cDNA (+/- 450 ng/µl) and 3.5 µl of H_2O. Non-template controls (NTC) using 1 µl of sterile H_2O to replace the cDNA template were included. The thermal cycle program used was as previously described [116]. All qPCR experiments were conducted using the CFX Connect ^{TM} Real-Time PCR detection system (Bio-Rad, CA, USA).

4.3.2.4 *Statistical analysis*

Gene expression analysis and all statistical analyses were performed using the CFX Connect ^{TM} Manager software (Bio-Rad, CA, USA). For qPCR, the raw data consisted of the PCR cycle number required to reach a fluorescence threshold (Ct). Gene expression fold-change was calculated using the 'normalization with identified control' technique (Bio-Rad, CA, USA). The $2^{-\Delta\Delta Cq}$ method was used for normalizing target genes to the reference genes. For gene expression analysis a change of two-fold was regarded as significant. Statistical differences were calculated using ANOVA to determine the significance of the samples occurring at random if no correlation exists, at a 0.05 level of significance.

4.4 Results

This study conforms with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [1]. RNA quality and all sample validations are as stated in Chapter 3, with details of individual samples provided in Supplementary Tables 3 and 4. All NTC were expressed at a level of >45Ct, confirming that reactions were not contaminated. All changes above the two-fold threshold were found to be significant based on ANOVA, confirming the reliability of the data. As an internal control, two primers sets were designed for the JHEH gene to assess whether the expression observed was consistent and independent of the primer used for amplification.

4.4.1 Primer optimization and amplification

All 15 genes were amplified with an efficiency between 90-110% and all correlation coefficients were r > 0.95 (Table 6). A single melt curve peak was present for all genes,

confirming that primer dimers were not present. All target gene amplifications values were between 11 and 26 Cq and well below the recommended cut-off value of 35 [1].

4.4.2 Virus infection at different time points

The change in insect gene expression over periods of 24, 48 and 72 h.p.i. as result of infection by CrleGV was measured at a constant temperature of 25°C. At 24 h.p.i. JHEHa (−2.14-fold) and JHEHb (−2.2-fold) were significantly down-regulated and were the only differentially transcribed genes after 24 h.p.i. After 48 h.p.i, JHEHa and JHEHb were further down-regulated −3.36-fold and −4.2-fold (Table 7). In addition COI (−2.03-fold), ENO (−2.31-fold), MAD (−2.48-fold), GEL (−2.72-fold) and GPO (3.58-fold) were also significantly down-regulated after 48 h.p.i. The only significant up-regulation at 48 h.p.i. was DI (2.1-fold). A large shift in expression was seen at 72 h.p.i (Table 7). Whereas most genes were significantly down-regulated after 48 h.p.i., after 72 h.p.i. no genes were down-regulated. However, five genes were significantly up-regulated after 72 h.p.i., including CD (3.22-fold), DI (2.84-fold), JHEHa (2.66-fold), JHEHb (2.75-fold) and HSP70 (2.05-fold)(Table 7).

4.4.3 Virus infection at different temperatures

Larvae infected with the virus at 15°C showed the fewest change in differentially expressed genes compared to uninfected larvae. Only DI (−2.04-fold) and TUB (−2.21-fold) were significantly different between infected and uninfected samples in larvae maintained at 15°C. Virus-infected samples at 25°C showed much higher levels of gene expression than at 15°C compared to uninfected samples. At 25°C significant down-regulation was found for COI (−2.03-fold), ENO (−2.31 -fold), MAD (−2.48 -fold), GPO (−3.58-fold), JHEHa (−3.36-fold) and JHEHb (−4.2-fold). Only GEL was found to be up-regulated in virus-infected samples, with a change of 2.72-fold. At 35°C there were fewer genes with significant changes. However, the changes seen with genes that regulate the cell basic processes were more extreme than those observed at 25°C. In addition, these changes all showed a significant up-regulation relative to uninfected larvae (Table 8).

4.5 Discussion

This study is the first analysis of gene expression of *T. leucotreta* larvae exposed to a baculovirus infection. These results are useful for understanding the interaction between CrleGV infection and *T. leucotreta* and provide a basis for further genetic studies on *T. leucotreta*. Thirteen genes were evaluated that provided insight into gene expression in *T. leucotreta*, which provides a platform for future gene expression research within the Lepidoptera. Eleven of the 13 genes had a significant change above the two-fold threshold. In this study, only two genes were not differentially expressed: HSP90 and TI5A. Although HSP90 is classically associated with stress responses, there is evidence that it is expressed at stable levels in certain species, and it has even been recommended as a potential reference gene [62]. This stability is confirmed by our results. The limited expression found in the TI5A gene could possibly be a result of an early expression peak, which can start as early as early as 6 h.p.i. with the latest time point being 24 h.p.i. [45], and therefore this gene might not be expressed at later time points [45,135,136]. Evidence suggests that baculoviruses rely on the insect host for translation and therefore, translation initiation factors and other translation supporting genes will be up-regulated in the host during infection. However, TI5A is not always a vital gene in relation to infection, and therefore it is not concerning that no changes were seen. The genes selected in this study provide good representation for assessing responses of *T. leucotreta* to CrleGV infections.

Reliability of gene expression data is crucial for understanding the host-virus interactions, as slight variations can alter the interpretation of the interaction. The similarity of expression results for both primers' for the JHEH gene supports the reliability of these results. Therefore, it can be assumed that the experiments showed no random expression values, and that the observed regulations were gene-dependent as opposed to primer-dependent. The reliability of the results presented in this chapter is underpinned by the compliance with the Minimum Requirements for Quantitative PCR Experiments (MIQE) guidelines, the experiment-specific reference gene selection presented in Chapter 3, the high quality of the RNA and primers used and the addition of internal controls.

4.5.1 Virus infection at different time points

Differential gene expression as a result of baculovirus infection in the midgut of tortricids was noted as early as 1.5 h.p.i. [44,135,136]. However, our results show little

differential expression between infected and control samples at 24 h.p.i. This could be a result of the first time point (24 h.p.i.) being too late to show the very early expression period. The only gene to show a change above the two-fold threshold at 24 h.p.i. was JHEH. JHEH was shown to be differentially down-regulated. This gene has a known association with a variety of different cellular functions [137], the most common of which is the degradation of juvenile hormone, which promotes insect maturation [138,139]. JHEH expression in *Manduca sexta* (Linnaeus, 1763) (Sphingidae: Lepidoptera) infected with baculovirus increased dramatically from 48-120 h.p.i. [140], which can be expected since accelerated maturity is a common defence response in stressed insects. JHEH was also found to be significantly down-regulated during baculovirus infection of *Epiphyas postvittana* (Walker, 1863) (Tortricidae: Lepidoptera) [46]. Since some baculoviruses are known to prevent pupation to counteract the insect defence response [141], this could explain the increasing down-regulation of JHEH found in *T. leucotreta* at 24 h.p.i. and 48 h.p.i.

The number of genes showing a change of two-fold or more increased from two to eight between 24 h.p.i. and 48 h.p.i. The JHEH gene was further down-regulated at 48 h.p.i. The lack of JHEH gene expression suggests that the CrleGV has an aggressive suppression of *T. leucotreta* maturation and that this is a key part of the infection process. Expression of the GEL gene was also decreased further at 48 h.p.i. As this would potentially increase apoptosis and cause major cell death events after 48 hours, it would be speculative to identify whether this is promoted by the insect, or the virus or is a passive side effect of infection.

GPO showed a significant decrease in down-regulation from 24 h.p.i. to 48 h.p.i. The GPO gene expression is known to be effected by virus infection with the major cause for down-regulation being oxidative stress [142–144]. The presence of oxidative stress in insect cells is a result of an increased oxygen demand created by the virus infection [145]. This oxidative stress is most likely the cause of the down-regulation found in the COI gene, which is involved in the oxidative phosphorylation metabolic pathway. This was consistent with the other two genes involved with metabolism, ENO and MAD, which were down-regulated slightly more than COI.

The only gene to be up-regulated across two different time points was DI, which showed a gradual increase in up-regulation, peaking at 72 h.p.i. DI is classically involved with insect defence against virus infections [146,147]. The DI gene counteracts viral attack by cutting double-stranded viral RNA, resulting in small interfering RNAs (siRNAs) [148]. These interfering siRNAs then degrade the viral transcripts to disrupt the virus infection process [45,148]. The up-regulation of DI therefore suggests a *T. leucotreta* defence response

to CrleGV infection. The increase in expression from 48 h.p.i. with the peak at 72 h.p.i. is consistent with literature indicating differential transcription of DI as a late infection response [45,148].

A major shift in gene expression was seen between 48 and 72 h.p.i. Five genes which were mostly down-regulated below the two-fold threshold after 48 h.p.i. were up-regulated above the two-fold threshold after 72 h.p.i. The greatest shift from down-regulation at 48 h.p.i. to up-regulation at 72 h.p.i. involved the JHEH gene, which is indirectly associated with insect defence response [141]. JHEH shifted 6.95-fold, which could potentially be a result of insect defences identifying the infection and accelerating maturation. It is important to note that, the other two genes directly associated with insect defence responses, CD and DI, were up-regulated at their highest levels at 72 h.p.i. This indicates that at the period between 48 h.p.i. and 72 h.p.i. there is a dramatic initiation of *T. leucotreta* genetic defence responses. Although HSP70 is commonly associated with stress responses in insects, its main cellular function is stabilizing stressed proteins for the maintenance of cellular processes. The up-regulation of HSP70 suggests an initiation of the insect's cell maintenance in the presence of damaged proteins [149]. Therefore, these results suggest mobilization of a variety of insect defence responses between 48 and 72 h.p.i. This delayed response to virus infection could be attributed to the success of the CrleGV infection process, as the virus may have suppressed some of the insects' defences at the earlier time points.

4.5.2 Virus infection at different temperatures

The influence of virus infection at different temperatures is largely affected by the speed of the metabolic processes of the insect [105,113]. This is supported by our results of CrleGV infection of *T. leucotreta* at 15°C, 25°C and 35°C. At 15°C there was limited change in gene expression for all genes except DI and TUB, in relation to viral infection. The down-regulation found with the DI gene is important as it is involved directly with insect defence responses by producing siRNAs. The down-regulation of DI would therefore suggest some breakdown in the hosts defences. This disruption suggests that the interaction between the virus and the insect at 15°C inhibits the insect defence response. This is supported by the up-regulation of DI at 25°C and 35°C [150]. Temperature dependency for host defences against virus attack has been observed in other organisms such as plants, where a direct correlation was shown between temperature increase and the presence of siRNAs [150]. The TUB gene showed similar up-regulated expression at 15°C and 35°C but not at 25°C. The up-regulation

of TUB could be the result of direct recruitment by the viral infection. It is well known that the host cytoskeletal network is responsible for the transport of viruses within the cell [151,152]. The up-regulation of TUB is therefore likely to the a result of direct recruitment of the virus. Viruses have been known to hijack molecular motors to migrate around subcellular structures [152]. The reduced expression level of TUB expression at 25°C indicates a reduction in viral manipulation of the cytoskeletal structures potentially as a result of some unknown host defences not shown with this study's target genes. The unidentified factors inhibiting the virus cytoskeletal manipulation at 25°C is confirmed by the constant expression of TUB below the two-fold threshold, found at all time points.

As with the dramatic shift seen in gene expression between 48 h.p.i. and 72 h.p.i. at 25°C, a similar change was seen in virus-infected samples maintained at 25°C and 35°C after 48 h.p.i. In larvae maintained at 35°C, the trend in expression change from down-regulation to up-regulation, with five genes up-regulated above the two-fold threshold. As seen with the 72 h.p.i. at 25°C results, 35°C had an up-regulation of JHEH at a very similar fold level. This again would suggest an indirect defence response from the host. However, the implications of this response on infection would most likely be irrelevant at this stage. It is known that the rate of infection is affected by metabolic rate and therefore directly affected by temperature. When taking the infection rate (metabolic rate) into account, the infection stage at 35°C after 48 h.p.i. is most similar to infection at 25°C after 72 h.p.i. However, unlike the gene expression observed at 25°C after 72 h.p.i, larvae at 35°C showed no up-regulation of genes directly associated with insect defence. This would therefore suggest that, at a similar stage of infection, *T. leucotreta* maintained at 35°C will have weaker defence against CrleGV than at 25°C.

4.6 Conclusion

The difference found between mid- and late–infection results reveal strong trends in the shift of insect gene expression between early and late stages of infection and the effects of temperature change. As only three temperatures were examined, it would be advantageous for future research to incorporate finer temperature intervals with the addition of developmental models to further understand the genetic interactions between *T. leucotreta* and CrleGV. At 15°C after 24 h.p.i. infected *T. leucotreta* showed limited gene expression changes above the two-fold threshold, with a mostly down-regulated trend. The lack of *T. leucotreta* virus defence genes shown at 35°C reveals that not only does the rate of infection increase due to

increased metabolism, but also that the high temperature stress inhibits the likelihood of *T. leucotreta* suppressing the CrleGV infection. The immediate implications of this finding could make temperature a more important factor when planning spray regimes for *T. leucotreta* pest populations, with future implications for improving virus efficacy and genetic resistance research.

Table 6. Primers used for qPCR gene expression analysis of *T. leucotreta.* Efficiency values (E (%)) represent the amplification efficiencies of the primer pairs. Coefficient of determination (R^2) values represent the goodness of fit of the primer pairs when amplifying 10x cDNA dilutions.

Gene name	Primer sequence (5'-3')	Amplicon length (bp)	Source	E (%)	R^2
Cell Death	F: TCTCCCAGCTGGAACTTCTG R: ATAGTGCTGGACGTGCCACT	80	*T. leucotreta*	91.4	0.993
Cytochrome oxidase 1	F: CCGGGATCTTTAATTGGTGA R: CATCCTGGTCCTGCTCCATT	171	Tortricidae	99.6	0.994
Dicer 2	F: GCTAGGCAGCATAGTCGCTTT R: GGGCGATGACAAACCTAAGA	65	*T. leucotreta*	100.8	0.995
Enolase	F: ACTTGGTGCTAACGCCATTC R: GCCAAGTCAGCCAAGTGTTT	59	Tortricidae	97.4	0.995
Juvenile hormone epoxide hydrolase (a)	F: CTGGCTACGACTTCGCTTTC R: ACTGCGCCATCAGTAAATCC	82	*T. leucotreta*	97.5	0.999
Juvenile hormone epoxide hydrolase (b)	F: TACCAGCATCAGACCGTTCA R: CGTCCATTGCGATGGTT	88	*T. leucotreta*	101.7	0.996
Gelsolin	F: GGCCTTACAAGCAGAAGCAC R: TTTGTTTCGGCGTCCACCTC	66	*T. leucotreta*	98.3	0.998
Glutathione peroxidase	F: AAGGGTGCCTCCTTGCTTAT R: GGGGATCCTGAGGCTATTGT	94	*T. leucotreta*	97.8	0.998
Heat Shock Protein 70	F: GGTGGAAGAGGAGAGGGTTC R: ACCTCACCACCAACAAGAGG	80	*T. leucotreta*	95.3	0.998

Gene name	Primer sequence (5'-3')	Amplicon length (bp)	Source	E (%)	R^2
Heat Shock Protein 90	F: AGATCGAGGATGTCGGTGAG R: CAGATGGGCTTGGTCTTGTT	78	*T. leucotreta*	100.5	0.999
Initiation factor 5a (ref AF109731)	F: CGAGAGCCGTATTTGCCTTA R: GATCCCTGATGGTGACTTGG	88	*T. leucotreta*	101.5	0.997
Mitochondrial aldehyde dehydrogenase	F: ATCTGCGAAGGTCCAAGATG R: TGCAGCATGCTCTTCAAGTC	76	*T. leucotreta*	97.2	0.999
α - Tubulin	F: ACCCGCGTATCCACTTCCC R: AACTCGCCCTCCTCCATACC	Not available	Lee *et al.*, 2007	97.8	0.986

Table 7: Fold change difference between control and CrleGV infected *T. leucotreta* at different time points post infection, with values shown for infected larvae. Bold values identify significant fold changes above the 2-fold threshold. P-values represent the likelihood of the value occurring at random.

Gene name	**Gene function**	**24hpi**		**48hpi**		**72hpi**	
		change	**p-value**	**change**	**p-value**	**change**	**p-value**
Cell Death	Defence	1.14	0.814	-1.26	0.126	**3.22**	**0.000**
Gelsolin	Cytoskeletal maintenance	-2.21	0.416	**-2.72**	**0.000**	1.12	0.755
Cytochrome oxidase 1	Metabolism	1.38	0.019	**-2.03**	**0.002**	1.28	0.065
Dicer	Defence	1.86	0.036	**2.10**	**0.000**	**2.84**	**0.000**
Enolase	Metabolism	-1.92	0.048	**-2.31**	**0.013**	-1.1	0.466
Juvenile hormone epoxide hydrolase (a)	Juvenility	**-2.14**	**0.001**	**-3.36**	**0.000**	**2.66**	**0.003**
Juvenile hormone epoxide hydrolase (b)	Juvenility	**-2.20**	**0.000**	**-4.20**	**0.000**	**2.75**	**0.010**
Glutathione peroxidase	Metabolism	-1.58	0.028	**-3.58**	**0.000**	1.25	0.004
Heat Shock Protein 70	Cell stabilization	-1.21	0.461	-1.53	0.015	**2.05**	**0.001**
Heat Shock Protein 90	Cell stabilization	-1.06	0.949	-1.59	0.000	1.17	0.450
Initiation factor 5a ref AF109731	Cell maintanence	1	0.942	-1.7	0.000	1.2	0.137
Mitochondrial aldehyde dehydrogenase	Metabolism	-1.04	0.976	**-2.48**	**0.000**	1.23	0.163
α-Tubulin	Cytoskeletal maintenance	1.36	0.246	1.18	0.199	1.1	0.795

Table 8. Fold change difference between control and CrleGV infected *T. leucotreta* at different temperatures, with values shown for infected larvae. Bold values identify fold changes above the 2-fold threshold. P-value represents the likelihood of the value occurring at random.

Gene name	**Gene function**	**15°C**		**25°C**		**35°C**	
		change	**p-value**	**change**	**p-value**	**change**	**p-value**
Cell Death	Defence	-1.59	0.074	-1.26	0.126	-1.19	0.000
Gelsolin	Cytoskeletal maintenance	1.52	0.248	**-2.72**	**0.000**	**4.69**	**0.000**
Cytochrome oxidase 1	Metabolism	1.09	0.452	**-2.03**	**0.002**	**2.28**	**0.000**
Dicer	Defence	**-2.04**	**0.003**	**2.1**	**0.000**	1.59	0.000
Enolase	Metabolism	-1.01	0.641	**-2.31**	**0.013**	**5.32**	**0.003**
Juvenile hormone epoxide hydrolase(a)	Juvenility	-1.85	0.247	**-3.36**	**0.000**	**2.62**	**0.000**
Juvenile hormone epoxide hydrolase (b)	Juvenility	-1	0.924	**-4.2**	**0.000**	**2.3**	**0.000**
Glutathione peroxidase	Metabolism	-1.41	0.002	**-3.58**	**0.000**	1.49	0.000
Heat Shock Protein 70	Cell stabilization	-1.16	0.294	-1.53	0.015	1.47	0.000
Heat Shock Protein 90	Cell stabilization	1.02	0.874	-1.59	0.000	-1.04	0.000
Initiation factor 5a	Cell maintenance	1.34	0.037	-1.7	0.000	1.38	0.000
Mitochondrial aldehyde dehydrogenase	Metabolism	-1.02	0.865	**-2.48**	**0.000**	1	0.000
α- Tubulin	Cytoskeletal maintenance	**2.21**	**0.000**	1.18	0.199	**2.11**	**0.000**

5

General Conclusion

Studies in gene expression provide detailed information on genetic interactions among species [45]. Over the last decade, these studies have become increasingly popular as the available technology to execute them has become more readily available. With the increased availability of these tools, the theoretical foundation of gene expression is advancing steadily towards greater application. This was demonstrated by *T. leucotreta* and its baculovirus CrleGV.

Understanding the genetic interaction between *T. leucotreta* and its baculovirus CrleGV is important for advancing the management of *T. leucotreta* populations in agricultural ecosystems. Therefore, the focus of this study was to examine the genetic response of *T. leucotreta* to CrleGV at different temperatures and at different points in the infection process. To address this aim, two preliminary objectives needed to be achieved. 1) Determination of the most suitable RNA extraction method for insect tissue; and 2) identification of the most suitable reference genes for *T. leucotreta* under various thermal and viral stresses. These preliminary objectives were completed using *T. leucotreta* and additional species, thus providing fundamental information for molecular entomology research with particular focus on agricultural pests in the Tortricidae.

5.2 Evaluation of RNA extraction methods

Four RNA extraction methods were evaluated: RNeasy® Mini Kit (Qiagen), SV Total RNA Isolation System (Promega), TRIzol® reagent (Invitrogen), and a CTAB-based method. These methods were used to extract RNA from four insect species, including two Lepidoptera (*Thaumatotibia leucotreta* and *Plutella xylostella*) and two Coleoptera (*Thanatophilus micans*

and *Tenebrio molitor*). By incorporating a number of insect species, the data could be evaluated to assess the most robust method for insect RNA extraction. No differences were found in the quality of isolated RNA from the four different species. For *T. Leucotreta, P. Xylostella, T.micans and T. molitor* the best RNA extraction method was the SV total RNA isolation system followed by the RNeasy® Mini Kit, TRIzol® reagent, and the CTAB-based method. Although the RNeasy® method provided high quality RNA, sometimes higher than the SV Total RNA Isolation System, the RNA isolated with this method contained higher levels of DNA contamination. The RNeasy® method therefore required an additional DNase step, decreased the RNA quality. Hence, the SV Total RNA Isolation System is recommended for RNA extractions from insect larvae including a range of species and this method was used throughout this thesis.

5.3 Reference gene selection

The first detailed evaluation of reference genes for qPCR was compleated using three different insect species: *Thaumatotibia leucotreta*, *Cryptophlebia peltastica* and *Cydic pomonella* (Totricidae: Grapholitini). This groundwork is key for future studies of gene expression within the Tortricidae because it provides candidate reference genes for members of this family. The six genes evaluated were ACT, AK, COI, EF1, ENO, and TUB. The most suitable reference genes were described for each insect species and each tissue type, (i.e. whole body, cuticle and midgut tissue). In addition, *T. leucotreta* was evaluated under viral and thermal stress. These results provided reference gene information for a broad range of uniquely different potential experiments. Trends showed that different species required different reference genes. Within each species, tissue differences were found, and cuticle tissue resulted in few suitable reference genes. For all conditions evaluated with *T. leucotreta*, ACT, AK and EF1 provided the most stable expression; hence, they were used for the normalization of qPCR throughout this study.

5.4 Target gene expression responses to *T. leucotreta* midgut CrleGV infection at different times and temperatures

A comparison of expression levels for 12 target genes showed important changes to key defence response genes in the midgut of *T. leucotreta* during the infection process. Most of the genes used in this study are known to be involved with insect-virus interaction are

activated only during the late stages of infection. Although there was the expected late stage infection gene activation at 35°C, at this temperature genes directly involved with defence were expressed at lower levels than at 25°C. This implies that *T. leucotreta* defence to CrleGV is reduced at higher temperatures an important consideration for pest management strategies.

The dynamic gene expression response of *T. leucotreta* to CrleGV infection under different temperature and different intervals not only provide insight into potential management strategies of this pest species, but they contribute to our understanding of insect virus interactions in general.

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Supplementary Table 1**.** Individual insect sample inoculated with CrleGV. Eight individual larva midgut were pooled into one extraction.

Sample	**Pre-inoculation weight (grams)**	**Post-inoculation weight (grams)**	**Liquid uptake weight (grams)**	**Occlusion bodies uptake**	**Sum of group occlusion bodies uptake**	**Mean group occlusion bodies uptake**	**Extraction number**
A-Cryptogran-24h-1	12.42	14.78	2.36	118000000	24h-Gran-ExA	90062500	RT1i
A-Cryptogran-24h-2	9.61	11.21	1.6	80000000			RT1i
A-Cryptogran-24h-3	12.57	13.78	1.21	60500000			RT1i
A-Cryptogran-24h-4	8.72	10.52	1.8	90000000			RT1i
A-Cryptogran-24h-5	12.03	14.07	2.04	102000000			RT1i
A-Cryptogran-24h-6	5.73	7.16	1.43	71500000			RT1i
A-Cryptogran-24h-7	10.34	12.16	1.82	91000000			RT1i
A-Cryptogran-24h-8	8.56	10.71	2.15	107500000			RT1i
A-Cryptogran-48h-1	6.73	7.74	1.01	50500000	ExA-Gran	82562500	RT5i
A-Cryptogran-48h-2	9.34	11.44	2.1	105000000			RT5i
A-Cryptogran-48h-3	7.59	8.63	1.04	52000000			RT5i
A-Cryptogran-48h-4	8.34	9.87	1.53	76500000			RT5i
A-Cryptogran-48h-5	7.12	8.51	1.39	69500000			RT5i
A-Cryptogran-48h-6	9.9	12.6	2.7	135000000			RT5i
A-Cryptogran-48h-7	6.94	8.59	1.65	82500000			RT5i
A-Cryptogran-48h-8	5.91	7.7	1.79	89500000			RT5i
A-Cryptogran-72h-1	6.77	8.92	2.15	107500000	72h Gran1	72312500	RT9i
A-Cryptogran-72h-2	5.92	6.85	0.93	46500000			RT9i
A-Cryptogran-72h-3	7.09	8.71	1.62	81000000			RT9i
A-Cryptogran-72h-4	5.98	7.56	1.58	79000000			RT9i
A-Cryptogran-72h-5	6.67	7.49	0.82	41000000			RT9i

Sample	Pre-inoculation weight (grams)	Post-inoculation weight (grams)	Liquid uptake weight (grams)	Occlusion bodies uptake	Sum of group occlusion bodies uptake	Mean group occlusion bodies uptake	Extraction number
A-Cryptogran-72h-6	4.6	6.01	1.41	70500000			RT9i
A-Cryptogran-72h-7	4.5	5.93	1.43	71500000			RT9i
A-Cryptogran-72h-8	7.34	8.97	1.63	81500000			RT9i
B-Cryptogran-24h-1	10.72	11.78	1.06	53000000	24-ExB	54375000	RT2i
B-Cryptogran-24h-2	9.67	10.84	1.17	58500000			RT2i
B-Cryptogran-24h-3	11.66	12.97	1.31	65500000			RT2i
B-Cryptogran-24h-4	8.54	9.23	0.69	34500000			RT2i
B-Cryptogran-24h-5	9.46	10.74	1.28	64000000			RT2i
B-Cryptogran-24h-6	10.62	11.77	1.15	57500000			RT2i
B-Cryptogran-24h-7	12.21	13.25	1.04	52000000			RT2i
B-Cryptogran-24h-8	11.17	12.17	1	50000000			RT2i
B-Cryptogran-48h-1	10.99	13.2	2.21	110500000	48h Ex B Gran	63812500	RT4i
B-Cryptogran-48h-2	6.93	8.1	1.17	58500000			RT4i
B-Cryptogran-48h-3	7.15	8.1	0.95	47500000			RT4i
B-Cryptogran-48h-4	6.3	7.34	1.04	52000000			RT4i
B-Cryptogran-48h-5	7.25	8.8	1.55	77500000			RT4i
B-Cryptogran-48h-6	6.38	7.68	1.3	65000000			RT4i
B-Cryptogran-48h-7	9.12	10.14	1.02	51000000			RT4i
B-Cryptogran-48h-8	7.01	7.98	0.97	48500000			RT4i
B-Cryptogran-72h-1	5.66	6.72	1.06	53000000	72h Gran 2	51750000	RT7i
B-Cryptogran-72h-2	9.38	10.68	1.3	65000000			RT7i
B-Cryptogran-72h-3	7	8.19	1.19	59500000			RT7i
B-Cryptogran-72h-4	6.09	7.02	0.93	46500000			RT7i

Sample	Pre-inoculation weight (grams)	Post-inoculation weight (grams)	Liquid uptake weight (grams)	Occlusion bodies uptake	Sum of group occlusion bodies uptake	Mean group occlusion bodies uptake	Extraction number
B-Cryptogran-72h-5	6.47	7.28	0.81	40500000			RT7i
B-Cryptogran-72h-6	5.98	7.35	1.37	68500000			RT7i
B-Cryptogran-72h-7	5.23	6.37	1.14	57000000			RT7i
B-Cryptogran-72h-8	6.55	7.03	0.48	24000000			RT7i
C-Cryptogran-24h-1	10.24	11	0.76	38000000	24-Gran-ExB	44125000	RT3i
C-Cryptogran-24h-2	12.49	13.21	0.72	36000000			RT3i
C-Cryptogran-24h-3	9.03	10.04	1.01	50500000			RT3i
C-Cryptogran-24h-4	9.43	10.09	0.66	33000000			RT3i
C-Cryptogran-24h-5	7.69	8.14	0.45	22500000			RT3i
C-Cryptogran-24h-6	11.25	12.38	1.13	56500000			RT3i
C-Cryptogran-24h-7	10.73	11.96	1.23	61500000			RT3i
C-Cryptogran-24h-8	7.44	8.54	1.1	55000000			RT3i
C-Cryptogran-48h-1	8.96	9.87	0.91	45500000	48h-ExC-Gran	41437500	RT6i
C-Cryptogran-48h-2	8.94	9.82	0.88	44000000			RT6i
C-Cryptogran-48h-3	8.74	9.42	0.68	34000000			RT6i
C-Cryptogran-48h-4	7.99	8.98	0.99	49500000			RT6i
C-Cryptogran-48h-5	6.96	8.05	1.09	54500000			RT6i
C-Cryptogran-48h-6	7.66	8.1	0.44	22000000			RT6i
C-Cryptogran-48h-7	8.8	9.55	0.75	37500000			RT6i
C-Cryptogran-48h-8	8.18	9.07	0.89	44500000			RT6i
C-Cryptogran-72h-1	11.08	11.78	0.7	35000000	72h-Gran 3	48750000	RT8i
C-Cryptogran-72h-2	10.63	11.86	1.23	61500000			RT8i
C-Cryptogran-72h-3	10.63	11.92	1.29	64500000			RT8i

Sample	Pre-inoculation weight (grams)	Post-inoculation weight (grams)	Liquid uptake weight (grams)	Occlusion bodies uptake	Sum of group occlusion bodies uptake	Mean group occlusion bodies uptake	Extraction number
C-Cryptogran-72h-4	9.37	10.7	1.33	66500000			RT8i
C-Cryptogran-72h-5	9.8	10.65	0.85	42500000			RT8i
C-Cryptogran-72h-6	7.42	8.27	0.85	42500000			RT8i
C-Cryptogran-72h-7	10.94	11.65	0.71	35500000			RT8i
C-Cryptogran-72h-8	10.03	10.87	0.84	42000000			RT8i
A-Cryptogran-48h-15°C -1	9.8	10.33	0.53	26500000	Infected15°C48h#2(4)	48125000	RT10i
A-Cryptogran-48h-15°C -2	10.6	11.02	0.42	21000000			
A-Cryptogran-48h-15°C -3	12.19	12.97	0.78	39000000			
A-Cryptogran-48h-15°C -4	10.12	11.1	0.98	49000000			
A-Cryptogran-48h-15°C -5	8.38	9.43	1.05	52500000			
A-Cryptogran-48h-15°C -6	10.32	12.18	1.86	93000000			
A-Cryptogran-48h-15°C -7	10.62	12.53	1.91	95500000			
A-Cryptogran-48h-15°C -8	10.63	10.8	0.17	8500000			
B-Cryptogran-48h-15°C -1	10.63	12.75	2.12	106000000	Infected 15°C 48h#1(2)	61437500	RT11i

Sample	Pre-inoculation weight (grams)	Post-inoculation weight (grams)	Liquid uptake weight (grams)	Occlusion bodies uptake	Sum of group occlusion bodies uptake	Mean group occlusion bodies uptake	Extraction number
B-Cryptogran-48h-15°C -2	10.64	11.6	0.96	48000000			
B-Cryptogran-48h-15°C -3	10.78	11.52	0.74	37000000			
B-Cryptogran-48h-15°C -4	10.86	11.92	1.06	53000000			
B-Cryptogran-48h-15°C -5	10.95	11.44	0.49	24500000			
B-Cryptogran-48h-15°C -6	11.84	13.36	1.52	76000000			
B-Cryptogran-48h-15°C -7	12.21	13.83	1.62	81000000			
B-Cryptogran-48h-15°C -8	12.22	13.54	1.32	66000000			
C-Cryptogran-48h-15°C -1	12.89	15.97	3.08	154000000	Infected 15°C 48h#2(5)	90187500	RT12i
C-Cryptogran-48h-15°C -2	13.07	13.82	0.75	37500000			
C-Cryptogran-48h-15°C -3	13.1	14.35	1.25	62500000			
C-Cryptogran-48h-15°C -4	13.42	15.01	1.59	79500000			

Sample	Pre-inoculation weight (grams)	Post-inoculation weight (grams)	Liquid uptake weight (grams)	Occlusion bodies uptake	Sum of group occlusion bodies uptake	Mean group occlusion bodies uptake	Extraction number
C-Cryptogran-48h-15°C -5	13.9	15.34	1.44	72000000			
C-Cryptogran-48h-15°C -6	14	17.08	3.08	154000000			
C-Cryptogran-48h-15°C -7	14.25	16.08	1.83	91500000			
C-Cryptogran-48h-15°C -8	15.38	16.79	1.41	70500000			
A-Cryptogran-48h-35°C -1	6.34	7.32	0.98	49000000	35°C Infected48h1	46125000	RT16i
A-Cryptogran-48h-35°C -2	6.32	7.58	1.26	63000000			
A-Cryptogran-48h-35°C -3	6.61	7.35	0.74	37000000			
A-Cryptogran-48h-35°C -4	6.41	7.82	1.41	70500000			
A-Cryptogran-48h-35°C -5	5.14	6.39	1.25	62500000			
A-Cryptogran-48h-35°C -6	6.55	7.28	0.73	36500000			
A-Cryptogran-48h-35°C -7	5.87	6.29	0.42	21000000			
A-Cryptogran-48h-	5.59	6.18	0.59	29500000			

35°C -8							
Sample	Pre-inoculation weight (grams)	Post-inoculation weight (grams)	Liquid uptake weight (grams)	Occlusion bodies uptake	Sum of group occlusion bodies uptake	Mean group occlusion bodies uptake	Extraction number
B-Cryptogran-48h-35°C -1	6.32	6.98	0.66	33000000	35°C Infected48h2	36875000	RT17i
B-Cryptogran-48h-35°C -2	5.93	6.84	0.91	45500000			
B-Cryptogran-48h-35°C -3	7.95	8.39	0.44	22000000			
B-Cryptogran-48h-35°C -4	7.8	8.23	0.43	21500000			
B-Cryptogran-48h-35°C -5	9.32	10.05	0.73	36500000			
B-Cryptogran-48h-35°C -6	7.14	8.09	0.95	47500000			
B-Cryptogran-48h-35°C -7	6.55	7.5	0.95	47500000			
B-Cryptogran-48h-35°C -8	7.29	8.12	0.83	41500000			
C-Cryptogran-48h-35°C -1	7.97	8.4	0.43	21500000	Infected48h35°C	51875000	RT18i
C-Cryptogran-48h-35°C -2	7.82	8.65	0.83	41500000			
C-Cryptogran-48h-35°C -3	7.51	8.72	1.21	60500000			

C-Cryptogran-48h-35°C -4	6.49	7.92	1.43	71500000
C-Cryptogran-48h-35°C -5	6.54	8.82	2.28	114000000
C-Cryptogran-48h-35°C -6	7.48	8.06	0.58	29000000
C-Cryptogran-48h-35°C -7	7.11	8.08	0.97	48500000
C-Cryptogran-48h-35°C -8	7.66	8.23	0.57	28500000

Supplementary Table 2. RNA and cDNA quality and concentration values for all *T. leucotreta* samples.

RT number	RNA 260/230	RNA 260/280	RNA conc.	cDNA 260/230	cDNA 260/280	cDNA conc.	Diluted cDNA conc.
RT1i	2.22	2.32	1052	1.56	1.86	1989.2	486.5
RT2i	2.23	2.38	498.7	1.55	1.9	702.2	504.1
RT3i	2.21	2.37	675.3	1.57	1.95	1203.1	527
RT1c	2.18	2.39	571.5	1.56	1.89	1277	528.4
RT2c	2.15	1.96	287.4	1.56	1.91	1312.8	513
RT3C	2.17	2.15	275.1	1.56	1.92	1282	330
RT4i	2.19	2.03	661.7	1.56	2	1349.6	499.4
RT5i	2.2	2.25	789.5	1.57	1.94	1046.8	563.4
RT6i	2.2	2.36	913.4	1.57	1.92	949.4	435.7
RT4c	2.21	2.27	1157.6	1.53	1.54	1541.6	477
RT5c	2.21	2.29	740.5	1.56	1.92	1284	463
RT6c	2.21	2.33	1122.8	1.57	1.92	1292.4	579.9
RT7i	2.2	2.28	374.6	1.56	1.94	1209.1	400
RT8i	2.16	1.8	574.1	1.56	1.91	1190.7	536.6
RT9i	2.15	1.92	408.8	1.5	1.92	1373.6	589
RT7c	2.19	2.19	204.8	1.56	1.98	1292.2	541.5
RT8c	2.19	2.24	263.5	1.56	1.93	1277.2	468
RT9c	2.17	2.19	312	1.56	1.9	1277.5	528.8
RT10i	2.19	2.22	389.2	1.57	1.87	1199.6	587
RT11i	2.19	2.24	413	1.57	1.91	1199.1	564
RT12i	2.21	2.35	740.5	1.57	1.81	1217.3	459.6
RT10c	2.21	2.27	381.8	1.57	1.92	800.2	515

RT11c	2.19	2.27	286.5	1.57	1.74	787.9	419.5
RT12c	2.2	2.28	710.1	1.55	1.89	808.9	461
RT13i	2.27	2.38	879.2	1.57	1.85	1349.3	-
RT14i	2.26	2.33	692.9	1.57	1.92	1222.7	-
RT15i	2.28	2.24	525	1.57	1.92	1206.6	-
RT13c	2.2	2.33	436.7	1.54	1.87	976.3	-
RT14c	2.24	2.4	1124.8	1.55	1.94	766.4	-
RT15°C	2.28	2.34	1003.6	1.55	1.89	774.4	-
RT16i	2.18	2.27	737.1	1.57	1.9	1199.7	554.8
RT17i	2.18	2.1	255.2	1.57	1.96	764	473.8
RT18i	2.18	2.22	777.8	1.65	1.85	1226.3	413
RT16c	2.18	2.26	197.5	1.57	1.89	1289.9	522.5
RT17c	2.19	1.96	268.6	1.58	1.91	1318.8	571
RT18c	2.18	1.94	274.8	1.57	1.9	1282.6	450
RT19i	2.25	2.3	353.7	1.55	1.92	1007.7	-
RT20i	2.22	2.33	800.2	1.56	1.92	1182.6	-
RT21i	2.26	2.25	281.6	1.56	1.92	1343	-
RT19c	2.2	2.04	380.9	1.56	1.93	1351.7	-
RT20c	2.26	2.25	272.5	1.56	1.93	1294.7	-
RT21c	2.18	2.29	187.7	1.55	1.92	1352.9	-