HONOURS RESEARCH PROJECT REPORT

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2015/2016

SCHOOL OF BIOLOGICAL SCIENCES

THE AGRICULTURAL PEST-CONTROL POTENTIAL OF RNA INTERFERENCE GENE KNOCKDOWN

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SUPERVISOR: DR AS BOWMAN

WORK COMPLETED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR A DEGREE IN HONOURS BIOLOGY

WORD COUNT: **8,283** (EXLUDING FULL REFERENCES & LEGENDS)

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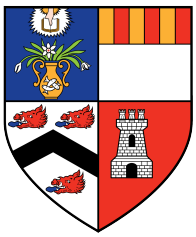

UNIVERSITY OF ABERDEEN

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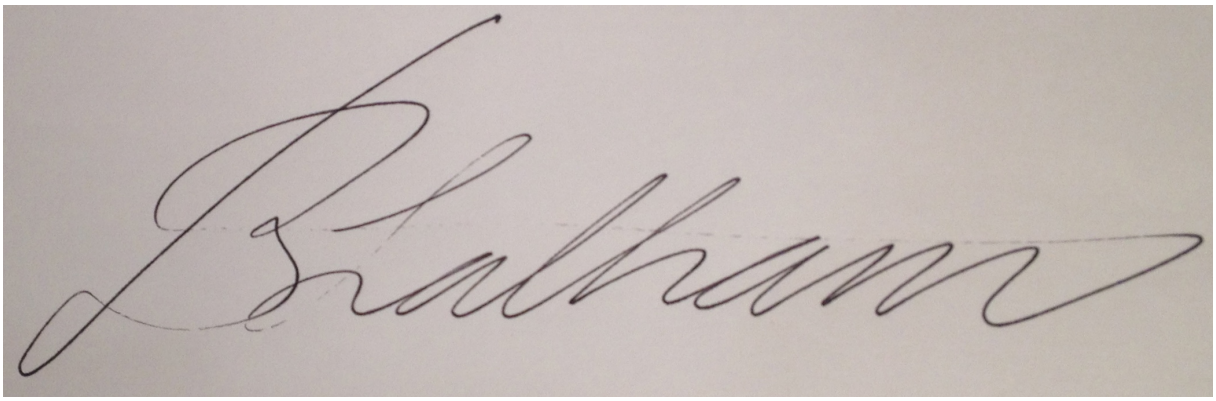
Contents

Declaration

2015-2016

I here declare that this research thesis entitled **The Agricultural Pest-control Potential of RNA Interference Gene Knockdown** is my own work unless otherwise stated. Wherever I have used the work of other persons I have clearly referenced the source. I have used in-text citations and I have included a list of full references at the end of this Honours dissertation. I have also made acknowledgments to those who particularly supported me during the research project.



BM Latham

Risk Assessment

Name of student: Brendan Michael Latham

Name of academic supervisor: Dr AS Bowman

Names of others involved in project: Dr EM Campbell

Description of work

Exploring the potential of gene knockdown by RNA interference as an agricultural pest control. Tested on the model organism *Tribolium castaneum*, the red flour beetle. This research endeavours to answer five main questions: 1) What is the risk of off-target effects using the trialled dsRNAs?; 2) How effective is dsRNAi knockdown at reducing survivorship?; 3) What is the expression level of the target genes at peak knockdown?; 4) Is mixed gene targeting more effective than separate gene targeting at increasing mortality?; 5) What are the relationships between: separate gene targeting, mixed gene targeting, mortality and knockdown? The first stage of this research, corresponding to the first question, will involve bioinformatics, and will be entirely computer-based work. The rest will be conducted within Dr AS Bowman's three laboratories in the University of Aberdeen Zoology Building. Here, the model organisms will be stored in controlled conditions and fed on flour. The dsRNA trigger for gene knockdown will be experimentally administered to the beetle larvae using microinjection. Mortality rates will be determined by daily counts and the effect of gene knockdown on gene expression will be assessed by RT-qPCR and gel electrophoresis densitometry.

Location: Laboratories of Dr AS Bowman in the University of Aberdeen Zoology Building

Start date: 7th of September 2015

TABLE OF POTENTIAL HAZARDS

Hazard Identification (Describe those aspects of the work that could create significant risks)	**Control Measures** (List those to be used to reduce the risks to an acceptable level)
Chemical Danger from chemicals used in molecular biology work, notably TRI reagent.	Wear Gloves when handling. Do not ingest. Perform work with TRI reagent in fume-hood.
Biological *Tribolium castaneum* beetles are pest organisms that pose a threat to the taxidermy specimens in the Zoology museum, if escaped	Beetles will be kept in closed boxes with thin meshing to prevent escape.
Sharps Microinjection glass capillaries	Care to be taken when handling. Performed under supervision until competent.
Electrical PCR machine, freezer, centrifuge, gel electrophoresis.	Be properly trained in use of equipment. Stay clear of wires.
Radiation NA	NA
Field NA	NA
Manual handling Microinjection, pipetting.	Perform under supervision until competent.
Workstation Extensive use of laptop and/or worktop. Laptop for bioinformatic analyses.	Ensure correct positioning of seat height. Take frequent breaks and walk about.
Animal House/Aquarium Access required No	

1. Abstract & Introduction

ABSTRACT

RNA interference (RNAi) gene knockdown promises many potential advantages over commercially valuable pesticides in controlling agricultural pests. To explore its promise as a future pest-control, ADP/ATP Translocase (AAT), Alpha-Tubulin (ATUB) and HSP90 genes were dsRNAi targeted in *Tribolium castaneum* via microinjection. A 1:1:1 mixed-targeted treatment of all genes was included to compare separate versus mixed targeted mortality and knockdown. Using bioinformatics, AAT was determined to be most off-target safe but all gene fragment specificities require improvement. Daily larval survivorships indicated significant ($P<0.05$) mortalities in all targets. A highly considerable >95% knockdown, derived from RT-qPCR, in all but mixed-targeted HSP90, was revealed. The strong mortality and knockdown data help secure the pest-control promise of RNAi. However, greatest knockdown produced lowest mortality, suggesting some genes are less important, and so care should be taken to select the most efficient targets. Mixed-targeting provided second lowest mortality, implying separate is more effective than mixed gene targeting as a means of controlling pests. Research should now focus on improving separate-targeting trials, particularly in reducing off-target risk. However, mixed-targeting should still be investigated by utilising a variety of dsRNA ratios on more functionally similar genes.

1.1 IMPORTANCE OF WORLD AGRICULTURE

The global agriculture industry is of huge economic importance. The combined world production of the ten most agriculturally important crops was valued at more than $624.9 billion in the year 2012 alone (FAO, 2014; Table 1.1). Its geographical scale is extensive. Approximately 10% of the planet's productive land surface is used for growing crops and a further 30% is used for raising livestock (FAO 1994). It provides food and employment for billions. Between 40% and 60% of total income in underdeveloped countries comes from

agriculture and it supplies work for up to 80% of the labour force (Johnston & Mellor, 1961). In 1997, 50% of the earth's population worked in farming (Johnson, 1997). More recently, it has been indicated, that out of all growing industries, agriculture has an especially beneficial effect in reducing poverty (Bresciani & Valdés, 2007; Ligon & Sadoulet, 2008). The industry is on the rise. Demand for crops is set to increase 100-110% between 2015 and 2050 (Tilman *et al*, 2011). This is correlated with a population inflation to approximately 9.2 billion people by the middle of this century (Bongaarts, 2009). According to Tscharntke *et al* (2012), the task for today's agriculture industry is to improve land productivity whilst conserving biodiversity on an increasingly populated planet.

Rank	**Crop**	**Production Value** (Int* $1000)	**Production Quantity** (MT*)
1	Rice	185,579,591	738,187,642
2	Wheat	79,285,036	671,496,872
3	Soybean	60,692,327	241,142,197
4	Tomatoes	59,108,521	161,793,834
5	Sugar Cane	57,858,551	1,842,266,284
6	Maize	53,604,464	872,791,597
7	Potatoes	48,770,419	365,365,367
8	Vegetables, fresh, NES*	46,143,720	269,852,343
9	Grapes	38,336,711	67,067,129
10	Cotton lint	37,095,127	25,955,096

Table 1.1 Global production of the 10 most important crop commodities in 2012 ranked by value. * Int = International commodity price. * MT = Metric Tonnes. * NES = Not Elsewhere Specified. Total value: $624,945,119,000. Total quantity: 5,255,918,361MT. FAO (2014).

1.2 IMPORTANCE OF PEST CONTROLS

1.2.1 The pests

Agricultural pests are mankind's most significant competitors for food. A predicted 25-50% of crops are removed by pests before and after harvesting. The majority of these are herbivorous insects (Pimentel, 1979). Approximately 10, 000 insect species feed on crops

worldwide (Ware & Whitacre, 2004). According to Oerke (2006), global agricultural pests from the year 2001 to 2003 - of rice, wheat, soybean, maize, potatoes and cotton - were responsible for between 50% potential loss in wheat production and up to 80% potential loss in cotton production.

1.2.2 The controls

Pesticides kill or repel pests, or interrupt their reproductive cycle (Gilden *et al*, 2010). Categories include: synthetic (Konwick *et al*, 2006); organic (Sherer *et al*, 2003); and inorganic (Weed, 1974). It is predicted that if pesticide use was to stop, 10% of the world's crop production as it is would be lost. And for particular crops, there would be almost 100% loss (Pimentel *et al*, 1992). Most agricultural pesticides are synthetic. Across the globe, approximately 2.5 million tonnes of synthetic pesticides have been used on agricultural crops every year (Pimentel *et al*, 1993). Synthetic pesticides are usually the most potent. In under 40 years following their discovery, modern agriculture improved its crop yields dramatically (Cheng, 1990). The economic value of the combined pesticide market is now considerable. In 2007 alone, the world spent approximately $9.4 billion on pesticides (EPA, 2011). Organic pesticides represent approximately 2% of that market with about 90% of those sales depending on bacterial proteins from *Bacillus thuringiensis* (Sanchis & Bourguet, 2008).

1.2.3 Pesticide problems

Despite the great need for pesticides, there are serious negative implications connected with their use. A growing problem is insect resistance (Roush & Tabashnik, 2012; Tabashnik *et al*, 2003). Resistance to pesticides has been recorded in over 440 insect and mite species (Roush & Tabashnik, 2012). Non-target kills by pesticides are also a well documented problem. Many cases of pesticides removing non-target, ecologically important organisms have been reported. For instance: in bees (e.g. Berry, 2009; Crane & Walker, 1983; Thompson, 2003); in fish (e.g. Dunier & Siwicki, 1993; Olafson, 1978); and in birds (e.g. Boatman *et al*, 2004; Senthilkumar *et al*, 2001). Non-target kills by pesticides can even create pests, by eradicating their natural predators (Naylor & Ehrlich, 1997). For example, the majority of the most harmful pests in 1970s California were generated by pesticides (National Research Council, 1989). Moreover, pesticides are often highly toxic to humans, causing a variety of diseases

(Eskenazi *et al*, 2007; Farr *et al*, 2004; Meeker *et al*, 2006), including cancer (De Roos *et al*, 2005). These harmful effects can be further exacerbated by the persistence and bioaccumulation of pesticides (Muir *et al*, 2003; Ribeiro *et al*, 2005; Senthilkumar *et al*, 2001 Willis & McDowell, 1982). For example, organochloride pesticides have been detected 20 years post-application (Kleka *et al*, 2001; Kuhard *et al*, 2004).

1.3 RNA INTERFERENCE

1.3.1 Background

RNA interference (RNAi) is a rapidly growing field of study indicating potential as a replacement to presently used commercial pesticides (Aronstein *et al*, 2011; Huvenne & Smagghe, 2010). It is a post-transcriptional gene-silencing system targeting specific sequences in response to double-stranded RNA (dsRNA) (Elbashir, 2001; Fire *et al*, 1998; Hammond *et al*, 2001; Hannon, 2002; Sharp, 2001). The phenomenon was first discovered and termed RNAi by Fire *et al* (1998) from experiments made on the nematode worm *Caenorhabditis elegans*. The studies progressed from earlier work that experimentally introduced RNA to disrupt gene function (Izant & Wientraub, 1984; Nellen & Lichtenstein, 1993), and were inspired by the discovery that sense RNA was as effective as antisense RNA at inhibiting gene expression (Guo & Kemphues, 1995). RNAi is thought to have evolved as an antiviral defence (Buchon, 2006; Cerutti & Casas-Mollano, 2006; Ding *et al*, 2004; Hammond *et al*, 2000).

1.3.2 dsRNA delivery & transport

A number of methods have been used to deliver dsRNA into the host organism. These include: microinjection (Acosta *et al*, 2005; Jaubert-Possamai *et al*, 2007; Ohrt *et al*, 2006); feeding (Araujo *et al*, 2006; Kamath & Ahringer, 2003; Turner *et al*, 2006); and immersion (Sawa *et al*, 2003; Sugimoto, 2004). Gene silencing has successfully been triggered by dsRNA throughout the host body in what is called systemic RNAi (Araujo *et al*, 2006; Miller *et al*, 2008; Posnien *et al*, 2009; Saleh *et al*, 2006; Walshe *et al*, 2009). It has also been triggered only locally, at the site of dsRNA entry, in what is called non-systemic RNAi (Dietz *et al*, 2007; Fire, 2007; Price & Gatehouse, 2008; Singh, 2008; Siomi & Siomi, 2009). Systemic RNAi has been demonstrated in many insect species (Miller *et al*, 2008; Posnien *et al*, 2009). These

include the model organism *Tribolium castaneum*, the red flour beetle - by microinjection, feeding and immersion (Aronstein *et al*, 2011). However, only cases of non-systemic RNAi have been reported in *Drosophila* (Roignant *et al*, 2003; Price & Gatehouse, 2008; Tomoyasu *et al*, 2008). Systemic transport of dsRNA is thought to be facilitated by *C. elegans* SID-1 and SID-2 transmembrane-protein homologues (Jose *et al*, 2009) and non-systemic transport by a form of endocytosis (Saleh *et al*, 2006; Ulvila *et al*, 2006).

1.3.3 RNAi mechanism

The first stage of the RNAi pathway is the cleaving of the introduced dsRNA by the RNase-II ribonuclease, Dicer (Bernstein *et al*, 2001; Billy *et al*, 2001; Boutla *et al*, 2001; Carmell & Hannon, 2004; Zamore *et al*, 2000). Cloven dsRNA is 21-23 nucleotides long and called short-interfering RNA (siRNA) (Caplen, 2001; McManus & Sharp, 2002; Kennedy *et al*, 2004; Kim *et al*, 2005; Shen *et al*, 2003). The double-stranded siRNA is then divided into a single passenger strand and a single guide strand according to base-pair asymmetry (Khvorova *et al*, 2003; Schwarz *et al*, 2003). The guide strand is integrated into the RNA-induced silencing complex (RISC) which the guide strand directs to mRNA possessing its sequence-compliment (Liu *et al*, 2003; Martinez *et al*, 2002). On reaching the target mRNA, the main active component of the RISC, the Argonaute protein, cleaves the mRNA, thus preventing mRNA translation (Kupferschmidt, 2013; Yigit *et al*, 2006).

1.4 RNAi AS A PEST CONTROL

1.4.1 Concept

There are a number of RNAi qualities that make it a promising pest control. Unlike today's pesticides, RNAi is exceptionally target specific (Qadota *et al*, 2007; Yang *et al*, 2001), even to the level of a single species (Whyard *et al*, 2009). And it could be a direct solution to the rise in pesticide resistance (Aronstein *et al*, 2011; Baum *et al*, 2007). It can also be completely non-toxic, including to humans (Wang *et al*, 2007). The main potential danger of using RNAi are 'off-target effects' - the silencing of random, non-target mRNAs that possess sufficiently high sequence identity (Federov *et al*, 2006; Jackson & Linsley, 2004). However, this is not prohibitive providing dsRNA specificity is carefully designed using bioinformatics sequence comparisons (Qiu *et al*, 2005; Xu *et al*, 2006; Yamada & Morishita, 2005). It should

also be noted that a harmful RNAi effect could not persist in the environment indefinitely due to the biodegradable nature of RNA. In *T. castaneum*, the RNAi effect only lasts a matter of days or weeks (Posnien *et al*, 2009).

1.4.2 RNAi in the red flour beetle, Tribolium castaneum

The red flour beetle is a widely used model organism and is a natural pest of stored grain (Champ & Dyte, 1977; Padin *et al*, 2002; Prates *et al*, 1998). It was the first coleopteran to have its genome sequenced (Aronstein *et al*, 2011; Hogenkamp *et al*, 2008; Richards *et al*, 2008). RNAi following microinjection of dsRNA has already been used extensively in *T. castaneum* for determining gene function (e.g. Arakane *et al*, 2009; Bucher *et al*, 2002; Choe *et al*, 2006; Tomoyasu & Denell, 2004). Potential pest control targets have thus been highlighted. These include: chitinase-like proteins and their moulting roles (Zhu *et al*, 2008); neuropeptide genes (Arakane *et al*, 2008); and chymotrypsin-like peptidases and their digestive properties (Broehan *et al*, 2010). According to Huvenne & Smagghe (2010), there have been no feeding experiments on this organism, apparently due to the success of microinjection studies. However, Whyard *et al* (2009) did describe using feed delivery of dsRNA to successfully kill *T. castaneum* by species-specific vATPase targeting.

1.5 AIMS & OBJECTIVES OF THIS RESEARCH

This research sought to enhance our understanding of the pest-control potential of RNAi. In light of the present demand for an agricultural pesticide replacement and considering the current momentum of RNAi research, this study was opportune. The project investigated this potential in the model organism *T. castaneum* by targeting three genes: ADP/ATP Translocase (AAT) (NCBI Accession No.: XM_968164.3); Alpha-Tubulin (ATUB) (XP_966492.1); and Heat Shock Protein 90 (HSP90) (NP_001094067). And by pursuing the following aims and objectives:

Aim 1	To investigate potential off-target effects of three dsRNAs.
Objective 1	To do so by bioinformatics.

Aim 2	To investigate the efficacy of dsRNAi knockdown at reducing survivorship.
Objective 2	To do so by measuring survivorship of microinjected larvae over 8 days.

Aim 3	To quantify gene expression at peak knockdown.
Objective 3	To do so by using real-time qPCR and gel electrophoresis densitometry**.**

Aim 4	To determine the relationships between separate gene targeting, mixed gene targeting, mortality and knockdown.
Objective 4	To do so by including in objective 2 experimental design a mixed-targeted treatment (MIX) of all three dsRNAs. And by statistical visualisation and comparison.

2. Materials & Methods

2.1 INVESTIGATING OFF-TARGET EFFECTS USING BIOINFORMATICS

2.1.1 Searching for alignments

Significant protein and nucleotide alignments were investigated for fragment sequences of the three target genes AAT (239 base pairs in length), ATUB (275bp) and HSP90 (520bp). These fragments corresponded to dsRNAs designed and patented by Dr AS Bowman (University of Aberdeen). Protein sequences were derived using EXpasy translate tool http://web.expasy.org/translate/, by selecting the longest open reading frame (Gasteiger *et al*, 2003). The first start methionine was selected to generate the FASTA format of the identified sequence. The fragment was then re-translated to include the nucleotide sequence. These protein and nucleotide sequences were placed into NCBI Basic Local Alignment Search Tools, BLASTs (protein BLAST & nucleotide BLAST): http://blast.ncbi.nlm.nih.gov/Blast.cgi. Default algorithm parameters were used for all BLAST searches except for nucleotide BLAST word sizes: AAT - 16; ATUB - 24; HSP90 - 28.

2.1.2 Visualising alignments: Phylograms

Significant and relevant alignments were downloaded and imported into MEGA (6.06) to generate phylogenetic trees (Hall, 2013). Determining relevancy was subjective. The concept was to exclude from analyses all species that would not appear within an agriculture ecosystem. This included all aquatic organisms and species endemic to wilderness regions. Species repeats were also removed. Exclusion was done in a conservative manner. Inputted MEGA sequences were aligned using default ClustalW parameters (Thompson *et al*, 2002). Overhanging portions unaligned with query sequence were discarded. Phylograms were then produced using the neighbour-joining algorithm (Saitou & Nei, 1987), except for a conservative (Pattengale *et al*, 2009) 5000 bootstrap iterations. Colour-coded higher taxonomic classifications were added to each species to indicate diversity within and between phylograms.

2.1.3 Visualising alignments: Identical hits

Nucleotide BLAST alignment outputs provided only overall percentage identity. To fine-tune visualisation of off-target hits, lengths and frequencies of perfect matches were counted. Stretches of perfect identity ≥15bp in length were considered to pose a risk of off-target effect. This was a conservative lower threshold: Previous research indicated 19 nucleotide-nucleotide matches as the minimum degree of identity required to potentially trigger an RNAi response (Czauderna *et al*, 2003; Kulkarni *et al*, 2006). And an earlier bioinformatics RNAi investigation used 17bp as the lowest parameter (Qiu *et al*, 2005). The percentage proportion of ≥15bp portions of perfect identity were also determined for each species alignment. All information was graphically summarised in bar graphs.

2.2 INVESTIGATING THE EFFICACY OF dsRNAi KNOCKDOWN BY MEASURING SURVIVORSHIP

2.2.1 Culture 1 experimental design

*T. castan*eum beetles were reared in whole wheat flour, sieved to remove bran, with 5% brewer's yeast (Arakane *et al*, 2009; Tomoyasu & Denell, 2004) and incubated at 33.7^{o}C. Six treatments were used: three separately gene-targeted, one mixed-targeted, and two control treatments - an uninjected control (Ralph *et al*, 2005) and a Green Fluorescent Protein (GFP) dsRNA control (Boutros *et al*, 2004). Each treatment had three replicates, one petri dish per replicate, with 10 larvae in each. (Note, for one of the three ATUB replicates, a larva escaped). Dead larvae were counted over eight days and survivorship was normalised to that of day one post-injection - As mortalities on that day were not considered to have resulted from any knockdown effects, due to the time-lag between gene knockdown and resultant mortality (Baum *et al*, 2007). To determine the day of most significant ($P<0.05$) reduction in survivorship between targeted and control treatments, a one-way ANOVA and Fisher's post hoc analysis (which conducted a series of t-tests between means) were utilised. Due to the time-lag, the preceding day was inferred to have been that of peak knockdown.

2.2.2 Microinjection

The dsRNAs were microinjected (Jaubert-Possamai *et al*, 2007) into third instar larvae (Miller *et al*, 2012). Larvae were anaesthetised on ice (Willis *et al*, 2011) 10 at a time for approximately four minutes prior to being placed on the back of a petri dish using double-

sided tape (Tan & Palli, 2008). Masking tape was then used to secure the larvae so that only abdomens showed (Figure 2.2.1) for microinjection using a Drummond Scientific Nanoject II microinjector (Yokoi *et al*, 2015). Per larva, 60ng of dsRNA reagent was injected at 46nL/s (speed 5, Fast). Mixed-targeted larvae were injected with 20ng of dsRNA reagent per target gene. Needles were 3.5" glass capillary tubes (Drommond Scientific) pulled by a Narishige needle puller (PN-3).

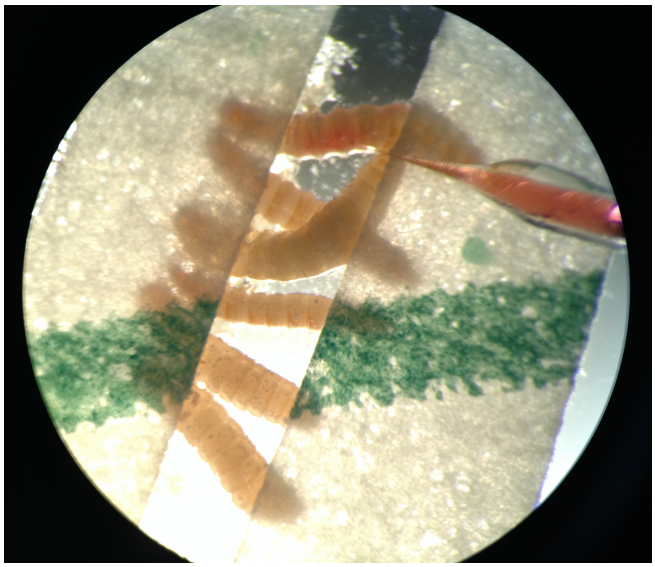


Figure 2.2.1 Microinjection set-up. Example injection using Neutral Red Solution and 1.8% saline to visualise successful entry. Layering: double-sided tape, larvae, then masking tape.

2.3 QUANTIFYING GENE EXPRESSION AT PEAK KNOCKDOWN

2.3.1 Culture 2 experimental design & harvesting

Culture 2 was as culture 1 but for one replicate per treatment. The sole purpose of culture 2 was to harvest live larvae on the day of peak knockdown: The larvae were placed in 1.5ml reaction tubes (Greiner Bio One) with 250µL of RNA*later*® stabilisation solution (Sigma-Aldrich). They were then stored at -77^{o}C for two days in a Jouan VXS570 ultralow freezer.

2.3.2 RNA extraction

Larvae were removed and placed on ice. Three larvae from each treatment were transferred to new labelled reaction tubes, one larva per tube. TRI reagent, or TRIzol, (containing Guanidinium thiocyanate and Phenol) was used to extract RNA (Chomczynski & Sacchi, 1987). RNA extraction was based on the Sigma-Aldrich TRI reagent® protocol: Step 1) Homogenisation. In a fume cupboard, 800μL of TRI reagent was added to each tube. Each larva was then crushed using a different decontaminated pestle (Oppert *et al*, 2010) per treatment. Step 2) Phase separation. In the fume cupboard, 80μL of 1-bromo-3-chloropropane was added to each tube. Lids were secured and tubes shaken. They were then centrifuged (model: Hettich-Zentrifugen Universal 32 R) at 12,000 x g for 15 minutes at 2-8^{o}C. Centrifugation divided the solutions into three layers: a red phase containing larval protein, an interphase containing DNA, and a colourless surface aqueous phase containing the RNA. Step 3) Formation of the RNA pellet. To each tube, 400 μL of 2-propanol was added. The tubes were again likewise centrifuged, but for 10 minutes, with hinges orientated upwards to allow for easier pellet locating. RNA precipitated and formed a pellet on the hinge-side of the bottom of each tube (Figure 2.3.1).

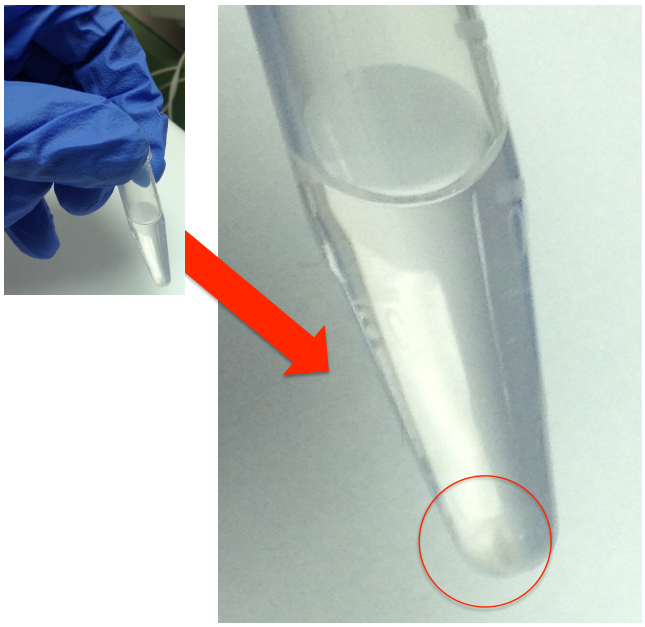


Figure 2.3.1 An RNA pellet (within red circle) precipitated on the hinge-side of the bottom of each reaction tube.

Step 4) Removing the supernatant. Using a P1000 pipette set to 800μL, the supernatant was removed. The isolated pellet was then washed with 800μL of 75% ethanol for every 800μL of TRI reagent. Samples were vortexed and centrifuged at 7,500 x g for 5 minutes at 2-8^{o}C before being stored in 75% ethanol and frozen at -77^{o}C overnight.

2.3.3 Spectrophotometry & reverse transcription

Pellets were removed and thawed. Ethanol was extracted and the tubes air-dried for 5-10 minutes. Drying for too long might have reduced pellet-solubility (Blackburn *et al*, 1999). RNA solutions were prepared by adding 80μL of nuclease-free H_2O (Sigma-Aldrich) to each tube. Prior to reverse transcription (RT), RNA solution concentrations were determined so as to derive the correct volume to add to each PCR well: Absorbance at 260nm, 230nm and 280nm was taken for each solution by applying 2μL to a NanoDrop^{TM} spectrophotemeter (ND-1000). The resultant concentration values in ng/μL were divided into 200μL and well volumes ascertained. A RT master mix was then prepared of 1μL iScript^{TM} Reverse Transcription Supermix (BIO-RAD) for every 2.75μL of nuclease-free H_2O, by pipette-mixing (iScript^{TM} RT protocol). Each reaction well in the 96-well plate contained 200μL of master mix and RNA solution. For every 5μL of RNA, 15μL of master mix was added. Finally, the plate was incubated in a traditional PCR machine for: 5 minutes at 25^{o}C, 30 minutes at 42^{o}C, and 5 minutes at 85^{o}C.

2.3.4 RT-qPCR

Quantitative real-time polymerase chain reaction (RT-qPCR) intercalated the reverse transcribed cDNA with fluorescent dye in each reaction cycle to produce relative quantification of culture 2 day 4 gene expression. The method was based on the iTaq™ Universal SYBR® Green Supermix (BIO-RAD) protocol. The iTaq™ Universal SYBR® Green components were thawed and mixed. A master mix of iTaq™ Universal SYBR® Green Supermix (2x) and primers was prepared. Two 96-well PCR plates were required, both including a housekeeping RP6 gene as a reference for gene expression (Radonić *et al*, 2004): One contained cDNA from separate gene targeted larvae and uninjected control larvae ('separate-targeting plate'). And the other the mixed-targeted cDNA and that of the GFP control larvae ('mixed-targeting plate').

Separate-targeting plate arrangement: 420μL of iTaq™ Supermix, 42μL of primers (four primers, for target genes and RP6) and 168μL of nuclease-free H_2O were added over 60 wells in total. RP6 was targeted in six wells per treatment (24 wells in total). And target genes were targeted each in 12 wells, six for its corresponding separately-targeted cDNA and six for uninjected larval cDNA. All primers were added to uninjected reactions.

Mixed-targeting plate arrangement: 300μL iTaq™ Supermix, 30μL of primers and 120μL of H_2O over 48 wells. There were 12 reactions per gene target (including RP6), six mixed-targeted and six from GFP control larvae, and all primers were added to each. The plates were then sealed with optically transparent film and vortexed for approximately 30 seconds. Then they were thermal cycled, including a melt point analysis, on a RT-qPCR machine (BIO-RAD CFX96) for: 40 cycles for 59 minutes at 42°C and 5 minutes at 85°C. RT-qPCR reactions were refrigerated at 1-8°C and all gene expression data accessed through CFX Manager (3.1).

2.3.5 Gel electrophoresis

To compare and contrast RT-qPCR gene expression results to that from a traditional method, gel electrophoresis was carried out. A 1% agarose gel was prepared for two electrophoresis trays, corresponding to the RT-qPCR plates: a 'separate-targeting tray' and a 'mixed-targeting tray'. 4.8g of agarose powder was added to 300ml of TBE buffer (containing Tris base, boric acid and EDTA) (Schuller *et al*, 2011). 30μL of SYBR® Safe DNA Gel Stain (Thermo Fisher Scientific) was then added. The solution was melted and 150ml poured into each tray and combs placed at the cathode end of the electrophoresis chambers. Gels were allowed to solidify and the RT-qPCR reactions were removed from the refrigerator. 4μL of Gel Loading Dye Blue (6x) (Thermo Fisher Scientific), which contained bromophenol blue, was added to 20μL of each RT-qPCR reaction and the solutions briefly centrifuged. TBE buffer was slowly poured until gels were covered, and then, after removing the combs, the reaction samples were pipetted into the wells. The dye gave the samples weight to sink to the bottom. The electrophoresis reactions ended when bromophenol blue had migrated approximately ¾ the length of the gel.

2.3.6 Densitometry

The electrophoresis gels were then removed from the chambers and photographed in a GelVue UV Transilluminator (GVM20). The photographs were scanned and snipped to isolate the cDNA gel bands. They were then combined lengthways into one Microsoft PowerPoint (Mac 2011, 14.1.0) graphic. Care was taken not to disproportionately edit the smaller images. The combined photograph was then imported into ImageJ (32) and the brightness intensity of each band was measured using equally sized densitometry rectangles that fell within the band horizontally but overlapped vertically. A plot of brightness was then generated for each band and the 'magic wand' tool used to measure trough-area of each plot. This area data was then exported and analysed in Microsoft Excel (Mac 2011, 14.1.0) to derive relative normalised percentage target gene expression.

2.3.7 Calculations & statistics

Relative normalised percentage values were calculated for target gene expression and similarly for separate- and mixed-targeted larval mortality. It was one of the main calculations used in this research:

$$Gene\ expression = \left[\frac{(Target\ gene\ mean\ trough\text{-}area/RP6\ mean\ trough\text{-}area)}{(Control\ gene\ mean\ trough\text{-}area/RP6\ mean\ trough\text{-}area)} \right] * 100$$

One-way analysis of variance (ANOVA), and where possible ($P<0.05$) a Fisher's post hoc analysis, were directed using Minitab Express (1.2.0) for: 1) Peak-knockdown determination; 2) Comparing gene expression results derived from RT-qPCR and gel electrophoresis densitometry; 3) Contrasting RT-qPCR Cq values; and 4) Comparing separate to mixed targeting for both larval mortality and RT-qPCR derived gene knockdown.

3. Results

3.1 INVESTIGATING OFF-TARGET RISK

3.1.1 ADP/ATP Translocase: Nucleotide alignments

The longest open reading frame from the EXpasy translation of the AAT fragment was 50bp in length. Despite adjusting algorithm word size, the AAT protein BLAST of this sequence produced only one species presenting a significant alignment: the hypothetical protein *Epinephelus bruneusi* [Sequence ID: AEB31312.1] [Sequence identity: 53%]. Therefore no protein phylogenetic tree was constructed. However, the AAT nucleotide BLAST produced the most significant alignments of all *T. castaneum* target genes (12 species in total) (Figure 3.1.1).

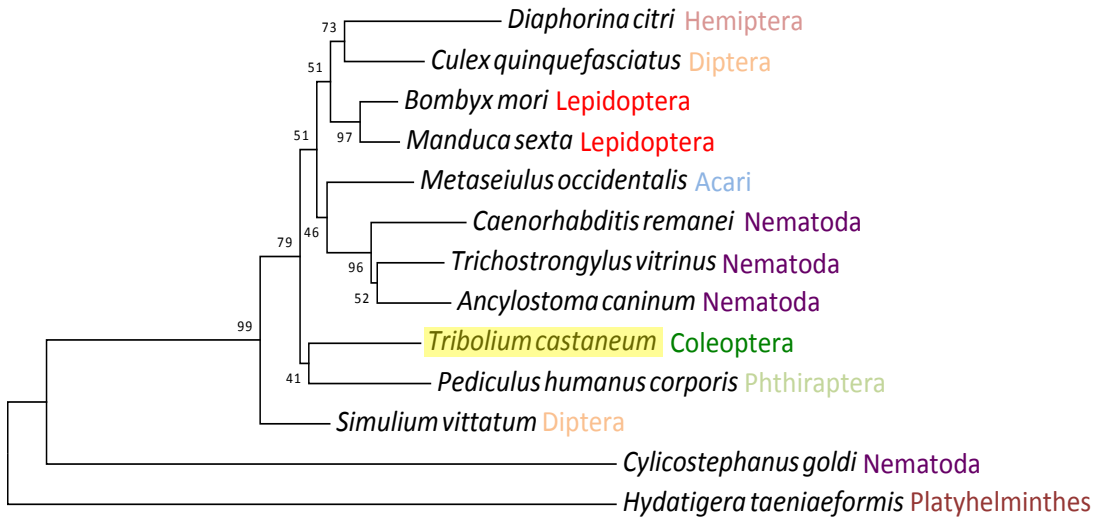


Figure 3.1.1 Phylogeny of ADP/ATP Translocase nucleotide fragment significant alignments. Phylogram of nucleotide sequences built with neighbour-joining method using MEGA (6.06). Numbers are bootstrap values. NCBI BLAST word size: 16. Database identifiable names and sequence IDs: *Diaphorina citri* ADP/ATP carrier proetin-like, mRNA [Sequence ID: XM_008474894.1]; *Culex quinquefasciatus* ADP/ATP carrier protein 2, mRNA [Sequence ID: XM_001847337.1]; *Bombyx mori* ADP/ATP carrier protein [Sequence ID: XM_004934186.2]; *Manduca sexta* ADP/ATP translocase mRNA, complete cds [Sequence ID: AY186577.1]; *Metaseiulus occidentalis* ADP/ATP carrier protein 1-like, mRNA [Sequence ID: XM_003748139.1]; *Caenorhabditis remanei* hypothetical protein mRNA, complete cds [Sequence ID: XM_003109090.1]; *Trichostrongylus vitrinus* ant-1 gene for ADP/ATP Translocase [Sequence ID: AM696281.1]; *Ancylostoma caninum* ADP/ATP Translocase ant-1 mRNA, partial cds [Sequence ID: KR676540.1]; *Tribolium castaneum* ADP/ATP carrier protein 1 [Sequence ID: XM_968164.3]; *Pediculus humanus corporis* ADP/ATP carrier protein, putative, mRNA [Sequence ID: XM_002432287.1]; *Simulium vittatum* clone SV-30 mitochondrial ADP/ATP carrier protein mRNA, partial cds [Sequence ID: EU930263.1]; *Cylicostephanus goldi* genome assembly [Sequence ID: LL384682.1]; *Hydatigera taeniaeformis* genome assembly [LL723352.1].

Yet these alignments proved to be the least taxonomically diverse: Unlike for ATUB, no vertebrate species gave significant alignments. Also, unlike HSP90, there were no representatives of a kingdom other than Animalia. Seven of the 12 species were of the phylum Arthropoda (six insects and one mite, *M. occidentalis*), four from the phylum Nematoda, and a single species from the phylum Platyhelminthes (*H. taeniaeformis*). According to the constructed nucleotide phylogeny, the AAT gene of the human parasitic body louse, *P. humanus corporis* (class Insecta), was most similar to the *T. castaneum* query sequence. The following were the node parameters: Number of nodes, 11; Bootstrap range, 58 (41-99); Mean bootstrap, 68.5.

3.1.2 ADP/ATP Translocase: ≥15bp identical matches

Of all target genes, AAT produced the most identical nucleotide matches ≥15bp in length (Figure 3.1.2). Nevertheless, in contrast to both the other target genes, only one AAT nucleotide hit reached the minimum threshold of 19bp proposed by Czauderna *et al* (2003) and Kulkarni *et al* (2006). None reached the normal threshold of 21 nucleotides (Caplen, 2001; McManus & Sharp, 2002; Kennedy *et al*, 2004; Kim *et al*, 2005; Shen *et al*, 2003). This 19bp identical hit came from the feline tape worm *H. taeniaeformis* and also accounted for the greatest overall proportion of total aligned sequence, at 59.38%. The ≥15bp portion from *P. humanus corporis* was the smallest proportionally, at 10.60%. There was one ≥15bp potential hit from all 12 species.

3.1.3 Alpha-Tubulin: Protein alignments

The BLAST ATUB protein sequence was 49bp long and did not produce *T. castaneum* Alpha-Tubulin as the query sequence (Figure 3.1.3). This was despite adjustments in word size. The query sequence was *Falco peregrinus* Alpha-Tubulin (XP_013160386.1). The constructed protein phylogram included considerably less significantly aligned and relevant sequences (27 species in total, excluding query sequence) compared to that of HSP90 (62 species). But the ATUB phylogeny was more diverse and included six classes: Mammalia; Sacernentia (phylum Nematoda); Arachnida; Insecta; Aves; and Reptilia. In contrast, despite how numerous, only one HSP90-aligned species was of a class other than Insecta: the Anuran (frog) *Pelophylax esculentis* (CAC29071.1). The ATUB protein phylogram also possessed weaker nodes according to bootstrap values. The strongest clade had a bootstrap value of

52. Which meant only 52% of the 5000 calculated tree iterations resulted in that clade (Berry & Gascuel, 1996). This was considerably weaker than the 100% most consistent HSP90 clade. Node parameters: Number of nodes, 4; Bootstrap range, 12 (52-40); Mean bootstrap, 44.

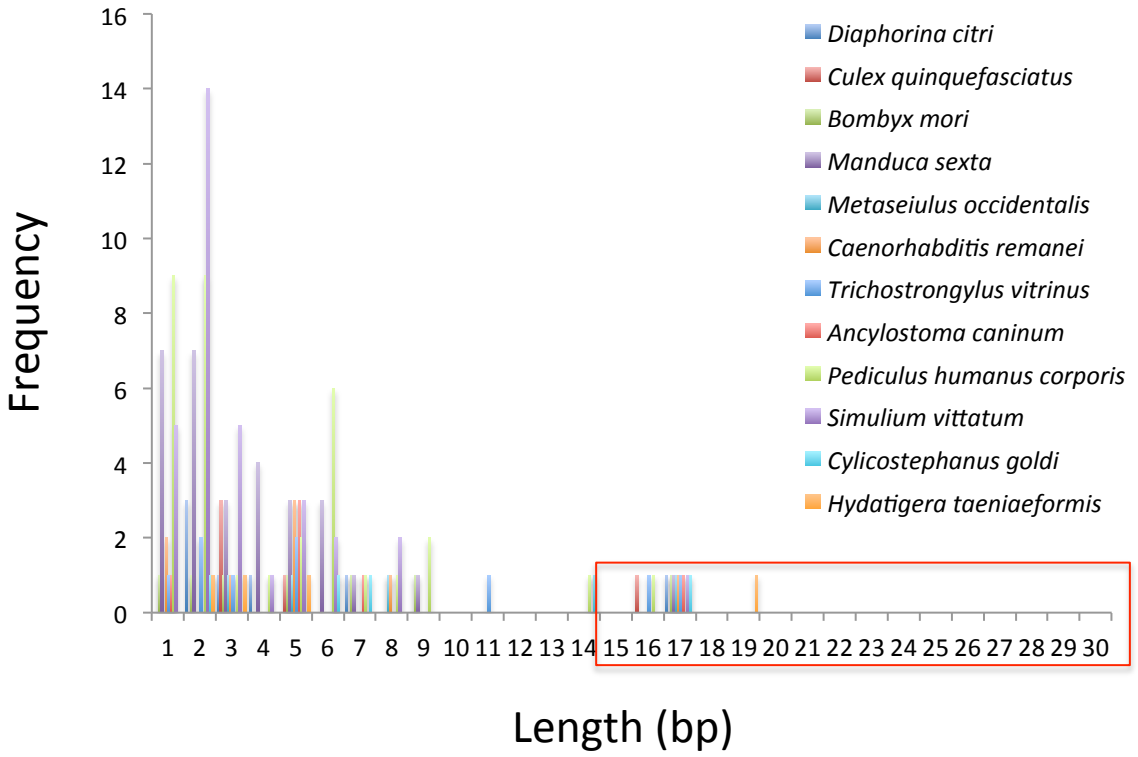


Figure 3.1.2 Lengths and frequencies of identical nucleotide portions from each species of the AAT nucleotide phylogeny (see Figure 3.1.1). Red box highlights identical portions ≥15bp considered to pose a risk of off-target effects. The following are the percentage cover of each ≥15bp portion of its corresponding species alignment: *Diaphorina* citri, 38.64%; *Culex* quinquefasciatus, 47.06%; *Bombyx mori*, 30.36%; *Manduca sexta*, 11.26%; *Metaseiulus occidentalis*, 44.74%; *Caenorhabditis* remanei, 32.08%; *Trichostrongylus* vitrinus, 46.48%; *Ancylostoma caninum*, 32.08%; *Pediculus humanus corporis*, 10.60%; *Simulium* vittatum, 11.26%; *Cylicostephanus* goldi, 34%; *Hydatigera* taeniaformis, 59.38%.

3.1.4 Alpha-Tubulin: Nucleotide alignments

The same ATUB fragment that produced the protein alignments, revealed substantially fewer significantly aligned and relevant nucleotide sequences (Figure 3.1.4). Nine species

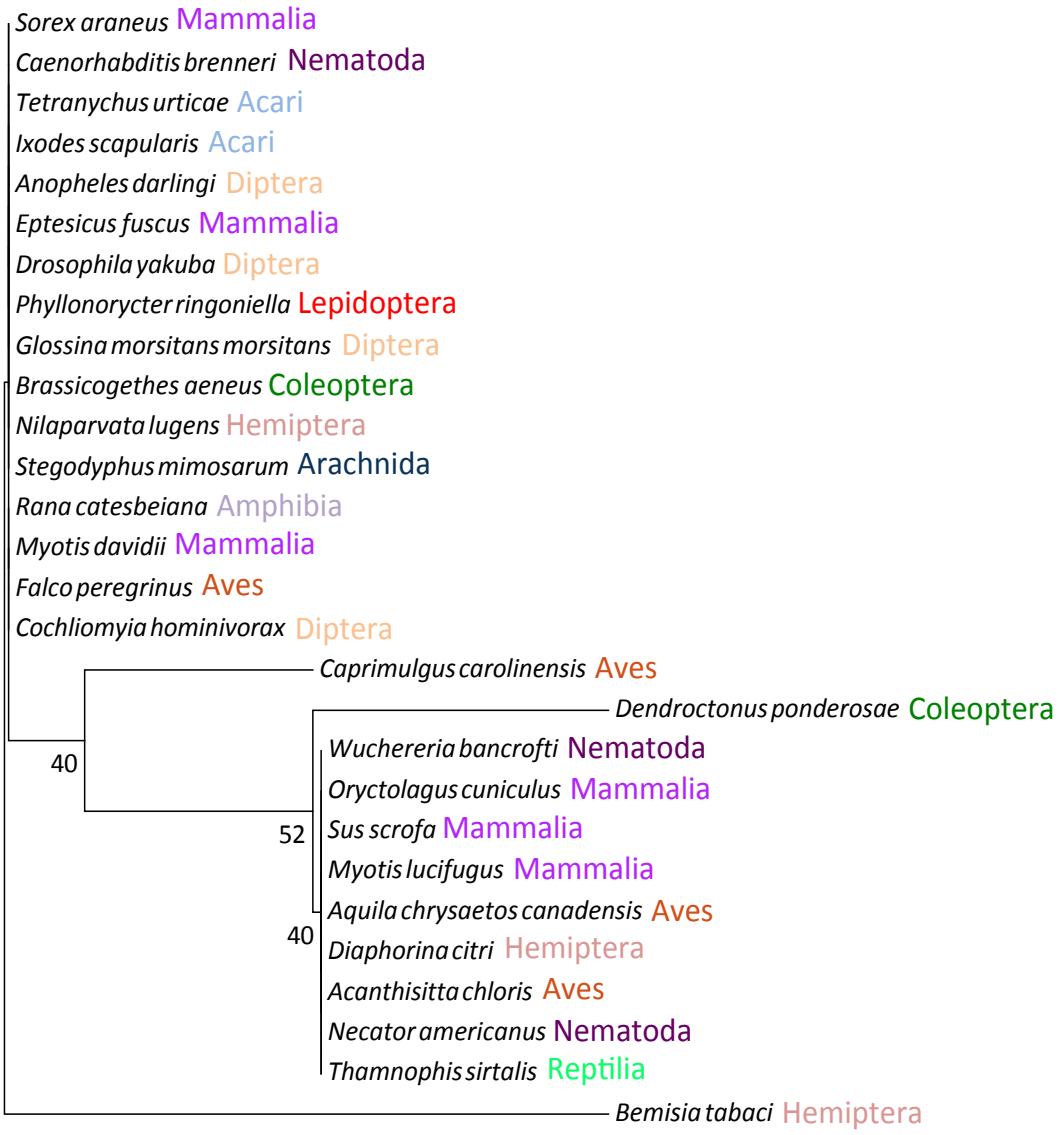


Figure 3.1.3 Phylogeny of significant and relevant amino acid alignments derived from *T. castaneum* ATUB fragment protein BLAST. Produced query sequence from *Falco peregrinus* not *T. castaneum*, which is not present. Built with neighbour-joining method using MEGA (6.06). Numbers are bootstrap values. NCBI database identifiable names and sequence IDs: *Sorex araneus* Tubulin Alpha-8 chain-like [Sequence ID: XP_004622462.1]; *Caenorhabditis brenneri* Alpha Tubulin [Sequence ID: ACD12703.1]; *Tetranychus urticae* Alpha Tubulin, partial [Sequence ID: AFU35093.1]; *Ixodes scapularis* Alpha Tubulin, putative [Sequence ID: XP_002416660.1]; *Anopheles darlingi* Alpha Tubulin chain [Sequence ID: ETN59126.1]; *Eptesicus fuscus* Tubulin Alpha-3 chain-like [Sequence ID: XP_008141103.1]; *Drosophila yakuba* similar to *Drosophila melanogaster* AlphaTub848, partial [Sequence ID: AAR09810.1]; *Phylonorycter ringoniella* Alpha Tubulin, partial [Sequence ID: AHW81488.1]; *Glossina morsitans morsitans* Alpha Tubulin [AAG49533.3]; *Brassicogethes aeneus* Alpha Tubulin, partial [Sequence ID: AGQ51765.1]; *Nilaparvata lugens* Alpha-2 Tubulin [Sequence ID: ACN79512.1]; *Stegodyphus mimosarum* Alpha Tubulin chain, testis-specific, partial [Sequence ID: KFM72982.1]; *Rana catesbeiana* Tubulin Alpha-1 chain [Sequence ID: ACO51588.1]; *Myotis davidii* Tubulin Alpha-3 chain-like, partial [Sequence ID: XP_006777768.1]; *Falco peregrinus* Tubulin Alpha-4 chain-like, partial [Sequence ID: XP_013160386.1]; *Cochliomyia hominivorax* Alpha-Tubulin [Sequence ID: AEK48259.1]; *Caprimulgus carolinensis* Tubulin Alpha-8 chain-like, partial [Sequence ID: XP_010166619.1]; *Dendroctonus ponderosae* hypothetical protein YQE_01314, partial [Sequence ID: ENN82309.1]; *Wuchereria bancrofti* Alpha-Tubulin, partial [Sequence ID: EJW71301.1]; *Oryctolagus cuniculus* Alpha-Tubulin [Sequence ID: AAG24288.1]; *Sus scrofa* Alpha-Tubulin [Sequence ID: ABB58920.1]; *Myotis lucifugus* Tubulin Alpha-1D chain-like, partial [Sequence ID: XP_006109205.1]; *Aquila chrysaetos canadensis* Tubulin Alpha-1C chain-like, partial [XP_011597615.1]; *Diaphorina citri* Tubulin Alpha-1A chain-like [Sequence ID: XP_008475044.1]; *Acanthisitta canadensis* Tubulin Alpha-8 chain-like, partial [Sequence ID: XP_009067679.1]; *Necator americanus* Tubulin/FtsZ family, GTPase domain protein [Sequence ID: XP_013304811.1]; *Thamnophis sirtalis* Tubulin Alpha-1B chain-like [Sequence ID: XP_013918978.1]; *Bemisia tabaci* Alpha-Tubulin [Sequence ID: AEV42053.1].

were nucleotide-aligned in total, exactly a third the number of protein sequences. In the phylogenetic tree constructed of these nucleotide alignments, *T. castaneum* was set in a clade of its own. None of the nine nucleotide-aligned species appeared in the 27 protein sequences. But as its protein phylogeny, the nucleotide alignments for this target gene were again the most diverse, including six classes: Mammalia; Sacernentia; Insecta; Aves; Parabasalia (kingdom Protozoa); and *H. vulgare*, barley (kingdom Plantae). It also specified stronger node bootstrap values than for AAT, including 100% for one clade. However, the overall nucleotide phylogeny was still considered weaker than that for AAT, as its most basal node was 37% compared to 99%. Node parameters: Number of nodes, 8; Bootstrap range, 76 (100-24); Mean bootstrap, 51.7

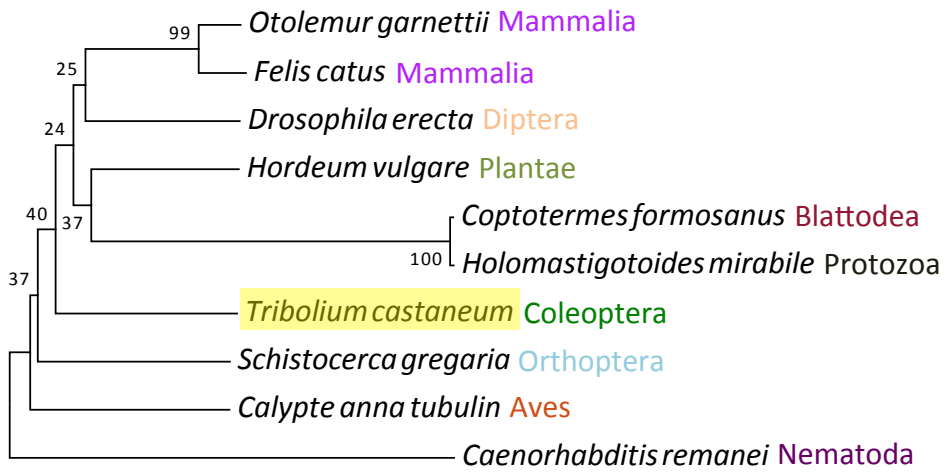


Figure 3.1.4 Phylogram of relevant species significantly nucleotide-aligned with *T. castaneum* Alpha-Tubulin. Built with neighbour-joining method using MEGA (6.06). Numbers are bootstrap values. NCBI BLAST word size: 24. Database identifiable names and sequence IDs: *Otelemur garnettii* tubulin alpha-3 chain-like, mRNA [Sequence ID: XM_012812666.1]; *Felis catis* tubulin alpha-3 chain, mRNA [Sequence ID: XM_011287925.1]; *Drosophila erecta*, mRNA [Sequence ID: XM_001980733.1]; *Hordeum vulgare* subsp. mRNA for predicted protein, complete cds, clone [Sequence ID: AK372321.1]; *Coptotermes formosanus* clone Alpha-Tubulin mRNA, partial cds [Sequence ID: KC632465.1]; *Holomastigotoides mirabile* Alpha-Tubulin mRNA, partial cds [Sequence ID: AB221062.1]; *Tribolium castaneum* Alpha-Tubulin 1-like protein, mRNA [Sequence ID: XM_961399.2]; *Schistocerca gregaria* Alpha-Tubulin mRNA, complete cds [Sequence ID: HQ851397.1]; *Calypte anna* Alpha-8 chain, mRNA [Sequence ID: XM_008499402.1]; *Caenorhabditis remanei* CRE-TBA-9 protein mRNA, complete cds [Sequence ID: XM_003117923.1].

3.1.5 Alpha-Tubulin: ≥15bp identical matches

All nine ATUB nucleotide-aligned species possessed one identical portion that was ≥15bp in length (Figure 3.1.5). Furthermore, all of these matches exceeded 21bp. However, in contrast to 59.38% for *H. taeniaeformis* AAT, the greatest proportion for identical matches was from the barley crop species *H. vulgare*, at 29.21%, for a match 26bp long. But the

longest identical match was not from *H. vulgare* but from *C. remanei*, at 29 nucleotides in length - Which accounted for the second smallest proportion value of 20.57%.

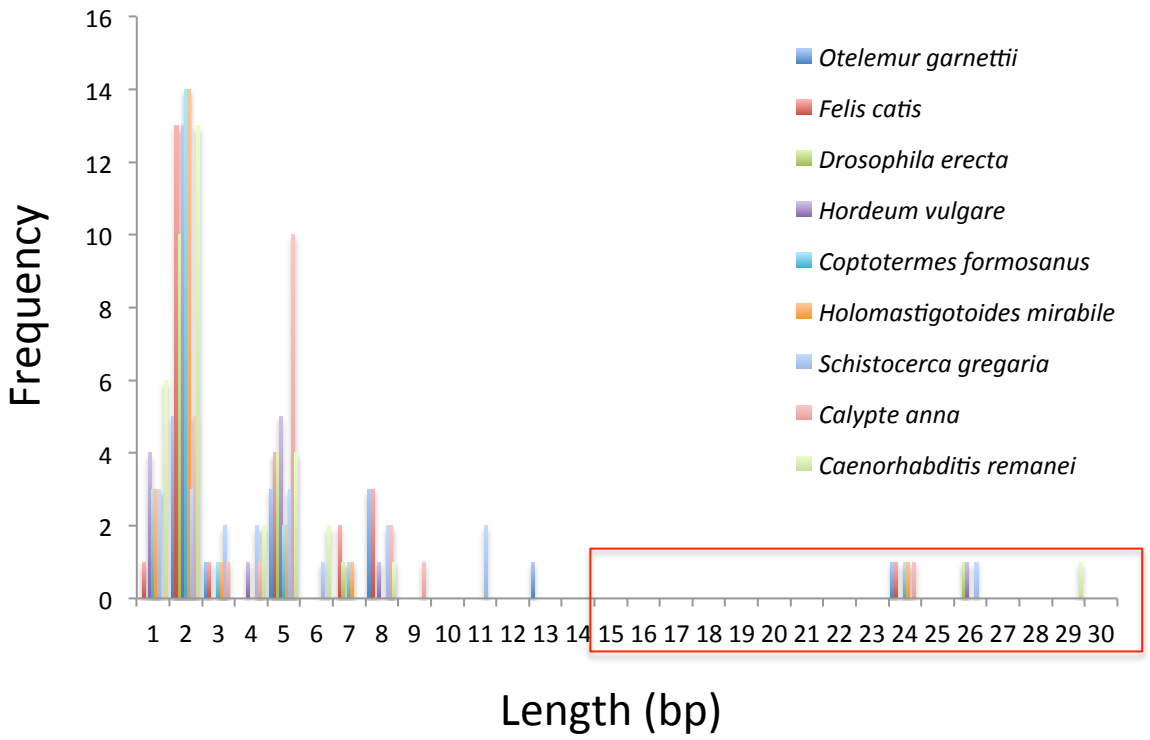


Figure 3.1.5 Lengths of identical nucleotide portions from each species sequence in Alpha-Tubulin nucleotide phylogeny (see figure 3.1.4). Red box indicates those identical portions 15 nucleotides in length or longer considered to pose a risk. The following are the percentage cover of identical nucleotide portions equal to or greater than 15, and equal to or greater than 21, nucleotides in length for each sequence: *Otelemur garnetti* ≥15n = 22.64%; *Felis catis* ≥15n = 17.39%; *Drosophila erecta* ≥15n = 29.21%; *Hordeum vulgare* ≥15n = 19.55%; *Coptotermes formosanus* ≥15n = 24.49%; *Holomastigotoides mirabile* ≥15n = 24.49%; *Schistocerca gregaria* ≥15n = 17.57%; *Calypte anna* ≥15n = 17.39%; *Caenorhabditis remanei* ≥15n = 20.57%.

3.1.6 HSP90: Protein alignments

The longest open reading frame of the HSP90 fragment was 133 amino acids in length. This BLAST sequence was considerably longer than for the other target genes (AAT: 50; ATUB: 49) and produced the most significant and relevant alignments, at 62 species (Figure 3.1.6).

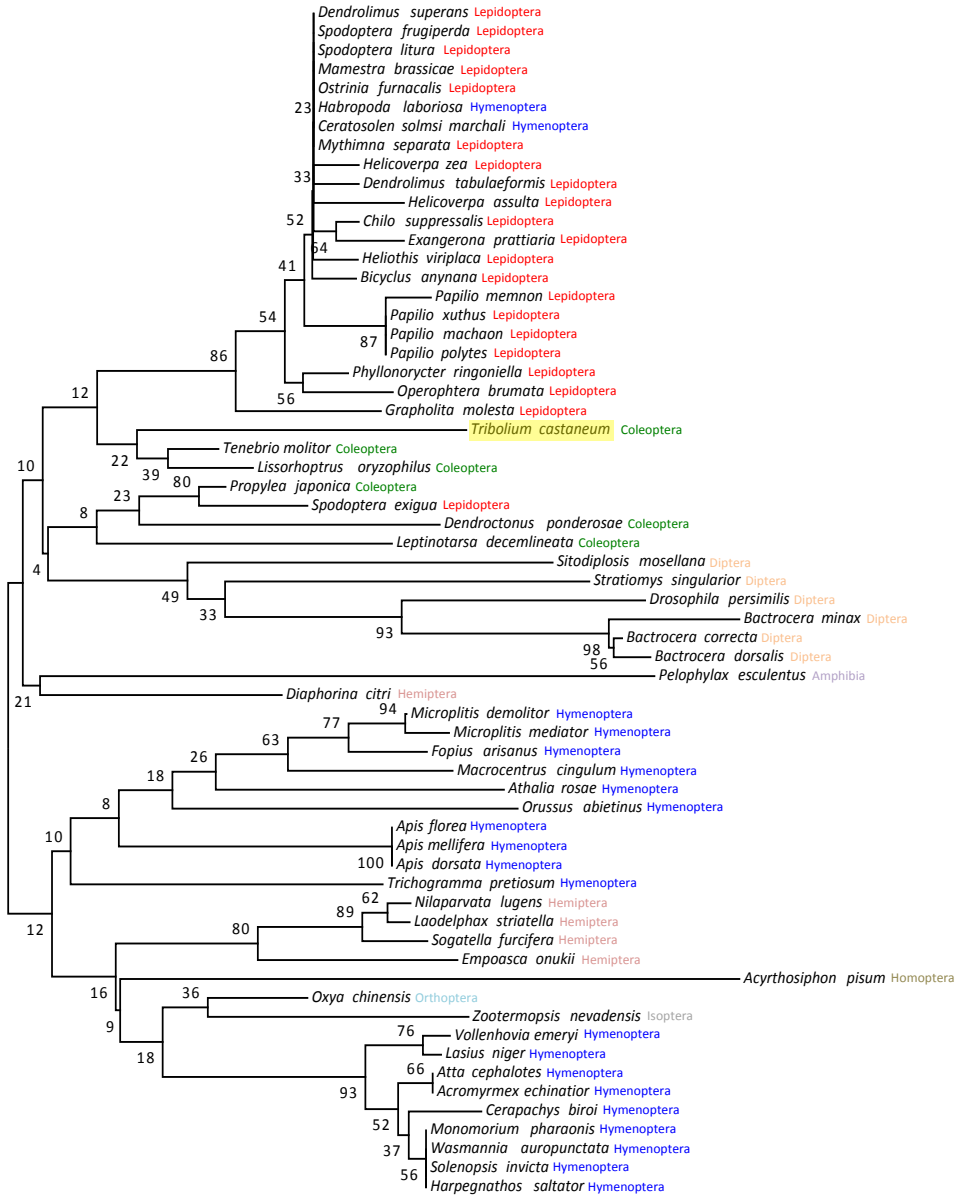


Figure 3.1.6 Phylogeny of Heat Shock Protein 90 significant and relevant amino acid alignments. Phylogram of amino acid sequences built with neighbour-joining method using MEGA (6.06). Numbers are bootstrap values. All species in this phylogeny are of the class Insecta, except for the frog species *Pelophylax esculentis*. Database sequence IDs: [*Helicoverpa zea*: ACV32639.1]; [*Dendrolimus tabulaeformis*: ABM89111.1]; [*Spadoptera frugiperda*: AAG44630.1]; [*Dendromlimis superans*: ABM89112.1]; [*Spadoptera litura*: ADK55516.1]; [*Mamestra brassicae*: BAF03554.1]; [*Ostinia furnacalis*: ADM26737.1]; [*Habropoda laboriosa*: KOC68123.1]; [*Ceratosolen solmsi marchali*: XP_011495774.1]; [*Mythimma separata*: ABY55234.1]; [*Helicoverpa assulta*: ADM26742.1]; [*Chilo suppressalis*: BAE44307.1]; [*Exangerona prattiaria*: ADM26739.1]; [*Heliothis viriplaca*: AHB33569.1]; [*Bicyclus anynana*: AFM73650.1]; [*Papilo memnon*: ADM26736.1]; [*Papilio xuthus*: BAM17884.1]; [*Papilio machaon*: XP_014355020.1]; [*Papilio polytes*: NP_001298475.1]; [*Phyllonorycter ringoniella*: AKN35066.1]; [*Operophtera brumata*: KOB64540.1]; [*Grapholita molesta*: AFV09397.1]; [*Tribolium castaneum*: ABR32189.1]; [*Tenebrio molitor*: AFN02498.1]; [*Lissorhoptrus oryzophilus*: AHE77376.1]; [*Propylea japonica*: AHW57925.1]; [*Spodoptera exigua*: ACL77779.1]; [*Dendtroctonus ponderosae*: AEE61673.1]; [*Leptinotarsa decemlineata*: AHB18587.1]; [*Sitodiplosis mosellana*: AIS72814.1]; [*Stratiomys singularior*: AER28025.1]; [*Drosophila persimilis*: XP_002021279.1]; [*Bactrocera minax*: AIA62362.1]; [*Bactrocera correcta*: AGU42456.1]; [*Bactrocera dorsalis*: ADO30471.1]; [*Pelophylax esculentus*: CAC29071.1]; [*Diaphorina citri*: XP_008487009.1]; [*Microplitis demolitor*: XP_008551765.1]; [*Microplitis mediator*: ABV55506.1]; [*Fopius arisanus*: XP_011310275.1]; [*Macrocentrus cingulum*: ACE77780.1]; [*Athalia rosae*: XP_012250991.1]; [*Orussus abietinus*: XP_012270106.1]; [*Apis florea*: XP_003694932.1]; [*Apis mellifera*: XP_006571335.1]; [*Apis dorsata*: XP_006616995.1]; [*Trichogramma pretiosum*: XP_014223994.1]; [*Nilaparvata lugens*: ADE34169.1]; [*Laodelphax striatella*: AHB63833.1]; [*Sogatella furciera*: AFK64820.1]; [*Empoasca onukii*: AIM18803.1]; [*Acyrthosiphon pisum*: BAH71458.1]; [*Zootermopsis nevadensis*: KDR11182.1]; [*Vollenhovia emeryi*: XP_011879750.1]; [*Lasius niger*: KMQ95531.1]; [*Atta cephalotes*: XP_012059177.1]; [Acromyrmex echinatior: XP_011060405.1]; [*Cerapachys biroi*: XP_011345590.1]; [*Monomarium pharaoinis*: XP_012539357.1]; [*Wasmannia auropunctata*: XP_011691196.1]; [*Solenopsis invicta*: XP_011171324.1]; [*Harpegnathos saltator*: XP_011154034.1].

However, the constructed phylogram was the least diverse. Except for one Anuran, all were insects, representing a total of nine orders: 21 Lepidoptera species; 21 Hymenoptera; six Coleoptera (including *T. castaneum* query); six Diptera; five Hemiptera; one Homoptera; one Orthoptera; and one Isoptera. At 47 nodes, the tree exhibited the most clades. The nearest node to *T. castaneum* was 22%, which compared to 100% for the three honey bees, indicated no distinct closely related species. Node parameters: Number of nodes, 47; Bootstrap range, 96 (100-4); Mean bootstrap, 45.4.

3.1.7 HSP90: Nucleotide alignments

Despite producing the most protein alignments, the same HSP90 fragment produced the fewest on the nucleotide-level (Figure 3.1.7).

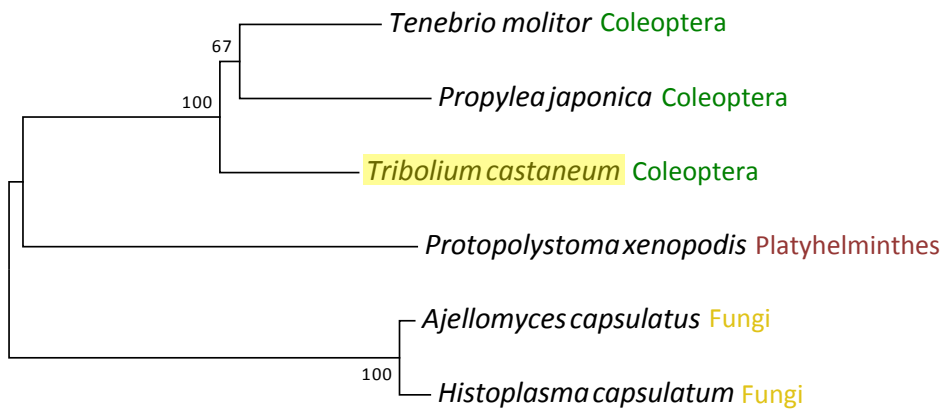


Figure 3.1.7 Phylogeny of Heat Shock Protein 90 significant and relevant nucleotide sequence hits. Phylogram of nucleotide sequences built with neighbour-joining method using MEGA (6.06). Numbers are bootstrap values. NCBI BLAST word size: 28. Database identifiable names and sequence IDs: *Tenebrio moliter* clone HSP90 mRNA, complete cds [Sequence ID: JQ918768.1]; *Propylea japonica* strain HSP90 mRNA, complete cds [Sequence ID: KF792063.1]; *Tribolium castaneum* HSP90 gene, complete cds [Sequence ID: EF633444.1]; *Protopolystoma xenopodis* genome assembly [Sequence ID: LM846143.1]; *Ajellomyces capsulatus* NAm1 ATP-dependent molecular chaperone, partial mRNA [Sequence ID: XM_001540796.1]; *Histoplasma capsulatum* HSP82 gene, complete cds [Sequence ID: M55629.1].

In total, five relevant species were significantly aligned (0.08% the number of protein alignments). Unlike no commonality between the ATUB phylogenies, two species appeared in both HSP90 phylograms, the coleopterans *T. moliter* and *P. japonica*. The other nucleotide-aligned species represented the phylum Platyhelminthes and the kingdom Fungi, making the nucleotide-level alignments for this target gene more diverse than on the

protein-level. But on the nucleotide-level, it was still second to ATUB in diversity. Node parameters: Number of nodes, 4; Bootstrap range, 33 (100-67); Mean bootstrap, 89.

3.1.8 HSP90: ≥15bp identical matches

As for the other target genes, all HSP90-aligned nucleotide sequences possessed identical portions ≥15bp long (Figure 3.1.8).

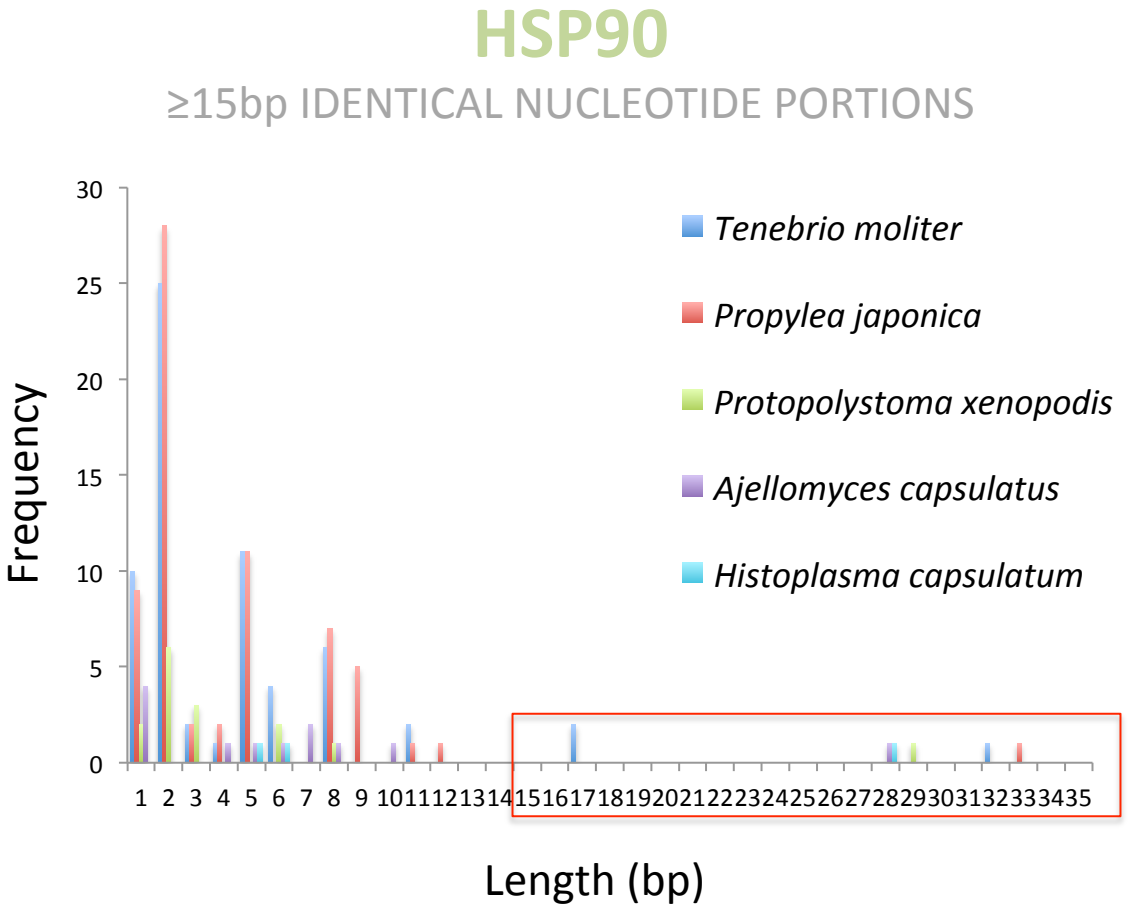


Figure 3.1.8 Lengths of identical nucleotide portions from each species sequence in heat shock protein 90 nucleotide phylogeny (see figure 3.1.7). Red box indicates those identical portions 15 nucleotides in length or longer considered to pose a risk. The following are the percentage cover of identical nucleotide portions equal to or greater than 15 nucleotides in length for each sequence: *Tenebrio moliter* ≥15n = 18.03%; *Propylea japonica* ≥15n = 9.19%; *Protopolystoma xenopodis* ≥15n = 31.18%; *Ajellomyces capsulatus* ≥15n = 28.87%; *Histoplasma capsulatum* ≥15n = 68.29%.

These five alignments had portions ≥21bp. Four of the five had one potential off-target hit and, unlike for the other genes, the beetle *T. moliter* possessed more than one: two 17 nucleotides long and one 32 nucleotides in length. This target gene produced the smallest and the greatest ≥15bp proportions: 9.19% in *P. japonica* and 68.29% in *H. capsulatum*. But although the smallest in proportion, the *P. japonica* identical match was also the longest of all potential hits, at 33bp.

3.2 SURVIVORSHIP

3.2.1 Trends in survivorship

On most days, mixed-targeted larvae did not exhibit lowest survivorship (Figure 3.2.1).

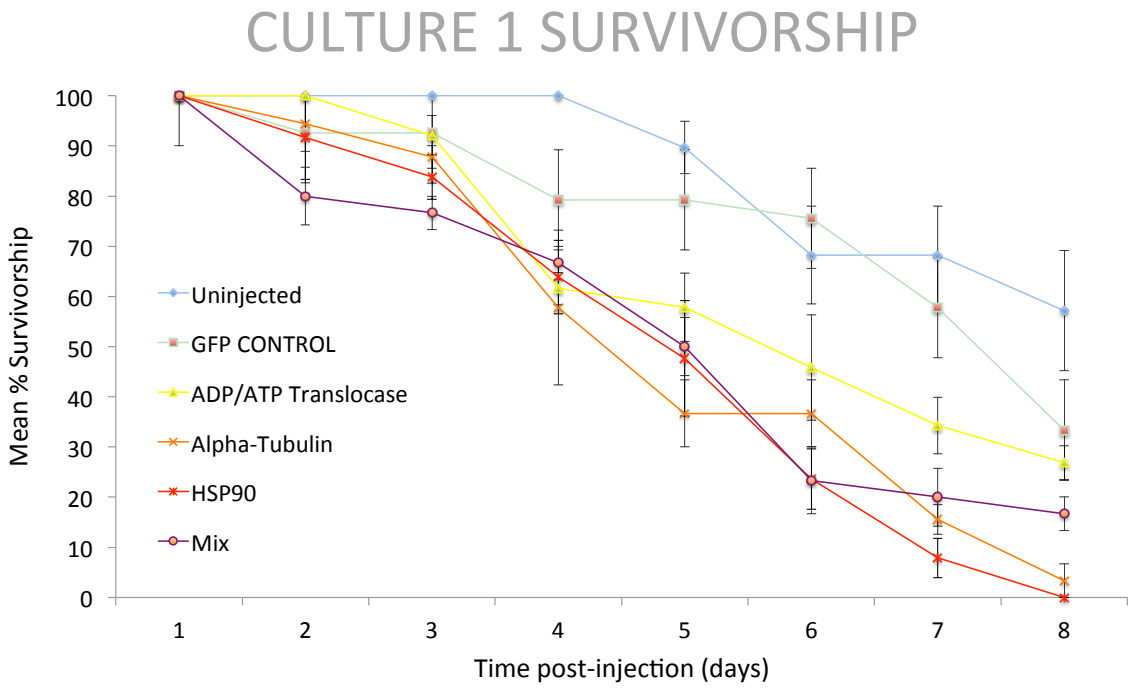


Figure 3.2.1 Culture 1 mean percentage survivorships of microinjected larvae. With SD error bars. Day 1 post-injection normalised to 100% to account for lag in knockdown effect.

Also, for most days, control survivorship was greatest. Mean percentage survivorship was normalised to that of day 1, which was: Uninjected 63%; GFP 77%; AAT 87%; ATUB 89%; HSP90 83%. After day 1, no uninjected larvae died until day 5 (two larvae, one from replicates one and three). Except for day 6, uninjected larvae showed greater survivorship than those of the GFP control. Lowest survivorship (greatest mortality) was displayed in the

following treatments over the following days: MIX, days 2-3; ATUB, days 4-5; MIX and HSP90 on day 5; and HSP90 from days 6-8. Larvae separately targeted for HSP90 were the only to reach 0% survivorship by day 8. Post day 2, except for equal survivorship for AAT- and GFP-targeted larvae on day 3, control larval survivorships were greatest. Larvae separately targeted for AAT revealed consistently greatest survivorship of all targeted larvae, except on day 4. Day 4 exhibited greatest clumping of targeted larval survivorships.

3.2.2 Peak knockdown

In culture 1, the day that presented the most significant ($P<0.05$) disparity between treatments (separate and mixed targeting) and controls (uninjected and GFP) was day 5 (Figure 3.2.2) (n=3) [$F_{(6,11)} = 5.06$, $P = 0.01$].

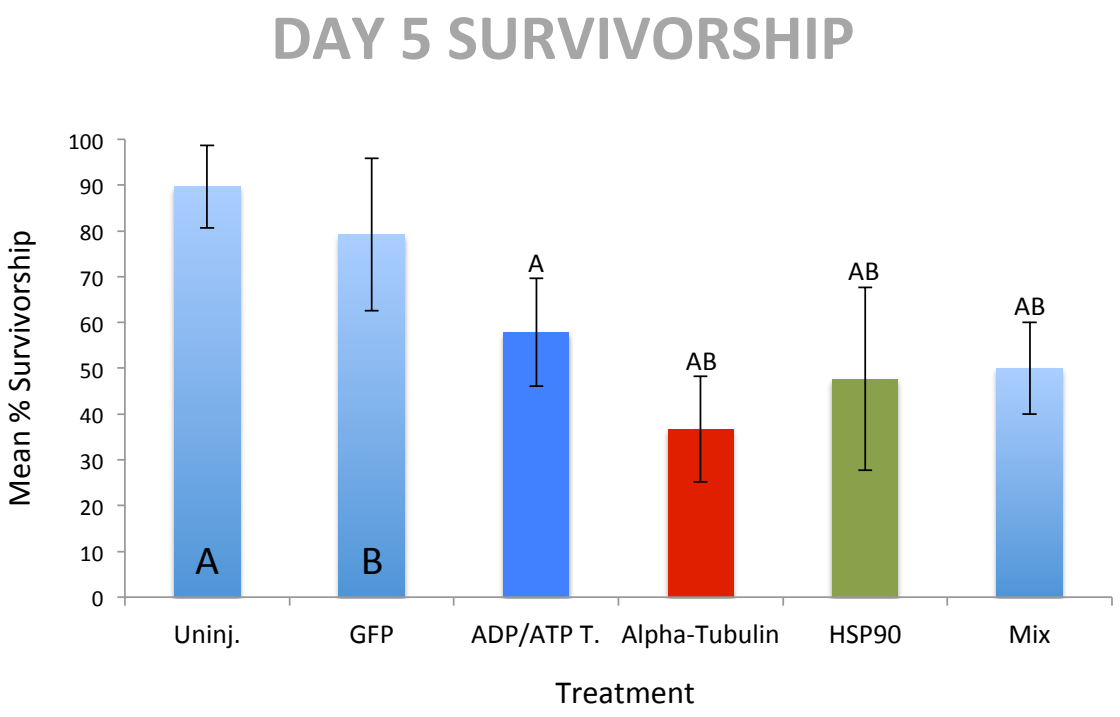


Figure 3.2.2 Day 5 mean survivorship for each treatment with SD error bars. Letters above error bars indicate treatments statistically significantly different, as calculated using a one-way ANOVA Fisher's post hoc comparison, Minitab Express (1.2.0). Day 5 survivorship exhibited greatest disparity between treatments and controls. Due to lag-effect, day 4 was inferred to have been day of peak knockdown and therefore day for optimum harvest.

All separate and mixed targeted survivorships on this day were significantly different to that of the uninjected control (Bar A). And AAT was the only treatment not significantly different in survivorship to the GFP control (Bar B). More mixed-targeted larvae survived to day 5 than two of the three separately-targeted treatments, but the difference was not significant. Due to the lag-effect, day 4 knockdown was inferred to have been responsible for day 5 mortality and therefore the day of peak knockdown. Day 4 of culture 2 was thus chosen as the day for optimum RNA harvesting.

3.3 GENE EXPRESSION

3.3.1 RT-qPCR quantification cycles & phases

The quantification cycle (Cq) threshold lines were generated by CFX Manager (3.1) and demonstrated the fluorescence levels necessary for detection (Schefe *et al*, 2006). The Cq values per reaction corresponded to the number of cycles required for generated fluorescence to cross the threshold. RT-qPCR amplification curves charted the build-up of fluorescence emission at each cycle (Figure 3.3.1). The level of fluorescence corresponded to the level of gene expression. A one-way ANOVA and Fisher's post hoc comparison were applied to Cq values corresponding to mixed-targeted and GFP control gene expression. Grouping information indicated that control ATUB Cq values (18.33 ± 1.66 SD) was significantly ($P<0.05$) different to that of all other targets, bar that of AAT control larvae (20.29 ± 0.35), which was significantly ($P<0.05$) different only to targeted AAT. GFP control HSP90 Cq values were second highest (22.5 ± 1.47) and the only control result, of both plates, not significantly different to its corresponding targeted treatment. All separate-targeting plate targets were significantly ($P<0.05$) different except for separately-targeted ATUB and HSP90 (25.31 ± 1.73 & 24.42 ± 0.18) [$F_{(5,28)} = 90.29$, $P < 0.0001$]. Targets with lower Cq values suggested greater gene expression by reaching threshold fluorescence earlier. The following are separate and mixed targeted, and control, ranked gene expression lowest to highest, as indicated by Cq findings (not normalised): Separate-targeted [$ATUB_{(uninjected\ control,\ UC)}$, $AAT_{(UC)}$, $HSP90_{(UC)}$, $HSP90_{(knocked\text{-}down,\ KD)}$, $ATUB_{(KD)}$, $AAT_{(KD)}$]; Mixed-targeted [$ATUB_{(GFP\ control,\ GC)}$, $AAT_{(GC)}$, $HSP90_{(KD)}$, $ATUB_{(KD)}$, $HSP90_{(GC)}$, $AAT_{(KD)}$]. Reactions from both plates exhibited considerable overlapping and cross-over in the plateau phase, as visualised for the mixed-targeting plate in Figure 3.3.1.

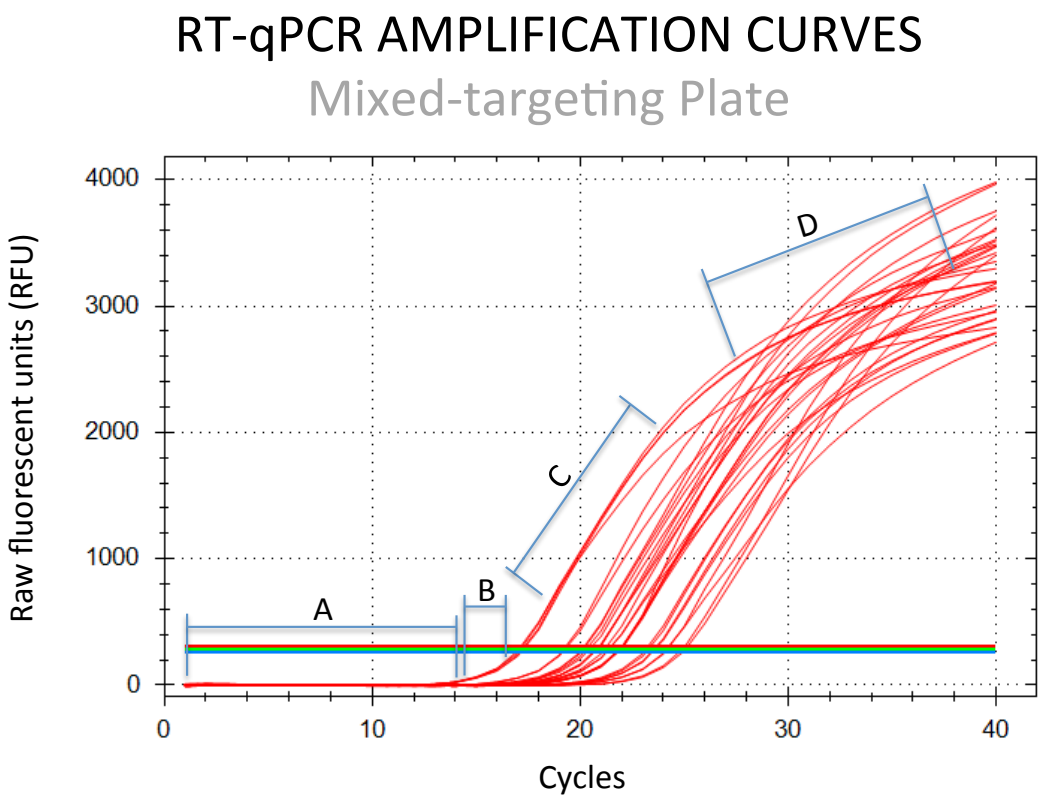


Figure 3.3.1 RT-qPCR gene expression profiling for three target genes from mixed-targeting plate. Includes target gene expression results from GFP control larvae. Does not include RP6 gene expression. Showing Cq threshold lines: AAT (blue); ATUB (red); HSP90 (green). Curves chart build-up of fluorescence emission at each cycle. Letters denote phases: (A) Linear ground phase; (B) Early exponential phase; (C) Log-linear phase; (D) Plateau phase. Curves in plateau phase are closer together and overlap. Graph formed using CFX Manager (3.1).

3.3.2 Melt curve product validation

RT-qPCR intercalating dye bonded to any cDNA and was not sequence-specific. To ascertain whether or not a single product was amplified per amplicon, per well, melt curves were produced. A single melt curve peak for an amplicon indicated a single amplified product. The results indicated that a single product was formed per amplicon, per reaction well, for both the separate- and the mixed-targeting plate assays. And the three gene products each had a different mean melting point (Figure 3.3.2). Mean melting temperatures per target gene, per plate, for knocked-down and control amplicons were: Separate-targeted [AAT 81.25 ^{o}C ± 0.27 SD, ATUB 80.5 ^{o}C ± 0, HSP90 77.65 ^{o}C ± 0.24]; Mixed-targeted [AAT 81.19 ^{o}C ± 0.26, ATUB 80.04 ^{o}C ± 0.14, HSP90 77.72 ^{o}C ± 0.26].

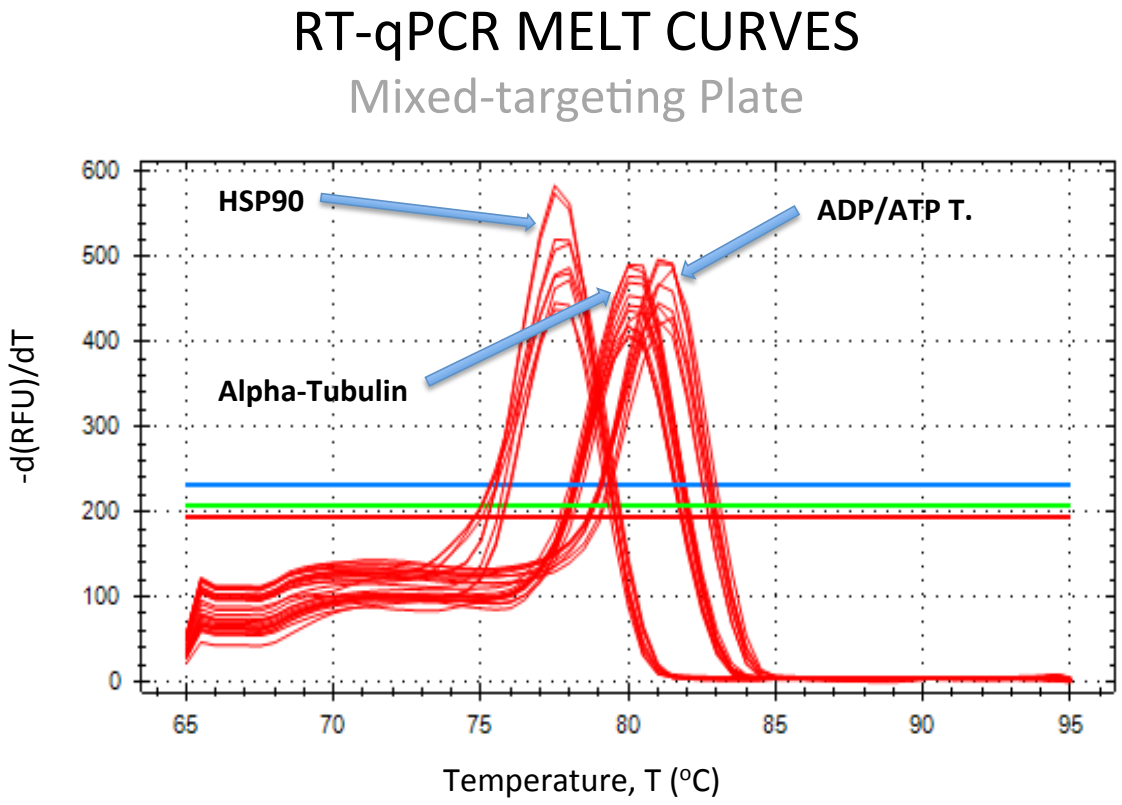


Figure 3.3.2 RT-qPCR melt curves of mixed-targeted trialled genes. A single peak formed per target gene amplicon, indicating a single product. Mean peak melting point per gene: AAT 81.19 °C ± 0.26, ATUB 80.04 °C ± 0.14, HSP90 77.72 °C ± 0.26. Graph formed using CFX Manager (3.1).

3.3.3 Gene expression according to RT-qPCR

RT-qPCR results for separate gene targeting (Figure 3.3.3A) indicated very considerable gene knockdown. Compared to uninjected control larvae, separately-targeted larvae produced a >99.9% reduction in AAT and ATUB gene expression (0.082% expression ± 0.027 SEM & 0.083% ± 0.042, respectively) and a >98% reduction in HSP90 gene expression (1.602% ± 0.111). Individual gene expression in mixed-targeted larvae was also considerably reduced, when compared to GFP control larvae (Figure 3.3.3B). Expression level ranking was different for mixed-targeted expression, with ATUB displaying a >97% reduction (2.977% ± 1.848); then AAT a >95% reduction (4.221% ± 2.981). But reduction in mixed-targeted HSP90 gene expression was again least. However, mixed-targeted HSP90 expression was exceptionally greatest at 70.518% (± 10.613). It was the only not to demonstrate at least a >95% reduction, and instead provided a considerably lesser >29% loss in expression.

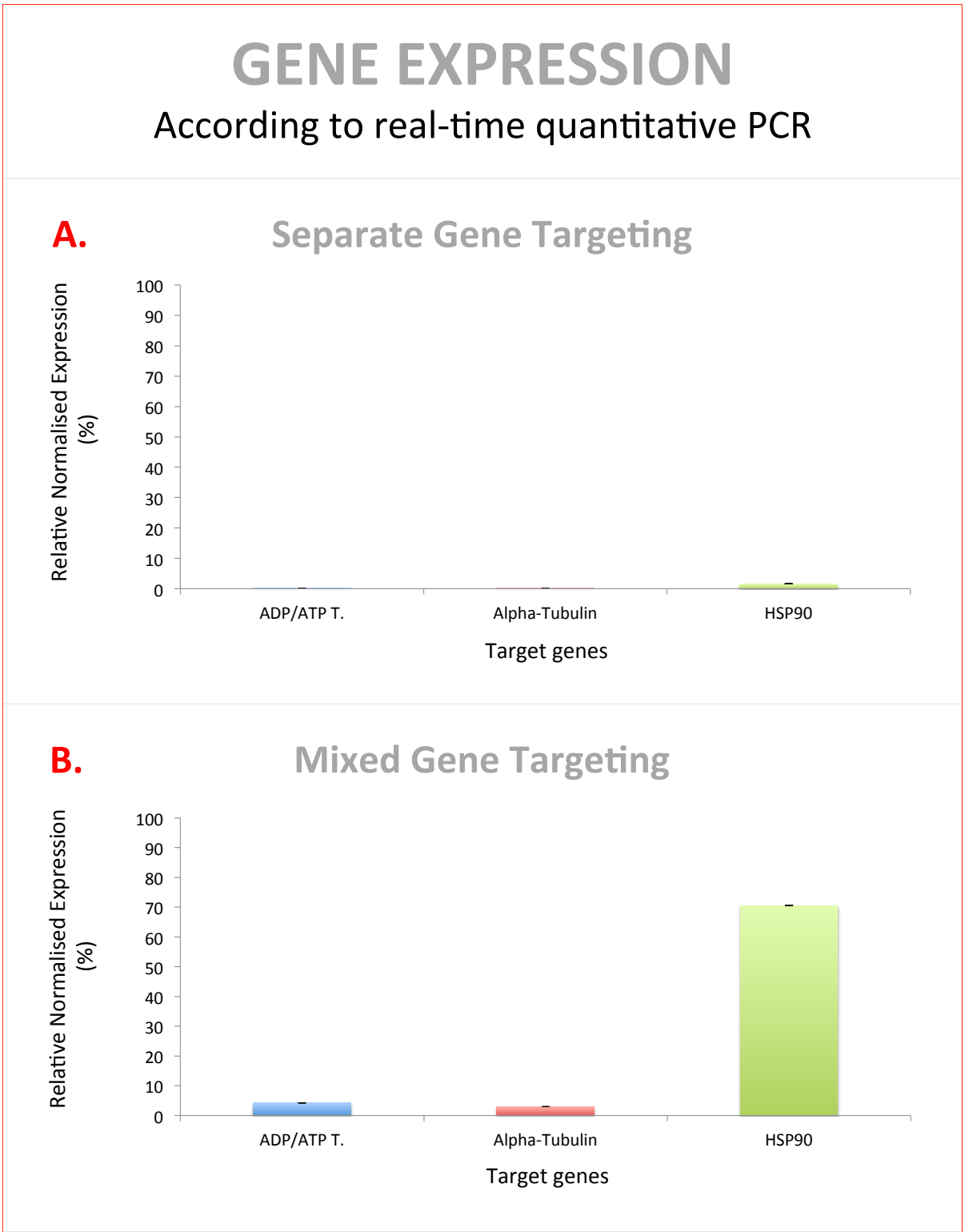


Figure 3.3.3 Relative normalised gene expression according to RT-qPCR data. (A) Separate-targeted gene expression normalised to uninj. control (uninj. gene expression = 100%). From larvae microinjected with 60ng of dsRNA, 60ng of reagent per gene. (B) Mixed-targeted gene expression normalised to GFP control (GFP = 100%). From larvae microinjected with 60ng of dsRNA mixture, 20ng of reagent per gene. With percentage expression SEM error bars. Mean relative normalised expression results were computed by CFX Manager (3.1).

3.3.4 Gene expression according to gel electrophoresis densitometry

Both separately and mixed targeted gene expression, according to gel electrophoresis densitometry (Figure 3.3.4), were significantly ($P<0.05$) greater overall than according to RT-qPCR [Separate-targeting $F_{(1,4)} = 136.74$, $P = 0.0003$] [Mixed-targeting $F_{(1,4)} = 5.33$, $P = 0.0821$]. Furthermore, unlike according to RT-qPCR, densitometric analysis indicated expression was greater in mixed-targeted larvae than in those separately targeted, for all but the ATUB target gene. However, separate-targeted expression level ranking was the same between methods: AAT (89.87%), ATUB (90.29%), then HSP90 (115.13%). But was not for mixed-targeting: AAT (71.51%), ATUB (80.34%), then HSP90 (82.09%). Separate and mixed targeted HSP90 expression was again greatest in both. Unlike from RT-qPCR, an expression of 115.13% for separately-targeted HSP90 suggested greater HSP90 expression in microinjected larvae than in uninjected larvae. However, gel electrophoresis densitometry was considered a substantially less accurate method. Moreover, the results were not as representational, because the RT-qPCR reactions, used at the beginning of electrophoresis, were accessed in the plateau phase (see Figure 3.3.1). In which the control and targeted amplicons were considerably closer together and crossed-over. Accordingly, only gene expression results derived from RT-qPCR were analysed in section 3.4.

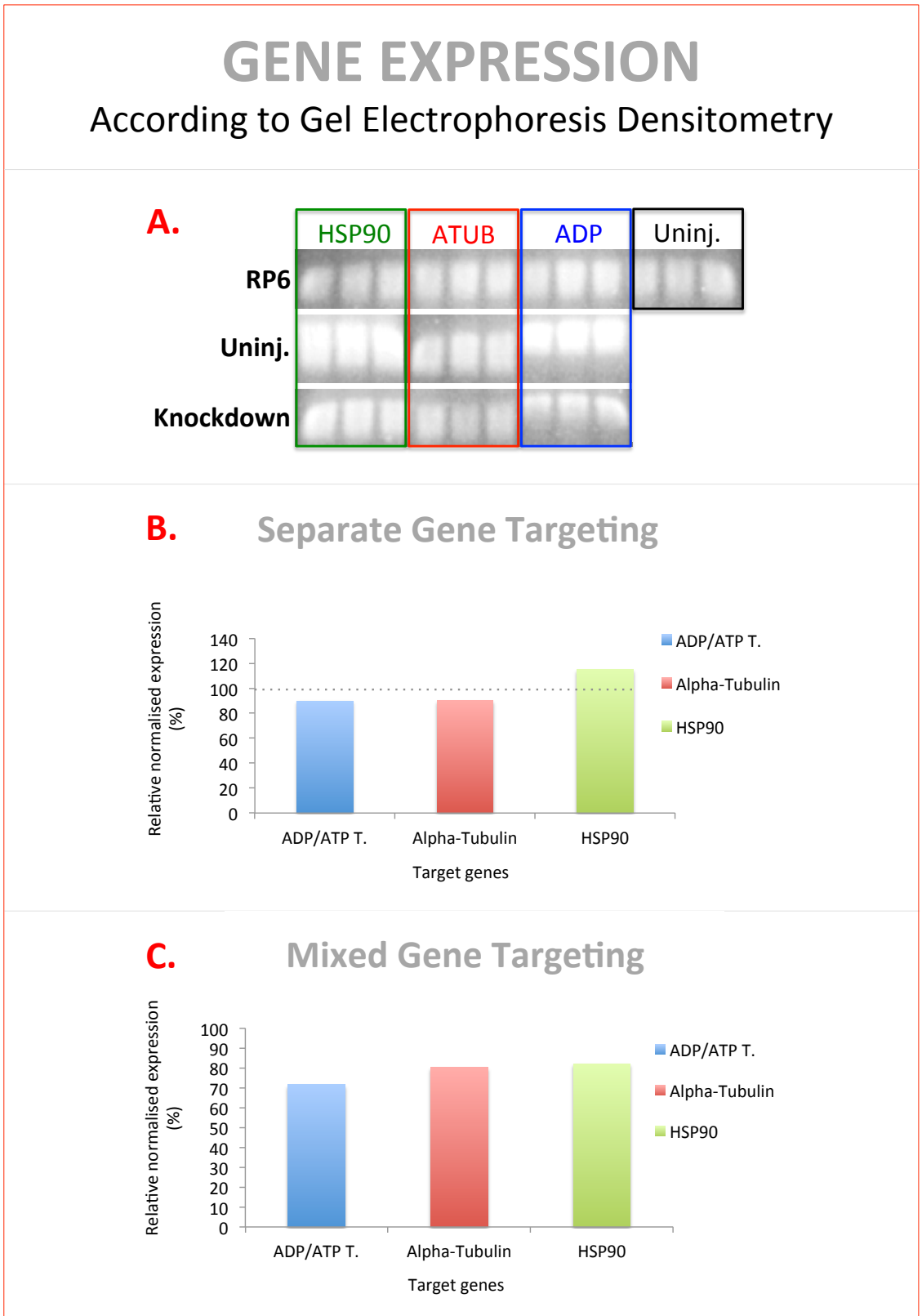


Figure 3.3.4 Visualising gene expression from gel electrophoresis data. (A) UV photographs of separate-targeting tray gel bands showing differences in band brightness corresponding to differences in gene expression. (B) Separate-targeted gene expression according to densitometry analysis of band brightness normalised to RP6 housekeeping gene and compared to normalised uninj. control (uninj. = 100% expression). (C) Mixed-targeted gene expression compared to normalised GFP control (GFP = 100%) Densitometry analysed using imageJ (32).

3.4 RELATIONSHIPS: SEPARATE GENE TARGETING, MIXED GENE TARGETING, MORTALITY & KNOCKDOWN

3.4.1 Analyses of variance

Separate-targeting percentage mortalities (Figure 3.4.1A) from culture 1 day 5 larval survivorships were normalised to that of uninjected larvae, in keeping with gene expression normalisation. Likewise, mixed-targeting mortality was normalised against that of GFP control larvae. Mixed-targeting mortality was the second smallest both when normalised (36.53% ± 6.27 SD) and as an absolute (50% ± 10). Only mean knockdown values were accessed as the inverse of the CFX Manager (3.1) expression outputs (see Figure 3.3.3). To compare separate- to mixed-targeting mortalities, separate-targeting values were considered a single sample, as well as individually. Correspondingly, so was separate-targeting knockdown (Figure 3.4.1B).

Mixed-targeting mortality per larva resulted from combined knockdown of the three target genes. Therefore, mixed-targeting mean knockdown per gene was stacked to reveal a value of 222.28% overall knockdown per larva. Mixed-targeting mortality did not significantly ($P>0.05$) differ to separate-targeting mortality [$F_{(1,10)} = 0.82$, $P = 0.3851$]. In contrast, mixed-targeting knockdown, additive, did significantly ($P<0.05$) differ to that when separately targeted [$F_{(2,1)} = 4901.19$, $P = 0.0101$]. However, mixed-targeting, non additive, was not significantly ($P>0.05$) different to that of separately targeted larvae [$F_{(2,3)} = 0.48$, $P = 0.6579$].

Separately-targeted AAT knockdown was greatest at 99.918%, but by only 0.001%. However, ATUB was highest of mixed-targeting knockdown at 97.023%, by a margin of 1.244%. All separately and mixed targeted genes exhibited >95% knockdown, except mixed-targeted HSP90 at 29.482%. HSP90 knockdown was considerably least by both targeting methods. When mixed-targeted, HSP90 knockdown was 3.25x less than AAT (16.71x greater expression) and 3.29x less than ATUB (23.69x greater expression). When separately targeted, it was 1.015x less than AAT (19.94x greater expression) and 1.015x than ATUB (19.03x greater expression). Despite such lowest knockdown, corresponding HSP90-targeted mortality was an insignificant ($P>0.05$) intermediate 46.76% (±23) [$F_{(3,8)} = 1.34$, $P = 0.3269$].

Separate vs. Mixed Gene Targeting

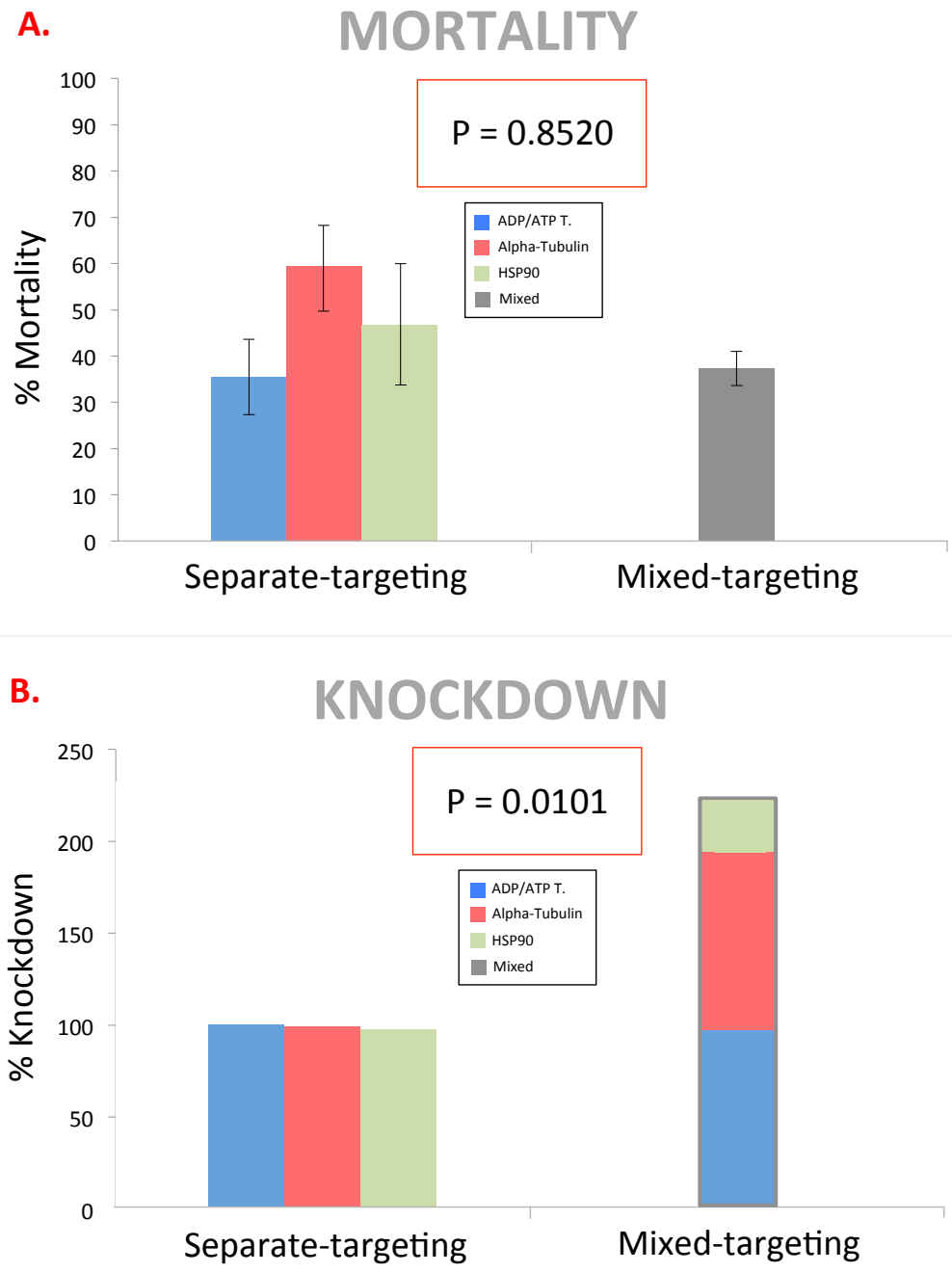


Figure 3.4.1 Mean separate and mixed targeted mortality and knockdown per larva. (A) Culture 1 day 5 post-injection mortality. Percentage mortality normalised to uninjected control for separate-targeting (coloured bars) and to GFP control for mixed-targeting (dark grey bar). Includes SD error bars. Results of a one-way ANOVA of separate vs. mixed targeting (Minitab Express, 1.2.0): $P>0.05$. (B) Culture 2 day 4 knockdown. Mixed-targeting is additive. Percentage knockdown normalised to uninj. control for separate-targeting (coloured bars) and to GFP control for mixed-targeting (dark grey frame containing coloured individual knockdown). Results of a one-way ANOVA of separate vs. mixed targeting: $P<0.05$.

3.5 DEFORMED PUPAE

Deformed pupae developed from cultures 1 and 2 (Figure 3.5.1).

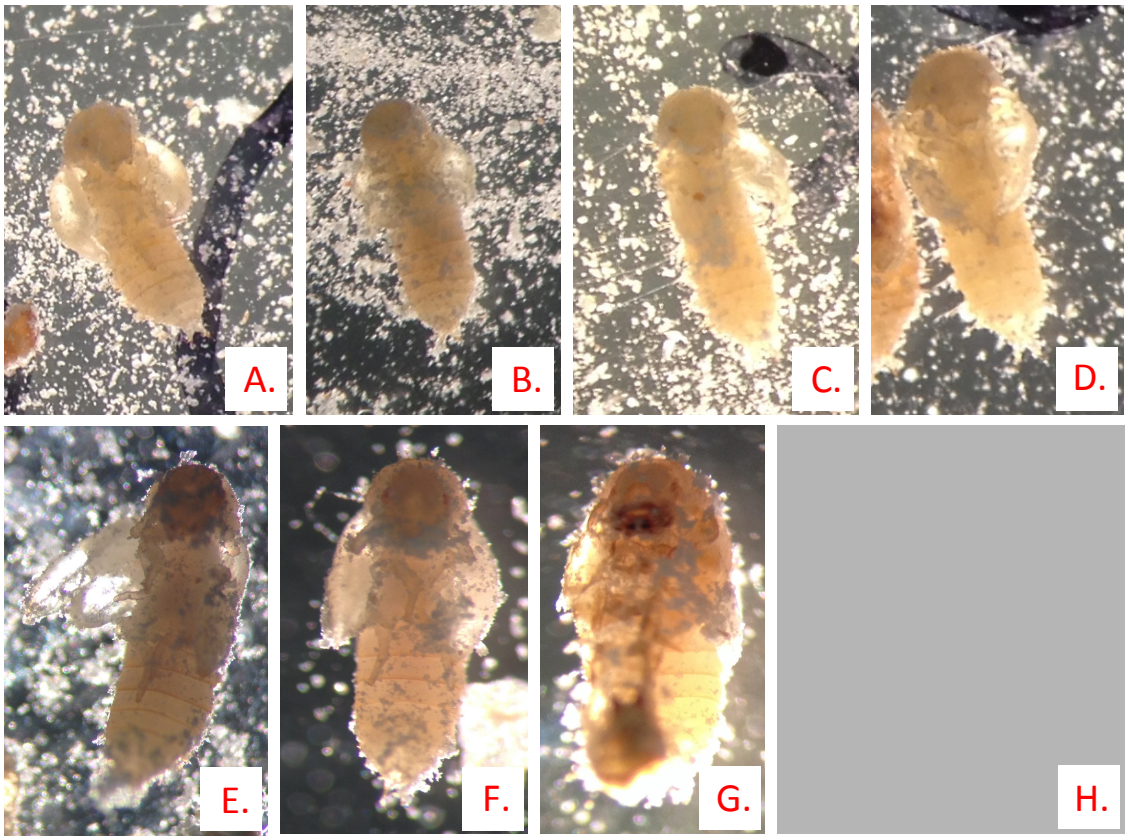


Figure 3.5.1 Photographs of deformed pupae. Details: (A) ATUB-targeted, culture 1, day 3 post-injection, replicate 2, 23.10.2015. (B) ATUB-targeted, culture 1, day 3 post-injection, replicate 3, 23.10.2015. (C) Mixed-targeted, culture 1, day 4 post-injection, replicate 1, 24.10.2015. (D) Mixed-targeted, culture 1, day 5 post-injection, replicate 3, 25.10.2015. (E) ATUB-targeted, culture 1, day 1 post-injection, 03.11.2015. (F) ATUB-targeted, culture 2, day 2 post-injection, 03.11.2015. (G) ATUB-targeted, culture 2, day 2 post-injection, 03.11.2015. (H) Not photographed. ATUB-targeted, culture 1, day 6 post-injection, 26.10.2015. Note, pupa G appeared to not have fully shed, apparently due to deformed appendages.

A total of 67 deformed and non-deformed pupae were observed: 49 from the culture 1 starting population of 179 larvae and 18 from the 60 culture 2 larvae (Figure 3.5.2). Of these, eight exhibited the deformation (11.94% of total). The deformation only arose in ATUB- and mixed-targeted pupae. The majority of ATUB-targeted pupae displayed the deformity. This was exactly five-fold more than amongst those mixed-targeted, which were mostly non-deformed. On the iBeetle database for *Tribolium* RNAi phenotypes, 826 pupal appendages have been reported, most of which were of denatured wings and elytra. The 'elytra

blistered' deformation accounted for 15 of these reports and was the most similar in appearance, particularly in iBeetle screen iB_00101 (http://ibeetle-base.uni-goettingen.de/details/iB_00101), which was derived from the fly ortholog tubulin-binding cofactor B (TBCB, CG11242).

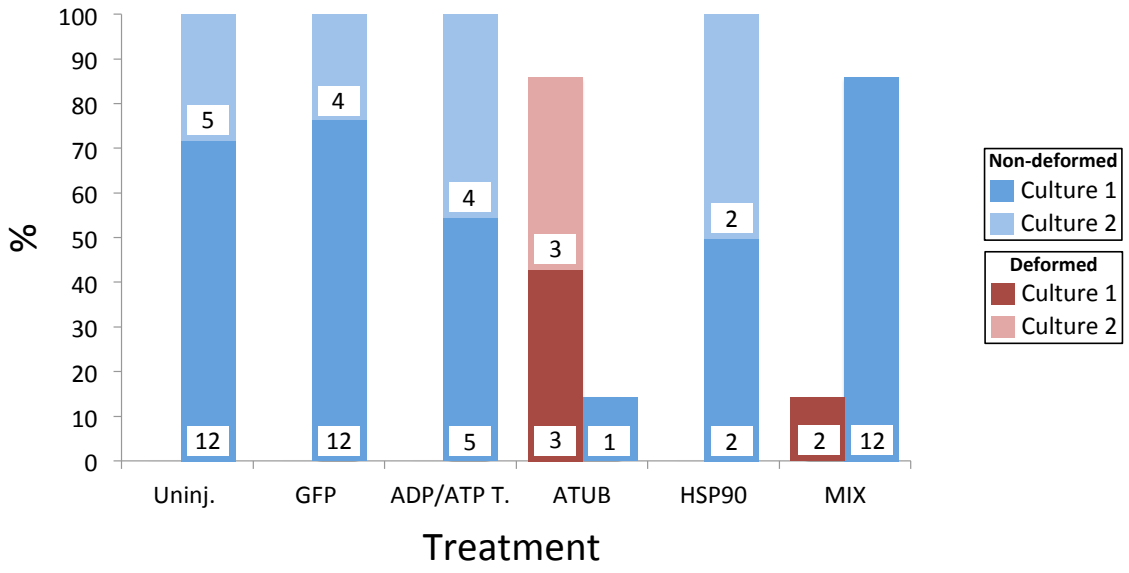


Figure 3.5.2 Percentage bar graph of deformed and non-deformed pupae from cultures 1 and 2. Deformed pupae only developed from ATUB- or mixed-targeted larvae. Absolute numbers of pupae are displayed within bars.

4. Discussion

4.1 CONCLUSIONS, THEIR SIGNIFICANCE & FUTURE RESEARCH

The main pest-control selling point of RNAi is its exceptional specificity (Aronstein *et al*, 2011; Baum *et al*, 2007; Yang *et al*, 2001; Qadota *et al*, 2007; Whyard *et al*, 2009). Therefore, the potential of non-target hits should be the main benchmark for determining the best gene choice. The minimum threshold for off-target hits is normally 21bp identical matches (Caplen, 2001; McManus & Sharp, 2002; Kennedy *et al*, 2004; Kim *et al*, 2005; Shen *et al*, 2003). Thus AAT, the only trialled gene not to have revealed any identical matches ≥21bp, was the most off-target safe. Moreover, its least nucleotide-level diversity compliments this conclusion. Despite this, the 19bp longest match (which was also proportionately greatest at 59.38%) was still on a proposed threshold (Czauderna *et al*, 2003; Kulkarni *et al*, 2006). Thus AAT was still not specific enough. Interestingly, this most probable hit with AAT came from one of the least related species, a Platyhelminth. This suggests less related organisms are not necessarily safer. Accordingly, BLAST alignments should be explored with more caution.

The longer the dsRNA the greater chance of off-target effects (Qiu *et al*, 2005). However, despite producing the most protein-level alignments (62), possibly due to having been over twice the length of both other fragments, the HSP90 BLAST sequence was the second most off-target secure. This was because ATUB demonstrated the highest protein and nucleotide diversity. Also, because it indicated a ≥21bp potential hit with the highly relevant and agriculturally important species, barley (Ullrich, 2011). None of the target fragments were safe enough, as an off-target free reagent should be the pest-control aim. As such, care should be taken to further fine-tune the trialled gene specificities by removing identical match sequences from dsRNA design.

Despite requiring improvements in specificity, all trialled dsRNAs rendered significant ($P<0.05$) mortality. Furthermore, most strikingly, in all but larvae mixed-targeted for HSP90, expression was reduced by a dramatic >95%. These findings certainly help to highlight the

potency of RNAi as a potential pest-control. However, some intriguing results were exposed. Despite exhibiting a noteworthy 99.918% greatest reduction in expression, separate-targeted AAT consistently (but one day) provided the lowest target mortality. It was also the only gene to produce a survivorship insignificantly ($P>0.05$) different to that of GFP control larvae, on the day of peak mortality. What is more, separately targeted HSP90 expression was >19x greater than that of both AAT and ATUB. Yet its corresponding survivorship was not the least. Indeed, larvae separate-targeted for HSP90 were the only ones to reach 0% survivorship by day 8. These findings are interesting and indicate that knockdown does not directly relate to mortality. As such, it should not be used entirely as an indicator of a successful pest-control. It also suggests some genes are more important for larval survival. If so, in this case it would appear HSP90 was more critical than AAT. If some genes do indeed cause greater mortality with less knockdown, care should be adopted to focus on these as pest-control targets.

In a previous study, mixed RNAi gene targeting was shown to have been more potent than separate-targeting (Kim *et al*, 2004). This would perhaps be intuitive. Nonetheless, the results of this research suggest separate-targeting may be surprisingly more efficient. Mixed-targeted mortality was the second lowest of all target treatments on the day of peak mortality, both absolute (50%) and normalised (36.53%). Focus should be predominantly directed therefore to improving separate-targeting trials, particularly in reducing off-target risk. However, the differences between separate and mixed targeted mortalities were not significant ($P>0.05$). Furthermore, mixed-targeting still significantly ($P<0.05$) lowered larval numbers. The reasons for lesser mixed-targeted mortality are unknown. Considering that each gene was targeted with a third of the dsRNA than when separately targeted, this may have had an influence. Perhaps future investigations using a variety of ratios, e.g. 1:1:2 and 1:2:3 etcetera, may provide greater mortality than separate-targeting.

Additionally, it may be that the functional dissimilarities between this study's trial genes contributed to a dilution effect of RNAi potency when mixed-targeted. It may be that simultaneously targeting more similar genes would have greater focused the RNAi influence, even synergistically. AAT silencing was shown to produce significant host mortality. It is known that AAT is responsible for ATP retrieval from the location of synthesis, the

mitochondrial matrix (Saraste & Walker, 1982). The energy derived from the hydrolysis of this ATP is crucially applied to proton transport in sustaining many organelles, by an enzyme known as v-ATPase (Dow *et al*, 1997). RNAi knockdown of vATPase in the western corn rootworm (*Diabrotica virgifera virgifera*) was shown to induce significant host mortality (Baum et al, 2007). The same was discovered, in a species-specific capacity, in *T. castaneum* (Whyard *et al*, 2009). As such, these two genes are not only both potential pest-control targets but are both functionally related. Therefore, the next step in trialling mixed-targeted RNAi gene knockdown in insects, should be to address both these genes using a variety of dsRNA doses.

HSP90 expression was considerably the highest both when separately-targeted (1.602%) and when mixed-targeted (70.518%). Perhaps this was due to an environmental stressor. HSP90 expression has been shown to be significantly upregulated in insects in response to heat stress (Colinet *et al*, 2010); cold stress (Singh *et al*, 2013); and prior to pupal diapause (Miyoshi *et al*, 2010). All larvae were daily removed from the incubator (33.7^{o}C) and perhaps the change in temperature had a heat stress effect. An alternative hypothesis is that HSP90 expression was modulated during the recorded (Figure 3.5.2) pupal development. These environmental conditions were experienced by all larvae. However, if the immobilising on ice, during the microinjection process, acted as a cold stress, this did not apply to uninjected larvae.

What is more interesting is that when separately targeted, AAT and ATUB knockdown were almost identical, with AAT slightly exceeding ATUB (0.001%). On the other hand, when mixed-targeted, ATUB exceeded AAT by 1.244%. It seems a third the dsRNA dose per gene, 20ng, may lower the degree of knockdown, but not necessarily in proportion with using 60ng. It may have been that AAT required a further 40ng of dsRNA trigger to bring AAT and ATUB knockdown levels together. If genes respond differently to different quantities of reagent, they might have different optimum doses for producing sufficient mortality. This again may be tested using a variety of dsRNA ratios and comparing resultant mortalities. Finally, although mixed-targeted knockdown was less for all genes, >95% knockdown was still revealed in two of the three targets. This suggests 20ng may have been enough to

induce adequate mortality, and using a third of the reagent would mean considerable financial savings in a future commercial context.

4.2 FINAL CONCLUSIONS

In conclusion, this research has indicated separate and mixed RNAi gene targeting can reduce gene expression by a very considerable >95% and significantly ($P<0.05$) increase larval mortality. These findings help to further secure the RNAi promise as a future pest-control. However, a number of important further findings have been disclosed. Knockdown may not be an indicator for mortality and it appears separate may be more effective than mixed gene targeting. Future studies should therefore focus on improving separate-targeting trials, particularly by reducing off-target risk. However, it would be interesting to try and improve mixed-targeting using a variety of dsRNA doses to target more functionally similar genes and to test for optimum doses. Lastly, these dose ratios should be based on the separate targeting information from this study, which suggest some genes such as AAT may be less important and require more dsRNA reagent.

4.3 IMPROVEMENTS

Were I to do this research again, the following is a list of improvements I would make:

4.3.1 In determining off-target risk

1. Investigate further algorithm adjustments to identify *T. castaneum* ATUB protein sequence as query.
2. Research why no such query was produced.
3. Likewise, investigate why only one AAT protein alignment was identified, despite many more on the nucleotide-level, and the possible implications this may highlight.
4. Construct minimum evolution and maximum parsimony trees, as well as neighbour-joining phylograms. Compare and contrast.
5. Add to colour-coded higher taxonomic classifications, alignment percentage identity values.
6. Standardise nucleotide BLAST word size.

4.3.2 In researching survivorship

7. Instead of percentage normalising against day one survivorship, start with twenty larvae, and on day 1 post-injection, remove surplus, so that each treatment replicate has 10 live larvae on day 2.
8. Standardise error bar type and research pros and cons - SEMs or SDs?
9. Use different ratios of dsRNA doses: Example, 20% for one gene, 40% each for other two. Then 30% for the same gene and 35% for the other two. Then 40%, and 30% for other two. Continue as such for all three targets.
10. Target v-ATPase with AAT, using a variety of doses, and determine the optimum ratio based on resultant mortalities. Hypothesis: more AAT required for optimum.
11. Quantify the lag-effect and investigate any disparity depending on targeted gene and dsRNA transport.
12. More precisely determine when larvae die, within the hour.
13. Investigate mechanisms for doing so. Suggestion: can larval oxygen output be monitored and used to determine how many larvae are alive within each petri dish?
14. Standardise time of survivorship count more precisely.

15. Investigate apparent clumping of day 4 post-injection survivorship.
16. Harvest larval RNA from all days and compare and contrast gene expression results with survivorships.
17. Use only one culture, with more replicates. Example: eleven replicates in total, eight of which are pre-assigned each a day for live-harvesting.

4.3.3 In quantifying & analysing gene expression

18. Preferably, normalise separate- and mixed-targeted gene expression to both controls.
19. Isolate individual amplicons. Compare and contrast.
20. Colour-code each amplicon according to target gene (suggestion for CFX Manager).
21. Include amplification and melt curves for separate as well as mixed targeting plate.
22. Display and analyse Cq values from separate-targeting plate as well as mixed-targeting plate.
23. Using above data, investigate any differences in gene expression between controls, especially for HSP90.
24. Merge data from both plates or invest in machine with larger plate size. Likewise, use only one gel tray and not two.
25. Construct bar graphs of knocked-down vs. control Cq values, with presented Fisher's grouping information, as in Figure 3.2.2.
26. Display gene expression SD values according to RT-qPCR
27. Display gene expression SD values according to densitometry results.
28. Arrange Figure 3.3.4A in the standard order: AAT, ATUB, then HSP90.
29. Obtain original individual gene expressions from each reaction well from CFX Manager (3.1). Use this data in a one-way ANOVA to statistically compare separate vs. mixed gene targeting - Instead of considering separate-targeting knockdown a single sample.
30. Decrease the number of RT-qPCR cycles if electrophoresis is to be done again. Based on Figure 3.3.1, I would choose 26 cycles.
31. Run standard curves and obtain percentage efficiencies for primers.

Acknowledgements

This research has been the highlight of my life, in education, to date. I count the experience as a privilege. I would like to thank the following people for helping me with it: Dr AS Bowman, especially for listening to my early research ideas and allowing me to express my enthusiasm and eccentricity; Dr EM Campbell; C McIntosh; and CR Christie. I particularly thank Dr EM Campbell for his extensive guidance, without which the data-collection would not have been possible. And for his friendly attitude which made my time in the laboratory all the more enjoyable. Thank you.

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