Functional analysis of the insulin/IGF signalling pathway and the infective larva developmental switch in *Parastrongyloides trichosuri*

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy
In
Molecular Genetics

at Massey University, Palmerston North,
New Zealand.

Susan Josephine Stasiuk

2010

ABSTRACT

Parasitism, in nematodes, is a very successful life strategy which has evolved throughout the Nematoda phylum in several independent events. However, the genetic basis for parasitism remains unknown. *Parastrongyloides trichosuri* is a facultative parasitic nematode of the Australian brushtail possum. This parasite has retained the unusual ability to sample its environment at each generation, and make the developmental decision to develop either into a free-living nematode or, in response to environmental stress, develop into an infective larva, which must then seek out a host in order to complete its life cycle.

The nematode model organism, *Caenorhabditis elegans*, also responds to environmental stresses by developing into a dauer larvae. The dauer hypothesis proposes that dauer larvae and infective larvae are homologous and that dauer larvae may be an evolutionary pre-adaptation that facilitated the evolution of parasitism in nematodes. One of the signalling pathways which control dauer larva development in *C. elegans* is the Insulin/IGF signalling pathway.

Gene orthologues of the insulin/IGF signalling pathway were cloned from *P. trichosuri*: the *daf-2* tyrosine kinase receptor, the *age-1* phosphatidylinositol 3′ kinase and the *daf-16* FOXO forkhead transcription factor. The expression profiles of these genes were characterized by q-PCR which determined that they were differentially expressed during the developmental switch to infective larva. Rescue by complementation showed that a *P. trichosuri daf-16* transgene was able to recover both stress and developmental phenotypes in *C. elegans daf* mutants, suggesting it might perform an orthologous role in *P. trichosuri*.

This research also demonstrated that the biology of *P. trichosuri* infective larvae and *C. elegans* dauer larvae are quite similar. Some of the environmental signals which control the free-living/infective larva developmental switch in *P. trichosuri* were characterized in this study and found to be similar to the environmental signals which trigger dauer larval development. These are: population density, food availability and temperature.

There is a genetic component to the ability to respond to the environmental signals and inbred lines which display diverse developmental plasticity were isolated.

ACKNOWLEDGEMENTS

With much appreciation, I would like to thank the following people; first and foremost, Dr. Warwick Grant, my supervisor, for his support and willingness to let me explore unusual avenues in order to solve problems and satisfy my curiosity. Thank you also to my co-supervisor Max Scott, for all his practical advice and support during my time at Massey.

The following people of the "Worm Lab" team contributed in some very practical and hands on ways for which I am very grateful. Kirsten Grant for worm microinjections, Mark Ralston and Robyn Hirst for the animal husbandry of the possums, Jan Newton-Howes for collaboration on some of the RNAi and quantitative RT-PCR work, Matt Crook, Tina Englbrecht and Marleen Richter for collaborating on the *P. trichosuri* chemical mutagenesis screening and to the AgResearch statisticians and bioinformaticians: Fred Potter, John Koolaard and Roger Moraga Martinez.

I am deeply grateful for the helpful discussions and moral support of Jan Newton-Howes, Jacqui Knight and all the members of the 'Worm Lab" team. And to all my family and friends, who eventually stopped asking: "is it done yet?" and continued to offer their support and love. Finally, thanks to the *P. trichosuri* larvae: several hundred thousand gave up their lives in the biological assays, in order to ensure that the frontiers of science could be pushed back.

I would also like to thank both my employer AgResearch and the FoRST Enterprise Scholarship and the Royal Society Marsden Fund for their financial support throughout this PhD. Approval for the experiments described in this thesis was granted by the Wallaceville and Grasslands Animal Ethics committee AEC # 273.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

Abbreviations:

aa	amino acid
BAC	bacterial conditioned medium
BLAST	Basic Local Alignment Search Tool
BLAST X	Basic Local Alignment Search Tool for translated sequence
bp	base pair
^{o}C	degrees Celsius
cDNA	copy deoxyribonucleic acid
cds	coding sequence
CM	conditioned medium
DMSO	dimethyl sulfoxide
daf	dauer formation
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
F_1	First generation
F_2	Second generation
FCS	Foetal Calf Serum
g	gram
gfp	green fluorescent protein
hr	hour
HCl	hydrochloric acid
IIS	Insulin/IGF Signalling
iL3	infective larva
kb	kilobase-pairs
kD	kilodalton
KOAc	potassium acetate
L	litre
L1	first larval stage
L2	second larval stage
L4	fourth larval stage

LD_{50}	Lethal dose at which there is 50% survival
LC_{50}	Lethal concentration, point at which there is 50% survival
LB	Luria-Bertani media
M	molar, moles per litre
mg	milligram
µL	microlitre
mL	millilitre
Milli-Q water	water purified by Milli-Q ion exchange column
µM	micromolar, micromoles per litre
mM	millimolar, millimoles per litre
mRNA	messenger ribonucleic acid
NaOAc	sodium acetate
NGM	Nematode Growth Medium
nmol	nanomole
nt	nucleotide
NTC	No template control
ORF	open reading frame
PCR	polymerase chain reaction
pH	-Log $[H^+]$
PI3′K	Phosphatidylinositol 3′ kinase
P_0	Parental generation
q-PCR	quantitative reverse transcriptase PCR
RACE	Rapid amplification of cDNA ends
RNA	ribonucleic acid
RNase	ribonuclease
RNAi	RNA interference
RT-PCR	reverse transcription-polymerase chain reaction
rpm	revolutions per minute
SDS	Sodium dodecyl sulphate
SL1	Splice leader one
SL2	Splice leader two
5′ or 3′ UTR	5′ or 3′ untranslated region
ts	temperature sensitive

CHAPTER 1
INTRODUCTION

1.1 *Parastrongyloides trichosuri*

1.1.1 *P. trichosuri*, a parasite of mammals; its life cycle and its placement in the current Nematoda phylogenetic tree

Nematoda is a diverse and evolutionary ancient phylum, divided into five clades, each clade contains many parasitic members (1995; BLAXTER 1998; BLAXTER *et al.* 1998; DORRIS *et al.* 2002; 1997). Parasitism - deriving nutrients and habitat, usually at the expense of the host, must be a very successful life strategy for nematodes, because molecular phylogenetic inference suggests that parasitic nematodes have risen from free-living forms by several independent events throughout the phylum (BLAXTER *et al.* 1998; DORRIS *et al.* 2002). The clade of nematodes I am particularly interested in is clade IVb, which contains *Strongyloides* and *Parastrongyloides*, genera with remarkable life history strategies (DORRIS *et al.* 2002).

First described by Grassi in 1879, as cited in (VINEY and LOK 2007), the genus *Strongyloides* has a complex life cycle, illustrated for a number of species in Figure 1.1; the life cycle of this genus consists of parasitic parthenogenetic females within the host or free-living males, females and infective larvae in the environment. The early larval stage make a developmental decision to develop either into free-living male and female larvae (which is termed heterogonic, or indirect development) which reproduce sexually in the environment or alternatively, the larva may develop directly into female infective larvae (termed homogonic development), which must then seek out a host to complete its life cycle (HARVEY *et al.* 2000). *Strongyloides* species generally have a single heterogonic cycle and are then committed to develop into the infective form. One exception to this, *Strongyloides planiceps,* may have a maximum of 9 heterogonic cycles before becoming committed to homogonic development (YAMADA *et al.* 1991). In 1928 an intestinal nematode was isolated from the gut of a shrew, this parasite had similar characteristics to the *Strongyloides* species, however, the parasitic morph of this new species consists of males and females rather than parthenogenetic females only (MORGAN 1928). It was classified as a new genus: *Parastrongyloides;* a species of this genus isolated from the Australian brushtail possum (*Trichosurus vulpecula*) is *Parastrongyloides trichosuri* (MACKERRAS 1959; MORGAN 1928).

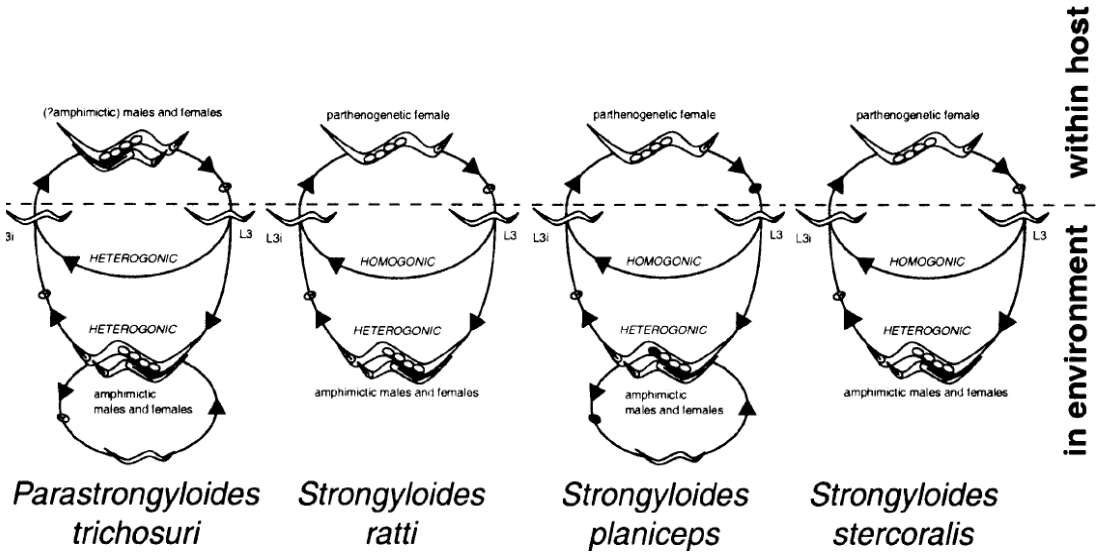


Figure 1.1: Life cycle variation in *Strongyloides* and *Parastrongyloides* (DORRIS *et al.* 2002). The life cycles of *P. trichosuri, S. ratti, S. planiceps and S. stercoralis*, illustrating the direct (homogonic) and indirect (heterogonic) development.

1.1.2 The free-living versus parasitic life cycle switch of *P. trichosuri.*

P. trichosuri hatch from eggs produced by either the parasitic or the free-living worms; at the L1 stage the larvae make a developmental decision, based upon signals from the environment (food and population density), to develop into either short lived, free-living nematodes or into long lived, parasitic nematodes. The L1 larvae entering the parasitic life cycle become developmentally arrested at the infectious third larval stage (iL3) until they encounter an appropriate host, which they enter by skin penetration and migrate to the gut, presumably through the lungs (CROOK *et al.* 2005), to develop into parasitic adults. Free-living *P. trichosuri* have an average life span of 6 days at 20°C and the parasitic morphs have a life span in excess of 120 days. This 20 fold difference in lifespan between the two developmental morphs is almost certainly a direct result of differential gene expression (Figure 1.2).

P. trichosuri is the only known parasitic nematode capable of an apparently unlimited number of free-living generations. This unique characteristic of *P. trichosuri* makes it ideal for the study of parasitism, because the short lived, facultative free-living life cycle, enables it to be cultured on agar or liquid media in a laboratory setting throughout its entire life cycle, and gives researchers direct access to its reproductive stage of

development for both classical and molecular genetics (GRANT *et al.* 2006b). The development of this nematode can be controlled, so that following genetic manipulations, researchers can direct development towards either the free-living life cycle or to the parasitic life cycle.

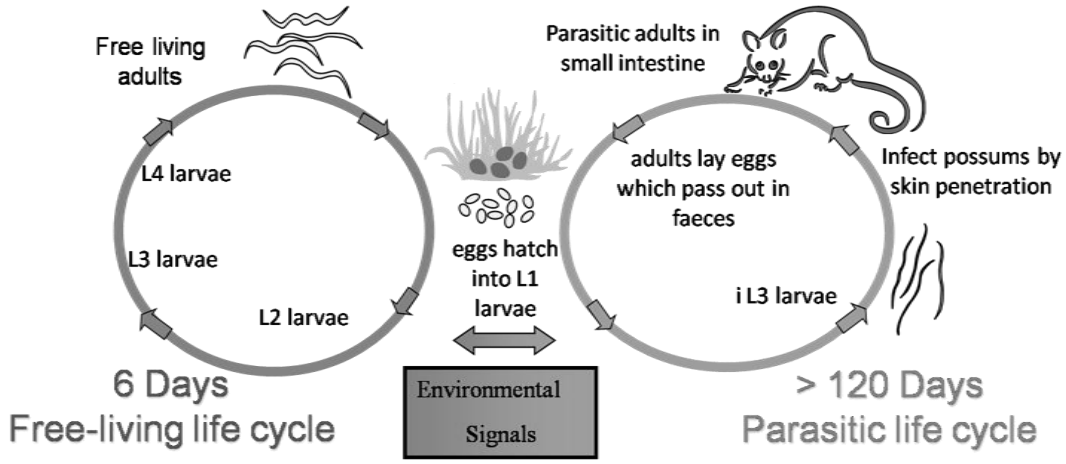


Figure 1.2: Life cycle of *P. trichosuri.*
P. trichosuri first larval stage may develop to either short lived free-living nematodes or into infective larvae which require an appropriate host in order to complete their parasitic life cycle. Environmental factors influence this developmental switch.

Parasitic nematodes have evolved from free-living nematodes, eventually culminating to an absolute requirement of a specific host in order to complete their life cycle. Therefore one can speculate that *P. trichosuri*, with its unique developmental plasticity, represents an intermediate step in the evolution of parasites, one that has retained the ability to develop into a free-living nematode. If this is so, have the developmental trigger(s), for the switch between free-living and parasitic life cycles, and the molecular mechanisms by which these triggers are sensed and transduced, also been conserved throughout evolution within this clade of nematodes?

This thesis describes the investigation of the environmental signals that trigger parasitic rather than free-living development, and tests the hypothesis that the insulin/IGF signalling pathway (IIS) plays a role in this developmental choice. This hypothesis is based on the striking resemblance between the dauer larva stage of development of many free-living nematodes and the infective larva stage of many parasitic nematodes, including *P. trichosuri*.

1.2 *Caenorhabditis elegans*

In this section I examine the free-living nematode model organism, *Caenorhabditis elegans* - its role as a model in parasitic research and its developmental switch to the facultative diapause stage: dauer. I examine the environmental cues which trigger this developmental switch, and the signal transduction pathways which process these signals, in particular the insulin/IGF signalling transduction pathway.

1.2.1 *C. elegans*, a free-living nematode model organism used extensively in developmental biology and its use in parasitic nematode research.

The free-living nematode, *C. elegans* has been extensively studied for over 4 decades. It is used extensively in biological research of because its short life span and simplicity combined with its conservation of basic biology with humans makes it a good model. The entire genome sequence of 96.89 Mb has been sequenced, the adult contains <1000 cells and the entire cell lineage has been determined (RIDDLE 1997) .

C. elegans belongs to the order Rhabditida and is most closely related to the free-living *Diplodasterida* and the vertebrate parasitic order *Strongylida* (small gut nematodes). It was suggested by Bürglin *et al.* that *C. elegans* should make an excellent model for these important veterinary and human parasites (BURGLIN *et al.* 1998). The *C. elegans* genome sequencing project allows parasitologists to relate the possible function of hypothetical genes from nematode parasites by comparison with the hypothetical *C. elegans* orthologue (BIRD *et al.* 1999; BURGLIN *et al.* 1998).

C. elegans moults through 4 larval stages prior to becoming a reproductive adult in approximately 51 hours at 25^{o}C on a diet of *Escherichia coli*. The adult stage consists of hermaphrodites and males (1995) (Figure 1.3). When *C. elegans* L1 larva encounter a stressful environment, such as low food resources and high population density, which indicate to the worm that there are insufficient resources to develop to a reproductive adult, it will respond by entering diapause to become a non-eating, non-ageing, developmentally arrested "dauer" larva stage (CASSADA and RUSSELL 1975) (from the

German word for "enduring"). When food becomes abundant again, dauer larvae will resume development at the L4 stage (CASSADA and RUSSELL 1975; GOLDEN and RIDDLE 1984b). Dauer larvae are termed non-ageing because whether the duration of the dauer larvae stage lasts for few days or for several months it will not affect the length of the adult life span once development has resumed, which is approximately 14 days (GOLDEN and RIDDLE 1984b).

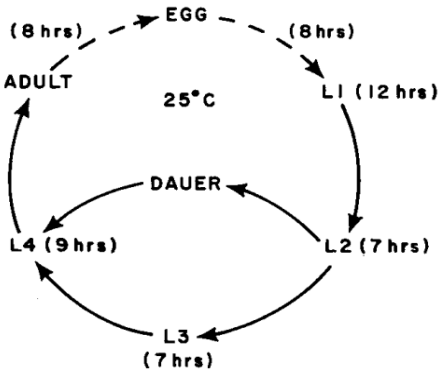


Figure 1.3: The life cycle of *C. elegans* grown at 25°C with *E. coli* OP50 as food source (RIDDLE 1997).

The dauer larval stage shares many characteristics with the infective third larval stage (iL3) of many parasitic nematode species (BIRD *et al.* 1999; BURGLIN *et al.* 1998; RIDDLE 1987; 1997). Dauers and infective larvae (both of which are called the dispersal stage) are both developmentally arrested, long-lived, resistant to environmental stress, motile, and their metabolism is based upon stored lipids (BIRD *et al.* 1999). In addition to these biological characteristics, dauer larvae and iL3 share many of the same cellular features. The chemosensory neurons which detect the environmental signals that trigger the dauer larval developmental choice in *C. elegans* have orthologues that are also required for the iL3 development in *S. stercoralis* (ASHTON *et al.* 1998). Also of note, homologues of many of the genes involved in dauer larval formation (*daf* genes) have been isolated from parasites, to be discussed in Section 1.6 herein.

The biological similarities observed between *C. elegans* dauer larvae and the iL3 stage of many parasitic helminth species has led to the "dauer hypothesis", which states that the dauer larvae stage is analogous to the infective larvae stage and that it has served as an initial stage in the evolution of parasitism (BIRD *et al.* 1999; BLAXTER *et al.* 1998; BROOKS and ISAAC 2002; BURGLIN *et al.* 1998; HASHMI *et al.* 2001).

However, there are also significant differences between *C. elegans* and many parasite species, such as the difference in codon usage between the *Strongyloides* and *Caenorhabditis* species, which may make attempts to identify genes orthologues involved in development difficult. In particular, the A/T rich nature of *Strongyloides* genes makes aligning orthologous genes difficult (MOORE *et al.* 1996). Free-living nematodes and parasites inhabit very different environments and it has been highlighted by Geary *et al.*, that there is the further complexity of the parasitic process which are likely to require a very different set of genes not found in the free-living nematodes (GEARY and THOMPSON 2001). Comparison of the EST database from *S. stercoralis* iL3 and the dauer larvae stage of *C. elegans* suggests that the majority of sequence matches between the two nematodes are for genes required for normal cellular processes such as metabolism, cell structure and protein synthesis (MITREVA *et al.* 2004). There are less matches between the *S. stercoralis* infective larvae stage and the C. *elegans* dauer larvae stage than between the other larval stage (L1), compared in this study (MITREVA *et al.* 2004), suggesting that the transcriptional signature of dauer larvae is perhaps not as analogous to iL3 as was previously suggested.

Genes in which mutations give rise to changes in dauer larvae formation are given the name of *daf* genes, which is an abbreviation for dauer formation genes. Nomenclature of *C. elegans* genes is as follows: the gene or gene activity is given as three letters in italics followed by a dash and a number (*daf*-2). The corresponding protein product is given by the same three letters in capitals followed by the number (DAF-2). The mutant or mutant phenotype is given by the same three letters with the first letter capitalized (Daf-2), for further clarification see website www.wormbase.org/wiki/index.php/Nomenclature. Over 30 mutants have been found affecting dauer larvae formation. These mutants fall into two categories, dauer defective mutants (Daf-d) and dauer constitutive mutants (Daf-c). Daf-d mutants fail to form dauer larvae in conditions that would normally induce wild-type worms to become dauer larvae, and Daf-c mutants forms dauer larvae under conditions where wild-type worms should develop normally (BRAECKMAN *et al.* 2001; GEMS *et al.* 1998; 1997).

Mosaics develop from a single fertilized egg, but have two populations of cells each with different genotypes; mosaics are a useful method for analysis of gene function. Mosaic can be created artificially by transforming mutants with a wild-type copy of the

mutant gene; if, at an early embryonic cell division there is a spontaneous loss of the extrachromosomal array from one of the dividing cells and the embryo continues to develop, cells eventually differentiating into tissues, one can examine the tissue specific function of the transgene. Depending upon which early cell division this loss occurs, animals can be screened to detect the loss of a gene of interest in almost any tissue type.

An actual parasite model is essential and will provide a much needed resource with which to study parasitism. Key features of a parasite model might include the ability to compare transcriptional profiles between the parasitic and free-living stages within a single species, rather than by indirect comparison to a different species (*C. elegans*) which makes it difficult to determine if one is comparing like with like. Another key feature of a good parasite model would be an adequate "tool kit" of molecular and genetic techniques with which to determine gene functions.

1.2.2 *C. elegans* larval development is influenced by environmental signals

C. elegans develop to dauer larva when environmental cues signal to the worm that there is a reduced chance of reaching reproductive adulthood: i.e.: either limited food; high population density or increased temperature (GOLDEN and RIDDLE 1982; GOLDEN and RIDDLE 1984a). The environmental cue for determining population density was originally termed "dauer pheromone". It is secreted from *C. elegans* and can be isolated from liquid cultures and partially purified by ethanol extraction (GOLDEN and RIDDLE 1982; GOLDEN and RIDDLE 1984b). The structure was initially determined to be a hydroxylated short chain fatty acid with a molecular weight of less than 1000kDa. It is insensitive to drying, autoclaving or treatment with 1N acid or base for 1 hr at room temperature (GOLDEN and RIDDLE 1982) or with proteinase K, RNase and DNaseI (GOLDEN and RIDDLE 1984a). Chemical analysis has recently elucidated the structure of dauer pheromone, it consists of a mixture of three ascarosides, chemical derivatives of a 3,6-dideoxyhexose ascarylose (BUTCHER *et al.* 2007; JEONG *et al.* 2005).

Whether the "dauer pheromone" should be considered a pheromone has been debated (VINEY and FRANKS 2004). Its apparent function better fits the definition of an environmental cue, as there is no evidence for natural selection and therefore dauer

pheromone might be better considered a cue, like a waste product (VINEY and FRANKS 2004).

Preparations of natural dauer pheromone from spent cultures influence the development of *C. elegans* by inducing dauer formation (GOLDEN and RIDDLE 1982; GOLDEN and RIDDLE 1984b; GOLDEN and RIDDLE 1984c; GOLDEN and RIDDLE 1985). This dauer pheromone has also been shown to inhibit the resumption of development even after *C. elegans* dauer larvae are moved to an abundant food supply (GOLDEN and RIDDLE 1982; GOLDEN and RIDDLE 1984b; GOLDEN and RIDDLE 1984c). A food signal purified by organic extraction from yeast extract or cultures of *E. coli* cause dauer larval recovery in the absence of additional dauer pheromone (GOLDEN and RIDDLE 1984a). This food signal is antagonistic to the pheromone signal; it is the relative proportion of food: pheromone that influences dauer larval recovery rather than the specific concentration of either (GOLDEN and RIDDLE 1982; VINEY *et al.* 2003).

Dauer pheromone activity appears to be species specific as different species of Rhabditida produce a pheromone that is active only upon themselves; the closely related *C. elegans* and *C. briggsae* are the exception to this, as they each produce dauer pheromone which is able to influence the development of the other species (GOLDEN and RIDDLE 1982).

1.2.3 *C. elegans* dauer pheromone is detected by chemosensory neurons

Animals must respond to their environment and make appropriate changes in order to thrive. *C. elegans* mutants that do not respond to the environmental signals that regulate dauer larvae formation are often defective in chemotaxis or have abnormal sensory neurons (GOLDEN and RIDDLE 1982). This suggests that the environmental signals for dauer larvae formation are detected by the nervous system (BARGMANN and HORVITZ 1991b) which then produces the ligands which trigger the signal transduction pathways controlling development. Bargmann and Horvitz used laser ablation to kill individual or groups of chemosensory neurons to determine their role in dauer larvae development. The neurons ADF and ASI are necessary for dauer larvae formation because when both are killed the worm cannot enter the dauer stage; if either is left

intact the animals develop normally (BARGMANN and HORVITZ 1991a). When ADF, ASG and ASI are ablated but ASJ is left intact, the animal becomes a dauer larva transiently and then recovers to develop normally. If ASJ is ablated in dauer larva, they do not exit dauer stage effectively (BARGMANN and HORVITZ 1991b) which suggests that ASJ plays a role in dauer larva maintenance by sensing when resources are suitable for normal development to adult.

Parasitic nematodes must also be able to detect environmental signals to thrive. Infective larvae of *Ancylostoma caninum* and *Strongyloides stercoralis* use chemical signals to detect a host and *Haemonchus contortus* infective larvae use environmental signals in order to position themselves on the grass stalks to increase the likelihood that they will be ingested by a host (ASHTON and SCHAD 1996). Upon entering the host the parasite encounters a new environment which triggers resumption of development. These environmental signals are detected by the chemo- and thermosensory neurons located in amphids or nerve clusters (ASHTON and SCHAD 1996). *S. stercoralis* larvae have the capacity to make the developmental choice to become free-living or infective larvae, but when laser ablation was used to destroy neurons ASF (which is equivalent *to C. elegans* neuron ADF) and ASI this resulted in homogonic, infective larva formation (ASHTON *et al.* 1998).

There is indirect evidence that the muscarinic receptor of the interneuron plays a role in this signal transduction in *A. caninum*. Muscarinic receptor agonists (such as: carbachol; oxotremorine; pilocarpine and arecoline) promote infective larva recovery in *A. caninum* (as demonstrated by resumption of feeding) these agonists are also able to promote recovery of pheromone induced *C. elegans* dauer larvae; further, muscarinic antagonists such as atropine are able to inhibit dauer larvae recovery in both *C. elegans* and *A. caninum* (TISSENBAUM *et al.* 2000). These same muscarinic agonists are able to promote dauer larvae recovery of mutants of the TGF-β pathway but not in *C. elegans* mutants of the insulin/IGF pathway (TISSENBAUM *et al.* 2000). This suggests that chemosensory signalling, through the muscarinic receptor, is involved in dauer larva development and recovery; it also suggests that the insulin/IGF signalling pathway is required for dauer recovery. It is possible that certain parasitic nematodes may also use this pathway for diapause regulation.

1.3 *C. elegans* DAUER DEVELOPMENT IS MEDIATED BY THREE SIGNAL TRANSDUCTION PATHWAYS.

In *C. elegans* there are three signal transduction pathways (cyclic GMP; TGF-β and the insulin/IGF) involved in dauer larvae development (1997). These act in parallel but appear to share at least some of the same targets.

The insulin/IGF signalling pathway (IIS) controls glucose metabolism, development, size and longevity (BARBIERI *et al.* 2003; LONGO and FINCH 2003; TATAR *et al.* 2003) in animals. Its importance is demonstrated by the evolutionary conservation that exists in nematodes, *Drosophila*, mice and human. The IIS pathway (BRAECKMAN *et al.* 2001) is illustrated in Figure 1.4.

When there is abundant food and low dauer pheromone levels, the chemosensory neurons stimulate the muscarinic receptor of the interneurons, this results in the release of insulin-like ligand(s) which binds to and activate DAF-2, an insulin receptor kinase. DAF-2 then activates a signalling cascade through AGE-1, a phosphatidylinositol 3′ kinase; the end result of this signalling cascade is the phosphorylation of the forkhead transcription factor DAF-16, through the activity of serine/threonine kinases (AKT-1/2) (BRAECKMAN *et al.* 2001). Phosphorylated DAF-16 is sequestered in the cytoplasm through association with 14-3-3 proteins (BERDICHEVSKY *et al.* 2006), this allows the expression of genes that are involved in metabolism and development. When there is decreased IIS signalling, DAF-16 is dephosphorylated and translocated to the nucleus where it down-regulates genes involved in reproductive development and activates genes involved in dauer larvae formation or enhanced life maintenance (BERDICHEVSKY *et al.* 2006; BRAECKMAN *et al.* 2001).

If *C. elegans* encounters environmental conditions which signal to the worm it is appropriate to enter diapause until conditions improve, there is a decrease in IIS. Otherwise, the IIS cascade is constitutively activated to maintain reproductive development. Only when there is a down regulation of IIS through RNAi or mutation (to be discussed herein) or there is disruption of the chemosensory neurons which detect

the environmental signals, such as was highlighted by the ablation experiments, is there a switch to the default dauer larvae development program.

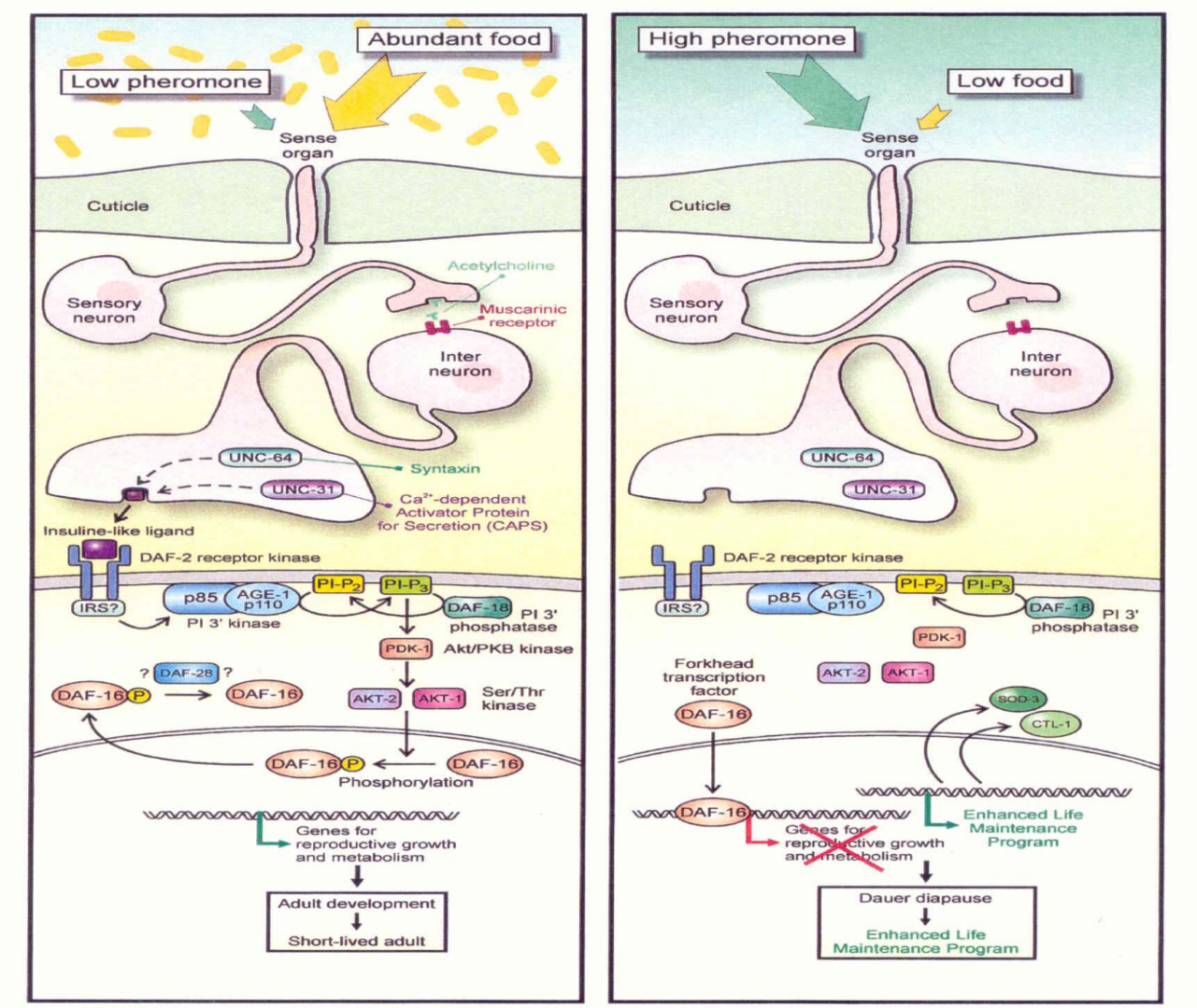


Figure 1.4: The insulin/IGF signalling pathway (BRAECKMAN *et al.* 2001).
The insulin/IGF signalling pathway, the left panel shows the pathway activated triggered by the environmental signals of low pheromone and food resulting in the DAF-16 transcription factor being phosphorylated and sequestered in the cytoplasm. The right panel shows the pathway inactivated and DAF-16 becoming nuclear localized and activating gene expression.

1.3.1 Receptor ligands in the insulin/IGF signalling pathway.

There are more than 38 insulin ligand proteins for the DAF-2 receptor, few of which have been functionally characterized (PIERCE *et al.* 2001). Some ligands appear to act as DAF-2 receptor agonists and some as antagonists, illustrating the complex and precise interaction which must exist between ligand and receptor.

Peirce *et al.* identified many *C. elegans* DAF-2 receptor ligands and reported *ins-1* as being most closely related, structurally, to mammalian insulin. *ins-1* over expression enhanced dauer phenotype in Daf-2 mutants and resulted in partially penetrant dauer larvae arrest in wild-type worms, suggesting INS-1 functions as a DAF-2 antagonist (PIERCE *et al.* 2001).

Murphy *et al.*, reported that INS-7 is a DAF-2 receptor agonist that can function cell non-autonomously to increase DAF-2 pathway activity (MURPHY *et al.* 2003). Expression of *ins*-7 is negatively regulated by the DAF-16 transcription factor: when conditions are at threshold levels for dauer larvae formation, some cells will have their insulin/IGF pathway interrupted resulting in the nuclear localization of DAF-16 and the down regulation of *ins*-7, thereby preventing the activation of adjoining DAF-2 receptors (MURPHY *et al.* 2003). This feedback regulation may explain why in wild-type worms, threshold levels of dauer pheromone do not result in a mosaic of dauer and non-dauer cells. Instead the entire animal reaches a consensus to become dauer larvae or to continue development. In accordance with this theory, down regulation of *ins*-7 by RNAi results in increased dauer larvae formation (MURPHY *et al.* 2003).

Li *et al.*, identified *daf*-28, which encodes an insulin-like protein that is expressed in the ASI and ASJ sensory neurons and is down regulated in the presence of dauer pheromone. Daf-28 mutants display, down regulation of the IIS pathway, nuclear localization of gfp tagged *daf-16* reporter constructs, and also have increased dauer larvae formation (LI *et al.* 2003). No direct evidence has been found that DAF-28 plays a role in longevity, which raises the possibility that different ligands are necessary for dauer larvae formation than are required for longevity. This is entirely possible, as it has been demonstrated that some DAF-2 ligands act as agonists and some as antagonists. Also of note, Daf-2 mutants which have different portions of the DAF-2 receptor inactivated, are incapable of responding to a specific signal from the gonad which also influences life span (HSIN and KENYON 1999; PATEL *et al.* 2008).

1.3.2 DAF-2, the Insulin Receptor kinase

The *daf*-2 gene encodes a receptor tyrosine kinase that plays a role in development, adult longevity, reproduction, fat storage and resistance to both bacterial infection and elevated temperature. DAF-2 negatively regulates the DAF-16 transcription factor by inducing its phosphorylation through the activity of AKT-1 and AKT-2 resulting in DAF-16 being sequestered in the cytoplasm (LEE *et al.* 2001; LIBINA *et al.* 2003; LIN *et al.* 2001). Daf-2 non-conditional mutants may be grouped into two classes: class 1 alleles are generally less severe, are suppressed by *daf-12* and map to either the furin-like cysteine rich domain, the ligand binding domain2 or the fibronectin III (FnIII); class 2 alleles are not suppressed by *daf-12,* are often more severe and have additional effects such as reduced feeding or reduced movement; these alleles map to the ligand-binding cleft or to the tyrosine kinase domain (PATEL *et al.* 2008).

DAF-2 is a membrane bound receptor, and might therefore be expected to only function cell autonomously. Apfeld and Kenyon examined mosaic animals of either the AB lineages (epidermis, pharynx, neurons, vulva) or the P_1 lineages (pharynx, gonad, sex muscles, intestine, germline, body muscles, epidermis) in three different Daf-2 mutant backgrounds for the dauer larvae and non-dauer larvae phenotype, and found that the removal of *daf*-2 from all but a subset of cells still results in the entire animal becoming dauer larvae or long-lived (APFELD and KENYON 1998; LIBINA *et al.* 2003). If *daf*-2(-) acts cell autonomously then one would expect to see a mixture of dauer and non-dauer tissues. This was not found, which suggests that DAF-2 stimulates secondary signals, potentially endocrine signalling, to allow all tissues to reach a consensus (APFELD and KENYON 1998; MURPHY *et al.* 2003).

1.3.3 AGE-1, the phosphatidylinositol 3′ kinase

The *age*-1 gene encodes a phosphatidylinositol 3′ kinase, which plays a role in metabolism, life span, dauer larvae formation, reproduction and embryonic development. It is downstream of the DAF-2 insulin receptor and upstream of the PDK-1 and AKT-1/2 kinases (Figure 1.4). AGE-1 negatively regulates the DAF-16 forkhead transcription factor (THOMAS and INOUE 1998; WOLKOW *et al.* 2000). Age-1

was the first longevity mutant to be identified and it was later discovered that Age-1 and the dauer larvae mutant Daf-23 are alleles of the same gene (1997). Different Age-1 alleles have different effects on life span, with the greatest extension being 2-3 times that of wild-type for the allele *age-1(mg44)* (TISSENBAUM and RUVKUN 1998).

The activity of AGE-1 is tissue specific. For example, restoring *age*-1 specifically to muscle cells in Age-1 mutants does not restore wild-type life span but does rescue metabolic defects (e.g. intestinal fat levels) and the Daf-c phenotype (WOLKOW *et al.* 2000) .

1.3.4 DAF-16, the FOXO Forkhead Transcription Factor

The major target of the insulin/IGF signalling pathway is DAF-16, a FOXO forkhead transcription factor (APFELD and KENYON 1998; APFELD and KENYON 1999; OGG *et al.* 1997). DAF-16 is both a negative regulator of genes involved in reproductive growth and a positive regulator of many genes involved in enhanced life maintenance (BRAECKMAN *et al.* 2001; HSU *et al.* 2003; MCELWEE *et al.* 2003; MURPHY *et al.* 2003). When the IIS pathway is activated, DAF-16 is phosphorylated by the activity of AKT-1/2 kinases and removed from the nucleus (Figure 1.4). When the IIS pathway is inactive, non-phosphorylated DAF-16 enters the nucleus to regulate gene expression.

C. elegans DAF-16 has seven isoforms, three of which (*daf-16a1*, *daf-16a2* and *daf-16b*) have been found to be active in development and life span regulation (OGG *et al.* 1997). The letter designations between the published and Wormbase versions differ (http://www.wormbase.org, release WS201). The published designations isoforms: "a1", "a2" and "b" are designated: "c", "b" and "a" respectively in Wormbase. For the purposes of this thesis we have used the designations consistent with the reported literature referenced herein, not Wormbase. The DAF-16a1 and DAF-16a2 isoforms are identical except for a differentially spliced 3rd exon, resulting in a two 2 amino acid addition to DAF-16a1. No difference in activity or specificity has been identified between these two isoforms to date. The C terminus of both *daf-16a* and *daf-16b* contains a Forkhead helix 3 DNA binding domain which determines specificity (OGG *et al.* 1997). The DAF-16A isoform is responsible for life span extension and dauer larvae

formation; no Daf-16b mutants have been found with these phenotypes to date (LIN *et al.* 2001). Differential splicing of *daf-16a* and *daf-16b* result in each isoform having its own promoter and a subtly different expression profile: *daf-16a* is expressed at all stages throughout the somatic tissue with the exception of the somatic gonad and pharynx; whereas, *daf-16b* is expressed at all stages in the pharynx and many neurons and from the L3 stage onward, is also expressed in the somatic gonads (LEE *et al.* 2001; OGG *et al.* 1997). DAF-16B is required for pharynx remodelling during dauer larvae formation. *daf-16a* transgene expression is able to restore Daf-2(-) life span to a Daf-2;Daf-16 double mutant, and complement the Daf-d(ts) phenotype of *daf-16* at the restrictive temperature. However, this rescue is incomplete and these dauer larvae have a pharynx that is not completely reformed (LEE *et al.* 2001).

In *C. elegans*, 14-3-3 scaffolding proteins: FFT-2 and PAR-5 are bound to phosphorylated DAF-16 to promote its retention in the cytoplasm. However, when the worm is exposed to environmental stress, phosphorylated DAF-16 becomes acetylated by CBP/p300 and localized to the nucleus where it binds to SIR-2.1, a NAD+ - dependent deacetylase, this prevents further acetylation of the transcription factor and promotes the transcription of genes involved in stress response and long life (BERDICHEVSKY *et al.* 2006; VAN DER HEIDE and SMIDT 2005). There is a JNK-1 pathway independent of the IIS pathway which also results in the nuclear localization of DAF-16 and up regulation of stress response genes and genes involved in longevity (OH *et al.* 2005). The phosphorylation activity of JNK-1 is known to target different phosphorylation sites on DAF-16 compared to the IIS phosphorylation activity of AKT-1/2 (OH *et al.* 2005). It is interesting to note that it is phosphorylation of DAF-16 by AKT-1/2 which results in retention in the cytoplasm by 14-3-3 proteins, whereas phosphorylation by JNK-1 results in nuclear localization of DAF-16.

Lin *et al.* created a DAF-16A::GFP reporter construct with all of the AKT-1/2 four phosphorylation sites mutated, and found the reporter construct was transported to the nucleus despite the IIS pathway being active (LIN *et al.* 2001). However, the constitutive nuclear localization of the DAF-16A construct did not result in an extension of life span nor in dauer larvae formation (LIN *et al.* 2001). This mutated constitutively active form of DAF-16 was able to rescue dauer larvae phenotype of Daf-16 null mutants suggesting it was able to function correctly. This result suggests that IIS does

more to inhibit longevity than just preventing DAF-16 nuclear localization (LIN *et al.* 2001). In contrast, Lee *et al.* determined that a constitutively nuclear localized DAF-16A transgene was able cause dauer larval formation or larval arrest so long as care was taken to establish strongly expressing lines, which was achieved by maintaining lines on RNAi plates that silenced the transgene until the experiments were performed (LEE *et al.* 2001) .

The *daf-16* forkhead transcription factor orthologues have been cloned from *S. stercoralis,* named *Ss fktf-1a* and *Ss fktf-1b*. These orthologues contain three domains that are conserved with *C. elegans daf*-16: an N-terminal domain, a central forkhead DNA binding domain and the C-terminal domain. The DNA binding domain shares 79.5% amino acid identity with DAF-16a1. In addition, there are four putative Akt/protein kinase B phosphorylation sites. RT-PCR showed constant levels of expression in all stages of development although it is possible to interpret the figure presented as showing that the *daf-16* expression in the iL3 stage was up-regulated (MASSEY *et al.* 2003) . The *S. stercoralis* orthologue *Ss* FKTF-1b is able to rescue *C. elegans* mutant dauer larvae phenotype in a complement assay, suggesting *Ss* FKTF-1b may perform a similar function in *S. stercoralis* development. *daf*-16 orthologues have also been recently cloned from the hookworms: *A. caninum* and *A. cylanicum*. Protein analysis shows that the *Ancylostoma* forkhead domains have 94% identity to *Ce*-DAF-16a, and there are three conserved Akt phosphorylation motifs. These orthologues are transcribed throughout hookworm development, and in tissue culture, *Ac*-DAF-16 can drive transcription of a reporter construct which is under the control of a *Ce* DAF-16 family DNA binding element (GAO *et al.* 2009).

1.3.5 Pleiotropic effects of the IIS pathway

All Daf-2 mutants display to some extent the following characteristics: a delay in reproduction, an extended life span, diapause entry and resistance to oxidative stress (DILLIN *et al.* 2002). The timing of IIS signalling determines which phenotype is displayed. By using RNAi to knock down *daf*-2 (or rescue of Daf-2 mutants with RNAi knockdown of *daf*-16) at various times in development it has been determined that extension of life span requires DAF-16 activity in adulthood only, DAF-16 activity

during development does not influence life span. Knock down of *daf-2* with RNAi in early larval stage results in increased dauer larvae formation; a delay in reproduction is influenced by *daf*-2 RNAi before the L4 stage and resistance to oxidative stress is affected by *daf*-2 RNAi in adults only (DILLIN *et al.* 2002).

1.3.6 Tissue specificity of IIS and the effects on development and life span

Many tissues express *daf*-2 and *daf*-16, but the developmental and longevity activities of these genes are tissue specific (APFELD and KENYON 1998; LIBINA *et al.* 2003; WOLKOW *et al.* 2000). Wolkow *et al.* found that *daf*-2 and *age*-1 act mainly through the nervous system to regulate longevity, as illustrated by restoration of wild-type life span in a Daf-2 or Age-1 mutant background using neuron specific tissue expression of *daf*-2 or *age*-1 transgenes respectively. In contrast, Libina *et al.* found that restoration of *daf*-16 activity to a Daf-2;Daf-16 double mutant using neuronal tissue specific transgene expression, resulted in only a 20% increase in life span, while intestine specific *daf-16* expression resulted in a 50-60% increase in life span (LIBINA *et al.* 2003). Libina *et al* went on to confirm this strong and specific control of ageing by the IIS pathway through the intestine using mosaics (LIBINA *et al.* 2003).

C. elegans Daf-2 mutants which are mosaic for Daf-16(-) age more slowly, no matter which tissues lack *daf*-16 (LIBINA *et al.* 2003). This suggests that *daf*-16(+) cells acts cell non-autonomously to signal *daf*-16(-) cells to age more slowly. However, different mosaics have different life spans: mosaics that have DAF-16 present only in the AB lineage (neuron, epidermis, pharynx and vulva) have small extensions in life span, whereas mosaics with DAF-16 present in the P_1 lineage (gonad, pharynx, body muscle, intestine and germline) have longer extensions in life span. This is consistent with work described previously using tissue specific restoration of *daf*-16 activity resulting in greater extension in life span with gut specific *daf*-16 expression (LIBINA *et al.* 2003).

Intestinal IIS has the greatest influence on ageing whereas neuronal IIS has the greatest effect on dauer larvae formation. The dauer larvae formation phenotype of Daf-2 mutants is most effectively rescued by *daf*-2 signalling in neurons (WOLKOW *et al.*

2000). Restoring *daf*-16 in neurons of Daf-2;Daf-16 double mutants resulted in complete dauer larvae recovery whereas there was little effect on dauer larvae recovery with *daf*-16 intestinal expression and no effect with muscle expression (LIBINA *et al.* 2003). The Daf-c phenotype of *age*-1 mutants is rescued by *age*-1 expression in muscle, intestine or neurons (WOLKOW *et al.* 2000). Wolkow *et al.*, found that expression of *daf*-2 pathway genes in muscle, intestine or subset of neurons can regulate metabolism and dauer larvae arrest but not life-span (WOLKOW *et al.* 2000).

Hsin and Kenyon found that signalling from the germline also influences ageing in *C. elegans*, when the germline cells are laser ablated in wild-type worms there is an increase in life span of 60%, when the entire gonad (germline plus somatic gonad cells) are ablated there is no change in life span (HSIN and KENYON 1999). This signal from the germline requires the activity of *daf*-16, as ablation of germline cells in Daf-16 mutants did not result in life span extension. However, when the entire gonad is removed from Daf-16 mutants, life span decreases (HSIN and KENYON 1999). A model proposed to explain these results is that the germline cells normally produce a signal to shorten life span, which acts through DAF-16 and that the somatic gonad cells also produce a signal, that normally lengthens life span which is independent of DAF-16 (HSIN and KENYON 1999).

The picture that is starting to emerge from this research suggests that DAF-16 is a master regulator of stress response, diapause and longevity. Dauer larvae formation signals act most effectively through the neurons and longevity signals through the intestine. Also, that the DAF-16 transcription factor activity is dependent upon its phosphorylation and acetylation state and upon many as yet unknown cofactors in order to act as a positive and negative regulator of gene expression.

1.3.7 Downstream targets of IIS involved in development

The developmental and longevity activities of IIS signalling may be tissue specific, as suggested by the research mentioned above. But the worm must have ways of acting cell non-autonomously to transmit these tissue specific signals to every tissue, so that

the entire worm becomes dauer or long lived, this suggests hormonal signalling plays a role in this processing.

The sensory neurons are triggered by favourable environmental signals and release signalling peptides that trigger the IIS or TGF-β pathways. These pathways conclude at either DAF-16 of the IIS pathway (inactivating it), or DAF-14 DAF-8 (Smad proteins transcription factors of the TGF-β pathway which activate reproductive program and DAF-9 (cytochrome P450) (LUDEWIG *et al.* 2004). DAF-9 is thought to catalyse the limiting step in the biosynthesis of a lipophilic hormone, now known to be Δ4 and Δ7 dafachronic acids (DA). These DA are ligands for DAF-12, a nuclear hormone receptor (LUDEWIG *et al.* 2004). DAF-12 is both a positive and negative regulator of genes involved in reproductive development or dauer larvae formation (APFELD and KENYON 1998; BAUMEISTER *et al.* 2006; FIELENBACH and ANTEBI 2008; GERISCH *et al.* 2001; MURPHY *et al.* 2003). The Δ4 and Δ7 dafachronic acids (DA), are derived from cholesterol which must be supplied to *C. elegans* cultures as they are unable to synthesis sterols *de novo*; *C. elegans* deprived of cholesterol for two generations constitutively form dauer larva (MATYASH *et al.* 2004).

When DAF-9 is not stimulated to synthesise the dafachronic acids, DAF-12 regulates genes involved in diapause (LUDEWIG *et al.* 2004). The DA-DAF-12 system appears to be conserved in *P. pacificus, C. elegans* and *S. papillosus,* and it has been determined that one of the steroid hormones, dafachronic acid Δ7-DA, controls entry into the infective larval stage of *S. papillosus* (OGAWA *et al.* 2009).

1.4 DEVELOPMENTAL PLASTICITY

1.4.1 Phenotypic plasticity and evolution

Organisms live in a variable world and must be able to respond to changes in their environment. Phenotypic plasticity is "the ability of a single genotype to produce more than one alternative form of morphology, physiological state and/or behaviour in response to environmental conditions". Until recently phenotypic plasticity, or the

range in environmentally induced phenotypes (noisy data), was considered a nuisance by biologists. It wasn't until the 1980s, that evolutionary developmental biologists fully embraced phenotypic plasticity as a major driving factor in evolutionary theory (PIGLIUCCI 2005; STEARNS 1989; WEST-EBERHARD 2005).

As reviewed in (STEARNS 1989), Wright (1932) theorized that increased plasticity reduces the requirement for genetic change. In contrast, Waddington (1953) suggested that a greater degree of plasticity extends the ecological range of a species thereby creating the opportunity for evolutionary change to occur through natural selection, which acts on the mutational variation which arises in the population (STEARNS 1989).

West-Eberhard expands on this, suggesting that adaptive evolutions drives species differentiation through natural selection. This process starts with a population of variable developmental organisms; this variability arises from both the genetic diversity which exists within all populations and also the variability in the phenotypic plasticity of their responses to environmental signals. Then a new genetic input or an environmental input results in developmental recombination. Developmental recombination is the "origin of a new trait via the altered pattern of expression of old traits" an example of one possibility of this may be developmental switches which are modular and are controlled by many environmental or genetic inputs, altering these inputs may result in altering the pattern of the developmental switch. Genetic accommodation may then occur to stabilize this new phenotype (WEST-EBERHARD 2005).

1.4.2 Reaction Norms

Phenotypic plasticity is the ability of a single genotype to display variable phenotypes in different environments (PIGLIUCCI 2008) and can be visualized by reaction norms, which are usually displayed as plots of phenotype versus two or more different environmental conditions. Each genotype gives different responses to environment. Polyphenism is the term used when the phenotypic plasticity results in two or more discrete phenotypes such as different morphs (i.e.: queen bee versus worker bees) (STEARNS 1989).

1.5 PARASITE *in vitro* CULTURING AND INFECTIVE LARVAE SWITCHING.

Strongyloides parasitic females lay eggs which hatch to become either free-living males, free-living females (heterogonic development), or infective larvae (homogonic development). As in dauer larvae development, the proportions of homogonic or heterogonic morphs may be influenced by a number of environmental factors: temperature was found to influence homogonic development in: *S. planiceps, S. ratti, S. papillosus* and *S. stercoralis* (ARIZONO 1976b; MINATO *et al.* 2008; NWAORGU 1983; SHIWAKU *et al.* 1988), pH influences developmental morphs in *S. planiceps* and *S. ransomi* (ARIZONO 1976a; MONCOL and TRIANTAPHYLLOU 1978), in *S. ratti*, development is influenced by CO_2 or O_2 levels (TAYLOR and WEINSTEIN 1990), fatty acids and food resources also influences homogonic development in *S. ratti* (MINEMATSU *et al.* 1989). Intra-host factors, for example: the immune status of the host or age of the parasitic female, were investigated in *S. ratti, S. ransomi* and *S. stercoaralis* (HARVEY *et al.* 2000; MONCOL and TRIANTAPHYLLOU 1978; SHIWAKU *et al.* 1988), it was found that the proportion of free-living males is not influenced by culturing conditions but is correlated to the immune status of the host and the duration of the infection, suggesting that sex determination takes place before hatching and that the host may provide signals which influence sex determination (HARVEY *et al.* 2000; MONCOL and TRIANTAPHYLLOU 1978; SHIWAKU *et al.* 1988; STREIT 2008). Environmental conditions, while not affecting the numbers of genetically female larvae, do influence the proportion of females that become either free-living or infective larvae.

Culture pH was determined to influence development of *Strongyloides ransomi* and *Strongyloides planiceps*; more extreme pHs increase the proportion of infective larvae and decrease in the proportion of free-living females in an inverse relationship (ARIZONO 1976a; MONCOL and TRIANTAPHYLLOU 1978). pH does not influence the proportion of male *S. ransomi*, although the number of males was shown to increase with the duration of the infection (HARVEY *et al.* 2000; MONCOL and TRIANTAPHYLLOU 1978). Reducing the nutritional value the food source by autoclaving or washing the faecal matter results in an increased proportion of iL3s at the expense of free-living females (MONCOL and TRIANTAPHYLLOU 1978). High population density or starved

cultures of *S. planiceps* result in increased proportion of iL3 (ARIZONO 1976a). The environmental conditions mentioned above (i.e.: washed, autoclaved or depleted culture conditions, even extremes in pH) are likely indicators of quality and quantity bacterial growth in the faecal cultures and hence food availability. Population density is an indirect indicator of food availability as it is a gauge of competition for food resources. Together these signal to the worm the likelihood of having the food resources necessary to reach reproductive adulthood. It should be noted that these same signals, food and population density trigger the dauer/non-dauer larva switch in *C. elegans*.

Temperature is another environmental factor that influences heterogonic (free-living) versus homogonic (iL3) fate of female offspring while not effecting male numbers. In *S. planiceps, S. papillosus* and *S. ratti,* high incubation temperatures result in a higher proportion of free-living females to iL3, whereas low incubation temperatures resulted in more iL3 formation (ARIZONO 1976b; MINATO *et al.* 2008; NWAORGU 1983). It was noted that this temperature dependent developmental fate was reversible but only until the first larval moult (ARIZONO 1976b; MINATO *et al.* 2008; NWAORGU 1983; VINEY 1996). Extreme temperature also influences the developmental fate of *S. stercoralis* but with an opposite result, at high temperatures there is an increase in proportion of iL3 development to free-living females (SHIWAKU *et al.* 1988). To explain this disparate result, while keeping with the hypothesis that extreme culture conditions result in the homogonic developmental route, it was noted by Shiwaku *et al.* that *S. planiceps* and *S. papillosus* are found in equatorial regions and therefore an "extreme" culture condition for them would be colder temperatures (SHIWAKU *et al.* 1988).

Additional fatty acids in the culture medium of *S. ratti* also cause an increase in iL3 formation with free-living female number decreasing in direct response, the proportion of males is not influenced (MINEMATSU *et al.* 1989). In axenic culture *S. ratti* is influenced by the proportion of CO_2 and O_2 in culture with low CO_2 favouring iL3 formation and high CO_2 resulting in higher proportions of free-living females (TAYLOR and WEINSTEIN 1990).

Either increased immunity in the host or environmental conditions with abundant food resourses will result in an increased proportion of heterogonic females and males.

Whereas, culture conditions with low food resources or extremes of temperature or pH have increased proportion of homogonic development, this may signal to developing larvae of the increased risk of reaching adulthood and successfully reproducing, thereby influencing the developmental decision to develop directly into iL3 (MONCOL and TRIANTAPHYLLOU 1978; STREIT 2008; YAMADA *et al.* 1991).

One of the difficulties of culturing parasites is the absolute requirement of an appropriate host to complete the life cycle. In order to eliminate this constraint, efforts were made to culture multiple free-living generations of *S. stercoralis* and *S. planiceps* by removal of uterine eggs from the free-living females into fresh faecal cultures. Despite repeated efforts it was determined that *S. stercoralis* go through only one free-living generation, the progeny of a free-living female are always committed to development into infective larvae (YAMADA *et al.* 1991). By transplanting uterine eggs of *S. planiceps* into fresh faecal cultures, nine free-living generations were possible, although the fecundity of each successive generation drops off rapidly (YAMADA *et al.* 1991). Recently, it has been discovered that addition of the dafachronic acid Δ7 is able to suppress what would normally be constitutive development to infective larva from free-living generation of *S. papillosus* (OGAWA *et al.* 2009) and a second free-living generation was possible.

Tsuji and Fujisaki claim to have successfully cultured infective larvae to the parasitic stage of *Strongyloides venezeulensis* by *in vitro* culture in a Dulbecco's Modified Eagles medium. Despite this claim there were incomplete morphological changes and only a number of the parasitic stage specific proteins were induced as shown by Western blot of a 2D SDS-PAGE probed with immune sera from rats (TSUJI and FUJISAKI 1994). These results suggest only partial development towards parasitic adult stage had occurred.

1.6 THE INSULIN/IGF SIGNAL TRANSDUCTION PATHWAY IN *C. elegans* AND OTHER PARASITIC HELMINTHS.

1.6.1 Parasitic helminths with *daf* gene orthologues

As reviewed in Beall and Pearce (2002), several parasitic species may have orthologues to the genes involved in the IIS or the TGF-β developmental pathways (BEALL and PEARCE 2002). *Brugia malayi* and *B. pahangi* contain ligands from the TGF-β family that are differentially expressed during developmental switching. *B. pahangi* also contains an orthologue to one of the SMA-6 receptor proteins of the TGF-β signalling pathway (BEALL and PEARCE 2002). A range of parasites have a *daf-7* gene, an apparent orthologue of the TGF-β ligand, although its expression pattern differs to the *C. elegans* expression pattern (BRAND *et al.* 2005; CROOK *et al.* 2005; FREITAS and ARASU 2005; MASSEY *et al.* 2005; VINEY *et al.* 2005) .

Muscarinic agonists promote diapause recovery and muscarinic antagonist will block this recovery in wild-type *C. elegans* and *A. caninum* but not in IIS mutants, suggesting a functional IIS pathway is required for diapause recovery (TISSENBAUM *et al.* 2000). The requirement of the IIS pathway in diapause recovery in the hookworms *A. caninum* and *A. ceylanicum*, was corroborated by chemical inhibition of AGE-1 of the IIS pathway using a chemical inhibitor of PI3′K activity, LY294002. *A. caninum* and *A. ceylanicum*, treated with LY294002 did not demonstrate resumption of feeding behaviour upon stimulus with the appropriate signals (BRAND and HAWDON 2004). Two DAF-16 transcription factor orthologues have been cloned from *S. stercoralis* (MASSEY *et al.* 2003). *C. elegans* Daf-16 dauer larvae defective phenotype was rescued by transgenesis with the *S. stercoralis fktf-1b* (*daf-16b* orthologue) (MASSEY *et al.* 2006). Two *H. contortus daf-16* orthologues have recently been cloned, and one: *Hc daf-16.2*, was able to recover dauer larvae defective phenotype in a *C. elegans* mutant (HU *et al.* 2009). DAF-16 orthologues cloned from *A. caninum* and *A. ceylancium* were used in an *in vitro* assay to drive expression of a reporter gene from a conserved DAF-16 binding element (DBE) (GAO *et al.* 2009).

1.6.2 TGF-β regulated dauer larvae development and vulva development of *C. elegans* and nematode species

A comparison of dauer larvae of *C. elegans* and the infective larval stage of *Strongyloides* and *Parastrongyloides* species show some common biological characteristics, which fits the hypothesis that these stages are analogous and that the dauer larvae stage may have served as a starting point in the evolution of parasitism. If these stages are analogous then one can extrapolate that the molecular processes that control entry and exit to the dauer larvae stage would by analogous to the control of infective larva entry and exit in the *Strongyloides* and *Parastrongyloides* species. One of the goals of this research is to test the hypothesis that orthologues of the IIS genes of *C. elegans* are the key genes in the developmental regulation and longevity in *P. trichosuri*. It should be noted that investigation into other developmental processes has highlighted some striking differences between *C. elegans* and the nematodes species, discussed below.

One such difference is dauer larvae development through the TGF-β signalling pathway. In *C. elegans*, *daf*-7 is expressed in the amphids at the early larval stages to produce the ligand which activates the TGF-β cellular receptor, the end result of which is normal reproductive growth, *C. elegans daf*-7 is not expressed in the dauer larvae stage. In contrast to this, *daf-7* expression in several parasitic species including *A. caninum* (FREITAS and ARASU 2005*)*, *S. ratti* (CROOK *et al.* 2005), *S. stercoralis* (MASSEY *et al.* 2005) and *P. trichosuri* (CROOK *et al.* 2005) is not at the early larval stages, but rather at the arrested infective stage of development. It has been speculated that whereas in *C. elegans* TGF-β signalling results in normal reproductive growth, in these parasites TGF-β signalling results in developmental arrest (VINEY 2009; VINEY *et al.* 2005).

Another example of developmental processes that diverged evolutionarily is vulva development between the nematodes *C. elegans* and *P. pacificus*. The Wnt pathway controls vulva development in both species however the cell fates of non-vulva cells are quite different between the species: the cells fuse with the hypodermis in *C. elegans* and die in *P. pacificus* (SOMMER and STERNBERG 1996); a single anchor cell controls vulva cell fate in *C. elegans* whereas in *P. pacificus* there are signals from multiple cells of the somatic gonad (SIGRIST and SOMMER 1999); in *C. elegans* Wnt signalling has a positive

role in vulva induction, as *bar-1*(encoding β-catenin) mutations result in hypoinduction of the vulva, in contrast, *P. pacificus, bar-1* knockdown results in multi vulva phenotypes (ZHENG *et al.* 2005). So while the developmental machinery remains conserved, the downstream processes have diverged.

1.7 FUNCTIONAL ANALYSIS OF HELMINTH GENES

1.7.1 Mechanism of RNAi in *C. elegans*

Since it was first identified, RNAi has become a powerful reverse genetics tool to test gene function. Post-transcriptional gene silencing or "cosuppression", was first identified in plants over a decade ago (as described: (BERNSTEIN *et al.* 2001; HUTVAGNER and ZAMORE 2002; TOMARI and ZAMORE 2005; ZAMORE *et al.* 2000)), and was first reported in *C. elegans* in 1998 when the introduction of antisense RNA resulted in the specific down regulation of the homologues endogenous gene (FIRE *et al.* 1998; GUO and KEMPHUES 1995). It has since been identified in a variety of organisms including fungi, *Drosophila*, zebrafish, mice and mammalian cell cultures (BERNSTEIN *et al.* 2001; HUTVAGNER and ZAMORE 2002; TOMARI and ZAMORE 2005; ZAMORE *et al.* 2000). Double stranded RNA (dsRNA) is the trigger that initiates the RNAi gene silencing mechanism. Since dsRNA is the genomic material of certain viruses, RNAi is thought to have developed from a defence mechanism to protect cells from viral invasion (TIJSTERMAN *et al.* 2004).

Dicer and RISC are components found in all classical RNAi pathways. Dicer forms a complex with several proteins (RDE-1 and RDE-4), which together function to dice the dsRNA into small interfering RNA (siRNA) of 19 -21 nucleotide length with a 2 nucleotide 3' overhang (SCHWARZ *et al.* 2003; ZAMORE *et al.* 2000). The siRNA forms a complex with RISC (RNA-Induced Silencing Complex). The RISC complex will incorporate the strand that is less tightly base-paired at the 5' end, and only if the anti-sense strand binds to the complex is there silencing of the endogenous target mRNA (SCHWARZ *et al.* 2003; TOMARI and ZAMORE 2005). After incorporation of the

antisense into the RISC complex, an RNA dependant RNA Polymerase (RdRP) may amplify the small interfering RNA (siRNA), perhaps by using endogenous mRNA as a template and the siRNA as primer, thereby creating more dsRNA and amplifying the RNAi effect from a few molecules of siRNA (TOMARI and ZAMORE 2005). Alternatively, the siRNA:mRNA duplex may be degraded by RISC. The latter degradation is an evolutionarily conserved process that appears to function in many species, whereas the former, amplification of RNAi effect from a few molecules by RdRP, has only been described in *C. elegans* and plants (TOMARI and ZAMORE 2005; WANG and BARR 2005). See figure 1.5.

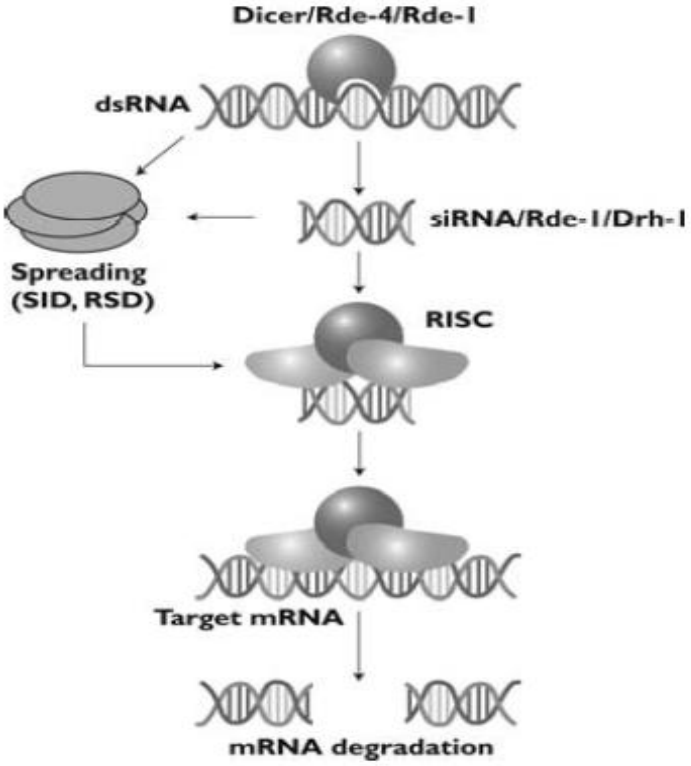


Figure 1.5: Cellular Mechanism of RNAi in *C. elegans* (GELDHOF *et al.* 2007)
Double stranded RNA is diced into small inhibitory RNAs (siRNA) by Dicer; the siRNA serves as a template for the RISC complex of proteins, to degrade target mRNA which is complementary to the siRNA sequence.

C. elegans is extremely susceptible to RNAi and the triggering dsRNA may be delivered to the worm in a variety of ways. It may be in microinjected into any part of the body, it may be fed to *C. elegans* in bacteria expressing dsRNA, or it may be transformed into *C. elegans* and expressed as transgenes. Alternatively, the worms may

be soaked in *in vitro* transcribed dsRNA. For a review see May and Plasterk (MAY and PLASTERK 2005).

The amplification of dsRNA by RdRP is thought to be responsible for part of the "spreading" effect that is seen in *C. elegans*. A few molecules of dsRNA may be introduced to a single tissue of *C. elegans*, but the effect of RNAi is seen in almost the entire worm, (neurons being the notable exception to this). The "spreading" effect in *C. elegans* also requires cellular uptake of the dsRNA by SID-1, a transmembrane protein. Sid-1 mutants do not take up dsRNA expressed from bacteria. Furthermore tissue specific transgene expression of dsRNA in a Sid-1 mutant background resulted in cell autonomous effects of RNAi (FEINBERG and HUNTER 2003; WINSTON *et al.* 2002), which is consistent with a role for SID-1 in RNAi spreading.

The *C. elegans* genome contains several RNA-directed RNA polymerases (RdRP). Mutations in one of these genes: *rrf-3*, results in *C. elegans* which are hypersensitive to RNAi, even in neurons. Screening of genes in a Rrf-3 mutant background resulted in detection of genes that do not display an RNAi phenotype in wild-type worms (SIMMER *et al.* 2002). As the genes involved in the RNAi pathway become elucidated in other organisms, down regulating the *rrf*-3 orthologue becomes a potential way to increase the sensitivity of RNAi.

1.7.2 RNAi in parasitic species

RNAi in nematode parasites has been reported in relatively few cases, despite RNAi technology being available since 1998. This suggests that RNAi in parasitic helminths is not as straight forward as has been demonstrated in *C. elegans*. The plant parasites to be discussed do not feed until established within the host plant, which makes an RNAi feeding assay extremely difficult to perform, and parasites, as a rule, are not amenable to transgenesis making RNAi by transgene expression technically challenging. The most successful delivery method of dsRNA for parasitic nematodes appears to be soaking, or electroporating, the nematode in highly concentrated solutions (mg/mL range) of *in vitro* transcribed dsRNA.

Some RNAi effect in *Meloidogyne artiellia, Heterodera glycines, Globodera pallida* and *B. malayi* have been shown to be long lasting; transcript knockdown and the associated phenotype persisted for days after treatment (FANELLI *et al.* 2005; URWIN *et al.* 2002). However, the effectiveness of RNAi in parasitic nematodes is inconsistent and appears to be not only gene specific but also dependent upon: length of exposure to the dsRNA, the delivery method, and length of the dsRNA (LILLEY *et al.* 2007). Effectiveness may have to be determined on a gene by gene basis as soaking may be more effective then electroporation and vice versa depending upon the gene of interest.

For the plant parasite, *M. artiellia,* soaking of egg masses in *in vitro* transcribed dsRNA derived from the chitin synthase gene resulted a reduction in chitin protein in the egg cases, knockdown in transcript abundance, and a delay in the release of the juvenile larvae 2 (J2) stage from the eggs, although not in a reduction in J2 numbers (FANELLI *et al.* 2005). Soaking of J2 stage of plant parasite has been used with success in a variety of plant parasite species, reviewed in (LILLEY *et al.* 2007) and the results to date for RNAi in plant parasites have highlighted some common features and constraints; the size of the dsRNA molecule must be between 42-1300 bp; in general, the longer the soaking time the greater the gene silencing effect and once removed from exposure to dsRNA the gene silencing effect is time limited (LILLEY *et al.* 2007) .

To promote dsRNA uptake, Urwin *et al.* used octopamine to induce pharyngeal pumping of the plant parasites *H. glycines* and *G. pallida* (J2) soaked in a concentrated solution of *in vitro* transcribed dsRNA (URWIN *et al.* 2002). Uptake of dsRNAi derived from cysteine protease and major sperm protein (MSP) genes resulted in a reduction of the transcripts of these genes and/or a change in phenotype (URWIN *et al.* 2002). J2 stage uptake of MSP dsRNA into the mouth and stylet lumen resulted in a loss of MSP expression in the male adult sperm 6 days after development to the parasitic stage, which may be indicative of spreading and longevity of the RNAi effect in these species.

Lustigman *et al.* reported that soaking of third larval stage (L3) human filarial parasite *Onchocerca volvulus* in *in vitro* transcribed dsRNA cathepsin L and Z-like cysteine proteases genes resulted in incomplete moulting of the L3 stage. However, no molecular work was done to confirm a knockdown in gene transcript from the RNAi treatment (LUSTIGMAN *et al.* 2004). The adult stage of *B. malayi*, a human filarial

parasite, is also amenable to soaking RNAi, such that soaking in dsRNA derived from the RNA polymerase II subunit *(ama-1),* beta tubulin (*tub-1*) and sheath protein (*shp-1*) genes resulted in reduction of transcript and, in the case of *ama*-1 and *tub*-1 RNAi, death of the worms (ABOOBAKER and BLAXTER 2003). There was also an RNAi effect on the embryos within the fecund parasitic females, as parasitic females treated with *shp*-1 dsRNA gave rise to offspring with malformed sheaths (ABOOBAKER and BLAXTER 2003). Hussein *et al.* demonstrated that soaking adult *Nippostrongylus brasiliensus* in dsRNA derived from the gene encoding the secreted form of acetylcholinesterases (AChE) resulted in a knockdown in protein levels (estimated from polyacrylamide gels) although no phenotype was reported and knockdown of gene transcript was not confirmed (HUSSEIN *et al.* 2002).

Issa *et al.*, reported on the efficiency of RNAi phenotype in *Trichostrongylus colubriformis*, a sheep gastrointestinal parasite, by a variety of delivery methods (feeding, soaking and electroporation of dsRNA). However although a phenotype was reported, knockdown of the target gene expression was not confirmed by molecular means. Feeding L1-L3 *T. colubriformis* with bacteria expressing dsRNA tropomyosin gene gave a strong phenotypic response, while a feeding assay with dsRNA ubiquitin gene gave no phenotypic response, leading these authors to conclude that feeding dsRNAi may result in false negatives if this delivery method is used. Soaking L1 worms in *in vitro* transcribed dsRNA exhibited a size preference -small siRNA giving a stronger response then larger fragments of dsRNAi. Electroporation of L1 larvae with *in vitro* transcribed dsRNAi gave both the strongest and most consistent RNAi response in this parasitic nematode (ISSA *et al.* 2005).

RNAi in *H. contortus* was performed by soaking exsheathed L3 and L4 stage larvae in solution of dsRNA of the β-tubulin gene. A reduction in gene repression resulted after 24 hours along with reduced motility, both of which were eventually recovered by the time worms developed to the adult stage (KOTZE and BAGNALL 2006). Of note, the RNAi effect apparently does not require ingestion as the iL3 stage used in these soaking experiments have no working mouth parts; and the adults were treated with ivermectin to paralyze pharynx, yet both these stages were susceptible to RNAi from soaking

As has been observed in other parasitic species (GELDHOF *et al.* 2007), RNAi effect in *H. contortus* appears to be gene specific. Geldhof *et al*. tested 11 genes by soaking and 4 of these same genes by electroporation; of these only 2 genes (β-tubulin and *sec-23),* showed consistent knockdown in expression. It was observed however, that at electroporation voltages high enough to cause significant larval death in the controls, there was a decrease of expression levels of an additional 3 genes. Visser *et al*. tested 8 genes for RNAi knockdown in *Ostertagia ostertagi* by both electroporation of L1 and soaking of L3, only 5 out of 8 genes showed an RNAi effect for soaking of L3 whereas, there was less effect, 2 out of 8 genes, for electroporation (VISSER *et al.* 2006).

Bioinformatic searches of *H. contours* and *B. malayi* genomes (95% and 98% coverage respectively) have failed to find the full complement of RNAi pathway genes homologues, raising the question whether the classical RNAi pathway exists in mammalian parasites (KNOX *et al.* 2007). It was conjectured by these authors that both the delayed responses and the stronger silencing observed with short dsRNA or siRNA was due to transcriptional silencing only.

1.7.3 Chemical Mutagenesis

Reverse genetics is a powerful tool for determining gene function. Whereas RNAi results in a transient "knock down" in expression of a gene, the "knock out" of a gene function, for example by, deletion mutation due to chemical mutagenesis, result in permanent removal or mutation of the gene. Chemical mutagenesis, followed by PCR screening to find deletion mutants, has been successfully used in *C. elegans* (GENGYO-ANDO and MITANI 2000; JORGENSEN and MANGO 2002; LIU *et al.* 1999).

Simplified, the procedure is to treat a parental population of *C. elegans* (generation P_0) with a chemical mutagen at the L4 to early adult stage, when the gonads and germline cells are developing. Examples of mutagens used are ethyl methane sulfonate (EMS), ethylnitrosourea, diepoxyoctane and ultraviolet-activated trimethylpsoralen. The offspring of the mutagenized P_0 (generation F_1) are held at a lower temperature to prolong their life after having lain eggs (generation F_2), so that it is possible to go back to isolate the mutant after screening by PCR for deletion mutations in a gene of interest.

Viney *et al*. have reported on the only successful chemical mutagenesis of a parasitic helminth. In this experiment, *S. ratti* were screened phenotypically after mutagenesis to select for worms with resistance to ivermectin at low concentrations (of 10 ng/mL). By treating the P_0 larval stage with the chemical mutagen EMS, as the reproductive organs develop, and then allowing mating to occur, a proportion of the F_1 generation will be heterogyous for mutations, these mutant offspring obigately form iL3, which are used to infect a rat. These iL3 all develop to parasitic females which reproduce by mitotic parthanogenosis, the eggs of which are genetically equivalent to the adult. The eggs develop to both free-living males and females which are allowed to mate, and the offspring screened for mutations which confer resistance to low levels of ivermectin (VINEY *et al.* 2002). Resistance to low concentrations of ivermectin is an indicator of amphid neuron integrity and in *C. elegans;* such mutations would result in worms unable to initiate dauer larvae development. The mutant *S. ratti* worms isolated from this experiment with a resistance to ivermectin were unable to produce a patent infection, which gives indirect evidence for the importance of amphid integrity in infective larval development (VINEY *et al.* 2002).

1.7.4 Transformation of parasitic nematodes

The complex life cycle of most parasitic nematodes means that direct genetic manipulation by transgenesis or mutagenesis has been limited to a handful of special cases.

If the parasitic gene in question has a *C. elegans* orthologue, then rescue by complementation is possible by microinjecting the parasite gene into the relevant *C. elegans* mutant, and then assessing for recovery of the wild-type phenotype (HASHMI *et al.* 2001). Analysis of parasite gene function using the *C. elegans* model system has been achieved by microinjecting *C. elegans* gonads with the foreign parasite DNA expressed on a transgene. This results in the formation of a heritable extrachromosomal array of the exogenous DNA and has allowed for the functional study of several parasite genes (BRITTON *et al.* 1999; GRANT 1992; GRANT and VINEY 2001; HASHMI *et al.* 1995). Careful assessment must be made as spurious results may occur; for example, a *C. elegans* collagen mutant, transformed with a *T. colubriformis* collagen gene (*Tc-dpy-*

13), resulted in an exaggeration of the mutant phenotype rather then rescue (GRANT 1992).

C. elegans may also be used to assess the spatial and temporal expression pattern of a gene of interest using the parasite gene promoters in a reporter construct. *H. contortus* promoters for the genes AC-2 and pep-1 were studied in this way, and although the spatial expression pattern correlated with expression patterns in *H. contortus*, the temporal pattern differed (BRITTON *et al.* 1999). These examples highlight the drawback of using a different species: even a closely related free-living nematode, in order to study gene function, because the control elements or protein folding may not be processed as they would be within the original species.

Transgenic analysis of gene expression directly within the parasite is a developing field. Biolistic transformation (bombardment with particles coated in transgene reporter constructs) was first shown to produce transient expression in the parasitic helminths *Ascaris* spp., *B. malayi* and *Litomosoides sigmodontis* and Trematode *Schistosoma* spp. as reviewed in (KALINNA and BRINDLEY 2007). As the gonad structures of *C. elegans* and the free-living stages of *Strongyloides* and *Parastrongyloides* are similar, microinjection of transgene constructs in these species was demonstrated as a possibility.

Heritable transgenesis of a transgene has been reported in *P. trichosuri* (GRANT *et al.* 2006a), as demonstrated by β-gal staining and PCR screening for the presence of the transgene through multiple generations. Lok *et al.* also demonstrated heritable expression of a transgene encoding a gfp marker in the F_1 generation in *S. stercoralis* although the gfp was only expressed in the first generation, the presence of the transgene was detected by PCR for multiple generations (LI *et al.* 2006). These authors also showed that expression of the gfp marker required parasite endogenous regulatory sequences such as the *S. stercoralis era-1* 3′ UTR. In both *P. trichosuri* and *S. stercoralis*, there is evidence of gene silencing of the transgene, as the transgene remains detectable by PCR for several generations after transgene expression is no longer detectable. With biolistics, the transformation particles penetrate only as far as the hypodermis and the transgene expression is only found in these tissues, whereas microinjection delivers the transgene to the gonad syncytium of the female where it is

incorporated into the nuclei of the developing embryo, the possibility of tissue specificity exists. Tissue specific expression was achieved in *S. stercoralis* with a panel of several endogenous promoters (JUNIO *et al.* 2008).

*****

The aims of this project are to investigate the environmental signals which influence the *P. trichosuri* iL3 developmental switch, and to test whether insulin/IGF signalling plays a role in the transduction of these signals. In order to achieve the first aim, I will investigate the effect of a population density signal on *P. trichosuri* iL3 development. I will also investigate how temperature and food availability modulate the effect of the population signal to influence iL3 development. To achieve the second aim, I will clone relevant insulin/IGF signalling orthologues from *P. trichosuri*, such as the tyrosine receptor kinase (*daf-2*); the phosphatidylinositol 3′ kinase (*age-1*), and the FOXO forkhead transcription factor (*daf-16*). These will be characterized bioinformatically, and their function will be tested either directly in *P. trichosuri* or indirectly in transgenic *C. elegans*.

CHAPTER 2

MATERIALS AND METHODS

2.1 BACTERIA AND WORM STRAINS

2.1.1 *Escherichia coli* strains

E. coli stains were maintained on LB plates at 37°C or grown in LB broth at 37°C with shaking at 230 rpm. Antibiotics, (100mg/L Ampicillin or 50mg/L Neomycin Sulfate) were used for selection where necessary [Sigma, Aldrich]. *E. coli* glycerol stocks were cryopreserved in 15% glycerol at -80°C.

Table 2.1: *Escherichia coli* strains used

	Genotype	Supplier	Ref.
XL-2	endA1 gyrA96(nalR) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 proAB+ lacIq Δ(lacZ)M15 Amy CmR] hsdR17(rK- mK+)	Stratagene	(GREENER 1993)
DH5a	F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r_K^- m_K^+), λ–	Invitrogen	(GRANT *et al.* 1990)
TOP10	F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ^-	Invitrogen	(CASADABAN and COHEN 1980)
Nova Blue	endA1 hsdR17($r_{K12}^-m_{K12}^+$)supE44 thi-1 recA1 gyrA96 relA1 lac F' [$proA^+B^+$ $lacI^q$ ZΔM15::Tn10(Tet^R)]	Novagen	*
SURE	recB, recJ, sbcC201, phoR, uvrC, umuC::Tn5, mcrA, mcrB, mrr, Δ(hsdRMS), endA1, gyrA96, thi, relA1, lac*, supE44, (F'proAB, $lacI^Q$ ZM15, Tn10)	Stratagene	(GREENER 1990)
BL21 (DE3)	F^- ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$ λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])	Invitrogen	(STUDIER *et al.* 1990)
HT115 (DE3)	F^- mcrA mcrB IN(rrnD-rrnE)1 rnc14::Tn10 (DE3 lysogen: lacUV5 promoter T7 polymerase (IPTG inducible T7 polymerase) RNaseIII-.	FireLab	(TIMMONS *et al.* 2001)
HB101	F^- mcrB mrr $hsdS20(r_B^- m_B^-)$ recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 $rpsL20(Sm^R)$ glnV44 λ^-	Invitrogen	(BOYER and ROULLAND-DUSSOIX 1969)
OP50	*E. coli* that is uracil auxotroph (Undefined genotype preferred food source of *C. elegans*)	CGC	(BRENNER 1974)

*Note: source, parentage, and provenance of NovaBlue™ are proprietary, and would not be released by Novagen.

2.1.2 *Parastrongyloides trichosuri*

P. trichosuri were incubated on NGM low peptone agar plates or in NGM low peptone broth. HB101 *E. coli* bacteria was used as the food source and supplemented with dried possum faecal extract where appropriate. For long term storage of *P. trichosuri* the first

larval stage was cryopreserved in a 10% vol/vol of DMSO. See cryopreservation procedure Section 2.4.9.

2.1.3 *Caenorhabditis elegans* strains

C. elegans strains were obtained from the CGC (Caenorhabditis Genetics Centre) and incubated on NGM agar plates or in NGM broth. Either *E. coli* HB101 or *E. coli* OP50 bacteria were used as the food source. For long term storage of *C. elegans* the first larval stage was cryopreserved in worm freezing solution (Appendix 2). See cryopreservation procedure Section 2.4.10.

Table 2.2: List of *C. elegans* strains used:

Strain	Linkage groups and genotypes	Reference
N2 Bristol	Wild-type	(Maupas, 1900)
DR26	*daf*-16(m26)I	(RIDDLE *et al.* 1981)
CB1370	*daf*-2 (e1370)III	(RIDDLE 1977)
TJ1052	*age*-1 (hx546)II	(JOHNSON *et al.* 1993)
DR1309	*daf*-16(m26); *daf*-2(e1370)	(RIDDLE *et al.* 1981)
DR1408	*daf*-16(m26); *age*-1(m333)	(RIDDLE *et al.* 1981)

2.2 PARASITOLOGICAL PROCEDURES

2.2.1 Possum animal husbandry

Australian brushtail possums (*Trichosurus vulpecula*) possums were maintained as described in (GRANT *et al.* 2006b). All procedures in animal husbandry were in accordance with the Wallaceville and Grasslands Animal Ethics Committee's requirements. Animal Ethics Approval was obtained: AEC # 273.

Wild possums were captured in cage traps from bush/pasture margins in areas surrounding Upper Hutt or Palmerston North, New Zealand. Following capture, animals were housed individually in mesh cages 58 x 58 x 800 cm containing a 30 x 24 x 24 cm nest box and were fed daily a selection of fresh fruit. Water and possum pellets [Sharpes Grain and Seeds Ltd, Lower Hutt, New Zealand] were available *ad libitum*. Possums in New Zealand are known to be infected with three species of nematode

(*Paraustrostrongylus trichosuri, T. colubriformis* and *P. trichosuri*) so all wild caught animals were treated orally with 2 doses, one week apart, of a mixture of albendazole at 23.5mg/kg and levamisol at 37.5mg/kg [Arrest® Ancare, Ltd], which has been shown to effectively remove all three species (RALSTON *et al.* 2001). When necessary, animals were then anaesthetised by intramuscular injection of either 25 mg /kg live weight Zoletil 100 (Virbac Laboratories, Auckland, New Zealand) or intramuscular injection of 50 mg/kg of Ketamine hydrochloride [Phoenix Pharm, Auckland, NZ]. Euthanasia was by intracardiac injection of sodium pentobarbitone following Zoletil or Ketamine anaesthesia. All procedures were approved by either the Wallaceville Animal Research Centre or Grassland's Animal Ethics Committee.

Infections were initiated by transdermal infection with 3,000-5,000 infective larvae, applied to previously wetted belly fur of anaesthetised animals. To determine patency, (which occurs at ~14 days post infection) overnight faecal samples were collected between 0800 and 0900 h and nematode eggs per gram (EPG) were determined using a modified McMaster technique with a sensitivity of 50 EPG. Parasites from the small intestine were recovered as described previously (STANKIEWICZ 1996).

2.2.2. Egg extraction of *P. trichosuri*

Faecal pellets from infected possum were soaked in a saturated salt solution and broken up into slurry. Coarse debris was removed by sieving through a kitchen sieve and the liquid slurry poured into the lid of a large petri dish. Eggs float to the surface of the due to the increased density of saturated salt solution. The base of petri dish was floated on this liquid faecal slurry for several minutes to allow the eggs floating on the surface to adhere to the glass surface. The petri dish base was then lifted and eggs washed off onto a 106µm screen to remove large debris. Eggs and small particulate material that washed through this screen was washed onto a 20µm screen to allow for liquid and small debris to be washed away. Cleaned eggs were then layered onto a 22µm mesh suspended over a shallow layer of distilled water. After 5 hours incubation at room temperature the newly hatched *P. trichosuri* larvae, which pass through the mesh into the water, were pelleted by gentle centrifugation (<200xg for 2 minutes) and used in the developmental assays described herein.

2.2.3 *In vitro* culturing of free-living *P. trichosuri*

Synchronized *in vitro* cultures of free-living *P. trichosuri* were achieved by dispensing approximately 500-1,000 L1 larvae onto 10mL Petri dishes containing NGM low peptone agar. Each culture was supplemented with 5-10 mg of a possum faecal extract suspended in 25-50μL of a 10% (w/vol) broth culture of *Escherichia coli* strain HB101. The possum faecal extract consists of pooled fresh faeces suspended at 10% w/vol in water: 200 μL aliquots were dispensed into microcentrifuge tubes, dried in a SpeediVac and stored at -20^{o}C until being rehydrated.

Liquid cultures of free-living worms at one L1 larvae per μL were initiated by culturing newly hatched larvae, recovered by salt flotation (as described Section 2.2.2), into 1/10 liquid NGM supplemented with 0.3-1.0% w/vol *E. coli* HB101 as a food source and 50μg/mL of Gentamicin sulfate to prevent contamination and excess growth of the *E. coli* HB101 food source.

2.2.4 Synchronous cultures of *P. trichosuri*

Synchronous *in vitro* cultures of *P. trichosuri* were achieved by axenizing gravid adults to release internal eggs. 1 mL of worm axenizing solution (Appendix 2) was added to pelleted gravid adult worms, then briefly vortexed, incubated for 30 seconds and quickly diluted with 45mL of PBS. This was followed by 4 additional washes with 45mL of PBS each. The eggs released were replated as described above.

2.2.5 Infective larval culturing of *P. trichosuri*

Faeces from possums infected with *P. trichosuri* was mixed with vermiculite at approximately ratio of 50:50 and moistened with dH_20. This was layered onto Whatman® paper draped across a raised platform in a container of distilled water faecal: vermiculite. The mixture was kept moist and covered. After 9-12 days incubation at room temperature the infective larvae that crawled out of faeces and down the Whatman® paper into the water were collected by gravitational settling.

2.3 CONDITIONED MEDIUM PREPARATION AND EXTRACTION

2.3.1 *P. trichosuri* conditioned medium (CM)

The procedure for preparation of a *P. trichosuri* conditioned medium was based on the preparation of dauer pheromone for *C. elegans* (GOLDEN and RIDDLE 1982). In order to produce a preparation with increased concentration of biological factors liquid cultures of *P. trichosuri* free-living nematodes at ~1,000 worms/mL in 50-75 mL of liquid low peptone NGM, plus Gentamicin sulfate (50µg/mL) and *E. coli* HB101, were incubated with agitation at 180 rpm (on an orbital shaker) and 20^{o}C for 5-7 days. Worms and bacteria were removed by centrifugation at 3500g for 20 minutes and the supernatant passed through a 0.2µm filter to remove any residual worms, worm debris and bacteria. Fresh larvae and bacterial food source were re-inoculated into this medium and culturing and recovery of the medium repeated. This cycle of culture followed by nematode and bacterial removal was repeated 4 times to produce 4X conditioned medium. A parallel culture was prepared that had been subjected to 4 identical rounds of incubation with bacteria and antibiotic but no worms, this was named BAC control.

Two preparations of condition medium were prepared. CM Batch #1 was used for all biological assays and CM Batch #2 was used for creation of inbred lines by pre-selection (Section 2.4.8).

2.3.2 *C. elegans* conditioned medium extraction

An adaptation of the protocol described (OGAWA *et al.* 2009). Activated charcoal [AJAX Chemicals] was prepared by boiling in distilled water and removing supernatant. The charcoal was then washed several times to remove all floating particles. A 100mL culture of *C. elegans* in liquid NGM and 0.4% (w/vol) *E. coli* OP50 as food source was cultured for 5 days and then harvested. The worms and debris was removed by filtering through Whatman® filter paper #1 and then centrifuged at 17,000xg in Fiberlite F10-6X500Y rotor for 30 minutes. The supernatant was passed through a 0.2µm filter and added to beaker containing 15mL of the prepared charcoal and stirred with magnetic stirrer for 1 hour. The supernatant was discarded and the charcoal washed 4 times with 100mL distilled water. Charcoal bound pheromone was extracted, first using 20mL of ethanol by stirring for 1 hour followed by five extractions

using 15 mL of a 1:1:1 mixture of acetone:toluene:ethanol and the eluates pooled and dried. The residue was resuspended in 1 mL of H_2O, aliquoted and stored at -20^{o}C.

2.3.3 *P. trichosuri* conditioned medium extraction

As described above, with the exception that a 100mL culture of *P. trichosuri* in liquid NGM with 0.4% (w/vol) *E. coli* HB101 as food source was extracted.

2.4 *P. trichosuri* BIOASSAYS

2.4.1 Infective larval induction by conditioned medium

The ability of 4X conditioned medium to induce infective larval development was determined using a two fold serial dilution series with at least 5 replicate wells at each dilution of conditioned medium and approximately 100 freshly hatched L1 per 100µL low peptone liquid NGM *E. coli* HB101 (0.35%w/vol) and 50µg/mL Gentamicin sulfate were added per well. At day 5 the proportion of free-living and infective larvae were calculated for each treatment.

2.4.2 Infective larval induction by conditioned medium and mediated by food availability

A two-fold serial dilution series of conditioned medium with 8 replicate wells at each dilution containing approximately 100 freshly hatched L1 per 100µL low peptone liquid NGM and 50µg/mL Gentamicin sulfate per well was set up in 96 well plates. *E. coli* HB101, at a concentration of either 0.3% w/vol, 0.4% w/vol, or 0.5% w/vol was added to each well and the plates incubated at 20^{o}C. At day 5 the proportion of free-living and infective larvae were calculated for each treatment. Statistical analysis of iL3 development in response to conditioned medium at different food concentrations was as described Methods Section 2.9.

2.4.3 Infective larval induction by conditioned medium and mediated by temperature

A two-fold serial dilution series of conditioned medium with 8 replicate wells at each dilution containing approximately 100 freshly hatched L1 per 100µL in low peptone NGM broth with *E. coli* HB101 at 0.35% w/vol as the food source and 50µg/mL

Gentamicin sulfate per well was set up in 96 well plates. The plates were incubated at either: 14^{o}C, 20^{o}C and 26.5^{o}C. At day 5 the proportion of free-living and infective larvae were calculated for each treatment. Statistical analysis of iL3 development in response to conditioned medium at different temperatures was as described Methods Section 2.9.

2.4.4 Infective larval development in the absence of exogenous cholesterol

Adult *P. trichosuri* (approximately 5 females and males) were placed onto either: low peptone NGM agar plates, or low peptone NGM agar plates without cholesterol. *E. coli* HB101 and possum faecal extract was spotted onto plates as a food source. These plates were incubated at 20^{o}C. At day 3, approximately 5 females and males of the F_1 generation were replated (in triplicate) onto fresh low peptone NGM agar plates, or low peptone NGM agar plates without cholesterol with *E. coli* HB101 and possum faecal extract spotted onto plates as a food source. The F_1 adults were removed the next day after laying >30 eggs. At day 5 the proportion of F_2 free-living and infective larvae were scored for each treatment.

2.4.5 Brood size

Single *P. trichosuri* females that were late L4 larvae stage or early adult stage were plated individually into 24 well plates with NGM low peptone agar plates and with 5µL of 10% (w/vol) HB101 *E. coli* bacteria and possum faecal extract as the food source and incubated at 20ºC. Each female was paired with 5 early adult stage males. Each day the adults were transferred to new plate until they stopped producing eggs and the offspring were counted.

2.4.6 Sex ratio

As described above for determining brood size, however offspring were counted and scored as male or female when they reached maturity.

2.4.7 Free-living life span of *P. trichosuri*

P. trichosuri L1 stage worms were plated onto low peptone NGM agar plates with HB101 *E. coli* and possum faecal extract at 20^{o}C. The next day individual worms were plated into wells of a 48 well plate with low peptone NGM agar plug and seeded with

HB101 *E. coli* and possum faecal extract. Worms were observed daily and scored as dead when they did not respond to light touch.

2.4.8 Creation of inbred lines

In order to select for increased sensitivity to conditioned medium, *P. trichosuri* outbred population (KNP) was cultured in low concentration of conditioned medium (20%). The infective larvae population (G1) that developed i.e.: worms that were sensitive to a low concentration of conditioned medium, were used to infect a possum and eggs were collected. This next generation was subjected to incubation in 10% conditioned medium and the resulting population of iL3 that developed were used to infect a possum. The next generation (G2) was then subjected to incubation in 5% conditioned medium and the resulting population of infective larvae (G3) was used to infect a possum and the free-living progeny from this possum were used as the starting population of 10 generations of single pair brother/sister mating to create an inbred line. *P. trichosuri* were selected for resistance to conditioned medium by incubating in 80% conditioned medium for 36 hours, the few worms that failed to developed to iL3 but remained free-living served as a starting population for 10 generations of single pair brother/sister mating.

The majority of these single pairs produced progeny. From each well with progeny, a further 8 single pairs (in this case, the pairs were brother and virgin sister) were cultured. This process of single brother-virgin sister mating was continued for 10 generations. During this time, the majority of the "lines" died out leaving a small number of inbred lines surviving from the initial single pairs. Lines CM20 and CM1 were created from the larvae selected for sensitivity to conditioned medium and lines CM3 and CM13 were created from larvae that were resistant to the conditioned medium signal. Inbred lines were assayed for infective larvae development in response to serial dilution of CM batch #1. Each dilution series of conditioned medium had at least 5 replicates of each dilution and were repeated in 3 separate trials, the average proportion of iL3 development was graphed. Statistical analysis of iL3 development of the inbred lines in response to conditioned medium was as described Methods Section 2.9.

2.4.9 Cryopreservation of *P. trichosuri* L1

P. trichosuri eggs were extracted from possum faeces by salt floatation (as described previously). Eggs were laid over (0.4μm) Nitex mesh floating on distilled water in order to allow the first larval stage to wiggle through to distilled water. The first larval stage larvae were collected after incubation at RT for 5-6 hours. The larvae were then spun at 100 xg for 3 minutes, the supernatant was aspirated and an equal volume of 20% solution of dimethyl sulfoxide (DMSO) was added to produce a final concentration of 10% (vol/vol) DMSO. Cryovials containing 500uL of the worm DMSO mixture were slowly cooled to -80^{o}C at a rate of 1^{o}C/min in a "Mr Frosty" [Nalgene].

When removing strains from cryopreservation, vials were thawed as quickly as possible under warm running tap water and added to 12mL PBS, gently mixed by inversion several times and then spun at 300xg for 3 min. Most of liquid was aspirated and the worms were deposited onto NGM low peptone agar plates with faecal slurry and *E. coli* HB101 as food source. The L1's were picked onto clean plates as they recovered.

2.4.10 Cryopreservation of *C. elegans*

Starved *C. elegans* L1 stage larva were washed from culture plates with phosphate buffered saline (PBS) and washed 3 times with 2 mL of PBS, all spins are at 200 xg for 3 minutes and the supernatant aspirated off, leaving 1 mL. 1 mL of *C. elegans* worm freezing solution (Appendix 2) was added and aliquots of 500uL were slowly cooled to -80°C at a rate of 1°C/min in a "Mr. Frosty" [Nalgene].

When removing strains from cryopreservation, vials were thawed as quickly as possible under warm running tap water and placed onto a NGM plate with OP50 *E. coli* as food source.

2.4.11 SDS resistance of *P. trichosuri*

Free-living cultures consisting of either adult larval stage or cultures of infective larvae were washed from *in vitro* culturing plates, settled by gravitation and washed with PBS, the washed larvae were re-suspended to ~2000 larvae per mL in liquid low peptone NGM. An equal volume of 2% SDS (w/vol) (Sodium deodecyl sulfate) solution was added to achieve a final concentration of 1% SDS (w/vol). Aliquots consisting of

approximately 100 larvae were taken at timed intervals and assessed for motility and vitality. Worms were scored as dead when they no longer responded to touch.

2.4.12 Paraquat resistance of *P. trichosuri*

Paraquat (methyl viologen dichloride hydrate or N,N'-Dimethyl-4,4'-bipyridinium dichloride [Sigma Cat# M2254] was dissolved in liquid 1/10 NGM to various concentrations and aliquots of 100 μL per well were dispensed into 96 well plate in triplicate. Approximately 40 *P. trichosuri* of either adult stage or infective were added to each well. Worms were assayed for motility after 20 hours incubation at 20^{o}C. Those that showed movement were scored as alive (JEE *et al.* 2005).

2.4.13 Fatty Acid stain (Sudan Black) of *P. trichosuri*

Fresh culture of *P. trichosuri* infective larvae or free-living adults from well fed plates were washed with liquid low peptone NGM, and soaked for 30 minutes to empty gut contents. Worms were then fixed in 1% paraformaldehyde and subjected to 3 freeze thaws to disrupt sheaths and incubated on ice. This was followed by washes in 1X volume of 20% ethanol, 50% ethanol and 70% ethanol. Worms were stained overnight in a saturated solution of Sudan Black dissolved in 70% ethanol that had been filtered through a 0.2 μm filter. Sudan Black preferentially stains lipid deposits. The next day worms were subjected to a series of washes: 70%, 50% and 25% ethanol followed by two washes in PBS and then visualized on an Olympus BX-UCB microscope.

2.5 MOLECULAR BIOLOGY

2.5.1 IBSC approval for all genetic modifications was obtained:

GMO03/ARW027 (ERMA Approval code: GMD002615) and GMO05/ARW033 (ERMA Approval code: GMD004193).

2.5.2 Single worm lysis for PCR template

Single worms were picked into 8 well PCR strips and 25μL of lysis solution (Appendix 2) was added and strips incubated for 14 hours at 55^{o}C followed by 1 hour at 85^{o}C to inactivate the enzyme, the reaction was then allowed to cool to 4^{o}C for 1 minute. For

PCR this worm lysis mixture was diluted with sterile distilled water (1:10) and 1 μL used in a standard 20μL PCR reaction.

2.5.3 Isolation of genomic DNA from *P. trichosuri* or *C. elegans* worms

For isolation of genomic DNA from *P. trichosuri,* cultures of infective larvae were used and for *C. elegans,* liquid cultures of mixed population of worms were used. All manipulations were performed with wide bore pipettes to prevent shearing of DNA.

Worms were pelleted at 300xg for 3 minutes and the supernatant aspirated. The worm pellet was then ground under liquid nitrogen and 9mL of TE buffer (pH7.4), 0.6mL of 0.5M EDTA (pH8.0), 0.073mL of Proteinase K (16.4mg/mL) and 0.6mL of 10% SDS was added. This mixture was incubated at 50^{o}C overnight and then 2.2mL of 5M NaCl, and 1.8mL of a 10% (w/vol) CTAB/NaCl was added and incubated at 65^{o}C for 15 minutes. An equal volume of chloroform:isoamyl alcohol (24:1) was used to extract at room temperature for 1 hour. The solution was then centrifuged at 3000xg (6000rpm in a Sorval RC5B using an SS35 rotor) for 15 minutes and the aqueous phase removed. An equal volume of phenol: chloroform: is amyl alcohol (25:24:1) was used to extract at RT for 1 hour. The mixture was centrifuged as described above, the aqueous phase removed and an equal volume of chloroform:isoamyl alcohol (24:1) was used to extract for 1 hour at room temperature. The mixture was then centrifuged as described above and the aqueous phase removed. 2 volumes of ice cold ethanol was added and gently mixed. The DNA was centrifuged as described above, the supernatant removed and the pellet washed with 12 mL of 70% ethanol and then centrifuged as described above. The supernatant was aspirated and re-suspended in a small amount of 70% ethanol, transferred to an eppendorf tube and then centrifuged at 7500xg for 10 minutes. The supernatant was removed and the pellet air dried. The pellet was then re-suspended in 450μL of TE buffer (pH7.4) for 3-4 hours at 4^{o}C. An aliquot was run on an agarose gel to check quality and absorbance checked at A_{260}/A_{280}.

2.5.4 Isolation of total RNA from *P. trichosuri*

P. trichosuri at various developmental stages were collected from liquid culture and pelleted at 300xg for 3 minutes. The pellets were ground under liquid Nitrogen and total RNA was extracted from using the Trizol^{TM} Method [Invitrogen Cat# 15596-018]

as per manufacture's instructions. The RNA pellet was re-suspended in 10-20μL of DNase/RNase free distilled water [GibcoBRL Cat# 10977-015].

2.5.5 DNase I treatment of q-PCR template

Total RNA template for quantitative PCR was DNase I treated as per manufacture's instructions [Invitrogen Cat# 10481-018] before reverse transcription.

2.5.6 Quantification of RNA and DNA with Nanodrop

Nucleic acid was quantified using on the NanoDrop DN-1000 [NanoDrop Technologies] which can quantify nucleic acid in concentration range from 0 to 3700ng/μL without dilution. Good quality DNA should have an A_{260}/A_{280} ratio greater than 1.8 and good quality RNA should have a ratio greater then 2.0, the quantification was determined using the equations:

[RNA] (ng/μL) = 40 x A_{260} x dilution factor

[DNA] (ng/μL) = 50x A_{260} x dilution factor

2.5.7 Reverse Transcription

DNaseI treated total RNA was reverse transcribed using Superscript First Strand synthesis system for RT-PCR as per manufacture's instructions [Invitrogen, Cat# 11904-108].

2.6 MOLECULAR BIOLOGY – PCR METHODS

2.6.1 Routine PCR

Routine PCR was performed using 300 nM of each primer (see Appendix 3), 25 μM dNTP, 1x PCR buffer, 3 mM $MgCl_2$ and 0.2 units/μL of either Platinum Taq [Invitrogen Cat # 10966-083] or Expand High Fidelity Polymerase [Roche Cat #1732641]. The thermocycler profile incorporated a single cycle of enzyme activation at 95^{o}C for 5 min, followed by 20-35 rounds of 95^{o}C denaturation, 55^{o}C annealing and 72^{o}C extension at 1min per kbp.

2.6.2 Degenerate PCR

Degenerate PCR was performed using 400 nM primers, 25 μM dNTP, 1x PCR buffer, 3 mM $MgCl_2$ and 0.2 units/μL of Platinum Taq [Invitrogen, Cat # 10966-083] Enzyme activation was at 95^{o}C for 5 min, followed by 25 rounds of 95^{o}C denaturation, 48^{o}C annealing and 72^{o}C extension at 1min per kbp.

2.6.3 Long Range PCR

Long PCR products (greater then 7kbp) were amplified using Invitrogen Elongase Amplification kit [Invitrogen, Cat# 10481-018] as per manufacture's instructions.

2.6.4 Multiple-site mutagenesis of *P. trichosuri daf-16* genes using a modified overlap extension PCR (M)OE-PCR

Two *P. trichosuri daf-16A* vectors and two *P. trichosuri daf-16B* vectors were constructed (Figure 2.1). All were N terminal tagged with an HA epitope for immunohistochemistry (Methods Section 2.12). The HA epitope tag is the highly immunreactive peptide sequence: YPYDVPDYA from haemagglutinin influenza. This was inserted into the constructs in order to determine both the expression patterns and localization of these transgenes. In one set of vector constructs, the AKT-1/2 serine and threonine phosphorylation (ST phos) sites of the DAF-16 isoforms were mutated using a modification of the (M)OE-PCR protocol of An *et al*. (AN *et al.* 2005). PCR primers were designed to span the phosphorylation site, these primers incorporated a base substitutions T→C. See Appendix 3: Primer Table.

The construction of the vector which contained the mutated AKT-1/2 phosphorylation sites of *Pt daf-16*A isoform (aka: pSSD16A2) will be described below. The construction of the mutated isoform *Pt daf-16*B (aka: pSSD16B2) was made using the same protocol but with the appropriate primers to amplify the 5' end of the transgene.

In the first step, five parallel PCR reactions were performed to amplify DNA fragments using primers which contained the T→C nucleotide substitutions which results in a mutated AKT phosphorylation site. These were amplified using the proof reading Taq polymerase: KOD polymerase [Novagen Cat# 71086-3] as per manufacture specifications. To amplify the promoter regions PCR was performed with genomic DNA. For all other fragments, *P. trichosuri* cDNA was used as template. These PCR

products were gel purified to ensure complete removal of the cDNA template and were re-amplified using 1unit of proof reading Taq polymerase, KOD. In the second step, equimolar aliquots ($5x10^{10}$ DNA strands) of every two adjacent DNA fragment were amplified for 17 cycles with 1 unit of KOD polymerase, dNTP and buffer as per manufacture's instructions, but without primers. In the third step, 20µL of every product from the second step were added together with and amplified for 20 cycles with 1 unit KOD polymerase, dNTP, and reaction buffer as per instructions, but without primers. To this reaction, the outer primers (40pmol of each) were added along with 1 unit of KOD polymerase, dNTP and buffer as per manufacture's instructions and amplified for 30 cycles (Fourth and final step).

The final product was gel purified and ligated into pSTBlue-1 blunt cloning vector [Novagen Cat#70191-3] (Appendix 1) as per manufacture's instructions and cloned into either NovaBlue ™ *E. coli* cells [Novagen]. In the case of the mutated *Ptdaf-16*A transgene, SURE *E. coli* cells [Stratagene] cells were used to prevent recombination.

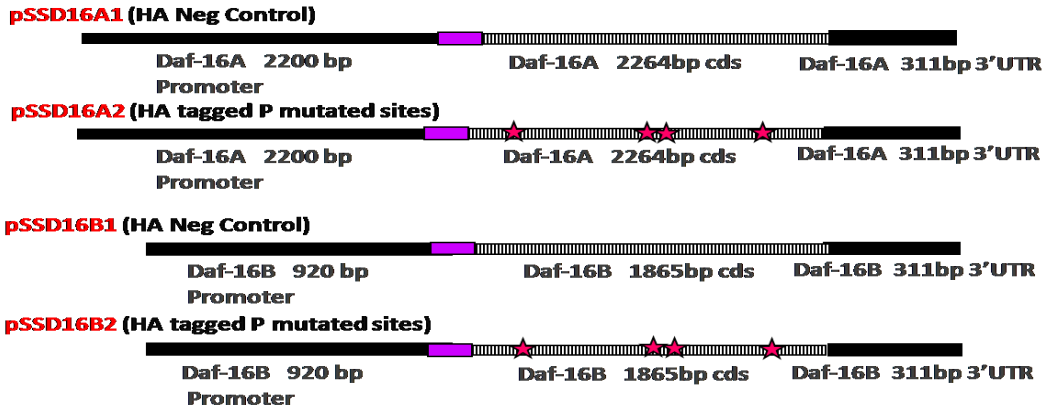


Figure 2.1: *P. trichosuri daf-16* constructs with mutated phosphorylation sites.
Solid black*: P. trichosuri* regulatory regions. Hashed section: coding region for *daf-16* isoforms. Red stars: mutated phosphorylation sites. Purple section: HA epitope protein tags for antibody detection by immunohistochemistry.

2.6.5 GenomeWalker™

To determine unknown genomic sequence up or down stream of a known sequence, the GenomeWalker™ Universal kit was used [Clontech. BD Biosciences, Cat # 638904]. Four GenomeWalker™ Libraries are first made by digesting *P. trichosuri* genomic DNA with blunt cutting restriction enzymes. The libraries created were: *EcoRV, DraI,*

PvuII and *StuI* libraries. *P. trichosuri* genomic DNA is digested to completion with each of the blunt cutting restriction enzymes in four separate reactions. Then a GenomeWalker^{TM} adaptor was ligated onto the ends of the digested DNA. This adaptor has binding sites for an Adaptor Primer 1 (AP1) and Nested Adaptor Primer 2 (AP2). Gene specific primers were designed to the *P. trichosuri* gene of interest. The gene specific primers must meet several criteria: they should be approximately 25-28 base pairs in length, have a GC content of 40-60% and an annealing temperature of approximately 67°C.

In separate reactions tubes, 1µL of each of the GenomeWalker™ Library template, the *P. trichosuri* gene specific primer and the AP1 primer were added along with Platinum Taq polymerase [Invitrogen Cat # 10966083] as per manufacture instructions. The two step Thermocycler program used was: 7 cycles: 94°C/25 seconds and 72°C/4 minutes followed by 32 cycles of: 94°C/25 seconds and 67°C/4 minutes followed by 67°C for an additional 4 minutes. A nested PCR reaction was performed using 1µL of the primary reaction in a 50µL secondary PCR reaction which incorporates a nested *P. trichosuri* gene specific primer, the GenomeWalker™ AP2 nested primer and Platinum Taq polymerase [Invitrogen Cat # 10966083] as per manufacture instructions and a two step Thermocycler program of: 5 cycles: 94°C/25 seconds and 72°C/4 minutes followed by 22 cycles: 94°C/25 seconds and 67°C/4 minutes followed by 67°C for an additional 4 minutes. Final products of greater then 500bp were gel purified, TA cloned into pGEMTeasy vector [Promega Cat# A1380] (Appendix 1) and sequenced.

2.6.6 5′ and 3′ RACE

In order to elucidate the coding region of a gene of interest the Roche kit for 5′ and 3′ RACE was utilized [Roche Cat # 03353621] as per the manufacture's instructions.

2.6.7 Quantitative Real Time PCR

To characterize the expression pattern of the putative IIS pathway genes, Quantitative Real Time PCR (q-PCR) was used to determine expression in different developmental stages. Three or two biological replicates of the following developmental stages chosen: free-living adults, parasitic adults, L1 stage, L1 which were exposed to conditioned medium for 36 hour, and therefore presumably initiated the developmental

process to becoming infective larva: L1+CM, iL3, infective larvae that have been collected after passing through a patch of possum skin into tissue culture medium: iL3+skin. To confirm that the L1+CM larvae were progressing along the developmental path to becoming infective larva, an aliquot of the culture was allowed to develop fully rather than being processed for total RNA and >90% developed to infective larva after 84 hours. The infective larva that were collected after *in vitro* skin penetration were presumably in the process of exiting from infective larva stage as judged by the presence of exsheathed skins in the media and infective larva that did not appear to have the presence of a sheath (pictures Appendix 4).

Template was generated by extracting total RNA, using TRIzol™ [Invitrogen] followed by DNAseI treatment as discussed in Methods Section 2.5.4 and Section 2.5.5 respectively. Concentration of total RNA was measure on Nanodrop and 20ng/μL of template was used in a reverse transcription reaction with Invitrogen Superscript RT as described in Methods Section 2.5.7. q-PCR was performed with *P. trichosuri gap3dh* primers on cDNA template and control template that was prepared identically but without reverse transcriptase (-RTase). The plotted C_T values are shown for some templates in Figure 2.2. The cDNA template used for q-PCR was checked for genomic contamination by PCR using primers to amplify a 760 bp section of genomic region of the *P. trichosuri* histone promoter (Figure 2.3 – top row). As a positive control, primers that amplify 500 bp of coding region of the *P. trichosuri tre1016e* gene were used (PCR was performed for 30 rounds of amplification) as shown in Figure 2.4 bottom row.

Quantitative real-time PCR was conducted using SYBER GREEN PCR Master Mix [AB Applied Biosystems Cat# 4309155] on a GeneAmp 5700 [PE Applied Biosystems], as per manufacturers instructions. 1μL of template, 12.5 μL of SYBER GREEN PCR Master Mix, 9.5 μL of PCR grade water and 10μM of each primer were used. Comparative C_T Method Relative for quantification of gene expression was used to analyse gene expression. A validation experiment was performed to determine that amplification efficiencies of the reference gene and gene of interest were approximately equal (Supplementary Material - CD). Results were analysed on a GeneAmp 5700 version1.6 software [PE Applied Biosystems]. The relative amount of the gene of interest was normalized to the endogenous reference gene: glyceraldehyde-3-phosphate

dehydrogenase (*gap3dh*) relative to a calibrator using the arithmetic formula: $2^{-\Delta\Delta C_T}$ (ABI User Bulletin #2, 1997) (Supplementary Material - CD).

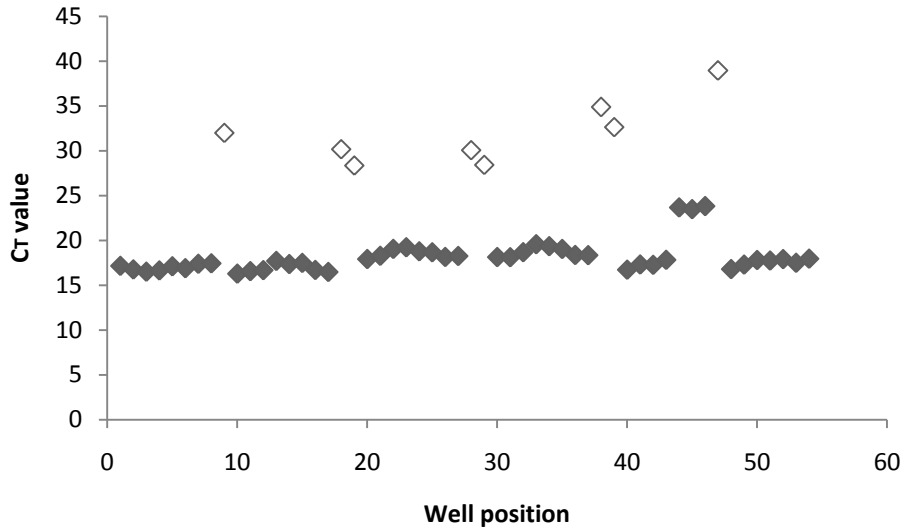


Figure 2.2: C_T values of Quantitative Real Time cDNA template with *gap3dh* primers.
q-PCR of templates with *P. trichosuri gap3dh* primers. Wells 1-9, free-living adult:Wells 10-19, L1; Wells 20-29, L1+CM; Well 30-39, iL3; Well 40-47, infective larva through skin; Well 48-53 parasitic adult. Solid diamonds are cDNA template and open diamonds are –RT control

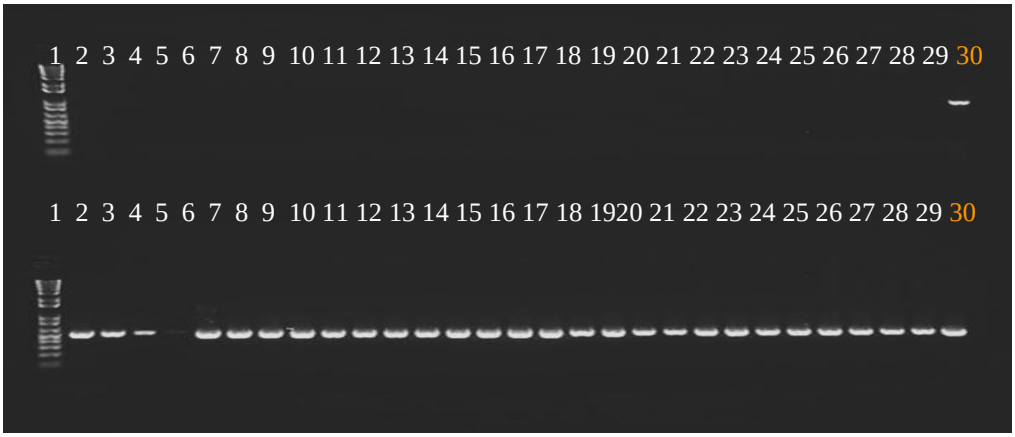


Figure 2.3: PCR of cDNA template from developmental stages of *P. trichosuri.*
Top row: genomic DNA *P. trichosuri* histone primers, expected size 760bp for genomic contamination Fwd primer: CACCCTTTACAGTCATTAATATATAATTTA Reverse primer: GTAACAAAATTAAA AACTCTGGTG . Bottom row: *P. trichosuri* cDNA *tre1016e* primers expect 500bp. Fwd primer: ATGCGCTTTAACATCTATTGCAC; Reverse Primer: GGAGGTTGTGATCTATTAAGAT 30 rounds of amplification Lanes 2-5, egg cDNA; Lanes 6-9 L1 cDNA; Lanes 10-13, L1+CM cDNA; Lanes 14-17, infective larva cDNA; Lanes 18-21, infective larva post skin penetration cDNA; Lanes 22-25, parasitic adult cDNA; Lane 26-29, free-living adult cDNA Lane 30 Positive control (*Pt-tre1016e* gene)

2.7 MOLECULAR BIOLOGY CLONING

2.7.1 Plasmid DNA purification

Plasmid purification was performed using the QIAprep Spin Miniprep kit [QIAgen, Cat# 27104] as per manufacture's instructions. Samples were eluted in 30µL of EB elution buffer and quantified on the NanoDrop™ as described above.

2.7.2 Gel purification

DNA bands were purified from agarose gels using the QIAquick Gel Extraction kit [QIAgen, Cat# 28704] as per manufacture's instructions. Samples were eluted in 30µL of EB elution buffer and quantified on the NanoDrop™ as described Section 2.5.6.

2.7.3 PCR purification

PCR products were column purified using the QIAquick PCR Purification kit [QIAgen, Cat# 28104] as per manufacture's instructions. Samples were eluted in 30 µL of EB elution buffer and quantified on the NanoDrop™ as described above.

2.7.4 Cloning of *P. trichosuri* genes and gene fragments.

Molecular biology procedures such as: PCR purification, restriction digest, alkaline phosphorylation of vectors, ligation and transformations were performed as per product specifications and according to general protocols in 'Molecular Cloning/ A Laboratory Manual' (Sambrook, Fritsch and Maniatis, Cold Spring Harbour Press (1989).

2.7.5 LiCl precipitation of plasmids prior to microinjection.

All manipulations performed on ice and centrifugations were at 4^{o}C.

To a plasmid prep (of <100µL) an equal volume of 4.28M LiCl was added and vortexed thoroughly. After which, the solution was incubated at -20^{o}C for 20 minutes and centrifuged at 14000 rpm for 5 minutes on a bench-top centrifuge. The supernatant was added to 1.5 mL of ice-cold 70% ethanol and vortexed thoroughly, followed by centrifugation at 14000 rpm for 20 minutes. The supernatant was discarded and pellet retained, air dried and then re-suspended in 20 µL distilled water, the concentration was measured on a Nanodrop™ as described and the concentration adjusted to 500 ng/µL with distilled water. Plasmids were stored at -20^{o}C prior to microinjection.

2.8 SEQUENCE ANALYSIS

2.8.1 DNA Sequencing

DNA sequencing was performed by the Allan Wilson Centre Genome Service, Massey University. DNA was sequenced by the dideoxynucleotide chain terminations method (SANGER *et al.* 1977) using BigDye™ Terminator Version 3.1 Ready Reaction Cycle Sequencing Kit on the capillary ABI3730 Genetic Analyzer, from Applied Biosystems Inc. Sequences and electropherograms were assembled using AB DNA Sequencing Analysis Software version 5.2. [Applied Biosystems Inc.]

2.8.2 Bioinformatic Analysis

Sequences were assembled and analysed using the Vector NTI package Version 10.1.1 (Invitrogen Corporation). Homologues in the GenBank database were searched using BLAST and FASTA programmes (ALTSCHUL *et al.* 1990) using NETSCAPE NAVIGATOR at the National Centre for Biotechnology Information, (NCBI) at website: (http://www.ncbi.nlm.nih.gov). Protein motifs were analysed using PROSITE (http://www.expasy.ch/prosite) and a local installation of InterProScan (ZDOBNOV and APWEILER 2001) was used to scan the translated sequences against the member databases of InterProScan. Phylogenetic guide tree was performed by Rogar Moraga-Martinez and was constructed using ProML from the PHYLIP (Phylogeny Inference Package) Version 3.6, which uses Maximum Likelihood to calculate to create evolutionary trees (FELSENSTEIN 2005).

2.9 STATISTICAL ANALYSIS

Statistical analysis for all of the conditioned medium bioassays was performed by AgResearch statisticians, Fred Potter and John Koolaard. The data from each dilution series was modelled with logistic regression where conditioned medium concentration was the explanatory variable and iL3 proportion was the response. This yields a simple linear model on the scale of the linear predictor (i.e. the logit (or logistic transformation) of the iL3 proportion is linearly related to conditioned medium concentration). The slope and intercept parameters from these straight-line regressions were separately

analysed to compare the five inbred lines using ANOVA. The dilution series to test the batches of conditioned medium was analysed by a GLM with complementary log-log link function LOGe(-LOGe(1-n/N)), where n is the number responding out of N examined; and binomial errors with the dilutions were transformed to logs. To determine whether different environmental conditions resulted in different proportions of infective larval development Parallel curve analysis using on-linear regression was used, and accumulated analysis of variance was used to assess the F probability score of the parameter tested.

To determine whether the rescue by complementation resulted in a greater proportion of mutants with wild type phenotype Logistic Regression was performed using a Generalized Linear Model (GLM) with the number responding out of a total, that is binomial data. The model was fitted using a logit link function (logit=LOGe(worms with wild type phenotype/total number – number of worms with wild type). Scaled deviances are calculated and approximate F-tests used to identify significant effects. The GenStat program was used (Payne, R.W., Harding, S.A., Murray, D.A., Soutar, D.M., Baird, D.B., Welham, S.J., Kane, A.F., Gilmour, A.R., Thompson, R., Webster, R., & Tunnicliffe Wilson, G. (2008). *GenStat Release 11.0 (PC/Windows)* VSN International, Hemel Hempstead)

The Student's t-test, using one-tailed analysis and assuming equal variance, was used to determine whether the level of gene expression of L1+CM was significantly less than the either the L1 stage or the iL3 stage for the qPCR analysis.

2.10 CREATION OF TRANSGENIC NEMATODES

2.10.1 Microinjection of *C. elegans*

Microinjections were performed by Kirsten Grant. *C. elegans* L4 stage larvae were picked onto fresh NGM agar plate with confluent lawn of *E. coli* OP50 and incubated at 16^{o}C overnight to ensure that they were at early adult stage the next day for microinjecting. Needles were pulled on Narashige PC10 needle puller using a two stage

temperature pull. Temperature of first stage of the pull was 96.7°C and temperature of the second stage of pull was: 94.5°C this was to ensure that needle point was fine enough to penetrate the nematode ovary. Needles were backfilled with the appropriate vector at concentrations ranging from 50ng/µL to 0.5ng/µL and checked to ensure that no bubbles blocked the needle. Glass coverslips were prepared prior to microinjection, onto which 2% agarose pads have been dried and covered with approximately 50µL of Voltalef oil 3S [VWR International, Cat# 24 626.185]. Early adult stage hermaphrodite worms were individually picked and laid onto the agarose pads to expose ovary. Microinjections were performed on an Olympus IX70 inverted microscope at an air pressure of 16-18psi. Injections were aimed at the distal arms of the nematode ovary (MELLO *et al.* 1991). After microinjection 4-5 worms were placed onto NGM agar plates with a confluent lawn of *E. coli* OP50 and incubated at 16°C. After 2 days F_1 generation worms were inspected for visual marker such as green fluorescent protein (gfp) and cloned onto fresh plates until stable lines were established.

With microinjection of *C. elegans*, the transgene is incorporated as an extra-chromosomal array, and therefore loss of the transgene can occur. Thus, the lines were examined weekly, and offspring that carried the visual marker were cloned onto fresh NGM plates.

2.10.2 Microinjection of *P. trichosuri*

Microinjection of *P. trichosuri* was performed as per protocol for microinjection of *C. elegans* described above with the following modifications: the coverslips onto which the worms were laid for microinjection were made from pads of 1.5% agarose that were dried down in a 55°C oven for 1 day. After microinjections 3-5 microinjected females and 5-7 males were placed on low peptone NGM agar plates with 10µL pool of *E. coli* HB101 and faecal extract slurry. Lines of transgenic worms were not established, the F_1 generation was screened for the presence of the transgene by PCR on single worms (Section 2.5.2) and by immunohistochemistry (Section 2.12).

2.11 RNA INTERFERENCE (RNAi)

2.11.1 RNAi of *P. trichosuri* by feeding assay

Inverted repeat gene constructs were made using the FireLab RNAi inducible expression vector pλ4440 (Appendix 1.14) or into pGWN2T7 (Appendix 1.15) and transformed into HT115 *E. coli*.

An overnight culture of the dsRNAi clone of interest was grown overnight at 37^{o}C (shaking) in LB/Amp (200μg/mL). The next day a fresh culture of LB/Amp (200μg/mL) for pL4440 or NGM low peptone/Neomycin (50μg/mL) was seeded with 1:50 volume of the overnight culture and transcription of the dsRNA induced with IPTG at a final concentration of 1mM for 4 hours at 37^{o}C shaking. The induced culture was pelleted and suspended to 10% w/vol in low peptone NGM and ~50μL of suspension was pooled into the centre of a low peptone NGM agar plate (with IPTG to 1.5mM) and approximately 20-30 L1 *P. trichosuri* were added. Observations were taken at 24 hours and 48 hours.

2.11.2 Induction of dsRNA

To assess whether induction of dsRNA transcription had occurred a 1mL aliquot of induced culture (and un-induced culture or vector control) was taken and centrifuged at 15000xg for 1 minute. The pellet was suspended in 750μL of solubilization buffer (Appendix 2) and sonicated [Sonics Vibracell VCX750] for 3 bursts of 20 seconds at setting amplitude 40%, (cooled on ice between bursts) and then centrifuged at 12000rpm for 5 minutes and the pellet suspended in 100μL of TE buffer (pH7.5) (Appendix 2). 20 μL was run on a 1% agarose gel and checked for a band of the appropriate size in the induced lane which is not present in the un-induced lane or vector control lane.

2.11.3 *In vitro* transcription

In vitro transcribed *P.t. hsp-70* (637 bp gene fragment) RNA for dsRNA Interference in *P. trichosuri* was produced using the mMESSAGE T7 kit [Ambion Cat#AM1345] and purified with the MEGAclear^{TM} kit [Ambion Cat#1908].

2.11.4 Silencer siRNA purification

To produce diced ~21mers of *in vitro* transcribed RNA in order to test siRNA silencing, the Silencer® siRNA Construction Kit [Ambion Cat# AM1620)] was used as per manufacture's instructions.

2.11.5 RNAi of *P. trichosuri* by electroporation

For electroporation, approximately 200 L1 larvae were suspended in 200 μL trehalose electroporation buffer (Appendix 2) containing 0.5mg/mL of double stranded RNA (dsRNA) then electroporated at 100 V by a single pulse in 0.2 cm cuvettes [BioRad GenePulser II]. The larvae were recovered by allowing sedimentation under gravity on ice then cultured on low peptone NGM agar plates with *E. coli* HB101 bacteria as a food source. Control worms were electroporated in trehalose buffer without RNA and then cultured in the same way.

2.11.6. RNAi of *C. elegans* and *P. trichosuri* by microinjection

Gene fragments were *in vitro* transcribed using mMESSAGE T7 kit [Ambion Cat#AM1345] and purified with the MEGAclear^{TM} kit [Ambion Cat#1908]. These purified *in vitro* transcribed fragments were then microinjected into the gonad of either *C. elegans* or *P. trichosuri* as described (Methods 2.10.1 and 2.10.2). Microinjected *C. elegans* or *P. trichosuri* were then plated at 1 worm per plate (the *P. trichosuri* microinjected females were plated with 5 adult males). The next day the surviving microinjected females were transferred to new plates, the progeny from the injection plates were counted and scored for live larva or unhatched eggs after 24 hours. Progeny from the transfer plates were counted and scored at 24 hours as either live larva or unhatched eggs (any hatched larvae and the original microinjected adult were removed), then at either 36 hours or at 48 hours eggs and the remaining larvae were counted and scored again.

2.12 IMMUNOHISTOCHEMISTRY

All manipulations were performed in Eppendorf tubes at 4^{o}C and supernatant was removed using a pipette under a stereo-microscope in order to avoid loss of worms.

Worms were fixed overnight at 4°C in 1 mL of 3.7% paraformaldehyde/PBS and then washed 3 times for 15 minutes with 1 mL of 4°C PBS. Worms were then incubated at 37°C overnight in 1mL 5% β-mercaptoethanol in BMB Buffer (Appendix 2) and washed as described above. Worms were permeabolized by adding 500µL collagenase mix (Appendix 2) and incubated at 37°C for 6 hours followed by 3 washes with cold PBS as described above. The anti-HA fluorescein tagged mouse monoclonal antibody [Roche, Cat # 11666878001] was diluted 1:100 in antibody diluent (Appendix2) and incubated at 4°C for 18 hours. Worms were then washed 3 times for 60 minutes each with 1mL of PBS and a final wash with 1mL of PBS/0.1% TritonX was performed overnight. Worms were visualized on an Olympus BX-UCB microscope at an excitation wavelength of 470-495nm and an emission wavelength of 510-550nm.

2.13 CHEMICAL MUTAGENESIS OF *P. trichosuri*

2.13.1 Chemical mutagenesis of *P. trichosuri* with EMS

All manipulations with ethyl methanesulfonate- EMS (CAS# 62-50-0) were done in a fume hood with appropriate protective gear and sterile technique used. Disposal of any liquid wastes was onto NaOH pellets and leaving the resulting slurry overnight, all equipment wiped with weak NaOH. Matt Crook performed the EMS mutagenesis of *P. trichosuri*. Tina Englbrecht and Marleen Richter performed the PCR screening for chemical mutagenesis.

Faecal egg extraction from *P. trichosuri* was performed as described previously. Approximately 10,000 L1s were plated onto 9cm NGM low peptone plates with a confluent lawn of *E. coli* HB101 and faecal extract and incubated until the late L4 larval stage or very early adult. Note: at 25°C this is approximately 30 hours, at 20°C this is approximately 40 hours. The L4 or early adults were washed off the plates with PBS and centrifuged at 300xg for 2 minutes. Larvae were suspend in 1 mL PBS and treated with 20mM EMS in PBS (note: negative control used PBS only) and incubated at room temperature for 3 hours (with rocking) in parafilmed 14mL container inside a 50mM parafilmed Falcon tube (double container). Mutagenized worms were then spun at 300xg for 2 minutes. Worms were washed 4 times with 9mL of PBS, then spread onto

9cm low peptone NGM plates with confluent lawns of *E. coli* HB101 and faecal extract and incubated at 25°C overnight.

The next day, after examining the plates for eggs, the mutagenized P_o adults were gently washed off the plates with 2 washes of 5mL PBS and then replated onto fresh low peptone NGM plates with a confluent lawn of *E. coli* HB101 and incubated at 25°C for a further 4 hours in order for more eggs to be laid. The P_o adults were then washed off as described above and discarded. Once all eggs had hatched the F_1 generation L1 larvae were washed, pooled and then spun at 300xg for 2 minutes to pellet. The F_1 generation L1 larvae were then plated in 96well plates containing 100µL NGM agar with *E. coli* HB101 lawn at 100 L1 larvae per well in 7µL PBS and incubated at 20°C for 3 to 4 days until this F_1 generation had laid eggs.

The F_1 generation adults were then gently washed from the 96 well culture plate, leaving the F_2 eggs sticking to the bacterial lawn, and deposited into the corresponding wells of a 96well PCR plate. Plates were spun and supernatant removed and the worms were then lysed as described previously (Section 2.5.2). 5µL of lysate from each well was pooled by both row and column to be used as template in order to screen for deletion mutants. Meanwhile, the 96well culture plate containing F_2 eggs were incubated at a lowered temperature of 12ºC in order to prolong life until screening was completed and confirmed.

2.13.2 Poison Primer screening for deletion mutants

In order to screen for a single deletion mutant gene within a population of several hundred wild-type copies Poison Primer Screening was used (EDGLEY *et al.* 2002). This is a nested PCR reaction in which the first round of PCR includes a third PCR primer (poison primer) that falls between the two external primers (see Figure 2.4). Amplification from the wild-type template leads to the production of two fragments: one full-length and one relatively short. The shorter fragment is amplified more efficiently than the longer. Amplification from a mutant template, in which the site for the third internal primer is deleted, leads to the production of a single mutant fragment from the normal external primers. In the second round of PCR, two internal primers are used. The shorter wild-type band from the first round cannot serve as a template for the second round PCR because it does not include the internal forward primer site. The

longer wild-type fragment can serve as a template, but there is less because its production was limited by competition in the first round. The lower level of effective wild-type gives the deletion fragment an advantage and thus increases the sensitivity with which a deletion can be detected in the F_1 lysate pools (EDGLEY *et al.* 2002).

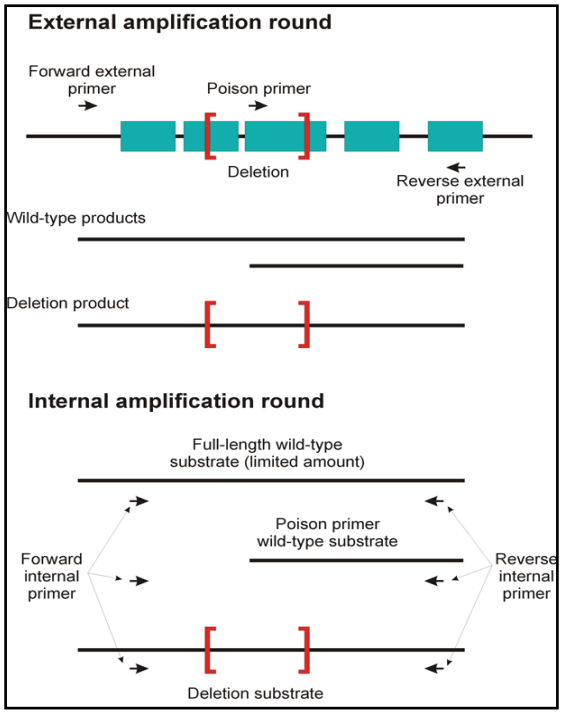


Figure 2.4: Poison Primer strategy (Edgley et al, 2002).
Two round PCR reaction, in the first round, full length wild-type, short wild-type and the deletion product is amplified, because the short full length out competes the full length product, fewer full length products are amplified. In the second round, nested primers are used, the poison product is not able to be amplified, and the ratio of deletion product to full length is slightly higher resulting in better amplification of the rare deletion product.

2.14 RECOVERY OF PHENOTYPE BY COMPLEMENTATION

2.14.1 Stress response

In order to determine if there is a recovery of wild-type stress phenotype by complementation with the *P. trichosuri* orthologue transgene, the transgenic worm's response to the oxidative stressor, Paraquat (methyl viologen dichloride hydrate or N,N'-Dimethyl-4,4'-bipyridinium dichloride) [Sigma Cat#M2254] was compared to mutant and wild-type (N2) controls. Some IIS mutant alleles of *C. elegans* display either a stress sensitive phenotype, such as the Daf-16 (m26) mutant which is sensitive

to the oxidative stressor: paraquat. The stress resistant mutant Daf-2 (e1370) has a stress resistant phenotype, and is able to survive longer in paraquat.

Synchronized *C. elegans* eggs were obtained by plating transgenic, fecund adults onto NGM agar plate for 2-6 hours and allowing them to lay eggs. Adults were removed and progeny grown until the L4 or early adult stage. Worms were washed off and re-plated, in triplicate, at 50 worms per well in a 96 well plate in 100µL of liquid low peptone NGM at either 0mM or 100mM Paraquat and incubated at 20°C for 20 hours. After 20 hours only transgenic worms (expressing gfp) were assessed for movement, those that failed to respond to touch were scored as dead.

2.14.2 Dauer larvae formation

Some IIS mutant alleles of *C. elegans* display either dauer larvae constitutive (Daf-c) or dauer larvae deficient (Daf-d) phenotype. In order to determine if there is a recovery of wild-type phenotype by complementation with the *P. trichosuri* orthologue transgene, the transgenic worm's response to semi-extracted *C. elegans* dauer pheromone (CeCM) (See Methods Section 2.3.2) this pheromone is able to induce dauer larvae formation in a wild-type worm or to a greater extent in some Daf-c mutants.

Synchronized *C. elegans* eggs were obtained by plating transgenic, fecund adults onto NGM agar plate for 2-6 hours and allowing them to lay eggs, adults were removed and eggs were washed from the plate. In triplicate wells, eggs at 1 egg/µL were plated into 75µL of liquid NGM plus Gentamicin at 50µg/mL and 0.35% (w/vol) HB101 *E. coli* as a food source with either 10% (vol/vol) CeCM or 0% (vol/vol) CeCM. After 5 days incubation at 20°C worms were assessed for dauer larvae phenotype: radial constriction of body wall and resistance to 1% SDS.

2.14.3 Life span

Synchronized *C. elegans* eggs were obtained by plating transgenic fecund adults onto NGM agar plates with HB101 *E. coli* as food source for 2-6 hours and allowing them to lay eggs, adults were removed and eggs incubated until they reached the L4/early adult stage. Worms were then individually plated into 96 well plates with agar plugs with *E. coli* HB101 as a food source and Gentamicin sulfate at 50µg/mL to prevent contamination and prevent overgrowth of *E. coli* HB101 food source. 5-fluoro-2′-

deoxyuridine FudR**;** [Sigma Chemicals Cat #0503] at 50 μM was added to the agar to prevent progeny production. Worms were assessed daily thereafter and worms that failed to respond to touch were scored as dead.

2.15 *P. trichosuri* infective larvae induction with LY294002

2-(4-Mopholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride: LY294002 [Sigma Cat #L9908] is a Phosphatidylinositol 3′ kinase inhibitor (such as the AGE-1 protein) and has been shown to induce dauer larvae formation in *C. elegans* (BABAR et al. 1999).

The effect of LY294002 in infective larva development in *P. trichosuri* was tested by incubating *P. trichosuri* in triplicate in a serial dilution of LY294002. LY294002 was dissolved in DMSO to a concentration of 100mM for a stock solution. Varying amounts (from 0mM to 0.05mM) was added to NGM low peptone with *P. trichosuri* L1 larva at 1 L1/μL and 0.35% HB101 and Gentamicin sulfate at 50μg/mL. The final concentration of DMSO did not exceed 0.4% (vol/vol). Worms were incubated at 20°C for 5 days and then scored for development to infective larval or free-living adult.

CHAPTER 3

BIOLOGY OF *Parastrongyloides trichosuri*

3.1 INTRODUCTION

The aim of this study was to characterize the free-living adult stage and the infective larval stage of *P. trichosuri* and, in addition, to define some of the environmental signals which influence the switch in development which occurs at the first larval stage and determines whether the L1 larvae enters the parasitic or free-living life cycle.

Previous studies have elucidated the anatomy of *P. trichosuri*. Briefly, the free-living phase consists of males and females, both of which go through four larval moults before reaching reproductive adulthood. The free-living adult male is approximately 0.7 mm long, the oesophagus is rhabditiform and the spicules are equal curved rods. The free-living adult female is approximately 0.8 mm long, the oesophagus is rhabditiform and the ovaries are didelphic. The infective larvae average approximately 0.57 mm in length. As the infective larva develops, a sheath forms and the oesophagus remodels and elongates to become one third to one half of the entire body length (MACKERRAS 1959). The infective larva enters its host by skin penetration and makes its way to the digestive tract, most likely via the lungs (CROOK *et al.* 2005). In contrast to the genus *Strongyloides,* the parasitic stage consists of both male and females, which are found in the upper part of the ileum of their host, the brushtail possum (*T. vulpecula*). The parasitic male is approximately 3.5mm long and the parasitic female is approximately 4.8mm long (MACKERRAS 1959). The basic biology has been described elsewhere (COWAN *et al.* 2006; GRANT *et al.* 2006b; STANKIEWICZ 1996).

In this study, I expand on the information which currently exists on the basic biology of *P. trichosuri* and examine the life history traits of the free-living morph such as: life span; brood size and the ratio of males to females. Of particular interest is an examination of the dauer hypothesis which suggests that the dauer larvae stage of *C. elegans* is analogous to the infective larval stage of parasites. In order to determine whether the infective larval stage of *P. trichosuri* share some of the defining characteristics of the *C. elegans* dauer larvae stage, I have examined some of the biology of the infective larva such as the elongation of the pharynx during development to the infective larva morph, resistance of the infective larva to environmental and chemical stress and storage of lipids in the infective larva. See Figure 3.1.

In addition to the biology of the *P. trichosuri* infective larva as it compares to the dauer larva, I have attempted to elucidate some of factors which control entry to the infective larval state, in order to determine if they are analogous to the factors which control entry to the dauer larvae state. The effects of population density, temperature, food availability and genetics were examined to determine what roles these environmental signals play in the nematode's developmental choice between entering either a parasitic or free-living life cycle.

Figure 3.1: *Parastrongyloides trichosuri* infective larva.
P. trichosuri infective larva, scale bars 100μm.

3.2 RESULTS: BIOLOGICAL CHARACTERISTICS OF *P. trichosuri* FREE-LIVING ADULTS.

3.2.1 Brood size and egg laying

In order to determine the brood size and the laying pattern of free-living *P. trichosuri*, assays were set up as described in the Methods (Section 2.4.5). Only wells in which the female body and at least two males were found to have died of apparent senescence were scored for this experiment. The average brood size for experiment #1 was 43 eggs per female (st dev 30.7, n=23) and the average brood size determined for experiment #2 was 67 eggs per female (stdev 41.6, n=32). Data from experiment #2 is shown. The egg laying pattern indicates there is a peak in egg production at the second day of egg laying (day 3 after egg stage) followed by a steady decline in fecundity. See Figures 3.2a & 3.2b.

3.2.2 Sex Ratio

P. trichosuri reproduces sexually. In order to determine the sex ratio of free-living *P. trichosuri* offspring, a sex ratio assay was set up as described in the Methods (Section 2.4.6). The sex ratio of male to female off spring of *P. trichosuri* free-living nematodes was determined, (error bars are stdev 0.849, n=32). See Figure 3.2c.

3.2.3 Life span of free-living *P. trichosuri*

The assay to determine the average life span of free-living *P. trichosuri* was performed as described in the Methods (Section 2.4.7). The mean life span of both male and female free living adult *P. trichosuri* was determined to be 7 days. The mean life span of was determined to be the lifespan when 50% of the population was alive, n=87. The lifespan of free living male and female *P. trichosuri* were analysed separately to determine lifespan. Statistical analysis was performed by probit analysis with the dispersion parameter fixed at 1 because of underspersion in order to estimate the average lifespan – i.e. point at which 50% of the population was alive, (with 95% confidence intervals). The tail end of the experiment, when 5% of the population remained alive (with 95% confidence intervals) was also examined. The confidence intervals at the average lifespan of males and females did not overlap, suggesting that

the males of this species have a slightly longer lifespan of approximately 7.7 days, compared to the females at 6.7 days. See Figure 3.2d.

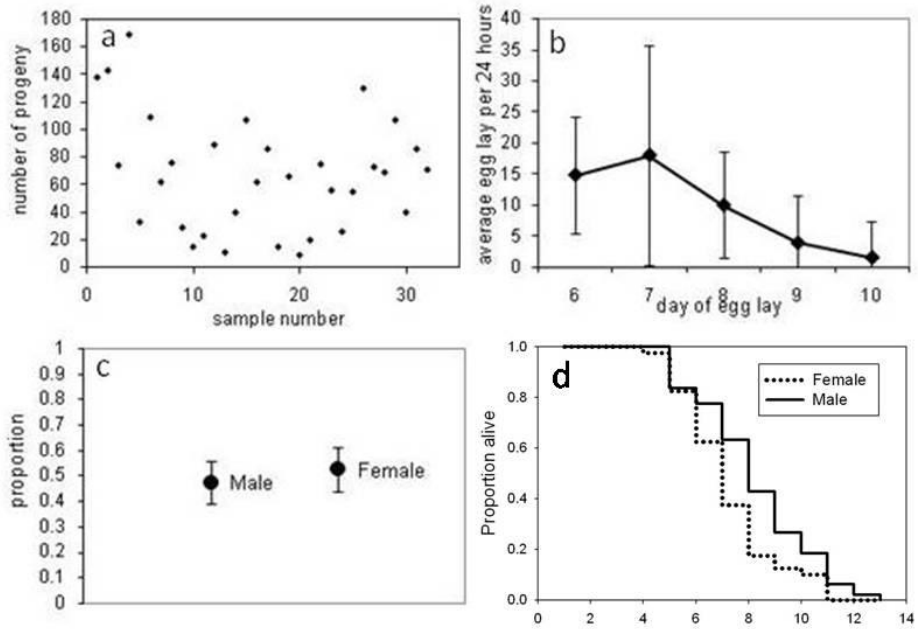


Figure 3.2: Brood size and Egg Lay pattern of free-living *P. trichosuri*.
(a) Brood size: one female and five males per plate were assessed daily and adult worms shifted to a fresh plate; the progeny were counted once they reached sexual maturity. Experiment #2, average brood size= 67 (stdev 41.6, n=32). (b) Egg Lay pattern: set up method described above, the average number of offspring measured per day (n=26) fecund free-living female *P. trichosuri.* (c) Sex ratio: set up method described above (stdev 0.849, n=32 females). (d) Life span: free living *P. trichosuri* on NGM low peptone agar plates

3.3 RESULTS: BIOLOGICAL CHARACTERISTICS OF *P. trichosuri* iL3

3.3.1 Resistance to 1% SDS

In order to assess whether the infective larval stage of *P. trichosuri* shares some of the defining characteristics of the *C. elegans* dauer larvae stage, *P. trichosuri* and *C. elegans* adults along with the infective larval stage of *P. trichosuri* and the dauer larvae stage of *C. elegans*, were incubated in 1% SDS as described in the Methods (Section 2.4.11). As shown in Table 3.1: *P. trichosuri* adults are sensitive to 1% SDS and survive less than 15 minutes whereas the infective larvae are resistant to incubation in 1% SDS and survive over 1 hour. *C. elegans* shows a similar trend: dauer larvae are

more resistant to incubation in 1% SDS than adults and are able to survive incubation for around 30 minutes.

Table 3.1: Resistance to chemical stresses: *C. elegans* dauer larvae and *P. trichosuri* iL3 larvae are resistance to 1% SDS

Stage	5 minutes	15 minutes	30 minutes	45 minutes	60 minutes
C. elegans dauer larvae	+++	+++	++	+	-
C. elegans adults	+++	+	-	-	-
P. trichosuri iL3	+++	+++	+++	+++	++
P. trichosuri free-living adults	+	-	-	-	-

Key
+++ healthy worms, normal sinusoidal movement
++ stressed worms, twitching movement
+ Intermittent trembling, many worms not moving
- Dead, completely unresponsive to gentle touch

3.3.2 Resistance to paraquat

In order to determine if *P. trichosuri* infective larvae are more resistant to chemical stress than the adult stage, *P. trichosuri* iL3 and free-living adults were exposed to various concentrations of paraquat for 20 hours at 20^{o}C as described in Section 2.4.12. Paraquat is a reagent which undergoes redox cycling *in vivo* to generate a superoxide radical ion and is used to test oxidative stress. Under these incubation conditions, the LC_{50} of *P. trichosuri* infective larvae is 170mM paraquat, whereas for the free-living adult stage the LC_{50} is 1mM. *C. elegans* shows a similar trend, with dauer larvae stage being more resistant to paraquat than the adult stage (Figure 3.3).

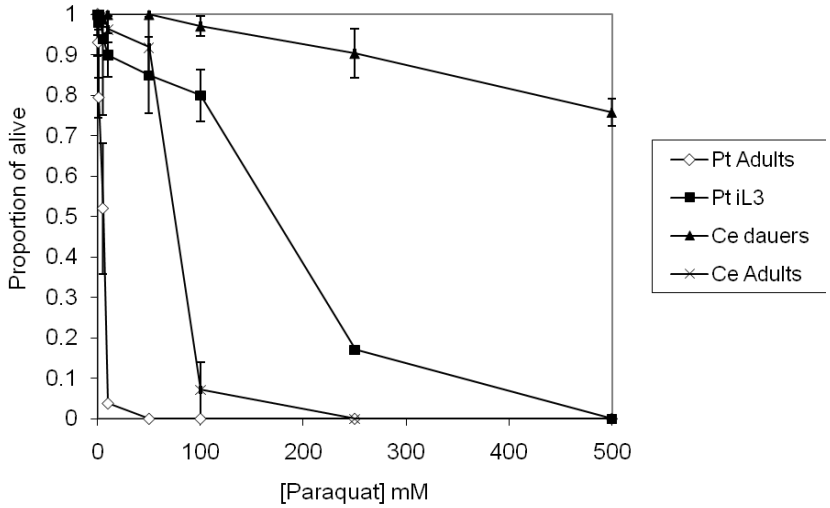


Figure 3.3: Resistance to chemical stress of *P. trichosuri* infective larvae and free-living adults. Paraquat was dissolved in liquid NGM (low peptone) to various concentrations, approximately 20 *P. trichosuri* iL3 or free-living adults were added to each well. After 20 hours of incubation at 20^{o}C worms were assessed for motility, and those that showed movement were scored as alive. Error bars are standard deveation

3.3.3 Resistance to heat

P. trichosuri infective larvae are able to withstand elevated incubation temperatures of 42°C for longer periods of time than the free-living adult stage. The LD_{50} of *P. trichosuri* adult stage when incubated at 42°C is 2 hours whilst the LD_{50} of infective larva stage is 23 hours (Figure 3.4).

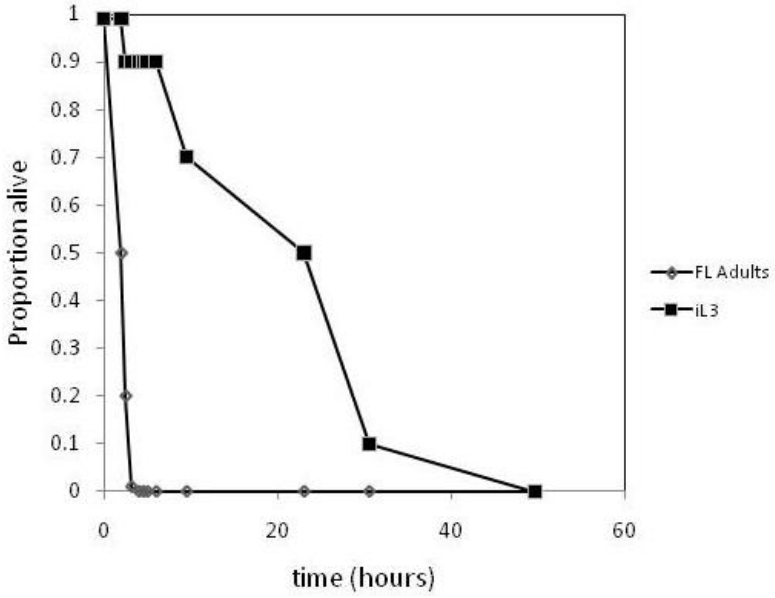


Figure 3.4: Stress response of *P. trichosuri* infective larvae and free-living adult stages to incubation (at 42°C).
Several thousand *P. trichosuri* free-living adults or infective larvae of were plated onto NGN (low peptone) agar plates with *E. coli* HB101 and faecal extract pellet. The plates were incubated at 42°C plates and examined at specified times and aliquots of approximately 20 worms were removed and observed for movement. If worms did not respond to touch they were scored as dead.

3.3.4 Lipid staining

In order to determine if the *P. trichosuri* infective larval stage has increased stores of lipids compared to the adult stage, *P. trichosuri* free-living adults and infective larvae were stained with Sudan Black, which preferentially stains lipids as described in Section 2.4.13. Figure 3.5 shows the increase in both size and number of Sudan Black stained globules, which are visible as dark blue deposits.

A.

B.

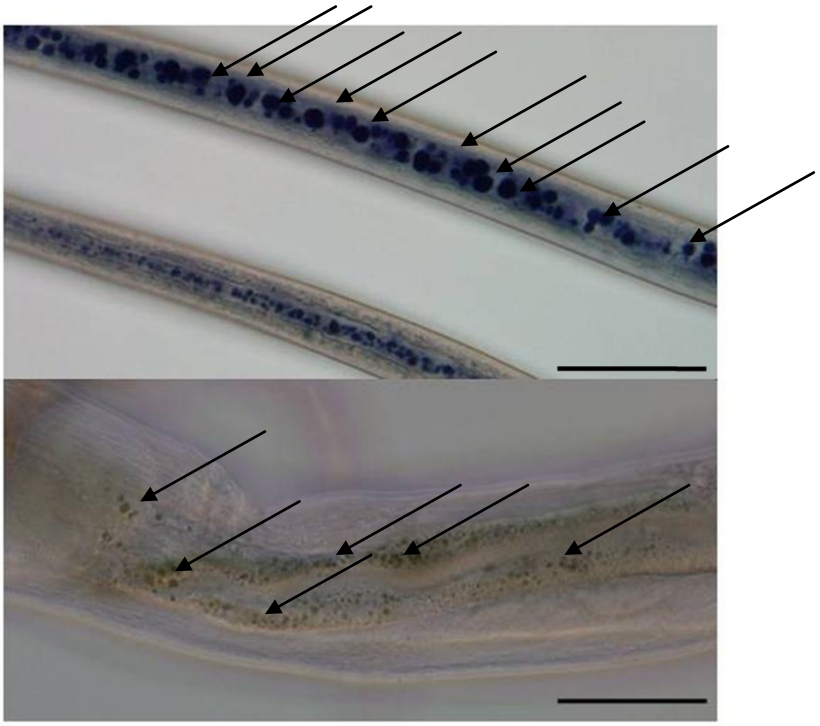


Figure 3.5: Sudan Black staining of *P. trichosuri* iL3 and free-living adults.
Worms were washed from well fed plates with liquid NGM (low peptone). Worms were then fixed in 1% paraformaldehyde, freeze thawed and washed in ethanol. Worms were stained with Sudan Black which preferentially stains lipid deposits. The next day, after destaining in ethanol washes, worms were visualized on an Olympus BX-UCB. Both images are through central midline of nematode Scale bar 50μm. Arrow point to some

A. *P. trichosuri* infective larvae Note two iL3 in this panel, the image is across the central midline
B. *P. trichosuri* free-living adult – central midline (Note the vulva opening)

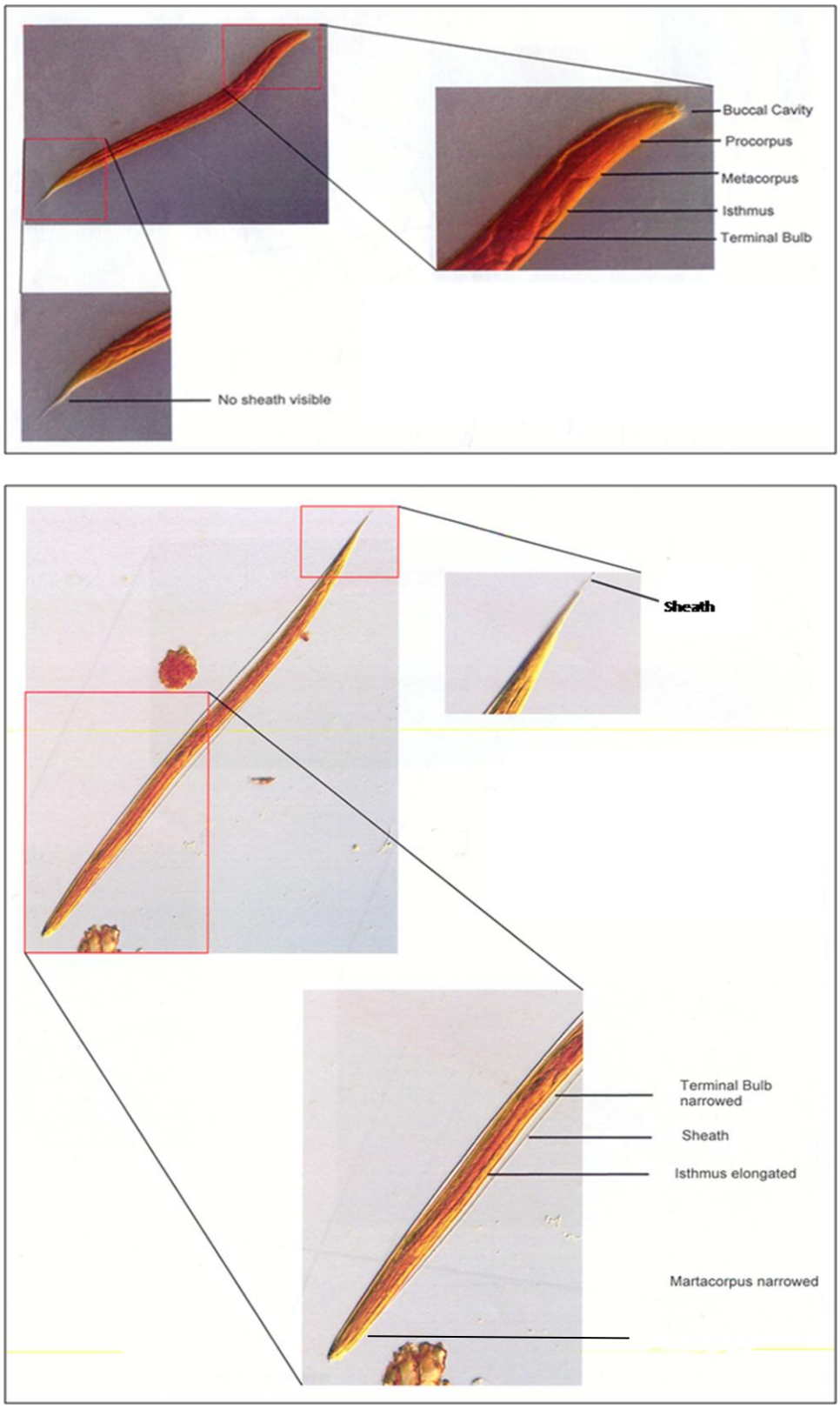


Figure 3.6: Pharynx remodelling of *P. trichosuri* during iL3 development
Top panel: *P. trichosuri* exposed to conditioned medium (CM) for 18 hours. Buccal cavity open and no sheath. Bottom panel: *P. trichosuri* exposed to conditioned medium (CM) for 84 hours during which, the pharynx becomes elongated and a sheath forms. Worms were stained with iodine and visualized with Digital Interference Contrast (DIC) Nomarski on an Olympus BX-UCB microscope.

3.3.5 Pharynx remodelling and sheath formation during development to infective larvae

During *P. trichosuri* development to the infective larval stage, the pharynx develops from a rhabdiform pharynx with distinct sections (top panel of Figure 3.6) to a filariform oesophagus, the features of which are that the isthmus becomes elongated and the metacarpus and terminal bulb become narrowed and indistinct (bottom panel Figure 3.6). The ratio of the metacarpus width to pharynx length decreases as the worm develops to infective larva (Table 3.2): the pharynx to body length in the infective larva is approximately 1/3rd to 1/2 while in the free-living adult stage the pharynx is approximately 1/5th the body length. When incubated at 20°C, the major morphological changes in development to the infective larval occur during the 40 to 60 hrs - the buccal cavity (mouth opening) closes over and the sheath forms (Figure 3.6).

Stage	Ratio Metacorpus width to pharynx length *	Buccal cavity	Presence of sheath
L1 in NGM	0.132179 std dev 0.013343	Open	No
Larvae at18hrs in NGM	0.147765 std dev 0.013579	Open	No
Larvae at 18hrs + conditioned medium	0.157938 std dev 0.013575	Open	No
Larvae at 40hrs + conditioned medium	0.146279 std dev 0.016267	Open	Yes
Larvae at 60hrs + conditioned medium	0.099852 std dev 0.020018	Closed	Yes
Larvae at 84hrs + conditioned medium	0.043638 std dev 0.014016	Closed	Yes

Table 3.2: Pharynx remodelling and sheath formation

**Average metacarpus width to total pharynx length averaged from ≥ 15 worm measurements.*

3.3.6 Cryopreservation of the free-living L1 stage

Cryopreservation ensures that specific lines or strains of *P. trichosuri* can be safely archived. Cryopreservation of the L1 stage of development was an adaptation of Nolan *et al*. (NOLAN *et al.* 1988). Freezing solutions of 10% DMSO supplemented with either 10% dextran (M250K) or 10% trehalose and a freezing solution of 10% trehalose supplemented with 10% dextran (M250K) were compared. The 10% DMSO freezing

solutions yielded the highest survival rate of approximately 75%. In order to determine whether pre-incubation in the solutions prior to freezing increased the survival rate, a 30 minute pre-incubation was compared to no pre-incubation. It was determined that pre-incubation in the freezing solutions did not increase the survival rate (results not shown). Important criteria for survival of the L1 larvae were the speed at which the thaw occurred and the inclusion of a wash step in greater than ten times volume of PBS or tap water to remove residual DMSO.

3.4 RESULTS: ENVIRONMENTAL SIGNALS WHICH PLAY A ROLE IN *P. trichosuri* iL3 DEVELOPMENT

3.4.1 iL3 developmental response to conditioned medium

In order to study the effect of various environmental signals on the developmental decision to enter the infective larval stage of *P. trichosuri*, conditioned medium (CM), which contains the biological factors produced by the worms, was prepared as described in the Methods section. Figure 3.7 shows that newly hatched *P. trichosuri* L1 larvae develop into infective larvae in a dose dependent manner when exposed to conditioned medium. Different preparations of conditioned medium gave different shaped curves, using logistic regression using Generalized Linear Model of the binomial data with a log-log link function and Accumulated analysis of deviance suggests that the lines have the same slope (approximate F probability <0.001, and a test statisitic of 428.00), but different asymptotes (approximate F probability of 0.010 and a test statistic of 7.02).

In order to eliminate the possibility that development is a stress response due to depleted media or bacterial waste products, L1 larvae were also incubated in a serial dilution of bacterial media control (BAC control) which was prepared in a similar manner to the conditioned medium, minus the worms see Methods (Section 2.3.1). *P. trichosuri* do not develop into iL3 in response to the BAC control, suggesting there is a biological factor in the conditioned medium that is produced by the worms which influences infective larval development in a dose dependant manner. This experiment served as a range-finding trial. At the highest concentration of conditioned medium in

these bioassays there was also evidence of death or delayed development of some of the free-living worms. Also, at the lowest concentration there was more variability in the developmental response. Therefore, in subsequent assays, the serial dilution was performed from 0.75 to 0.0468 fold dilution.

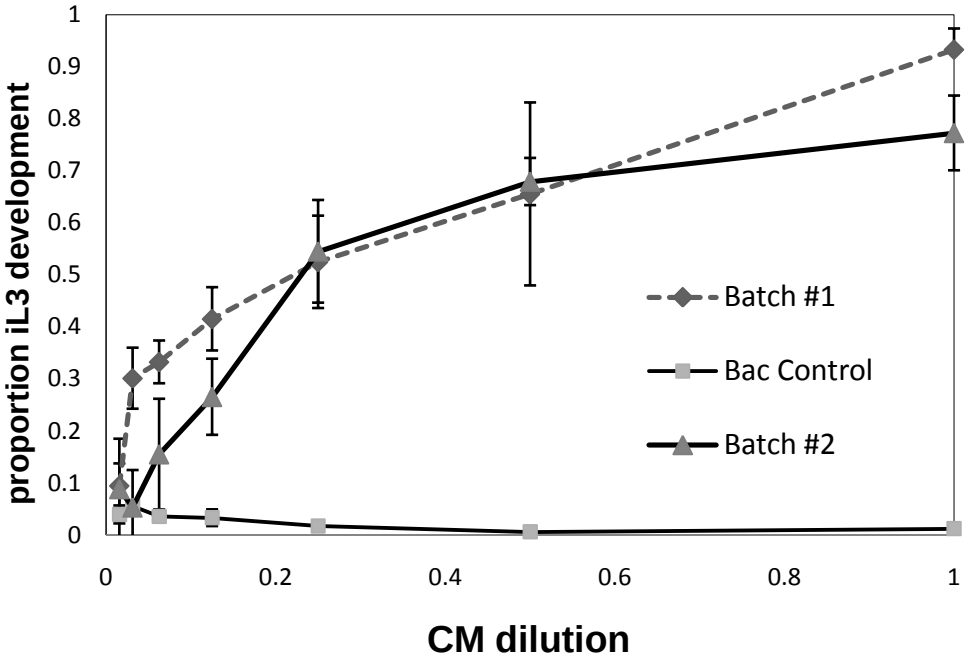


Figure 3.7: Proportion of *P. trichosuri* iL3 development at 20°C with various concentrations of conditioned medium in liquid NGM.
Conditioned medium (CM) was prepared as described in Methods section. The same procedure was performed without the addition of worms to create a bacterial control medium (Bac Control). L1 larvae at 1 L1/µL were incubated at 20°C in serially diluted conditioned medium and HB101 *E. coli* as food source. On day 5 the proportion of larvae that developed into iL3 were scored in 5 replicate wells. Error bars are standard deviation.

3.4.2 Infective larva development response to conditioned medium is mediated by temperature

In order to determine whether incubation temperature has an effect on the developmental choice of *P. trichosuri* L1, worms were incubated in a serial dilution of conditioned medium at various incubation temperatures, see Methods (Section 2.4.3). Figure 3.8 shows that incubation at lower incubation temperatures result in a higher proportion of *P. trichosuri* becoming infective larvae. Parallel curve analysis using non-linear regression was used to anaylse these results. Accumulated analysis of

variance suggests that culturing *P. trichosuri* at the different incubation temperatures resulted in statistically different proportions of infective larval development (F probability score of <0.001, and a test statistic of 249.74).

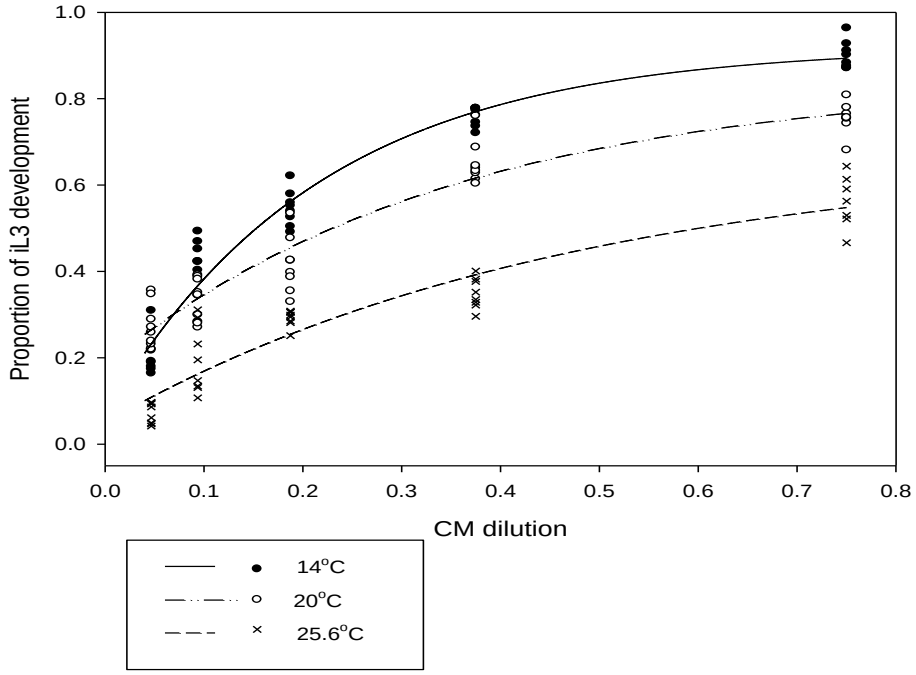


Figure 3.8: *P. trichosuri* iL3 development: the effect of incubation temperature versus conditioned medium.
P. trichosuri L1 larvae at 1 L1/µL were incubated at either: 14°C, 20°C or 26.5°C with Gentamicin sulfate at 50ug/µL, and 0.4% *E. coli* HB101 as food source and serially diluted conditioned medium. On day 5 the proportion of larvae that developed into iL3 were scored in 8 replicate wells of pooled biological replicates indicated by points on graph, lines are best fit lines. The lower the temperature that *P. trichosuri* larva are incubated at, within the temperature range tested, the greater the induction in infective larvae development.

3.4.3 Infective larval development response to conditioned medium is mediated by food availability

The effect of food availability on infective larval development in *P. trichosuri* was also examined, See Methods (Section 2.4.2). Figure 3.9 shows the effect of different concentrations of *E. coli* HB101 food source on the development of *P. trichosuri* larvae in serial dilutions of conditioned medium Batch #1. Parallel curve analysis using non-linear regression was employed to analyse the data accumulated analysis of variance

suggests culturing *P. trichosuri* L1 larvae at these different food concentrations resulted in statistically different proportions of infective larvae development (F probability score of <0.001, with at test statistic score of 204.11). Moreover, that there was a significant deviation from parallel of the reaction norms (F probability score of <0.001 with a test statistic of 7.68.

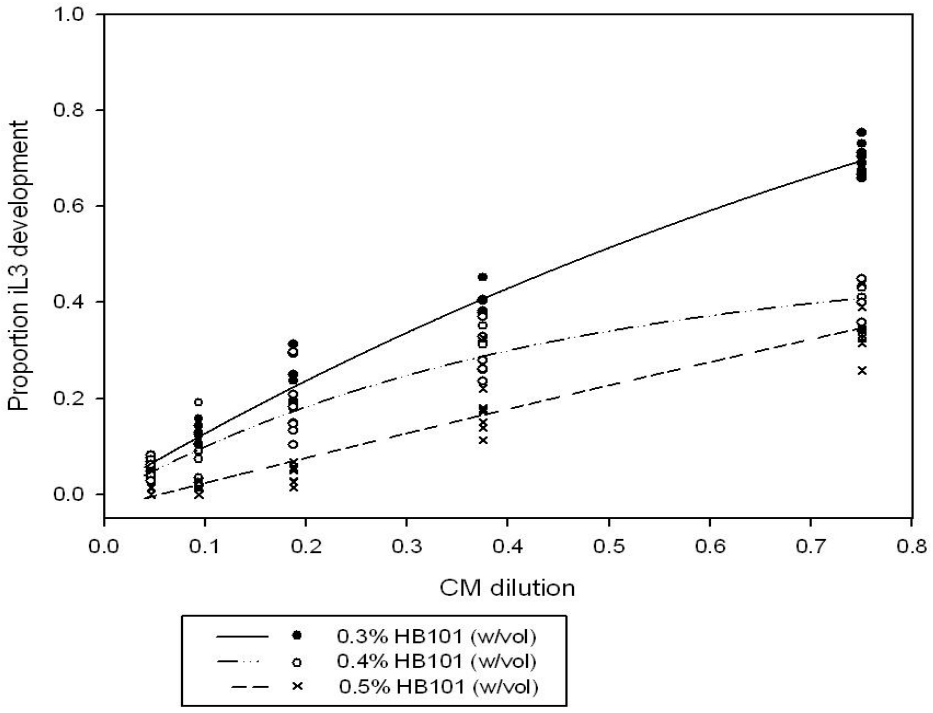


Figure 3.9: The effect of food concentration on *P. trichosuri* iL3 development. Food concentration versus conditioned medium
L1 larvae at 1 L1/µL were incubated at 20°C in various concentration of bacterial food source with Gentamicin at 50ug/µL and serially diluted conditioned medium. On day 5 the proportion of larvae that developed into iL3 were scored in 8 replicate wells of pooled biological replicates indicated by points on graph, lines are best fit. The lower the food concentration, the greater the induction of infective larvae development within the food availability range tested. Food treatments differs significantly at F probability of <0.001.

The range in proportion of infective larval development (y axis) from the highest dilution of conditioned medium to the lowest (x axis) is a measure of responsiveness of the larvae to conditioned medium, or the reaction norm. Treatments which lessen the sensitivity of the response would result in a decrease in the range of the proportion of infective larval development, whereas treatments that sensitise worms to conditioned

medium would increase the range. The range in the proportion of infective larval development for larvae exposed to 0.5% *E. coli* HB101 was 33%. (i.e. the range from the highest concentration of conditioned medium to the lowest concentration of conditioned medium). There was a difference of 36% in the range of infective larval development in the 0.4% HB101 *E. coli* food samples. In contrast, the lowest concentration of food availability (0.3% *E. coli* HB101 w/vol) had a difference in the range of response of 63%, almost double the response of the higher food concentrations.

In order to further analyse the relationship between the effect of food on response to conditioned medium and the effect of temperature on response to conditioned medium, for each set of exponential curves the starting point was subtracted from the asymptote, and variance for the sets of curves was analysed. To each set of data a family of c exponential curves is fitted by least squares, with the monotonic sense occurring to the right and rising. From c variates of fitted values within a family, c minimum fitted values m are found and each is subtracted from the asymptote estimate of its respective curve to give c heights (h(1…c)). The variance of the heights is then estimated:

$$\psi = \sigma^2(h(1\ldots\chi))....$$

A measure of the shape of the curves is given by the variance of the asymptotes: fan coefficient ψ = variance (asymptotes-minimum fitted values for each curve). To get an estimate of the range of ψ for each family of curves, all combinations of c-1 curves per family are used (Table 1); the bootstrapped values for each pair are lower case vis φ, and a set of re-sampled φ values along with ψ give an estimate of the range. If ranges fail to overlap, the fanning behaviour of each family of curves is considered different.

The fan coefficients (Table 3.3) for food are higher then the fan coefficients for temperature: i.e. the lowest fan coefficient for the food curves is larger then the highest fan coefficient for the temperature curves. Since there is no overlap in the fan coefficient values, this suggests the food response lines fan more than the temperature response lines. Thus food availability influences the developmental response of *P. trichosuri* to conditioned medium in a different manner than temperature does, which

suggests that a complex relationship exists between environmental factors and the developmental switches they modulate.

ψ for all food curves	0.05433
fan coefficient φ (for curves 0.3% w/vol and 0.4% w/vol)	0.1131
fan coefficient φ (for curves 0.3% w/vol and 0.5% w/vol)	0.0520
fan coefficient φ (for curves 0.4% w/vol and 0.5% w/vol)	0.1130
ψ for all temperature curves	0.003094
fan coefficient φ (for curves 14°C and 20°C)	0.004068
fan coefficient φ (for curves 14°C and 26.5°C)	0.015151
fan coefficient φ (for curves 20°C and 26.5°C)	0.00714

Table 3.3: Fan coefficients for the different incubation temperatures and food availability.
A measure of the shape of the curves is given by the variance of the asymptotes: fan coefficient ψ = variance (asymptotes-minimum fitted values for each curve). The best estimate of fanning is ψ for each data set, the bootstrapped values for each pair of curves is φ. There is no overlap between the two groups therefore the fanning of the food reaction norm lines is significantly greater then the fanning of the temperature reaction norm lines. Test of null hypothesis that mean fan coefficients of set1 is equal to the mean fan coeffiecient of set2. Test statistic t = 2.52 on approximately 3.01 degrees freedom. Probability = 0.086

3.4.4 Effect of cholesterol on infective larva development.

Exogenous cholesterol is required for *C. elegans* reproductive development as they are unable to synthesise steroids *de novo* (MATYASH *et al.* 2004). The cholesterol serves as a precursor for the production of dafachronic acid, which act as ligands for DAF-12 (nuclear hormone receptor) (MOTOLA *et al.* 2006). It has been demonstrated that *S. papillosus* also requires exogenous cholesterol for free-living development, as depletion of cholesterol results in 100% infective larval development (OGAWA *et al.* 2009). The effect of cholesterol depletion on infective larval development of *P. trichosuri* was examined, see Methods (Section 2.4.4). Figure 3.10 shows the effect on development of culturing *P. trichosuri* larvae on low peptone NGM agar plates for two generations with and without exogenous cholesterol. There was a significant increase (Student's t-

test, $p < 0.001$) in iL3 development for the F_2 generation of *P. trichosuri* cultured without exogenous cholesterol.

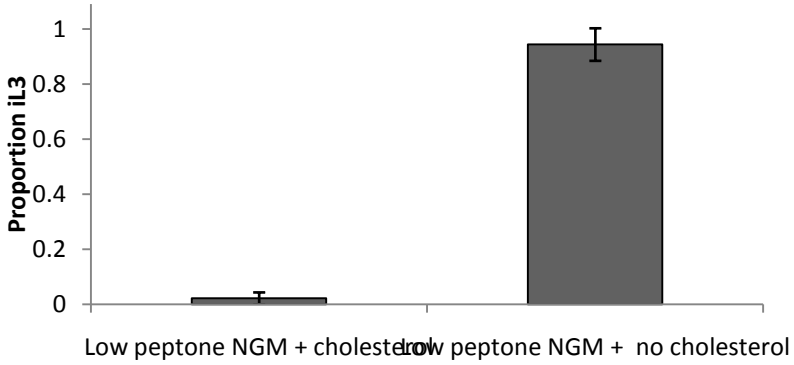


Figure 3.10: The effect of cholesterol on *P. trichosuri* iL3 development.
P. trichosuri adults were picked onto either low peptone NGM agar plates with or without cholesterol with *E. coli* HB101 and possum faecal slurry as the food source. F_1 generation worms were picked replated onto fresh NGM low peptone plates with or without cholesterol. These were allowed to lay eggs which were incubated at 20°C until day 5, the F_2 generation were then scored for free-living development or iL3 development. Bars equal standard deviation.

3.4.5 Effect of conditioned medium on the life span of adult *P. trichosuri*

The IIS pathway has been shown to influence life span in *C. elegans*, *Drosophila melanogastor* and possibly mammals (BELLINO 2006; MCCULLOCH and GEMS 2003; PIPER *et al.* 2008; RINCON *et al.* 2004; RINCON *et al.* 2005). In *C. elegans* biology, IIS controls both dauer larvae development and life span, and it has been shown by Kawano *et al*. (KAWANO *et al.* 2005) that a *C. elegans* conditioned medium contains a biological factor that acts pleiotropically to influence dauer larvae development at early larval stages and to extend life span when worms are exposed at later stages of development. In order to determine whether the *P. trichosuri* conditioned medium is able to influence life span as well as influence infective larval development, *P. trichosuri* at a late larval stage (L4 or early adult) were exposed to either conditioned medium or to the bacterial control medium (BAC). The results shown in Figure 3.11 indicate that there was no extension in the final life span, nor the midpoint lifespan at LD_{50}. CM lifespan

statistical test was nonlinear regression analysis with complementary log-log link to estimate the average lifespan (point at which 50% of the population was alive, with 95% confidence intervals). The tail end of the experiement when 5% of the population remained was also examined. The confidence intervals at both these time points overlapped, suggesting there was no extention of lifespan. The concentrations of conditioned medium used in the life span extension experiments were at concentrations that induce >90% infective larvae.

3.4.6 *P. trichosuri* inbred lines selected for sensitivity and resistance to conditioned medium

Inbred lines of *P. trichosuri* were established from an outbred starting population by selection at the extremes of response to conditioned medium as described (Methods Section 2.4.8). To determine whether this selection and subsequent inbreeding had resulted in lines that diverged significantly from the parental population and from each other, the data from each dilution series was modelled with logistic regression where conditioned medium concentration was the explanatory variable and iL3 proportion was the response. This yields a simple linear model on the scale of the linear predictor (i.e. the logit (or logistic transformation) of the iL3 proportion is linearly related to conditioned medium concentration).

The norm of reaction line for CM20's was significantly greater than KNP out bred line (F probability score of <0.001, with a test statisitic of 715.66). CM3's norm of reaction line was signaificantly less than the out bred KNP line (F probability score of < 0.001 with a test statistic of 198.29). The slope and intercept parameters from these straight-line regressions were separately analysed to compare the five inbred lines using ANOVA. CM3 had a significantly lower intercept then KNP, and CM20 had a significantly higher intercept than KNP, the other two lines had average intercepts similar to KNP (F probability score of <0.001 and a test statisitic of 16.44). Analysis of variance did not find any significant differences in slope between the lines (F probability score of 0.525 and a test statistic of 0.84).

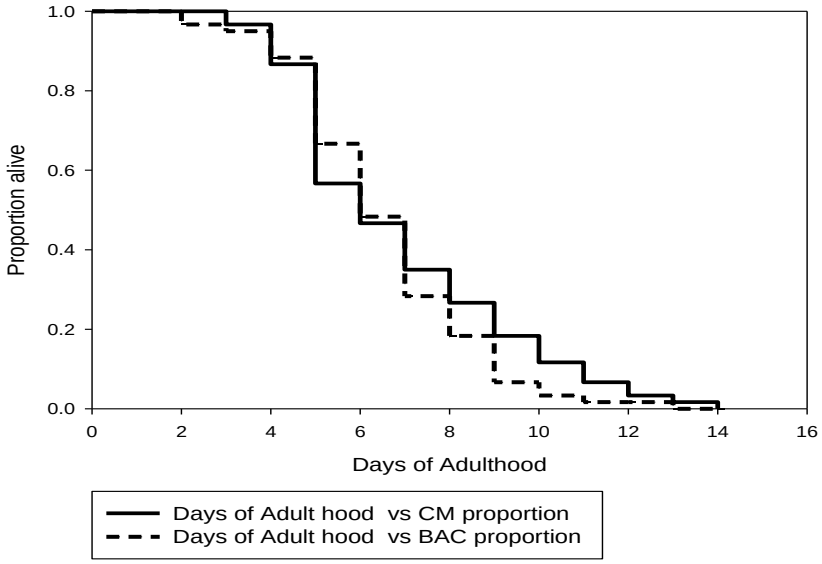


Figure 3.11: The life span of free-living *P. trichosuri* adults, in the presence of semi-extracted conditioned medium or bacterial control medium.

P. trichosuri free-living worms were until L4 stage (approximately 36-48 hours). Individual worms were plated in 96 well plates containing liquid NGM low peptone that was either 50% conditioned medium (supplemented with semi-purified conditioned medium to a ratio of 1:10) or in 50% bacterial conditioned media control (BAC). The worms were cultured at 20°C and observed daily. Worms were scored as dead when they no longer responded to touch.

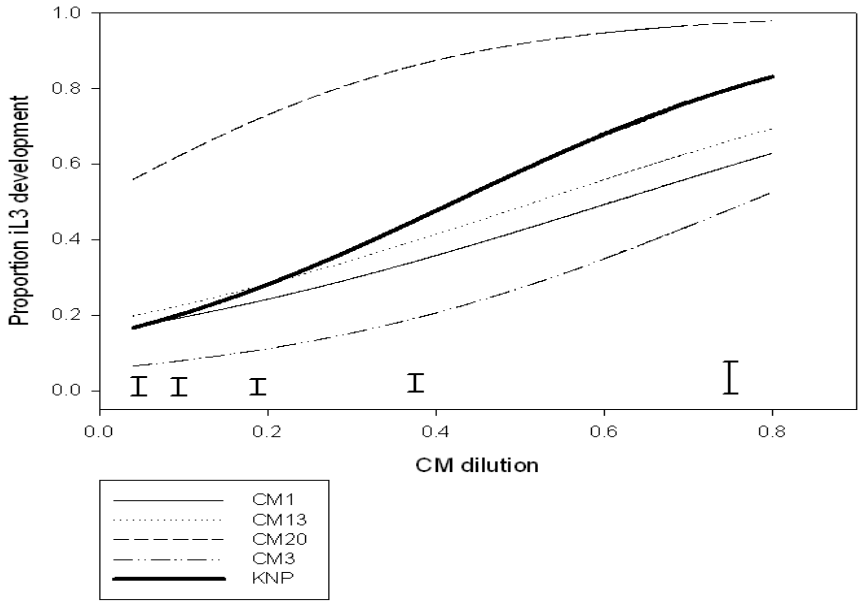


Figure 3.12: Infective larval development of *P. trichosuri* inbred lines grown in conditioned medium.

Inbred lines were created as described in Method Section 2.4.8. To determine developmental reaction norm of these inbred lines, a conditioned medium bioassay was performed. Each inbred line was tested in two possums, on three separate days, each with six replicate wells of each dilution of conditioned medium. The data was analysed by logistic regression and the fitted lines shown above. Inbred lines CM20 and CM3 differ significantly at all dilutions of the conditioned medium compared to the outbred line (KNP) ($P<0.001$). LSD error bars at the bottom of the graph represent the least significant difference (at 5% significance level) between any two inbred lines at the concentrations of conditioned medium tested.

3.5 DISCUSSION

Parasitism is a successful life history strategy that is thought to have evolved independently several times across the phylum Nematoda altering free-living ancestors to parasites with an absolute requirement for a host (BLAXTER *et al.* 1998). It is thought that in the evolutionary step from free-living nematode to parasite, the specialized dauer larvae stage served as the progenitor of the infective larva. This is based upon the apparent similarities in the biology of both the dauer larva and infective larva and has been described as the "dauer hypothesis" (ASHTON *et al.* 1998; BURGLIN *et al.* 1998).

The goal of this chapter was to examine the biology of the *P. trichosuri* infective larval stage and the *C. elegans* dauer larvae stage in the context of the dauer hypothesis. The environmental triggers of the developmental switch were also examined, along with the biology of free-living versus parasitic morphs. The signal transduction pathway which plays a role in development is examined in other chapters of this thesis.

3.5.1 Biological similarities between *P. trichosuri* infective larvae and *C. elegans* dauer larvae.

The *C. elegans* dauer larvae stage and *P. trichosuri* infective larvae stage display similar biologies. First, *P. trichosuri* infective larvae were able to withstand incubation at temperatures of 42^{o}C for ten times longer than the adult stage, whilst the *C. elegans* dauer larvae stage was able to survive 3 times longer then the adult stage at an elevated incubation temperature of 37^{o}C (1997). Second, the ability to withstand incubation in 1% SDS is a defining characteristic of the *C. elegans* dauer larvae stage (CASSADA and RUSSELL 1975; SWANSON and RIDDLE 1981); *P. trichosuri* infective larvae showed a similar ability to survive longer in SDS compared to adults. Third, both *P. trichosuri* infective larvae and the *C. elegans* dauer larvae stage survived longer in the oxidative chemical stressor, paraquat, compared to adult worms. Fourth, newly formed *P. trichosuri* infective larvae had large lipid stores, suggestive of the importance of lipids for metabolism during the infective stage of development. The *C. elegans* dauer larvae stage relies on lipid stores and increased gluconeogenesis for metabolism (MCELWEE *et al.* 2006; 1997). The infective larvae of *Ancylostoma tubaeforme, A. caninum* and

Uncinaria stenocephala have also been shown to rely on lipid stores for metabolism (CROLL 1972; HILL and ROBERSON 1985).

C. elegans enter the dauer larvae stage to enhance larval survival during periods of environmental stress. The cuticle thickens and buccal cavity closes off, so that this stage is physically unable to eat relying instead on lipid stores for energy (1997). A thicker cuticle protects the dauer larvae from environmental stresses such as desiccation, and may serve as an additional barrier to permeable toxins. Results presented in this chapter have shown that the *P. trichosuri* infective larval stage shares some of the defining biological characteristics of the *C. elegans* dauer larvae stage. *P. trichosuri* develops a sheath which entirely encloses the worm, including the buccal cavity during development to the infective larval stage and it is reasonable to speculate that a possible biological role of the sheath is to protect the infective larvae from some of the same environmental stresses encountered during diapause.

3.5.2 Environmental signals influence *P. trichosuri* infective larvae and *C. elegans* dauer larvae.

In species of parasites that have retained the developmental choice to enter either a parasitic morph or a free-living morph, such as species of the genera *Strongyloides* and *Parastrongyloides,* this developmental choice is influenced by environmental cues that are similar to those which influence dauer larvae development in *C. elegans*. These are population density, lack of nutrients or sub-optimal temperatures. The focus of this chapter was to investigate whether the environmental cues which influence *P. trichosuri's* development to the parasitic morphs, are similar to those which influence dauer larvae development in *C. elegans*.

Data presented here suggest that larvae develop to infective larvae in response to a biological factor found in conditioned medium. That it is not a stress response due to incubation in depleted media, nor of biological cues produced by the bacteria used to make the conditioned medium is illustrated by the fact that the larvae do not develop into infective larvae when exposed to a serial dilution of bacterial control medium, which was made by the same process as the conditioned medium without the addition of

the *P. trichosuri* nematodes. Thus, the larvae themselves produce a biological factor to which the developing larvae respond in a dose dependent manner reminiscent of the population indicator, dauer pheromone, of *C. elegans* (GOLDEN and RIDDLE 1982; GOLDEN and RIDDLE 1985). Population density was also determined to play a role in *S. planiceps* development (ARIZONO 1976a) but no attempts were made to determine if there was a biological factor produced by the worms that served as the signal. Two batches of *P. trichosuri* conditioned medium were made and it was determined that each had significantly different activity levels ($p<0.01$). This batch to batch variation in biological activity has been found in the production of *C. elegans* dauer pheromone batches (GOLDEN and RIDDLE 1982).

Food availability and temperature both play a role in *P. trichosuri* infective larval development: lower incubation temperatures or lower food concentrations result in a greater proportion of infective larvae. It has been found that lower temperature also results in more infective larval development in *S. ratti* (HARVEY *et al.* 2000; MINATO *et al.* 2008; VINEY 1996), *S. planiceps* (ARIZONO 1976b) and *S. papillosus* (NWAORGU 1983). This is in contrast to *C. elegans* and *S. stercoralis* where higher temperatures result in more dauer larvae or infective larvae formed respectively (GOLDEN and RIDDLE 1984b; GOLDEN and RIDDLE 1984c; SHIWAKU *et al.* 1988). The differential developmental response of these species due to temperature cues may be due to habitat, since *S. planiceps* and *S. papillosus* are found in equatorial regions may find cooler temperatures stressful (SHIWAKU *et al.* 1988). A food signal inhibits dauer larvae formation in *C. elegans* (GOLDEN and RIDDLE 1984b). Nutrition also effects the homogonic or heterogonic development of the parasitic nematodes *S. planiceps* (ARIZONO 1976a) and *S. ransomi* (MONCOL and TRIANTAPHYLLOU 1978). In these species, either a lower concentration of food, or food that has been depleted of nutritional value by washing and autoclaving, results in a higher proportion of infective larval development.

The developmental plasticity displayed by these parasites - in *Strongyloides* species in the first generation only (with the exception of *S. planicep* which have nine developmentally plastic generations), and in *Parastrongyloides* species at each generation, allows the worms to sample the environment; and when the environmental cues suggest the likelihood of successfully reaching reproductive adulthood, they make

the developmental decision to develop to the free-living morph, which results in an expansion of the population. Alternatively, when environmental cues such as increased competition for resources or non-optimal temperatures signal to the worm that it is in a stressful environment with decreased likelihood of successfully reaching adulthood, they enter diapause as infective larvae in the hope of eventually encountering a host to complete their life cycle.

In *C. elegans*, this developmental choice between diapause or reproductive growth is transduced through the IIS and TGF-β signalling pathways, which converge to regulate both the steroid hormone receptor DAF-12, and also the production of its ligands, the Δ4 and Δ7 dafachronic acids (FIELENBACH and ANTEBI 2008; MATYASH *et al.* 2004). Cholesterol serves as the precursor for the *de novo* synthesis of the dafachronic acids and must be supplied exogenously to *C. elegans* cultures for reproductive growth to occur (MATYASH *et al.* 2004). Application of dafachronic acids reduces infective larval formation in *S. papillosus* and *S. stercoralis* in favour of free-living morph (OGAWA *et al.* 2009; WANG *et al.* 2009). It was also demonstrated that these dafachronic acids bind to and activate DAF-12 orthologues of *S. stercoralis* and the hookworms *A. caninum*, *A. ceylanicum* and *Necator americanus* (WANG *et al.* 2009). Our data indicate that exogenous cholesterol must be administered to *P. trichosuri* for free-living development, which may suggest that a similar hormone receptor/steroid pathway may be conserved within this species.

Both the genotype of an organism and the physical environment in which that organism exists (G x E), influences its phenotype. One way to quantify the relationship between genotype, the environment and phenotype is by describing 'norms of reaction' of an organism. One way to do this is to describe the phenotype of an organism in a range of possible environments, when plotting these values the slope of the line is a measure of the plasticity (VIA 1993). The experiments in this study examined the proportion of two possible phenotypes of *P. trichosuri* larval development: development to infective larvae or development to free-living nematodes. This is referred to as a polyphenism: one genotype producing two discrete phenotypes in response to environmental signals. In order for evolution of a new reaction norms to occur, it is at the minium necessary that within a population, allelic differences produce different sensitivities to the environmental signal, alternatively, that some genetic loci influence the phonotype in

one environment only (i.e. environmental specific gene expression) (VIA 1993). Species which display diverse phenotypic plasticities have experienced different ranges of environmental variation upon which natural selection has acted {Via, 1993 #509}.

I have found that *P. trichosuri* larvae are able to integrate multiple environmental signals in order to make their developmental decisions. Temperature and food influence the developmental switch across the series of conditioned medium concentrations with different relationships (Compare Figure 3.8 and Figure 3.9). The shape of the reaction norms for infective larval development versus incubation temperature was the same for each of the temperatures tested, with the exception of the lowest conditioned medium dilution on the 14°C line. In contrast, low concentrations of food availability have a stronger effect on development than high concentrations of food, as illustrated by the steeper slope of the reaction curve at the lowest food availability. At the lowest concentration of food availability the resulting range in infective larval development is approximately double that of the larger food concentrations; one way of interpreting this is that low food availability makes the larvae more sensitive to the conditioned medium signal. It may be that food is an absolute requirement for development along the free-living life cycle, and low levels of food resources, coupled with high competition for those food resources as determined by high concentrations of conditioned medium (which is a population signal), would signal to the worm that its development to an infective larvae would be a better survival strategy.

P. trichosuri is a good candidate model organism for the study of evolutionary developmental plasticity; it has a short generation time; the polyphenic development to either infective or free-living larvae is easily scored; the free-living life cycle of *P. trichosuri* permits the creation of inbred lines for classical genetic analysis; while the huge reproductive output of this worm allows observations in large data set groups. It is possible to cyropreserve the first larval stage of *P. trichosuri* with ease, while cryopreservation of *P. trichosuri* infective larvae was resulted in few of the infective larvae (<30%) surviving the thawing process, and these survived for less then an hour (data not shown).

I have determined that within the outbred population of *P. trichosuri* (known as KNP), there is a broad range of responses to the infective larval inducing effects of the conditioned medium. These results have confirmed that there is a genetic component to susceptibility to the conditioned medium developmental signal. The nature of the genetic differences between these inbred lines is at present unknown. The difference may be in how the worms detect the biological signalling factor, the way in which this signal is transduced or even in the expression levels of a downstream gene involved in infective larval development. Of the four inbred lines of *P. trichosuri*, two of the lines showed a similar response to conditioned medium as the outbred line and two of the lines were at the extremes of response. Expression analysis of these inbred lines may elucidate some of these issues. Viney *et al.* have shown that different isolates of *C. elegans* also differ in their reaction norms to the dauer pheromone (VINEY *et al.* 2003). The differing responses to *C. elegans* dauer pheromone found by Viney *et al*, also suggests a genetic component to the developmental switch.

Harvey *et al*. found that for *S. ratti*, the host's immune status plays a role in the development of the parasite. Parasites from an immune host will have a greater proportion of heterogonic female and male offspring than parasites from a naïve host (HARVEY *et al.* 2000). To lessen the possibility that the differing responses to conditioned medium I saw in our inbred line development assays was due to the immune status of the host, rather then a genetic component of the inbred lines themselves, a new and different set of possums were infected with our inbred lines. The possums chosen for infection were all of similar age, weight and infection history (in that they were captured from a similar location and treated identically with anthelmintic dosage after capture). Figure 3.12 displays the combined data from both sets of possums. Results of the developmental response to conditioned medium from the second set of possums, which had similar immune status, showed no difference in the developmental response trends for each of the inbred lines tested. The two data sets were combined to produce Figure 3.12.

Within the parasitic life cycle there is a dispersal stage, usually the infective larval stage, during which the larvae seek a new host. Infective larvae are non-feeding, and therefore have a finite time in which to find a host and so there is always an element of uncertainty of encountering a host at the correct time. Therefore, it is good

evolutionary, bet-hedging strategy to have variable infectability within a population i.e. to have a proportion of both infective larvae and free-living larvae in order to prevent the entire population from developing to infective larva and not encountering a host (FENTON 2002). The data from the developmental bioassays of larvae exposed to conditioned medium have shown that even at the highest dose (1X) it is not possible to achieve 100% infective larval development, suggesting that within a population there is a reservoir of the population that do not respond easily to this signal. A semi purification and concentration (to 2X) of the conditioned medium, followed by exposure of larvae to this extract resulted in no further increase in the proportion of infective larvae development. The data from the inbred lines has also illustrated that within an outbred population there is genetic diversity within a population, as well as worms that display a range of responses to the developmental switch cues.

3.5.3 Biology of the free-living and parasitic morphs of *P. trichosuri*

Some of the basic biology of the free living morph of P. trichosuri reported here has been published in the paper (GRANT *et al.* 2006b). The average life span of free-living *P. trichosuri* was determined to be approximately 7 days. The method for estimating the life span of the parasitic morph is however indirect and measures the duration of time for which eggs can be found in possum faeces. Measurement of egg production from possum faeces is a conservative method of determining the life span of the parasitic stage, as this is only a measure of the reproductive age of the worms, and they quite possibly survive for a period of time after their reproductive capacity has been reached. An additional, important caveat is that this method is most likely measuring the effectiveness of the host's immune response; in other words, the host's ability to expel the parasites from the gut, rather than an actual measure of senescence of the parasites. The average patent period for parasitic infection of *P. trichosuri* was determined to be 74 days (GRANT *et al.* 2006b). This is a greater than 10 fold difference in life span compared to the free-living morph. This plasticity in life span has also been reported for *S. ratti*, a parasite that is capable of a single free-living life cycle before obligate development to infective larvae. *S. ratti* has a free-living life span of 3 days versus 271 for the parasitic morph in an immune compromised host (GARDNER *et al.* 2006). This is an almost 100 fold difference in life span. It is important to note that the

life span assays of *S. ratti* were performed in nude rats as the host's immune response affects life span, size, fecundity and heterogonic versus homogonic developmental response (GARDNER *et al.* 2004; HARVEY *et al.* 2000). Performing the *S. ratti* life span experiments in nude rats ensures that the host's immune response was not a concern. The experiments which determined the patency period of *P. trichosuri* were performed in possums with an unimpaired immune response; as such the host response would have had a significant effect on the patency and by association the life span measurement of the worms. Life span plasticity within a single species is almost certainly a result of differential gene expression between the morphs. One evolutionary theory of ageing is that organisms which face high rates of predation, disease, starvation or other harsh conditions such as temperature extremes will age more quickly than organisms which face low extrinsic mortality (KENNEDY 1976). The plasticity in life span may be an indicator of the extrinsic dangers faced by the different morphs. Whilst in the lumen of the host, parasites have to contend with the not insignificant effects of the host's immune response but are protected from the extremes in predation, starvation and temperatures encountered on the forest floor.

Another intriguing difference between the free-living and parasitic morphs of *P. trichosuri* is the difference in brood sizes: the free-living worms have an average brood size of 55, whereas the parasitic stage may display an estimated 200 fold higher fecundity. This estimate is based upon the following calculation: when the average parasite worm burden is approximately 100 females, the average egg count per gram of faeces is approximately 100, a possum might produce anywhere from 50 to100 grams of faeces per day. This means that each adult female is probably producing at least 100 eggs per day, which she does for at least 70-100 days. So, her lifetime reproductive output is of the order of 10,000 eggs.

High fecundity is a defining feature of many parasitic species. There are several theories for high fecundity in parasites, which are not mutually exclusive. Increased fecundity may serve to compensate for the risk of not finding an appropriate host, as more eggs mean a greater chance of some offspring surviving to find a host (KENNEDY 1976). Another theory is that high fecundity is simply an effect of the environment, intestinal parasites in the lumen are in a relatively protected environment, there are: no temperature extremes, an abundant food supply, no predation, the greatest (and certainly

not insubstantial!) threat is the host's immune response. The higher fecundity may simply be a result of the abundant resources and protected environment coupled with the longer life span. A positive correlation between size of female versus greater fecundity was found across 66 species of mammalian intestinal nematodes. Larger species tend to have longer prepatency periods and larger reproductive output and duration (SKORPING *et al.* 1991). The parasitic morph of *P. trichosuri* is 10 fold larger in size than free-living morph, and may lay more eggs simply because they are larger in size.

The life span of the free-living adult stage of *P. trichosuri* was assessed in the presence of the conditioned medium in an attempt to determine whether the biological factor produced by the worms was able to influence life span. *C. elegans* dauer pheromone is detected and transduced through the chemosensory neurons to regulate insulin signalling, which controls both dauer larvae development and life span in *C. elegans.* I wanted to determine if the biological factor contained within the *P. trichosuri* conditioned medium also had an effect on the life span of the free-living adult worms. I used conditioned medium at concentration that induces >90% infective larval development, and at this concentration should have stimulated the signalling pathway; however, I was not able to detect an effect on life span. The role of dauer pheromone in life span regulation in *C. elegans* is controversial. Alcedo and Kenyon found that *C. elegans* dauer pheromone at concentrations which induce 33% dauer formation did not extend life span (ALCEDO and KENYON 2004). The lab of Kawano *et al*. reported that a concentration of crude extract that induced 65-88% dauer larvae formation (a similar concentration to what I used) and were able to extend life span (KAWANO *et al.* 2005). Our data are in agreement with Alcedo and Kenyon; I did not observe a significant effect of conditioned medium on life span in *P. trichosuri*.

3.5.4 Summary

Results presented in this chapter have highlighted many intriguing similarities displayed between *C. elegans* dauer larvae and the *P. trichosuri* infective larvae, both in terms of biological characteristics and in the environmental signals which determine the developmental switch; these results appear to corroborate the dauer hypothesis.

Certainly, in terms of the biology of the infective larvae, it seems feasible that the dauer larvae stage of free-living nematodes may have served as a progenitor of the infective larvae.

An extension of this hypothesis would be that the molecular process which control dauer larvae development would be analogous to the molecular process which control infective larva development. Further investigation of the signalling pathways that are known to cause dauer larvae development in *C. elegans* are warranted to determine if they play the same sort of role in infective larval development. In Chapters 4 and Chapter 5 I investigate the structure of IIS gene orthologues and their role in infective larva development.

CHAPTER 4

CLONING AND CHARACTERIZATION OF THE *P. trichosuri* INSULIN/IGF SIGNAL TRANSDUCTION GENES

4.1 INTRODUCTION

In this chapter, I discuss three *P. trichosuri* gene orthologues of the IIS pathway; the tyrosine receptor kinase (*daf-2*), the phosphatidylinositol 3′ kinase (*age-1*), and also the FOXO forkhead transcription factor (*daf-16*). For each of the genes mentioned, I describe their cloning, sequence characterization and phylogenetic analysis. In the last section of this chapter I describe the expression pattern of these genes, as elucidated by Quantitative-PCR (q-PCR), for several different developmental stages of *P. trichosuri*.

Components of the IIS pathway are critical for cellular regulation and have been evolutionarily conserved from yeast to humans (BARBIERI *et al.* 2003). This signalling pathway controls a variety of processes such as growth, metabolism, reproduction, development and ageing (BARBIERI *et al.* 2003; FIELENBACH and ANTEBI 2008; LONGO and FINCH 2003; LUDEWIG *et al.* 2004; PARTRIDGE *et al.* 2005). Throughout evolution, the complexity of the IIS pathway has evolved and changed for instance: mice and humans have several insulin/IGF receptor genes, which form heterodimers, but only one ligand. In contrast, worms and flies have one receptor gene, which forms a homodimer, but a number of ligands: up to 39 insulin ligands have been discovered in *C. elegans* thus far (PIPER *et al.* 2008).

In *C. elegans*, DAF-2 is the IIS receptor. It is characterized by a signal peptide, two ligand binding domains separated by a furin-like cysteine rich domain, a fibronectin III domain, a transmembrane domain, and a tyrosine kinase domain with three tyrosine phosphorylation sites (YxxM) (KIMURA *et al.* 1997; PATEL *et al.* 2008).

In *C. elegans*, AGE-1 is a Phosphatidylinositol 3′kinase (MORRIS *et al.* 1996). *C. elegans* AGE-1 contains a p85binding domain, a PI3′kinase Ras binding domain, and a PI3′Ka accessory domain involved in substrate presentation and the catalytic domain.

In *C. elegans*, DAF-16 is a FOXO forkhead transcription factor (OGG *et al.* 1997). *C. elegans* DAF-16 is characterized by winged helix DNA binding domain and four conserved AKT/PKB phosphorylation sites.

IIS in *C. elegans* controls metabolism, life span, stress resistance and dauer larvae formation (BARBIERI *et al.* 2003; BRAECKMAN *et al.* 2001; FIELENBACH and ANTEBI 2008; GERISCH *et al.* 2001; MCCULLOCH and GEMS 2003). Research on insulin signalling in *C. elegans* has shown a complex picture: the effects of insulin signalling depend not only upon the ligand, but also upon the timing of the signal transduction and the tissue specificity of insulin signalling (PIPER *et al.* 2008).

There are conflicting reports in the literature on timing and tissue requirements of signalling. For example, Dillin *et al*. (2002) found in *C. elegans* that when IIS is interrupted in early development the result is increased diapause or dauer larvae development, but when insulin signalling is interrupted in the reproductive adult stage the result is a long lived adult (DILLIN *et al.* 2002). IIS signals that influence either life span or development are coordinated by different tissues. Wolkow *et al*. (2000), found by transformation rescue that when IIS is restored specifically to neuronal cells of a long lived mutant, the result is rescue of the wild-type life span. In contrast, Libina *et al*. 2003, found by transformation rescue and mosaic analysis that there was poor recovery of life span from neuronal specific IIS, while neuronal IIS has the greatest effect on dauer larvae formation. The dauer larvae formation phenotype of Daf-2 mutants are most effectively rescued by DAF-2 signalling in neurons (WOLKOW *et al.* 2000). Restoring *daf-16* in neurons of Daf-2;Daf-16 double mutants results in complete dauer larvae recovery whereas, there is little effect on dauer larvae recovery with *daf*-16 intestinal expression and no effect with muscle expression (LIBINA *et al.* 2003). The Daf-c phenotype of Age-1 mutants can be rescued by *age-1* expression in muscle, intestine or neurons (WOLKOW *et al.* 2000). Wolkow *et al*, also found that expression of *daf*-2 pathway genes in muscle, intestine or a subset of neurons can regulate metabolism and dauer larvae arrest but not life span (WOLKOW *et al.* 2000). Given the complexity of IIS system and the number of insulin genes, few of the insulin ligands have been characterized. However, it has been determined that at least some of the ligands act as antagonists of the insulin receptor while others are agonists (MURPHY *et al.* 2003; PIERCE *et al.* 2001).

The IIS cascade negatively regulates the FOXO forkhead transcription factor DAF-16. While the IIS pathway is activated, the *C. elegans* DAF-16 transcription factor is phosphorylated, through the activity of serine/threonine kinases (AKT1/2), and

sequestered in the cytoplasm. This allows normal reproductive growth to proceed. When the IIS pathway is inactivated, DAF-16 is dephosphorylated and enters the nucleus where it regulates gene expression to promote dauer larvae formation and repress sexual maturation (GOTTLIEB and RUVKUN 1994; LAMITINA and STRANGE 2005; LARSEN *et al.* 1995; LEE *et al.* 2003; OGG *et al.* 1997).

In summary: IIS maintains normal reproductive development - it is inactivation of the IIS pathway that results in a change of development to dauer larvae formation. In the context of parasite biology, if the hypothesis that infective larva are analogous to the dauer larva is correct, then an extension of this hypothesis might be that the IIS pathway may play a role in infective larva development and the *Parastrongyloides* free-living/parasite switch, if it plays an analogue role to dauer larvae development, than down regulation of IIS may result in infective larval development.

4.2 RESULTS: CLONING and SEQUENCING of *P. trichosuri daf-2*

Resources for *P. trichosuri* include an EST database that was derived from the parasitic adult stage, the infective larval stage and free-living stages. The library consists of 7963 sequenced clones and 2658 unsequenced clones which have been arrayed into 4324 contigs. These were sequenced as part of project funded by NIAID (National Institute of Allergies and Infectious Disease) to provide support for 125,000 ESTs from ~12 species of nematode parasites and are publically available at (http:www.nematode.net). In addition, the Bioinformatics Group, AgResearch Invermay has built contigs with the combined set of ESTs from *P. trichosuri*, and the closely related parasite species*: S. stercoralis* and *S. ratti* and these are available on the AgResearch EST database.

To isolate the *P. trichosuri daf*-2 gene orthologue, degenerate PCR primers were designed based upon protein alignment to full length *C. elegans (AAK29947), C. briggsea (AAQ90014), B. malayi (AAW50597), H. sapiens (PO8069) and X. laevis* (O73798) DAF-2 from the ENTREZ NCBI database (http://www.ncbi.nlm.nih.gov/entrez/) and the conceptual protein encoded by a *S .ratti*

translated EST(SR03316) with similarity to DAF-2 from the NematodeNet database (http://www.nematode.net/). The forward degenerate primer was designed to the conserved regions GQGSFGMV and the degenerate reverse primer was designed to the conserved region DVWSYGVV (Figure 4.1). PCR was performed on *P. trichosuri* cDNA from the iL3 stage using the degenerate Thermocycler conditions as described (Methods Section 2.6.2). The several PCR bands around the expected 636 bp region (Figure 4.2A) were first gel purified and then TA cloned into pGEMTeasy. The cloned genes were restriction mapped with *Eco RI* (Figure 4.2B) and sequenced using T7 and SP6 plasmid primers. A BLASTX search on website (http:www.ncbi.nlm.nih/gpvblast/Blast.cgi) showed that six clones had high similarity to *C. elegans* and *B. malayi* DAF-2 proteins. Primers were designed in order to walk out into unknown sequence using the GenomeWalker™ kit.

The full length *P. trichosuri daf-2* gene orthologue was cloned by sequential walk out steps from GenomeWalker™ Libraries. The method of *P. trichosuri* GenomeWalker™ Library generation, primer design and amplification are discussed in the Methods Section 2.6.5. For each walkout step, in both the upstream and downstream direction, clones were generated from one or more of the DraI, *EcoRV*, *PvuII* and *StuI* GenomeWalker™ Libraries. PCR fragments that were larger then 500 bp were chosen to be gel purified and TA cloned into pGEMTeasy. These were then sequenced and the sequences assembled using the Vector NTI (version 10.1.1) assembly program ContigExpress, which utilizes CAP3 consensus generation algorithms based on weighted sums of the quality value score (QVs) of assembled fragments (Figure 4.3). BLASTX was used to confirm that the sequence from the assembled gene fragments continued to show similarity to DAF-2 from other species. The nascent sequence thus generated was then used as template to design primers to walk further out into the genomic region. Multiple sequence alignment from sequencing clones in both directions, or multiple walkout fragments from the various GenomeWalker™ Libraries, were used to determine the genomic sequence. Where there were ambiguities, another PCR fragment was generated and used to confirm sequence. Analysis of the conceptual translation of the full length genomic sequence suggested that there were no introns and this was confirmed by a combination of RT-PCR and 5′ RACE. 3′ RACE and translation analysis and sequence alignment was used to deduce coding region and the putative stop codon. The putative coding region was amplified by PCR from both

cDNA and genomic DNA (Figure 4.4), which suggests genomic (or unspliced transcript) is of similar size to cDNA sequence.

Cloning *P. trichosuri daf-2* with GenomeWalker™ required a total of 4 walkout steps to generate 6,706 nucleotides of sequence, which includes 4464 bp of predicted coding region, approximately 2081 bp of promoter region and 161 bp 3′ UTR downstream of gene (Figure 4.5).

```
C elegans DAF-2(AAK29947)      (1248) QCGEGSFGKVYL--(n)--KSDVWSFGVVL
C briggsea DAF-2(AAQ90014)     (1208) VIGQGTFGKVYL--(n)--KSDIWSFGVVL
Human IGF1R(P08069)            (1004) ELGQGSFGMVYE--(n)--YSDVWSFGVVL
X laevis DAF2(O73798)          (1000) ELGQGSFGMVYE--(n)--NSDVWSFGVVL
S ratti daf-2 EST(SR03316)       (1)   -------------(n)--ASDVWSYGVTV
Consensus                      (1433) -GQGSFGMV----(n)----DVWSYGVV--
```

Figure 4.1: Design of *P. trichosuri daf*-2 degenerate primers
Degenerate primers designed to protein alignment of *C. elegans(Ce), C. briggsea, H. sapiens, IGF1R; X. laevis(Xl)* and translation of *S. ratti* DAF-2 EST , the conserved sequence identified for designing primers are shown in red. Forward primer: 5'-GGACAAGGATCWTTTGGAATGGT Reverse primer: 5'-CAACTCCRTAAGACCADACRT

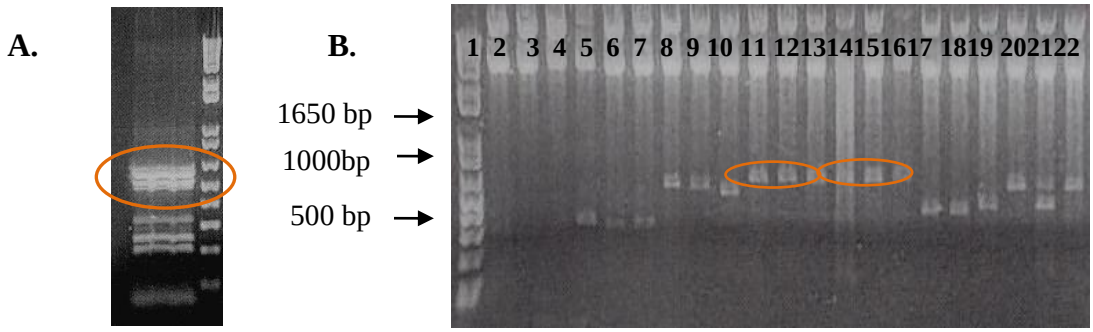


Figure 4.2: Agarose gel of *P. trichosuri* degenerate PCR for *daf-2* orthologue.
Degenerate PCR was used to amplify the 636 bp band of *P. trichosuri daf-2* gene orthologue. **A.** Several bands in this region were TA cloned (circled in red)**. B.** Clones of *daf*-2 degenerate PCR were restriction mapped with *EcoRI* and selected for sequencing with T7 primer : GTAATACGACTCACTATAGGG and BLAST X analysis. Clones 11- 16 (circled in red) contained the putative *daf*-2 orthologue.

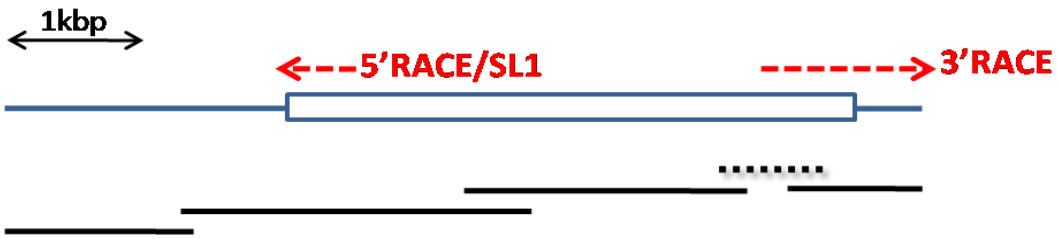


Figure 4.3: ContigExpress assembly of *P. trichosuri daf-2* walkout fragments derived using GenomeWalker™

Assembly of *P. trichosuri daf-2* orthologue sequences cloned from *P. trichosuri* genomic DNA using GenomeWalker™ as describe in the Methods section and assembled using Vector NTI (version 10.1.1) assembly program: ContigExpress. Black solid lines indicate GenomeWalker™ fragments; dashed black line is original degenerate PCR cloning fragment; red dashed lines indicate location of 5′and 3′RACE products and blue line is final assembly with the blue square indicating location of putative coding region. For primers see GenomeWalker primers in Appendix 3.

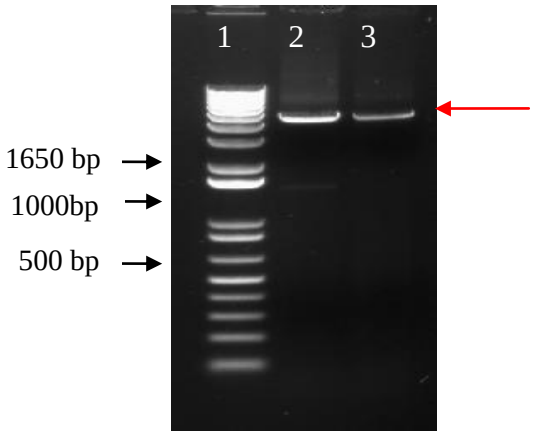


Figure 4.4: Agarose gel of *P. trichosuri daf*-2 putative coding region from genomic DNA and cDNA

The deduced coding region of the *P. trichosuri daf-2* orthologue, amplified by PCR, from *P. trichosuri* cDNA and genomic DNA template. Lane 1: 1KB Plus MW marker; Lane 2: *Pt daf-2* gDNA Lane 3: *Pt daf-2* from cDNA fwd primer: GCAAAACTAATGATTTATGCTATG Rev primer: CTATTTTT CATCAATTTCATCTG

4.3 RESULTS: ANALYSIS OF GENE STRUCTURE OF *P. trichosuri daf-2*

P. trichosuri daf-2 nucleotide and deduced polypeptide sequence is shown in Figure 4.5. The predicted coding region consists of a single open reading frame of 4464 bp. The translational start site was identified based upon a comparison of the protein sequences of DAF-2 from other organisms using BLASTX and Vector NTI sequence alignment program AlignX. The start site and 5′ UTR were confirmed with 5′ RACE and SL1

primer PCR on *P. trichosuri* cDNA, and the putative 3′ UTR region was determined by 3′ RACE. The 5′ UTR and 3′ UTR are highlighted dark gold, the coding region is highlighted yellow.

Alignment of *P. trichosuri daf-2* translated sequence to *B. malayi,* (Bm); *C. elegans,* (Ce); *D. melanogaster,* (Dm); *H. sapiens,* (Hs); and *X. laevis* (Xl) shows that *P. trichosuri daf-2* gene is similar of similar length to that of the *B. malayi,* (Bm); *H. sapiens,* (Hs); and *X. laevis* (Xl),whereas both *C. elegans* and *D. melanogaster daf-2* have longer 5′ and 3′ coding regions. See Appendix 5.

Figure 4.5: *P. trichosuri daf-2* gene sequence and structure:

```
   1  CTGCTTTCTC TTCTTCTAAT TTTTTAGGTT CATCCTTTTC TTTATTTTGT TGATGCTTCT
  61  TTTTTCTTGG ATCATATAAG AAAGCTTTTT TTTTTTCCAT CTCCAGTTGT AGTTTCTTGT
 121  TTTCCTTCAC GACTAGGTAC CTGTAAACCA CGGGCTTCTA ATAGTTCTCT AGCTTTTTGT
 181  TGAGCTAATC TTTGTGCTGG TGTCAACCAT TTACCTTCTC TTTTCAATTG TGCTTCACGT
 241  TCCTTCTTTT CAGCTTTAAT TCTAGCCTTT CTTTCTTCTT CTAATCTCGC TTCTTCTTCA
 301  CGTTTCTTTT CTAATGCCTC AAGCCTTTCT TCCTCTAATG CAGCTAATCT TAAAGCTTCT
 361  TCCCTTTCTT TCTCCTGTTG TTCTAAAAGT AATTTCAAAG CTGCCATCTT TTTATTTCCT
 421  TTTTTTTTCT TTTCAGATCC CTCTTTAATC TCTTCTTTTG TTTCCTCTTT TATAGTATCA
 481  TCATTACCGG GTTCTTCCTT TTTCTGAGCT TTTTTTGCTT TCATTTTTTC TCTTTTAGCT
 541  TTAGCTTTTT CACGTTTTAT ACGTTGTTCC TCTGTTTCTT CCTCTGGTGG TATTAATGAA
 601  GTTTTAGGTT CATTACTTTC CAATGCAGCT AAAATAGCAT CGATATCATC ATCAGCTTTT
 661  CTAACATTTC CTTTATTTTT TTTTGGTGGC ATCGTAAACA ATTTTCTAAA AAAAATTAAT
 721  ATTTTTAAAT TAAAGTTATT TCAAAATACA TAATATATTA AATTTATAAT AAAATATTTA
 781  AAAAAGCAAT CAAAATATAA TAAATTAAAA AGATAAAATA CGCTTTATGT GATTAAATCA
 841  TATGTTTATA CTTTAAAAAT AATAAATAAA TAAAGTCGTA AACATGAAAC TTGGTGCCTA
 901  CAAAATTATA TTATGAAACA GTTTACAAAT AATCGTGGTC AGAGAAAATG TACATATTTT
 961  TTAACAAATC TCATTTTCCT AAAGATAATG TTTTAATAAT TATTAAAATG TTACTTCCTT
1021  AAAACAATAA TAACAATATT TGTCATTAAA ATGAAGTTCG ATTCAAGAAA GTAATATTTA
1081  TGGAAATCTT TAATGATATA CATAAACCAT ATGTTTCTTG CCACCTAATA TGACAGTGTA
1141  ATAGTGAGTA ATATTTCTCC AAAATACAAA ATAACCTCTT ATACACAATT GTTTGATTAT
1201  TACTACACTT TTTCTATGTC ATATTAATTA TTTATCCAAT TATTATTTTT ATAATTCTCT
1261  TCATGTTATT TTCAATATTT GTCATAATGT AACACTATAA TTGTAATTTT TTTAATATTT
1321  TAATAATAAA GTTTTATTTT AAATATTTTT AAGATGAATA TTAAAAGATG ATATAAACTT
1381  AATTAAGCTT CAATTTTTCG TGAATATTTT TTGTTTTGAT AACAAGAAAT GTTAATTAAT
1441  TTTAAAAAAT ATTAACGTCA TAAATTTATA CGTATATGAA TTCATCTCTC TCCTATTCTA
1501  TAAGACGATT ATACATTATA TTTTGTATGA TTTTCAATCT TACCAACTAA GATGCTTTTA
1561  TCCGGCTTCC TATATGAATG AGAAATGATA ATATTAAATT CTTTTTCATT CTTTTTTAAC
1621  TACTTAATAT ATACATACCT CTTATTAATA TATATATCAT ACATTCATTA TTAATATAAA
1681  TATTTTTATT TTAAATATTA TTTAATCATA ATTTATTTCT TTAAATATAT ACAGACGTGC
1741  TTAAGTTTGA AATAGTTATT CTCACCTTAA TTGTCATTCA TTTTCATCTC TATATCTTTT
1801  GACTTCCTGT AATTTCATTA TTTTTATTTT TATACGTTAA AATTTAAAAA TTTTAATACT
1861  TTTTACGCGT GAAAAGATAT ATTAAATGAG AATATCGTTA ATGTTTTAAT TTTTCAGCAA

1921  AACTAATGAT TTATGCTATG CTTATTAATT TACTAATAAC TGCCTAATAT ATATTACATA
1981  AGATTCCTTA ATCAACAATT TTTAATGATA CTATTTTGAC TTGATACATT TGGGAGACAC

                                                        M  N  F  K  K  F  F
2041  CTTATTGTAA GGAAATTTTT ATTATATTTT ATTAAATATC AATGAATTTT AAAAAATTTT
      ·  R  E  C  Y  Y  L  K  H  F  I  L  I  T  I  L  F  I  S  Q  F  ·
2101  TTCGTGAATG TTATTATTTA AAACATTTTA TATTAATAAC TATACTATTT ATAAGTCAAT
      ·  H  C  H  I  N  D  T  I  C  A  G  V  S  I  K  F  K  L  F  E  ·
2161  TTCATTGTCA TATAAATGAT ACCATTTGTG CTGGGGTTTC AATAAAATTT AAATTATTTG
      ·  K  F  F  L  K  N  G  A  Y  N  A  K  I  Q  K  C  T  V  M  E  ·
2221  AAAAGTTTTT CCTCAAAAAT GGTGCATACA ATGCTAAAAT TCAGAAATGT ACTGTTATGG
      ·  G  D  L  V  L  A  E  P  G  I  P  E  N  T  I  T  F  S  N  L  ·
2281  AGGGTGATTT AGTGTTAGCA GAACCAGGTA TTCCTGAAAA TACAATTACA TTTTCAAATT
      ·  R  E  I  T  G  T  L  V  I  Y  G  F  H  H  I  N  S  L  T  S  ·
2341  TACGTGAGAT AACGGGTACA CTTGTAATAT ATGGTTTTCA TCATATAAAT TCATTGACGT
      ·  V  F  P  K  L  A  V  I  G  G  N  S  L  I  I  N  Y  A  F  I  ·
2401  CAGTTTTTCC AAAACTTGCC GTTATTGGTG GAAATTCTCT TATCATTAAT TATGCTTTCA
      ·  V  H  S  C  F  N  L  S  Y  V  G  I  P  T  L  K  L  I  K  K  ·
```

```
2461        TTGTTCATAG TTGTTTTAAT TTATCATATG TCGGCATACC AACTCTAAAA CTTATTAAAA
            · G  G  I  R  I  T  N  N  D  N  L  C  Y  T  Q  T  L  D  W  S ·
2521        AGGGTGGAAT ACGTATAACA AACAATGATA ACCTTTGTTA TACACAAACA CTAGACTGGA
            · H  I  A  D  G  R  V  G  N  I  I  V  E  D  S  S  K  T  R  C ·
2581        GTCATATTGC TGATGGAAGA GTAGGCAATA TAATTGTGGA AGACTCCTCA AAAACTAGAT
            · P  K  F  C  K  V  D  N  E  K  L  C  H  R  K  N  D  K  I  A ·
2641        GTCCTAAATT TTGTAAAGTC GATAATGAAA AATTGTGCCA TAGAAAGAAT GATAAAATTG
            · C  W  S  K  N  V  C  Q  S  K  C  E  Y  I  G  T  G  V  E  K ·
2701        CATGTTGGAG TAAAAATGTA TGTCAAAGTA AATGTGAATA TATAGGAACT GGAGTTGAGA
            · G  P  G  C  S  N  D  G  E  K  C  H  D  L  C  V  G  G  C  T ·
2761        AAGGCCCCGG ATGTTCAAAT GATGGAGAAA AATGTCATGA TCTTTGTGTT GGAGGATGTA
            · K  V  G  D  P  G  Y  C  N  S  C  R  Y  T  M  H  N  N  I  C ·
2821        CTAAAGTTGG AGATCCAGGT TATTGTAATT CTTGTAGATA TACTATGCAT AATAATATTT
            · V  K  K  C  P  Q  G  Y  Y  N  Y  M  N  H  R  C  V  T  K  E ·
2881        GTGTTAAAAA ATGTCCACAA GGATATTATA ATTATATGAA CCATAGATGC GTAACTAAGG
            · E  C  L  N  M  K  P  Y  L  D  L  A  S  G  L  N  I  L  E  E ·
2941        AAGAATGTTT AAATATGAAG CCTTATTTGG ATTTAGCGTC TGGATTAAAT ATATTGGAAG
            · Y  K  P  T  D  D  G  L  C  T  T  K  C  P  K  N  Y  E  E  D ·
3001        AATATAAACC AACAGACGAC GGATTATGTA CAACAAAATG CCCAAAAAAT TATGAAGAAG
            · K  A  D  P  K  H  C  V  S  C  K  G  N  C  L  K  R  C  F  S ·
3061        ATAAGGCAGA TCCAAAGCAT TGTGTATCGT GTAAAGGAAA TTGTTTAAAA CGTTGTTTCT
            · N  V  D  V  D  S  I  A  A  L  E  R  F  K  G  C  Q  I  V  E ·
3121        CTAATGTTGA TGTAGATTCA ATAGCTGCAT TAGAAAGATT TAAAGGTTGT CAAATTGTGG
            · G  N  F  T  L  K  L  I  A  G  T  S  D  L  S  P  E  K  L  E ·
3181        AAGGAAATTT TACTTTAAAA TTAATTGCAG GTACATCAGA TTTATCTCCG GAAAAACTTG
            · E  C  L  G  D  I  E  T  I  N  G  Y  L  Q  I  H  F  T  P  S ·
3241        AAGAATGTCT TGGTGATATT GAAACAATTA ATGGTTATCT ACAAATACAT TTTACTCCTT
            · V  I  S  L  H  M  F  K  S  L  R  E  I  R  G  E  F  L  Y  N ·
3301        CAGTTATATC ACTTCATATG TTTAAGAGTC TTCGTGAAAT AAGAGGAGAA TTTTTATATA
            · D  N  Y  A  L  V  I  E  Y  N  E  N  L  Q  T  L  F  P  A  D ·
3361        ATGATAATTA TGCGCTTGTA ATAGAATATA ATGAGAATTT ACAGACATTA TTTCCTGCAG
            · G  K  N  I  T  I  K  N  G  N  V  T  I  T  N  N  L  Q  L  C ·
3421        ATGGAAAAAA CATTACAATT AAAAATGGAA ATGTAACTAT AACTAATAAT CTCCAACTTT
            · E  D  K  A  K  A  F  L  R  V  V  G  K  Y  Y  N  N  T  E  Y ·
3481        GTGAGGATAA AGCTAAAGCA TTTCTGAGAG TGGTCGGTAA ATATTATAAT AATACAGAAT
            · L  N  A  L  K  T  N  G  E  R  A  I  C  N  E  R  I  L  N  L ·
3541        ATTTAAATGC ACTTAAAACA AATGGAGAAA GAGCTATTTG TAATGAAAGA ATTCTAAATC
            · T  A  I  N  Q  E  N  E  T  A  S  G  F  I  T  L  K  W  A  P ·
3601        TTACAGCAAT TAATCAAGAA AATGAGACTG CTAGTGGATT TATTACATTA AAATGGGCAC
            · L  N  I  T  E  M  D  Y  R  K  F  R  A  Y  Q  I  Y  Y  K  K ·
3661        CATTAAATAT TACAGAAATG GATTATAGAA AATTTCGTGC TTATCAAATA TATTATAAAA
            · V  P  D  N  V  T  K  V  D  I  F  A  N  R  S  A  C  G  D  A ·
3721        AAGTTCCGGA TAATGTAACA AAGGTGGACA TATTTGCAAA TCGTTCTGCT TGTGGGGATG
            · W  K  S  I  I  I  E  D  T  K  H  T  G  T  T  I  S  N  L  E ·
3781        CTTGGAAAAG TATTATTATT GAAGATACAA AACATACTGG AACAACAATT AGTAACTTGG
            · P  Y  T  W  Y  A  V  Y  V  E  T  K  V  M  P  H  N  T  A  F ·
3841        AACCATATAC ATGGTATGCC GTATATGTCG AAACTAAAGT TATGCCACAT AATACGGCTT
            · K  A  R  S  L  V  V  I  T  R  T  A  P  G  R  P  S  N  V  Q ·
3901        TTAAGGCAAG ATCTCTAGTT GTTATAACAA GAACTGCTCC AGGAAGGCCA TCAAATGTAC
            · N  V  D  I  K  V  S  N  N  R  E  I  D  I  K  W  S  P  P  S ·
3961        AAAATGTTGA CATTAAAGTT TCGAACAATA GAGAAATTGA TATAAAATGG AGTCCACCAT
            · K  P  N  G  I  I  A  Y  Y  E  V  S  W  K  L  I  P  H  T  L ·
4021        CTAAACCAAA TGGAATTATA GCATATTATG AAGTTTCATG GAAATTAATA CCTCACACAC
            · E  S  I  E  D  D  P  C  D  T  K  A  T  A  R  R  T  E  R  I ·
4081        TCGAGTCAAT AGAAGATGAT CCTTGTGATA CAAAAGCTAC TGCTCGTCGT ACAGAAAGAA
            · L  A  N  E  N  L  S  K  K  S  N  D  D  V  E  T  C  S  A  V ·
4141        TTTTGGCAAA TGAAAATTTA TCTAAAAAAT CAAACGATGA TGTTGAAACT TGTTCTGCTG
            · K  G  C  C  K  C  P  E  E  E  N  L  E  N  E  N  M  Y  T  K ·
4201        TCAAAGGATG CTGTAAATGT CCTGAGGAAG AAAACCTTGA AAATGAAAAT ATGTATACCA
            · Q  V  N  N  I  N  T  D  E  T  Y  K  K  E  F  E  N  H  D  F ·
4261        AACAAGTAAA TAACATTAAC ACTGATGAAA CTTATAAAAA AGAATTTGAA AATCATGATT
            · Q  D  K  M  Q  N  L  V  W  K  Q  R  V  K  K  H  I  L  S  L ·
4321        TTCAAGATAA AATGCAAAAT TTGGTTTGGA AACAAAGAGT TAAAAAACAT ATTTTATCAC
            · T  S  N  R  F  V  R  A  I  T  S  N  P  V  A  I  I  D  K  E ·
4381        TTACATCAAA TAGATTTGTT AGGGCAATAA CAAGCAATCC TGTTGCAATT ATTGATAAAG
            · K  K  K  N  Y  V  E  S  C  Y  G  S  T  R  S  P  D  T  E  G ·
4441        AAAAAAAGAA AAATTATGTT GAATCTTGTT ATGGATCAAC TAGGTCTCCC GATACTGAAG
            · N  K  D  E  N  I  G  E  I  I  E  K  L  D  V  P  V  N  Y  N ·
4501        GAAATAAGGA TGAAAATATT GGTGAGATAA TTGAGAAATT AGATGTACCA GTAAATTATA
            · D  E  G  K  I  R  I  N  E  S  S  E  L  Q  F  T  I  T  N  L ·
4561        ATGATGAGGG AAAAATAAGA ATTAATGAAA GTTCTGAACT TCAATTTACG ATAACGAATC
            · R  H  Y  G  E  Y  Y  I  V  I  N  V  C  L  I  G  I  Y  D  K ·
4621        TTCGTCATTA TGGAGAATAT TATATTGTTA TAAATGTTTG TCTTATTGGA ATTTATGATA
            · S  S  T  Q  D  K  N  D  F  C  C  K  Q  P  Y  H  T  S  Q  I ·
4681        AAAGTAGTAC ACAAGATAAA AATGATTTTT GTTGTAAACA ACCATATCAT ACATCACAAA
            · T  A  K  Q  L  N  F  D  K  I  N  E  D  S  I  F  A  L  N  T ·
4741        TTACAGCTAA ACAATTAAAT TTTGACAAAA TTAATGAAGA TTCAATATTT GCATTAAATA
```

```
          · T N E  Q N N  Q I V T  W N N  P T N  P N G P ·
4801      CTACAAATGA ACAAAATAAT CAAATTGTAA CGTGGAATAA TCCGACAAAT CCTAATGGTC
          · V L G  Y K V  T L K N  M D T  E Q T  P L Q Q ·
4861      CTGTTTTGGG ATATAAAGTA ACATTGAAAA ATATGGATAC AGAGCAGACA CCTCTTCAAC
          · C I S  V S N  L R V G  K N G  N R E  L P Y A ·
4921      AATGTATTTC AGTATCTAAT TTACGTGTTG GTAAAAATGG AAATCGTGAA TTACCTTATG
          · N F T  G L S  N G R Y  S I A  I R T  I S L A ·
4981      CTAATTTTAC TGGATTATCA AATGGAAGAT ATTCAATTGC TATAAGAACA ATTTCATTAG
          · G L S  D E V  V Y N D  L F T  I N V  P G I F ·
5041      CTGGATTAAG TGATGAAGTT GTGTACAATG ACTTATTTAC GATCAATGTC CCAGGAATCT
          · T P A  K I A  I I A S  L M I  C M I  L L M I ·
5101      TTACACCCGC AAAAATTGCA ATTATAGCTT CATTAATGAT ATGTATGATT CTTTTGATGA
          · L S I  Y Y Y  F N R S  F G K  K V T  E A V R ·
5161      TTCTATCAAT TTACTATTAC TTTAACCGTA GCTTTGGAAA GAAAGTTACT GAAGCCGTTC
          · Q T I  S S N  P E Y L  S Q F  D V Y  K Q D E ·
5221      GGCAAACAAT ATCATCTAAT CCTGAGTATC TTTCACAATT TGACGTTTAC AAACAAGATG
          · W E L  K R S  D I V L  E E Q  I G S  G T F G ·
5281      AATGGGAACT AAAACGTTCT GATATTGTTT TAGAAGAACA AATAGGTAGT GGAACATTTG
          · N V Y  K G F  G N N V  S T A  S G I  K F G P ·
5341      GAAATGTATA TAAAGGTTTT GGTAATAATG TATCAACAGC TTCTGGAATT AAATTTGGTC
          · C A I  K T V  R D S A  T P A  E K L  H F L F ·
5401      CTTGTGCTAT TAAAACTGTT AGAGATAGTG CTACGCCAGC AGAGAAACTT CATTTTCTTT
          · E A S  V M K  K F N T  A F I  V K L  Y G V V ·
5461      TTGAAGCTAG TGTTATGAAG AAATTTAATA CAGCATTTAT TGTTAAATTA TATGGTGTTG
          · S E G  Q P V  L V V M  E M M  E K G  N L R D
5521      TTAGTGAAGG ACAGCCTGTT CTGGTAGTAA TGGAAATGAT GGAAAAAGGA AATTTGAGAG
          · F L R  S H R  P D S E  E N V  D N R  P V P T ·
5581      ACTTTTTAAG ATCTCATAGA CCAGACTCAG AAGAAAATGT TGACAATAGA CCAGTACCAA
          · S Q K  L A N  W A A Q  I A D  G M A  Y L E S ·
5641      CATCACAAAA ACTTGCAAAT TGGGCAGCAC AAATTGCCGA TGGAATGGCT TATTTAGAAA
          · F K F  C H R  D L A A  R N C  L V H  R D E S
5701      GTTTCAAATT TTGTCACAGA GATTTAGCTG CCAGAAATTG CCTTGTTCAT CGTGATGAAA
          · V K I  G D F  G M A R  D I Y  Y H E  Y Y Q P
5761      GTGTTAAAAT TGGTGATTTT GGTATGGCTA GAGATATTTA TTATCACGAA TATTATCAAC
          · T G K  R L I  P V R W  M A Q  E S L  K D G K ·
5821      CAACAGGAAA ACGTCTTATT CCTGTAAGAT GGATGGCTCA AGAAAGTTTA AAGGATGGGA
          · F S V  K S D  V W S Y  G I V  L Y E  M L T L ·
5881      AATTTTCAGT GAAATCTGAC GTTTGGTCAT ATGGAATTGT ATTGTATGAA ATGCTAACAT
          · A Q Q  P Y A  G L D N  P D V  F D Y  I V T S ·
5941      TAGCACAACA ACCTTATGCT GGACTTGATA ATCCAGATGT CTTTGATTAT ATTGTTACAT
          · R R I  L S R  P Q G C  A D F  W [Y N I M] R S
6001      CGAGAAGAAT TTTATCAAGA CCACAAGGAT GTGCTGACTT TTGGTATAAT ATTATGAGAA
          · C W K  Y N P  S D R P  S F F  Q I L  M H L Q ·
6061      GTTGTTGGAA ATATAACCCA AGTGATAGAC CATCATTTTT TCAGATTCTT ATGCATCTTC
          · P Y T  T D E  F K Q Q  S F V  I N N  Y D K A ·
6121      AACCATATAC GACAGATGAA TTTAAACAAC AATCATTTGT TATTAATAAT TATGATAAGG
          · N N I  R D D  Y E F D  I P E  D E D  E E E E ·
6181      CAAATAATAT AAGAGATGAC TATGAATTTG ATATTCCAGA AGATGAGGAT GAAGAAGAAG
          · E V D  E E N  E E E N  E N N  N D I  D S E S ·
6241      AGGAGGTTGA TGAAGAAAAT GAAGAAGAAA ATGAAAATAA TAATGATATT GATAGTGAGA
          · D A M  L P E  N F F S  E T H  S T E  D Q T S ·
6301      GTGATGCAAT GTTACCGGAA AATTTTTTCT CTGAAACTCA TTCAACAGAA GATCAAACAA
          · T T D  K E I  L D I E  S N L  K H Y  N N E E ·
6361      GTACAACTGA TAAAGAAATA TTGGATATTG AAAGCAATTT AAAACATTAT AATAATGAAG
          · T S P  I L L  D D D D  I E L  M E L  E N T S ·
6421      AAACATCACC AATATTATTA GATGATGACG ATATAGAGTT GATGGAATTA GAAAATACAA
          · T V K  E F N  V N G Y  G R L  P T D  E I D E ·
6481      GTACTGTTAA AGAATTTAAT GTAAATGGTT ATGGTAGACT TCCTACAGAT GAAATTGATG
          · K *
6541      AAAAATAGAT TTCCTGAAAC ATTTTAAATT ATATATATTT ACTGGTATTT AAAAAAGGCA
6601      TATAGGATGA TTATTTTTAT GTTATAACTT CAATGTGTAT ATATTT[AATA AA]AAAAAAAA
6661      TTGATTTTAA TAAAAATAAT TTAGCTTAAA GAAAAAAAAA AAAAA
```

Coding region is highlighted in yellow
UTR regions are highlighted in dark gold
Introns are un-highlighted and lower case
Signal Peptide is highlighted in grey
IRS-1 [YxxM] motif is boxed
3′ UTR Polyadenylation site [AATAAA] motif is boxed

4.4 ANALYSIS OF THE DEDUCED PROTEIN STRUCTURE OF *P. trichosuri* DAF-2

4.4.1: Structure of *P. trichosuri* DAF-2 and motif analysis

The deduced amino acid sequence is 1488 aa and has an unmodified molecular weight of 162 kD. This polypeptide shares an overall 35.9% aa similarity with *C. elegans* DAF-2; the regions of highest similarity are in the protein kinase active domain, which has a local similarity of 72.3%. Protein alignment revealed that there are four cysteine residues conserved between *C. elegans*, *P. trichosuri* and *B. malayi*, which are thought to be involved in interchain disulfide bonds to form the holoreceptor (PATEL *et al.* 2008). A local installation of InterProScan (ZDOBNOV and APWEILER 2001) was used to scan the translated sequences against the member databases of InterProScan. A signal peptide was identified at amino acids 1-29, and the signal anchor is highlighted in grey (Figure 4.5), suggesting that this polypeptide is directed to a membrane surface.

Like *C. elegans* DAF-2, the putative *P. trichosuri* DAF-2 has two predicted ligand binding domains separated by a furin like domain, which has a local similarity of 44.6%. Motif analysis using InterProScan showed several significant differences between the *P. trichosuri* DAF-2 and *C. elegans* DAF-2 (Table 4.1), such as the presence of three fibronectin type III domains in *P. trichosuri* versus one fibronectin type III domain in *C. elegans*.

The predicted *P. trichosuri* DAF-2 protein has one IRS-1 site (YxxM), at position 1322 aa. IRS-1 sites are thought to be involved in binding PI3′ K adaptor subunits (WOLKOW *et al.* 2002). This IRS-1 site is conserved between *C. elegans*, *P. trichosuri* and *B. malayi* and is located within the C terminus of the pKinase domain (Table 4.1). While *P. trichosuri*, *B. malayi*, human and mouse each have one IRS-1 site, in contrast, both *D. melanogaster* and *C. elegans* DAF-2 have three such IRS-1 sites within the C terminal region of the pKinase domain. For a summary of the protein motifs of *P. trichosuri* and *C. elegans* DAF-2 orthologues see Figure 4.6 and Table 4.1.

Analysis of translated protein motifs using InterProScan		
Tyrosine Kinase Domain (pKinase domain)		
C. elegans	at position 1243-1517 aa	Similarity 72.3%
P. trichosuri	at position 1075-1346 aa	Identity 60.2%
L domain – Furin like – L domain		
C. elegans	at position 177-624 aa	Similarity 44.6%
P. trichosuri	at position 62-479 aa	Identity 30.8%
IRS-1 binding sites (YxxM)		
C. elegans	3	1474 aa, 1493 aa, 1623 aa
P. trichosuri	1	1322 aa
Fibronectin Type III domains * **using HMMPfam analysis**		
C. elegans	1	1070-1163aa
P trichosuri	3	504-605aa, 623-661aa, 899-990aa

Table 4.1: DAF-2 protein motifs of *P. trichosuri* and *C. elegans*

Protein motifs of *P. trichosuri* and *C. elegans* DAF-2 using the suite of analysis programs of InterProScan and Vector NTI. The more stringent analysis of HMMPfam was chosen for the identification of fibronectin type III domains. *Using HMMPfam for analysis P. trichosuri has 3 fibronectin Type III domains with e values of 0.39, 2.8e-06 and 0.11 respectively and *C. elegans* has 1 fibronectin Type III domain with an e value of 0.0024. The analysis program HMMSmart suggests that *C. elegans* has 3x fibronectin Type III domains with e values of 8.84, 7.57 and 12.43 respectively.

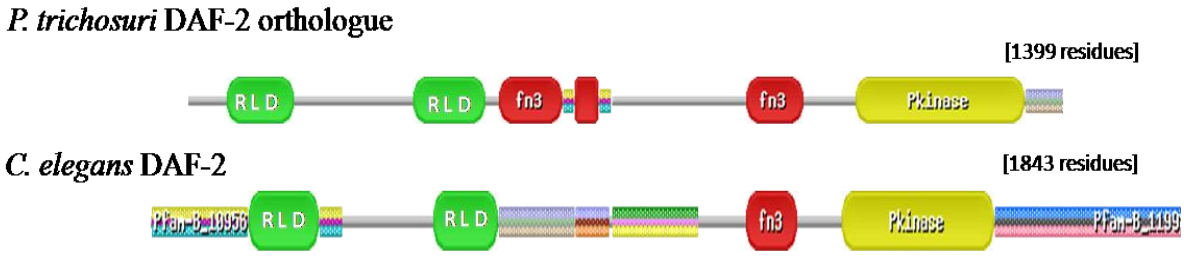


Figure 4.6: Prosite functional motif analysis of *P. trichosuri* DAF-2 orthologue and *C. elegans* DAF-2.
Prosite functional motifs as predicted by www.sanger.ac.uk/Software/Pfam. RLD (green): Receptor L Domain make up the bilobal ligand binding site; fn3 (red): fibronectin type III domain contain sites for binding DNA, heparin and cell surface interactions; Pkinase (yellow): catalytic core of protein kinase.

4.4.2 Phylogenetic tree

C. elegans has a family of tyrosine receptor kinases. In order to determine how closely related the putative *P. trichosuri* DAF-2 orthologue is to *C. elegans* DAF-2, a member of the IIS pathway, phylogenetic analysis of the entire polypeptide sequence of the

putative *P. trichosuri* DAF-2 orthologue was compared to receptor tyrosine kinases belonging to the IIS pathway of Ce, *C. elegans*; Bm, *B. malayi*; Dm, *D. melanogaster*; Hs, *H. sapiens*; Mm, *M. musculus*; Xl, *X. laevis*; along with several *C. elegans* tyrosine kinases (TRK) not associated with the IIS pathway (Figure 4.7). Analysis was performed using the ProML program of the PHYLIP (Phylogeny Inference Package) version 3.68. The putative *P. trichosuri* DAF-2 orthologue is most closely related to *B. malayi* DAF-2, the filarial parasitic nematode, followed by *C. briggsea* and *C. elegans.* Table 4.2 tabulates the evolutionary distances in the phylogenetic tree between sequences and the numbered nodes on the tree; the distance represents the estimated number of mutations per 100 aa necessary to change one sequence to the other (followed in brackets by the upper and lower limits of the branch length); the asterisks give the level of significance.

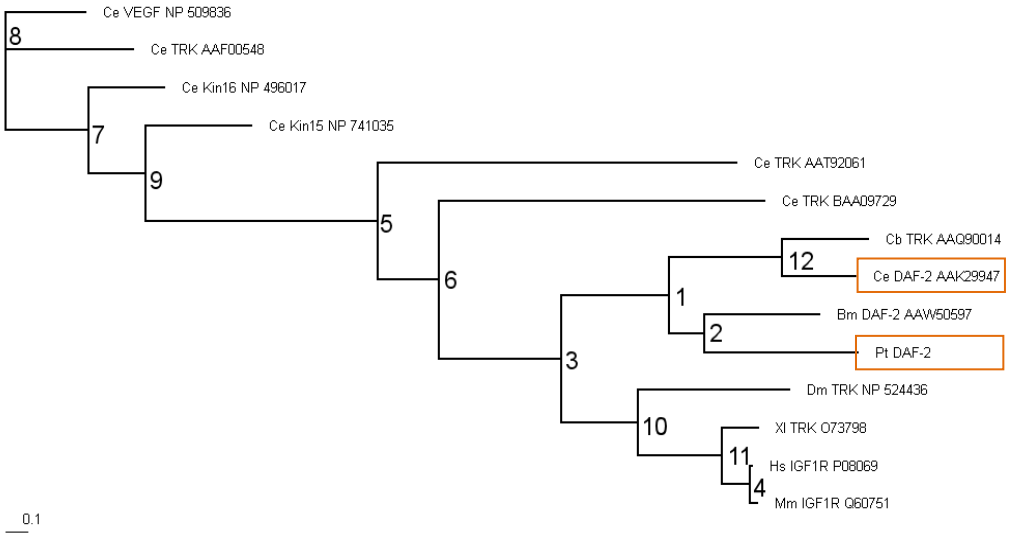


Figure 4.7: Phylogenetic analysis of Insulin/IGF receptor protein alignments.
Phylogenetic guide tree was constructed on full length proteins sequence, using the ProML program version 3.68 of the PHYLIP package (FELSENSTEIN 2005) This program uses Maximum Likelihood in order to create a tree with the highest probability. The protein sequences for this analysis were retrieved from a Pubmed/Entrez search the Dm, *D. melanogaster*; Hs, *H. sapiens*; Mm, *M. musculus*; Xl, *X. laevis*; Ce, *C. elegans*; Bm, *B. malayi*; Pt, *P. trichosuri*. GenBank or GenPept accession numbers follow two letter species designation.

Table 4.2: The distances between the nodes of the Phylogenetic analysis of Insulin/IGF receptor protein alignments are given in the following table followed by the upper and lower limits in parenthesis. Maximum Likelihood method was used to estimate distances and give confidence limits.

Between	And	Distance(upper, lower limits)	Confidence Limits
8	Ce_TRK_AAF00548	0.56141 (0.48644 0.63637)	**
8	Ce_VEGF_NP_509836	0.35260 (0.28683 0.41854)	**
8	7	0.36440 (0.26986 0.45893)	**
7	Ce_Kin16_NP_496017	0.33448 (0.25236 0.41677)	**
7	9	0.25004 (0.14469 0.35536)	**
9	5	1.02231 (0.82276 1.22180)	**
5	Ce_TRK_AAT92061	1.57259 (1.35641 1.78864)	**
5	6	0.26571 (0.11334 0.41808)	**
6	3	0.53713 (0.40827 0.66604)	**
3	1	0.47402 (0.38750 0.56054)	**
1	12	0.49605 (0.42712 0.56492)	**
12	Cb_TRK_AAQ90014	0.37641 (0.33177 0.42106)	**
12	Ce_DAF-2_AAK29947	0.32457 (0.28150 0.36772)	**
1	2	0.15606 (0.09995 0.21233)	**
2	Bm_DAF-2_AAW50597	0.50421 (0.44182 0.56644)	**
2	Pt_DAF-2	0.67479 (0.60421 0.74537)	**
3	10	0.34001 (0.25853 0.42134)	**
10	Dm_TRK_NP_524436	0.66461 (0.59094 0.73817)	**
10	11	0.36485 (0.30345 0.42627)	**
11	Xl_TRK_O73798	0.16246 (0.13347 0.19145)	**
11	4	0.12511 (0.09803 0.15236)	**
4	Hs_IGF1R_P08069	0.01034 (0.00310 0.01753)	**
4	Mm_IGF1R_Q60751	0.02999 (0.01987 0.04011)	**
6	Ce_TRK_BAA09729	1.43175 (1.26168 1.60168)	**
9	Ce_Kin15_NP_741035	0.46438 (0.35661 0.57229)	**

Distance = # of mutations per 100 aa to change one sequence to another
** = significantly positive, $P < 0.01$

4.5 RESULTS: CLONING AND SEQUENCING OF *P. trichosuri age-1*

Bioinformatics was used to assess whether ESTs to the any of the IIS pathway genes were contained within the AgResearch *P. trichosuri* CS 52 contig library. BLAST X results that showed the library contained an EST singleton sequence 011002CS5200001fa94 which aligned to part of the catalytic domain of *C. elegans* AGE-1 with an E value of 2e-44.

Primers were designed to amplify this 517 bp fragment from *P. trichosuri* cDNA prepared by RT-PCR from total RNA from the *P. trichosuri* infective larvae stage as described in the Methods (Section 2.5.4 through 2.5.7). The resulting PCR fragment from a $MgCl_2$ titration PCR (Figure 4.8) was column purified and then TA cloned into pGEMTeasy. The cloned gene was sequenced from the plasmid using T7 and SP6 primers. BLAST X search on the NCBI website (http:www.ncbi.nlm.nih/gpvblast/Blast.cgi) showed the sequence had high similarity to *C. elegans* AGE-1 and was subsequently used to design primers in order to walk out in both directions using the GenomeWalker™ kit.

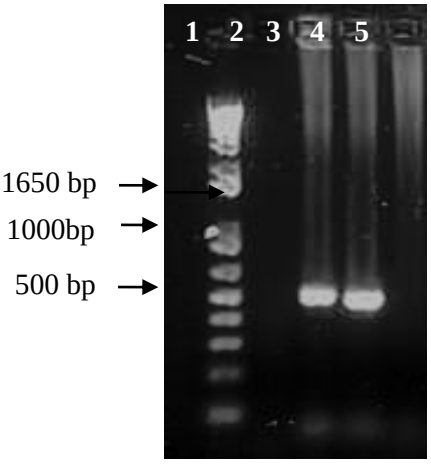


Figure 4.8: PCR $MgCl_2$ titration of *P. trichosuri age-1* gene fragment.
Primers designed to *P. trichosuri* EST singleton from the AgResearch *P. trichosuri* CS52 library which had high similarity to *C. elegans* AGE-1. Fwd primer: GTGTAACATTATTTGAGATTCAAAC Reverse primer: GCTTTTAAATAGTTCAAATCTTCC $MgCl_2$ titration PCR was performed on cDNA derived from *P. trichosuri* infective larvae total RNA. Lane 1: 1KB Plus MW marker; Lane 2: Pt-*age1cDNA* 1.5mM $MgCl_2$ Lane 3: *Pt-age1*cDNA 2.5mM $MgCl_2$ Lane 4: *Pt-age1*cDNA 3.5mM $MgCl_2$ Lane 5: Neg Control.

Primers were designed for GenomeWalking as described in the Methods (Section 2.6.5). For each GenomeWalker™ step in both the upstream and downstream direction, clones were generated from one or more of the *DraI, EcoRV, PvuII and StuI* GenomeWalker™ libraries. Fragments that were larger then 500 bp were chosen to be gel purified and TA cloned, these were then sequenced and the sequences assembled using the Vector NTI (Version 10.1.1) assembly program ContigExpress, as was described for *daf-2*. BLASTX was used to confirm that the new gene fragments cloned

continued to show similarity to AGE-1 sequence from other species. The resulting assembly was then used as template to design primers to walk further out into the flanking genomic region. Multiple sequence alignment from sequencing clones in both directions, or multiple walkout fragments from the various GenomeWalker™ Libraries, were used to determine sequence. Where there were ambiguities, another PCR fragment was generated and used to confirm sequence.

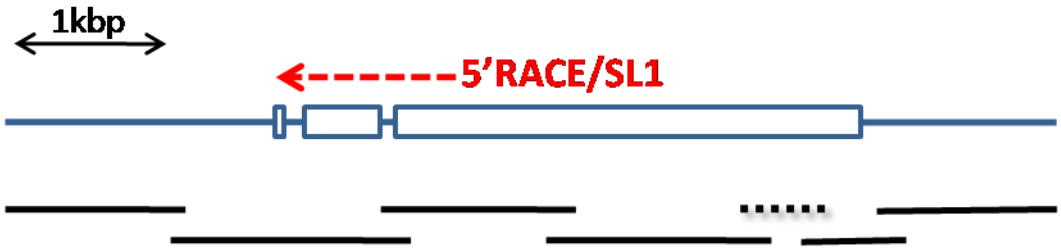


Figure 4.9: ContigExpress assembly of *P. trichosuri age-1*walkout fragments derived using GenomeWalker™
Assembly of *P. trichosuri age-1* orthologue sequences cloned from *P. trichosuri* genomic DNA using GenomeWalker™ as describe in the Methods section and assembled using Vector NTI (version 10.1.1) assembly program: ContigExpress. Black solid lines indicate GenomeWalker fragments; dashed black line is original degenerate PCR cloning fragment; red dashed lines indicate location of the 5′ RACE product and the blue line is final assembly with the blue square indicating location of putative coding region. For primers see GenomeWalker primers in Appendix 3.

4.6 RESULTS: ANALYSIS OF THE GENE STRUCTURE OF *P. trichosuri age-1*

A total of 6 GenomeWalker™ walkout steps were required to obtain 6865 bp of genomic sequence containing the *P. trichosuri age-1* gene. This sequence included the putative full-length coding sequence of 3707 bp and approximately 1900 bp of the promoter region and 1356 bp downstream (Figure 4.9).

The putative polypeptide sequence and stop codon of the open reading frame were determined by translational analysis and sequence alignment with other species. 5′ RACE and SL1 PCR of *P. trichosuri* cDNA were used to confirm the presence of two short introns of less than 50 bp located close to the 5′ end of the gene (Figure 4.10). The first intron is located just 7 bp from the putative start site of the *P. trichosuri age-1* gene, therefore, in order to compare size of the putative coding region from cDNA compared to genomic DNA different forward primers were required (Figure 4.11).

P. trichosuri age-1 nucleotide and deduced polypeptide sequence is shown in Figure 4.10. The cDNA is coded as dark gold. Sequence from *P. trichosuri age*-1 cDNA clones identified two introns (which are in lower case and not highlighted), the deduced amino acid sequence is 1207 aa and shown in capital letters above the coding sequence.

Figure 4.10: *P. trichosuri age-1* sequence

```
   1  TTTTATGAAT AATGTATAGA AAATCTATAC TTTATACATA TAAAAGCTTT CATAATATTT
  61  TTAATTATAA TAGATGCTGG CATGTAAAAG CTCCTCAAAT GATTCATCTT TTTAAAATTA
 121  TTATCCTTTA TTTATCAAGA GGATTAGATT GATAAGTTGG TGGACTTTTA GTTTTACGAT
 181  TCAATAACTT AACATAGTTA ATTGGGACTA AACCTACTCT ATTACCATCT TCTGAAGATG
 241  CTAACAACCA TCCACGTATT GGTGGCTGTT GGTCTTTAGG CGCAACTCTT AATTGATCGC
 301  CTTCATTAAA AGACAACTCA TGGTTCGATG CATTTCCTTG AAATGAAAAC AATGCACGAG
 361  CTGTATAATG ATCAGCCGCC CCAGTTGCCC ATTTTCTAGA TTCTTCAATT GTATTTACCA
 421  TACGGCTAAC AAATTTGTAA ATTAACCATG GACCACCTAT AGCAATGATC CAAAAAAGAA
 481  GTGCTGGCCA GTTGACACCA TCTCTTGTAT TACCACCTAA TTCTTGTGCA ATCCATTCAT
 541  TTGCTTTTAA TGGTTTGTGA GGTGTTTCCC ACGCAGTTTC ATTTATTAAG CTAGACTCAG
 601  GAAGTAATTT AAGTTTCACT AAAATGTATT TCCATAGCTT TTTGATACTT TTAAATATGA
 661  AAAATGTTTC TACTACTCCT ACTAATTGCA CTTTTAGTTT AGTGAACTGT TCAACTACAC
 721  CGATAACAGC TCGAAAAGAA CTAAATAATG CATTATGTGT TGAACTTAAC ATATGAGCAA
 781  CTGAAGCCAC TGCATTAACA ATTGACTCTA TAGATTGAAA AGCTCCTCTA GATGACTCTT
 841  CTGCTAATCG TACAAAATTG CTTTCAGGTG TAATATTATT ATTATTATTA TAAAGATAAT
 901  TATTATACAT ATTGTTTCCA TACCTAATAA AATATATTTG TTTACATAAA AATAAAAAAT
 961  ATAACTTACA TAGCGGGATA AATATTATTA TACATGTAAG GATTATTTAA TCCACTATAA
1021  TTAATATTAG GATTGAAAGA ATACACACCA TTGTTGGCTT CGTTGTTCAA TTGATTTGGT
1081  CTTGGTGGAA GTGGTGGTGG ATTCATTTTA TAGATTGATA AACAAATAAA TGAATTGTTT
1141  CTTATAAGGT AGTAAAAGGT ATTTCTAACT AAAGATACTA TTTATTGACA TCTTATCATA
1201  AGACGTGACG ATTTTGTCGA TAATATATTG TAGTAATGTG ATCAATTTTG ACTACAAAAT
1261  ACAATACAAA TTAATATAAA TTTATAAAGA TATGTTTTGT TTTATTAACA AAATTATTCA
1321  ATTTTATTCC AACTTGGAAG ATTATATGTA ATTGGTTTTA TAATAAAATT GATTGTGTCA
1381  AAGGCACAAT GTTGATCAGC GCATTATACA TTTATTTTAA AACTTTTATG TTTATATATC
1441  ATATAGCTGT ATGTTAATGA TAAGATAACG TGTGTTATCT AAAACGTTTA TTATTGTTAT
1501  CTATATATTA AGTAGTGCCA TTCTTAACAC ATCAACCATA TTGGATAAAA TAACCGGCAA
1561  TTATTATATG TCTCTCTTAA GATTACTGAC GTAATATGTT TGACTTATTT TATTCTAACA
1621  AAACATTTTA CATTGTTTTC CCTGTATGCA TAACGTTTAA TAACACATAA TAAACTGTAC
1681  ATATGCATTA TTTTCATAAA AAGTTGATGA TATATAGCGA CATACAATGA ATTATATTTC
1741  AGGTTTAAAA TATAATATAT ATATATCTTT CCATTAACTA TTTCATCACT ATTGTGATTT
1801  ATATATAAAA CTTAATTAAT TTTTCTTTTA TTAAAGTCTC AAAACGAAAA ATAGTGGATA
                                                  M  D
1861  GTACATTTAT TAATATATAT AGTTTTTCAA ACATATTGGC ATGGATGgta agtaaatatt
                                              G  S   S  S  K   Y  G  L  T ·
1921  atatattatt taaaatatta attttttta ttatagGTTC TTCATCAAAA TACGGTTTAA
      ·  P  E  P   P  D  P   F  K  S  K   R  R  H   E  E  N   K  K  R  K ·
1981  CACCGGAACC TCCAGATCCA TTCAAATCAA AGCGAAGGCA TGAAGAAAAT AAAAAAAGAA
      ·  N  E  S   K  N  K   R  V  V  P   K  N  I   N  Y  F   H  R  F  R ·
2041  AAAATGAATC AAAAAATAAA AGAGTAGTAC CAAAAAATAT AAATTATTTT CATAGATTTA
      ·  N  K  S   E  N  R   D  F  Y  S   E  I  K   N  K  T   L  Y  D  N ·
2101  GAAATAAATC TGAAAATAGA GATTTTTATA GTGAAATTAA AAATAAAACA TTGTATGATA
      ·  V  K  E   M  L  E   M  L  R  S   E  Y  E   I  N  E   F  V  G  K ·
2161  ATGTCAAAGA AATGTTAGAA ATGTTACGTT CTGAGTATGA AATTAATGAA TTTGTTGGAA
      ·  I  N  E   Y  S  F   R  H  N  V   W  K  H   F  V  K   Y  V  T  V ·
2221  AAATAAATGA ATATTCATTT AGACATAATG TTTGGAAGCA TTTTGTTAAA TATGTTACAG
      ·  K  E  F   C  L  A   E  H  E  Y   I  D  F   D  I  L   L  P  N  G ·
2281  TTAAAGAGTT TTGTTTAGCT GAACATGAAT ATATTGATTT TGACATTTTA TTACCAAATG
      ·  Y  M  I   T  I  N   T  S  T  R   S  T  L   E  Q  L   K  K  E  V ·
2341  GATATATGAT AACAATAAAC ACTTCTACTA GATCAACATT AGAACAATTA AAAAAAGAAG
      ·  F  F  Q   A  N  K
2401  TATTTTTTCA AGCAAATAAG Tatgtttata ataatatata tatttaaaaa taatatgtta
        L  K  I   N  K  R  L   L  S  M   D  N  Y   L  F  V  M   L  A  V ·
2461  ggTAAAAATT AACAAAAGAT TATTATCAAT GGATAATTAT CTTTTTGTTA TGTTAGCCGT
      ·  N  G  R   K  E  N  I   Y  D  E   S  C  Q   L  Y  V  Y   K  L  K ·
2521  TAATGGAAGA AAAGAAAATA TATATGATGA AAGTTGTCAA TTGTATGTTT ATAAATTAAA
      ·  S  P  I   L  A  L  H   Q  P  S   E  N  V   V  E  K  K   L  E  Q ·
2581  ATCACCAATA TTAGCACTTC ATCAACCTAG TGAAAATGTT GTTGAAAAAA AATTAGAACA
      ·  D  I  G   V  A  V  G   F  P  I   D  Q  L   D  Q  K  I   S  S  E ·
```

```
2641        GGATATTGGT GTAGCTGTTG GTTTTCCAAT AGATCAATTA GACCAAAAAA TATCATCAGA
            · A  K  L  F  R  V  S  L  F  E  F  C  V  T  T  I  A  D  R  C  ·
2701        AGCAAAATTA TTTAGAGTTT CTTTATTTGA ATTTTGTGTT ACTACTATAG CAGATCGATG
            · C  S  G  N  G  H  Y  A  F  F  E  D  N  I  L  E  L  E  Y  K  ·
2761        CTGTAGTGGA AATGGGCATT ATGCTTTTTT TGAAGATAAC ATTTTAGAAT TAGAATATAA
            · L  S  V  K  S  Q  N  K  I  E  D  K  K  M  I  A  K  V  Y  Y  ·
2821        ATTATCAGTA AAAAGTCAAA ATAAAATTGA AGACAAAAAA ATGATAGCAA AAGTATATTA
            · R  S  F  E  D  E  K  N  E  I  D  T  K  C  I  C  V  D  V  C  ·
2881        TAGATCATTT GAGGATGAGA AAAATGAAAT AGATACAAAG TGCATTTGTG TAGATGTATG
            · N  I  I  S  E  D  K  E  S  G  V  V  N  I  M  T  I  P  Q  L  ·
2941        TAATATCATA TCAGAAGACA AGGAATCTGG TGTAGTTAAT ATTATGACTA TACCACAGTT
            · I  N  F  T  L  K  E  L  K  S  M  G  N  D  I  T  E  E  S  K  ·
3001        GATTAATTTC ACATTGAAAG AGTTAAAATC AATGGGAAAT GACATCACAG AAGAGAGTAA
            · D  F  V  L  Q  I  V  G  Q  K  M  F  M  T  K  E  N  I  P  L  ·
3061        AGACTTTGTA TTACAAATAG TTGGACAAAA AATGTTTATG ACGAAAGAAA ATATTCCATT
            · T  S  F  R  Y  I  R  S  S  F  D  N  N  C  T  P  Q  L  I  L  ·
3121        GACATCATTT CGATACATTA GATCATCATT TGATAATAAT TGTACCCCTC AATTGATATT
            · C  R  K  K  L  I  Y  R  N  L  Q  P  F  V  N  M  H  V  P  Y  ·
3181        GTGTAGAAAA AAATTAATTT ATCGAAATTT ACAACCATTT GTTAATATGC ACGTTCCATA
            · Y  V  R  A  N  R  K  K  E  E  I  T  H  M  H  L  H  E  D  H  ·
3241        TTACGTAAGA GCAAACAGAA AGAAAGAAGA AATTACTCAC ATGCATCTTC ATGAAGATCA
            · G  D  T  R  Y  L  W  E  F  E  E  D  F  R  F  E  L  D  T  A  ·
3301        TGGTGATACA AGATATTTAT GGGAATTTGA AGAAGACTTT AGATTTGAAT TAGATACAGC
            · G  N  V  S  V  Y  D  S  E  Q  R  I  F  V  R  V  A  L  T  V  ·
3361        TGGAAATGTA TCAGTATATG ATAGTGAACA AAGGATATTT GTACGTGTTG CATTAACAGT
            · G  R  H  V  L  S  Q  R  D  S  T  Q  K  S  I  N  D  P  R  W  ·
3421        TGGAAGACAT GTTTTATCAC AAAGAGATTC TACACAAAAA TCAATAAATG ATCCAAGATG
            · R  G  Y  K  M  D  L  G  Y  Y  L  K  D  I  P  P  A  A  Q  L  ·
3481        GAGAGGTTAT AAAATGGATT TAGGTTATTA TCTTAAAGAT ATACCTCCTG CTGCACAATT
            · S  F  A  L  V  S  T  V  P  K  K  N  G  K  A  E  T  E  T  L  ·
3541        ATCATTTGCT TTGGTAAGTA CTGTACCAAA AAAGAATGGT AAAGCAGAAA CAGAAACATT
            · G  W  C  N  L  R  L  F  D  Y  K  N  R  I  V  Q  G  R  K  T  ·
3601        AGGATGGTGT AATTTAAGAT TATTTGATTA CAAAAATAGA ATTGTTCAAG GAAGAAAAAC
            · L  Y  L  H  T  P  T  T  Q  N  S  D  T  Y  I  N  P  S  G  P  ·
3661        ATTATATTTA CATACTCCAA CAACACAAAA TTCTGACACA TATATAAATC CAAGTGGCCC
            · E  G  M  N  M  Q  R  G  S  H  P  R  I  V  V  Y  F  K  D  Y  ·
3721        AGAGGGAATG AATATGCAAC GTGGTTCACA TCCACGTATT GTTGTTTACT TTAAAGATTA
            · S  S  R  S  R  K  I  H  V  K  Y  P  E  I  D  K  I  K  K  Y  ·
3781        TTCTTCAAGA AGTAGAAAAA TTCATGTAAA ATATCCAGAA ATTGATAAGA TAAAGAAATA
            · V  S  I  L  K  S  N  P  K  L  S  E  E  D  K  A  I  D  P  K  ·
3841        TGTAAGTATT TTAAAATCAA ATCCAAAGTT AAGTGAAGAA GATAAAGCTA TAGATCCAAA
            · T  V  S  I  E  E  G  K  K  L  K  K  Y  L  K  Y  L  D  G  T  ·
3901        AACAGTTAGT ATTGAAGAAG GTAAAAAATT AAAAAAATAT TTAAAATATT TGGATGGAAC
            · A  L  T  E  G  D  Q  L  Y  L  W  K  H  R  E  Y  I  C  T  H  ·
3961        GGCATTAACA GAAGGTGATC AACTTTATTT ATGGAAACAT AGAGAATATA TATGTACCCA
            · F  P  N  L  L  V  V  I  S  D  C  K  A  I  W  K  S  R  E  H  ·
4021        TTTTCCAAAT CTTTTGGTTG TTATAAGTGA TTGTAAAGCA ATATGGAAAT CCAGAGAACA
            · V  S  Q  F  Y  E  L  L  T  R  W  G  N  I  S  V  E  A  A  I  ·
4081        TGTTTCACAA TTTTATGAAT TATTAACTCG ATGGGGTAAT ATAAGTGTTG AGGCAGCAAT
            · E  L  L  D  N  R  Q  R  D  C  A  V  R  K  F  A  V  D  I  L  ·
4141        AGAGTTACTT GATAATAGAC AACGGGACTG TGCTGTACGA AAATTTGCGG TAGACATATT
            · D  N  W  L  N  D  E  R  F  K  L  F  I  M  H  L  I  Q  G  I  ·
4201        GGATAATTGG CTTAACGATG AGAGATTCAA ATTGTTTATT ATGCATTTGA TACAAGGAAT
            · K  Y  E  P  Y  Y  H  N  P  L  A  V  M  L  I  R  R  A  L  L  ·
4261        TAAATATGAA CCCTACTATC ATAATCCATT AGCAGTGATG CTTATTAGAA GAGCTTTATT
            · N  Y  Q  I  A  H  R  L  F  W  L  L  R  A  E  L  E  Q  E  I  ·
4321        GAATTATCAA ATAGCTCATC GACTTTTTTG GTTATTACGA GCAGAGTTAG AGCAAGAGAT
            · G  A  S  E  S  V  N  G  K  S  C  R  L  T  G  E  T  N  I  E  ·
4381        TGGTGCGTCA GAATCCGTAA ATGGAAAATC ATGCAGATTG ACTGGTGAAA CAAATATTGA
            · G  K  Y  T  L  S  V  V  R  C  T  I  M  L  E  C  L  L  R  A  ·
4441        AGGAAAATAC ACGTTATCAG TAGTAAGATG CACAATTATG TTAGAATGTC TATTAAGAGC
            · N  V  R  H  I  G  P  V  I  K  Q  V  R  M  V  N  E  L  F  K  ·
4501        AAATGTAAGG CATATAGGGC CTGTAATAAA ACAGGTAAGA ATGGTTAATG AATTATTTAA
            · I  S  N  E  V  K  N  N  T  S  K  E  S  N  T  K  F  L  Q  R  ·
4561        GATAAGCAAT GAAGTAAAAA ATAACACATC AAAAGAAAGT AACACAAAAT TTCTCCAAAG
            · R  L  K  S  V  V  H  D  M  E  H  V  E  S  P  L  N  P  V  V  ·
4621        AAGACTTAAA AGTGTTGTAC ATGATATGGA GCATGTTGAG TCTCCATTAA ATCCTGTAGT
            · I  L  G  E  L  C  I  E  E  C  R  V  L  S  S  A  K  Q  P  I  ·
4681        TATATTAGGA GAATTATGTA TTGAAGAATG TAGAGTACTA TCAAGTGCTA AACAACCAAT
            · K  L  V  W  N  N  S  E  P  L  A  R  L  S  K  K  T  H  Q  M  ·
4741        TAAATTAGTT TGGAATAATT CAGAACCATT AGCAAGGCTT TCTAAAAAAA CTCATCAAAT
            · I  F  K  N  G  D  D  L  R  Q  D  M  L  T  L  Q  V  M  K  I  ·
4801        GATATTTAAA AATGGAGATG ATTTAAGACA GGACATGTTA ACACTTCAAG TAATGAAAAT
            · M  D  A  F  W  K  S  M  G  Y  D  F  C  M  S  I  Y  E  V  L  ·
4861        TATGGATGCT TTTTGGAAAT CAATGGGATA TGATTTTTGT ATGAGTATAT ATGAAGTGTT
            · P  M  G  F  N  I  G  M  I  N  V  V  Q  K  C  V  T  L  F  E  ·
4921        ACCTATGGGA TTCAATATAG GGATGATAAA TGTTGTCCAA AAATGTGTAA CATTATTTGA
```

```
          · I  Q  T   N  E  K  K   R  S  V   S  L  A   M  E  T  A   C  V  N ·
4981      GATTCAAACA AATGAAAAAA AACGTAGCGT ATCATTAGCA ATGGAAACAG CCTGTGTAAA
          · K  W  I   G  K  Y  N   D  D  T   K  L  Y   L  E  A  V   D  R  F ·
5041      CAAGTGGATT GGAAAGTACA ATGACGATAC TAAATTATAT TTAGAAGCTG TAGATCGTTT
          · S  A  S   L  T  G  Y   C  V  A   T  Y  I   L  G  I  K   D  R  H ·
5101      TTCAGCTTCA TTGACCGGTT ATTGTGTCGC AACATATATA CTAGGAATAA AAGATCGTCA
          · Q  D  N   I  M  V  R   K  D  G   R  M  F   H  I  D  F   G  H  F ·
5161      TCAAGATAAT ATAATGGTAA GAAAAGATGG AAGAATGTTT CACATCGATT TTGGGCATTT
          · L  G  H   T  K  K  K   L  G  I   N  R  D   R  T  P  F   I  L  T ·
5221      TTTGGGGCAT ACAAAAAAGA AACTTGGTAT TAATCGTGAT AGAACACCAT TTATATTAAC
          · D  H  F   L  Y  V  I   A  K  G   R  S  Q   F  K  N  N   H  D  I ·
5281      AGATCATTTC TTATATGTAA TAGCAAAAGG GCGTTCTCAA TTTAAAAATA ATCATGACAT
          · M  K  F   R  E  N  C   K  D  A   F  L  I   L  Y  D  H   S  R  L ·
5341      AATGAAATTT AGAGAAAATT GTAAAGATGC ATTCTTAATT TTATATGATC ACTCTCGATT
          · F  I  S   L  F  R  M   M  L  S   I  G  L   P  E  C  S   T  Q  E ·
5401      ATTTATATCA TTATTTCGTA TGATGTTATC AATAGGTTTA CCAGAATGCT CTACACAGGA
          · D  L  N   Y  L  K  A   S  L  M   A  D  V   D  K  D  V   A  A  L ·
5461      AGATTTGAAC TATTTAAAAG CATCATTAAT GGCTGATGTT GATAAGGATG TAGCAGCTCT
          · Q  F  D   N  I  F  K   E  V  T   K  S  D   L  S  T  K   T  N  W ·
5521      TCAATTTGAC AATATATTTA AAGAAGTAAC AAAATCAGAT CTATCTACAA AAACGAATTG
          · F  F  H   S  V  K  H   M  *
5581      GTTTTTCCAT TCAGTTAAAC ATATGTAATA ATTATTTAAA TGCATTATCA TCGATGTAAT
5641      GTATGAAAAT TTATGTAAAA AAATACAATA ATAAATACGA TTAGTATAAA TCTAGAAAAA
5701      GATAATCCAT TTAGATTATG TACTCCTGTA TGTTAATAGT CATGTTCCGT TTTAAAGACT
5761      GTACTTGCTT TCGTATAAAG TAGTTTTATA ATGATATCAA TCACTTGCTC CTCCTTGGCC
5821      AATATTGCTT GATCCTCCCA AATGTAATTT GTATATATAG CTTGAAAATG ATACTTTCTC
5881      ATCAGTTTGA GTTATACGTT GCGTTTACTT AAATTTTTTT ACGTATATAT TATGTTATAA
5941      ATAATTGATT TAAAATAATA ATATTTATAT GAGATAAACC TTATAATTCT TAATCCGATA
6001      TTTTATTAAT ATTATTAATC AGCTTATATA AAATATTTTT GTAATTTTTA ATAAGCATCG
6061      TAACACAACT AGTTGATGGC AAGAAGGTTT ATTTCAAAAT TTGCATGTGA CAAGATTGTT
6121      TCCATTTGTA AATGCATCTT TTGGGATAGA TCTTTTTACT CATCATAATA ATATTATTGT
6181      TATTAAAAAC AGGATGACTT TTGTGTTAGT ACTTGCATTT ACATTTTTTA AAAATATACT
6241      TGTAAATGCA TCTCACTAGC ATAAATGTTA TCTATGTGAT CATTTATATT TTAAGAAAAT
6301      AATTTAATAA TATTTAAAAG TAAGATTTAT TAATTGAACC TCTGGATGGC AATTGGGTAA
6361      GCTGTTGGTT TTAAAAAGCA TCAACCAGCT AACTACACTG CCAACAAGGG ATTCAATTTT
6421      TTAATATATT AAGAATAGAT AATAAAATAT TGATATATGT ATATATAAAG ATAAAAATGG
6481      AGCACTAGGA GGCAGTTTAA TGAGCCGTTG AATTACAAGA TTTTTCAACC AGCTAATCAC
6541      ACTGCCATCT AGAGAACCAT TTTGAAACGT AATGCTAAAA ATTATTGAAA TATTTTTTAG
6601      TAAATAAAAG ATTATATATA TTTTTTAGTT GGTGAATGGA GAAAAATAAA AAGTGCATGT
6661      CACAAGATGT GTTGTACTTG CGACAATGAA ATTATTTAAT TCGGAGAATC ATTGTTAAAT
6721      GATACAACAA TCTAATGACA CATGCTTTAT TAAAAATTAA GGTTTTTTTT ATACATAAAT
6781      GATAATATGG TTGTTTTTTT AAATAAGAGA GCACATATTT TTGAAAAGAG ACAATATTTT
6841      GAAATAAAAA AAAAAAACGC TGCACATAAA ACCTATGTGT ATGTGTGATG ACAATCAACC
6901      TAAACAAATC ACTAGCATGG CAGTTGAGTG AGCTGTTGAG CATTTAATGA ATCACAACCA
6961      GCCA
```

Coding region is highlighted in yellow UTR regions are highlighted in dark gold Introns are un-highlighted and lower case 3′ UTR Polyadenylation site AATAAA is boxed

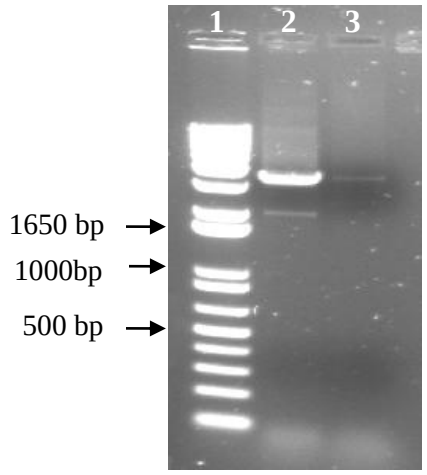


Figure 4.11: *P. trichosuri age-1* from genomic DNA and cDNA
The deduced coding region of *P. trichosuri age-2* orthologue was amplified by PCR from *P. trichosuri* cDNA and genomic DNA. Lane 1: 1KB Plus MW marker; Lane 2: Pt age-1 from gDNA Lane 3: Pt age-1 from cDNA. Fwd primer gDNA: ATGGATGGTAAGTAAATATTATATA; Fwd primer cDNA: ATGGATGGTTCTTCATCAAAAT Rev primer: TTACATATGTTTAACTGAATGGAA

4.7 ANALYSIS OF THE DEDUCED PROTEIN STRUCTURE OF *P. trichosuri* AGE-1

The predicted AGE-1 protein is 1207 a.a. (Figure 4.10) and has an unmodified molecular weight of 414.4 kD. This polypeptide shares an overall 42.7% similarity with *C. elegans* AGE-1. The region of highest similarity is the PI3′K catalytic domain which has a local a.a. similarity of 59.7%.

A local installation of InterProScan (ZDOBNOV and APWEILER 2001) was used to scan the translated sequences against the member databases of InterProScan, has shown that the putative *P. trichosuri* AGE-1 protein contains the same compliment of motifs that phosphatidylinositol 3′ kinase of the IIS pathway from *C. elegans* . A summary of the *P. trichosuri* AGE-1 motifs and their identity to *C. elegans* AGE-1 is shown in Table 4.3 and in Figure 4.12.

Table 4.3: Predicted protein motifs of *P. trichosuri* and *C. elegans* AGE-1 using the suite of analysis programs of InterProScan.

Analysis of translated protein motifs using InterProScan		
PI3′K catalytic domain		
C. elegans	871-1167 aa	Similarity 59.7%
P. trichosuri	936-1205 aa	Identity 49.5%
PI3′K ras binding domain		
C. elegans	226-346 aa	Similarity 38.8%
P. trichosuri	336-402 aa	Identity 19.0%
PI3′K accessory region		
C. elegans	595-783 aa	Similarity 47.8%
P. trichosuri	634-821 aa	Identity 35.5%
PI3′K C2 domain		
C. elegans	430-567 aa	Similarity 46.4%
P. trichosuri	465-560 aa	Identity 24.7%
PI3′K p85 binding		
C. elegans	83-162 aa	Similarity 37.3%
P. trichosuri	133-210 aa	Identity 25.3%

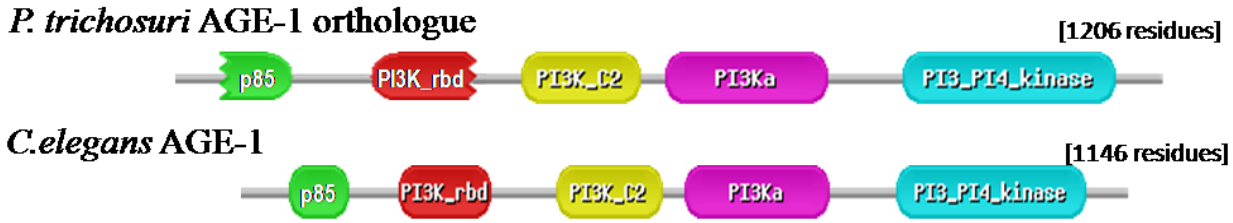


Figure 4.12: Structure of putative *P. trichosuri* AGE-1 protein.
Full length, translated sequence submitted for Prosite functional motifs as predicted by www.sanger.ac.uk/Software/Pfam. PI3′K_rbd (red): ras-binding domain; PI3′K-C2: C2 domain (yellow) may play a role in membrane association; PI3′Ka (pink): accessory domain may be involved in substrate presentation; PI3_PI4_kinase (blue): catalytic domain; p85b (green): region of the p110 phosphatidylinositol 3′kinase (PI3′K) that binds the p85 subunit.

4.7.1 Phylogenetic tree of protein alignments

Phylogenetic analysis was performed to determine the relatedness of the putative *P. trichosuri* AGE-1 to *C. elegans* AGE-1, a member of the IIS pathway, in order to determine whether the putative *P. trichosuri* AGE-1orthologue cloned is more closely related to *C. elegans* AGE-1 rather than other PI 3′ kinases not associated with the IIS

pathway. The entire polypeptide sequence of the putative *P. trichosuri* AGE-1 orthologue was compared to Ce, *C. elegans*; Hs, *H. sapiens*; Gg, *Gallus gallus;* Mm, *M. musculus* phosphatidylinositol 3′ kinases (PI3′K) of the IIS pathway from these species, along with several *Caenorhabditis* PI3′Ks partially confirmed by transcript evidence or not associated with the IIS pathway but with suppressor of morphological effect. Phylogenetic analysis was performed using PHYLIP (Phylogeny Inference Package) version 3.68, which indicates that *P. trichosuri* is most closely related to *C. elegans* AGE-1 and the nematode, *Pristionchus pacificus.* Table 4.4 gives a text version of the phylogenetic tree, tabulating the evolutionary distances between the sequences and the nodes of the number of mutations per 100 aa necessary to change one sequence to the other.

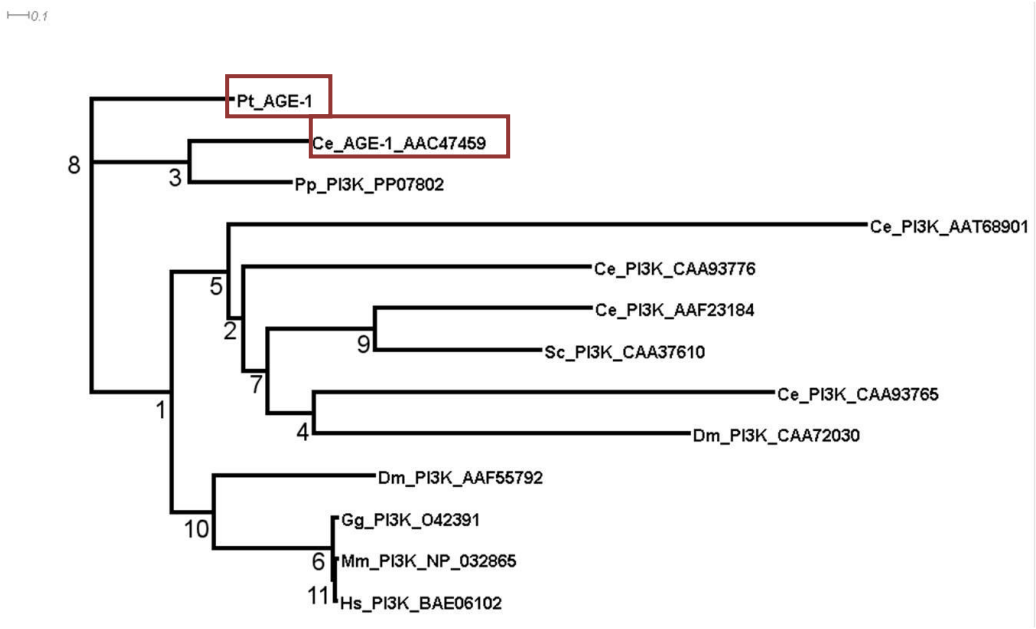


Figure 4.13: Phylogenetic analysis of phosphatidylinositol 3′ kinase protein alignments. Phylogenetic guide tree was constructed on full length proteins sequence, using the ProML program version 3.68 of the PHYLIP package (FELSENSTEIN 2005) This program uses Maximum Likelihood in order to create a tree with the highest probability. The species used were: Hs, *H. sapiens*; Mm, *M. musculus*; Gg *G. gallus* Ce, *C. elegans*; Pp, *P. pacificus*; Pt, *P. trichosuri*. GenBank or GenPept accession numbers follow two letter species designation.

Table 4.4: The distances between the nodes of the Phylogenetic guide tree of Insulin/IGF phosphatidylinositol 3′ kinase protein alignments are given in the following table followed by the upper and lower limits in parenthesis. Maximum Likelihood method was used to estimate distances and give confidence limits.

Between	And	Distance(upper, lower limits)	Confidence Limits
3	Ce_AGE-1_AAC47459	0.56526 (0.49075 0.63973)	**
3	Pp_PI3K_PP07802	0.48473 (0.41357 0.55575)	**
3	8	0.46535 (0.37992 0.55058)	**
8	1	0.38372 (0.29446 0.47285)	**
1	10	0.20102 (0.12724 0.27470)	**
10	Dm_PI3K_AAF55792	0.76444 (0.67115 0.85760)	**
10	6	0.56397 (0.48166 0.64628)	**
6	Gg_PI3K_O42391	0.02343 (0.01202 0.03483)	**
6	11	0.01365 (0.00291 0.02425)	**
11	Hs_PI3K_BAE06102	0.00364 (zero 0.00776)	**
11	Mm_PI3K_NP_032865	0.01102 (0.00439 0.01764)	**
1	5	0.26813 (0.16369 0.37403)	**
5	2	0.06959 (zero 0.17840)	
2	Ce_PI3K_CAA93776	1.65715 (1.48147 1.83300)	**
2	7	0.11852 (0.00849 0.22871)	*
7	9	0.51156 (0.37831 0.64465)	**
9	Ce_PI3K_AAF23184	1.03264 (0.88831 1.17697)	**
9	Sc_PI3K_CAA37610	0.79161 (0.66206 0.92101)	**
7	4	0.22309 (0.02989 0.41565)	**
4	Dm_PI3K_CAA72030	1.78741 (1.47289 2.10190)	**
4	Ce_PI3K_CAA93765	2.18872 (1.94368 2.43376)	**
5	Ce_PI3K_AAT68901	3.04296 (2.71219 3.37353)	**
8	Pt_AGE-1	0.67488 (0.58648 0.76337)	**

Distance = # of mutations per 100 aa to change one sequence to another

* = significantly positive, P < 0.05; ** = significantly positive, P < 0.01

4.8 CLONING AND SEQUENCING OF *P. trichosuri daf-16*

An internal fragment of the putative *P. trichosuri daf-16* gene was cloned with degenerate PCR primers designed from a protein alignment of the full length *C. elegans* DAF-16A protein sequence and *S. stercoralis* FKTF-1A, a putative *daf-16* orthologue of the closely related *Strongyloides* species. These sequences were obtained from the ENTREZ NCBI database (http://www.ncbi.nlm.nih.gov/entrez/). Primers were designed to the conserved regions QVYEWMV and DFNIPANS, and the resulting 1100 bp PCR product was gel purified (Figure 4.14A) and then TA cloned into pGEM Teasy vector and sequenced. The BLAST X search showed high similarity to the DAF-16 proteins of *S. stercoralis* and the FOXO transcription factor of mouse and human. This

gene fragment was used to design primers for the GenomeWalker™ kit in order to walk upstream and downstream into unknown regions of the genome.

The *C. elegans daf-16* and *S. stercoralis daf-16* gene both utilize differential splicing events to generate isoforms which have a common C terminal region and alternative N terminal regions. 5′ RACE was used to determine if *P. trichosuri daf-16* also has different isoforms. 5′ RACE was performed with total RNA from two different life stages: egg and infective larva. Bands were gel purified and TA cloned for sequencing (Figure 4.14B). Sequences were generated from the 16 clones which were sent away for sequencing, sequence analysis suggested two alternative isoforms for the *P. trichosuri daf-16* gene. One of the 5′ RACE clones was long enough to determine the putative *Pt daf-16b* start codon and 5′ UTR. GenomeWalker™ was then used to walk out in a series of sequential steps in each of the isoforms.

The full length *P. trichosuri daf-16* gene orthologue was cloned by sequential walk out steps from GenomeWalker™ Libraries. The method of *P. trichosuri* GenomeWalker™ library generation, primer design and amplification are discussed in the Methods (Section 2.6.5), and described previously. Contigs of the cloned fragments were assembled using the Vector NTI (Version 10.1.1) assembly program: ContigExpress, which utilizes CAP3 consensus generation algorithms based on weighted sums of the quality value score (QVs) of assembled fragments. *P. trichosuri daf-16a* had a total of 5 walkout steps and *P. trichosuri daf-16b* had a total of 4 walkout steps (Figure 4.15).

The *C. elegans daf-16* gene has an extremely long 3′ UTR, approximately 1159 bp long, which contains a confirmed intron (spliced for the b isoform); this unusually long 3'UTR is thought to contain, as yet, unidentified regulatory sequences. Two separate clones from a 3′ RACE were used to determine that the 3′ UTR of *P. trichosuri daf-16* is 397 bp long. This suggests that any putative regulatory elements which *C. elegans daf-16* 3′ UTR may contain are not present or are more densely organized in the *P. trichosuri daf-16* 3' UTR.

Sequence alignments of the translated transcripts to other species or RACE were used to confirm the putative start site and the 3′ UTR of the gene. Primers were designed to

clone the putative coding regions and PCR was performed on both genomic DNA and cDNA (Figure 4.17) which shows the different sizes of the spliced and unspliced forms.

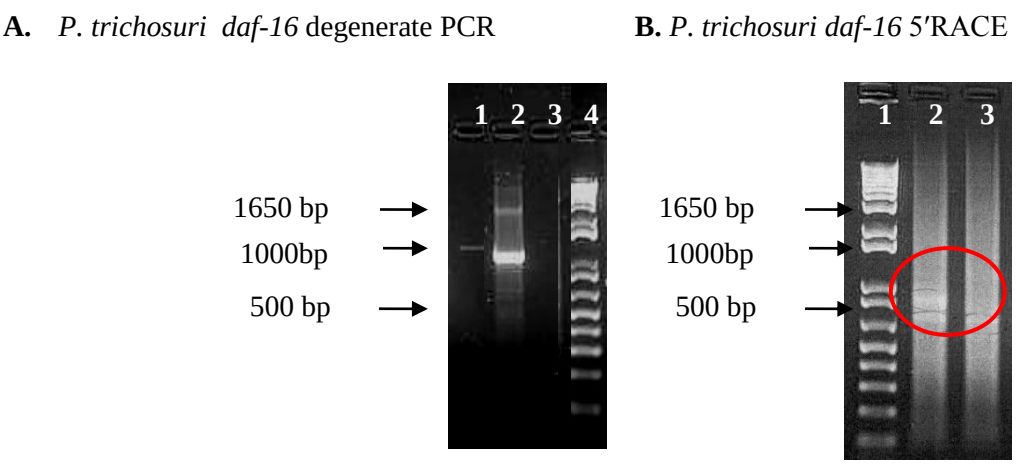


Figure 4.14: Degenerate PCR and 5' RACE of *Pt daf-16*
A: Agarose gel of *P. trichosuri* degenerate PCR $MgCl_2$ titration with primers designed to a conserved region of the gene based upon alignment of *C. elegans DAF-16A* and *S. stercoralis FKTF-1A*. Fwd primer: GGACATCCATATTCWCAACARTAYAC; Rev primer: ACTAATAATTCWGGAAA YAAYCAR; Lane 1: *Pt daf-16* degen 2.5mM; Lane 2: *Pt daf-16* degen 3.5mM; Lane 3: Negative Control; Lane 4: 1KB Plus MW marker Panel B; 5′ RACE of *P. trichosuri daf-16* gene on cDNA derived from the egg stage and infective larval stage of development. The fragments that were chosen to be cloned and sequenced are within the red oval. Lane 1: 1KB Plus MW marker; Lane 2: 5′ RACE on egg stage total RNA; Lane 3: 5′ RACE on iL3 stage total RNA. 5′RACE primer SP1: CCATTCCAC CATCACCTAATTGC; 5′RACE primer SP2: GCATCTGGATTGATTACCCACC

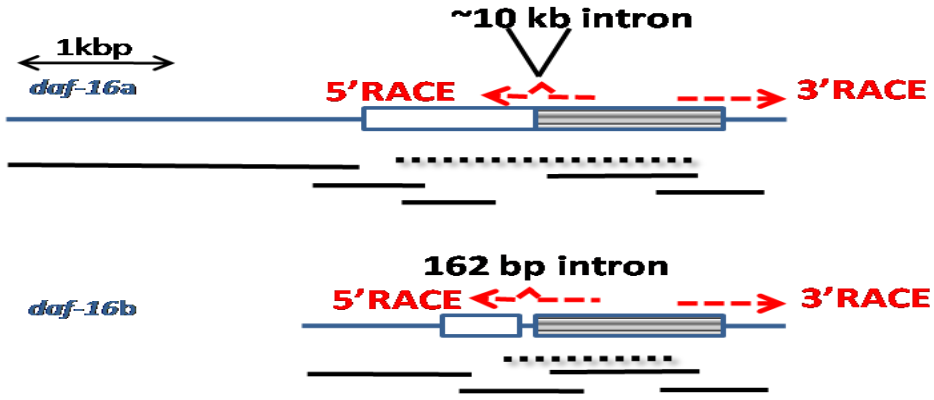


Figure 4.15: ContigExpress assembly of GenomeWalker™ *Pt daf-16*
Assembly of *P. trichosuri daf-16a* and *daf-16b* orthologue sequences cloned from *P. trichosuri* genomic DNA using GenomeWalker™ as describe in the Methods section and assembled using Vector NTI (version 10.1.1) assembly program: ContigExpress. Black solid lines indicate GenomeWalker fragments; dashed black line is original degenerate PCR cloning fragment; red dashed lines indicate location of 5′ and 3′ RACE products and blue line is final assembly with blue square indicating location of putative coding region. For primers see GenomeWalker primers in Appendix 3.

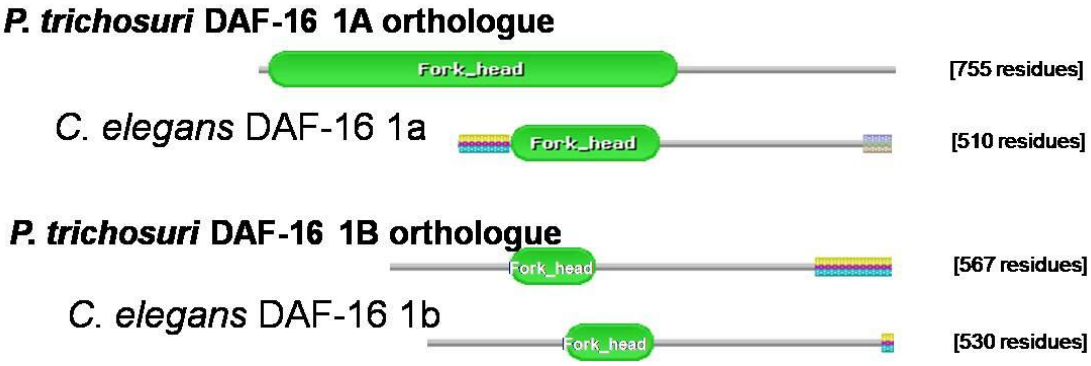


Figure 4.16: Structure of putative *P. trichosuri* DAF-16 structure
Full length, translated sequence submitted for Prosite functional motifs as predicted by www.sanger.ac.uk/Software/Pfam. Fork_head (green): winged helix B-DNA binding domain.

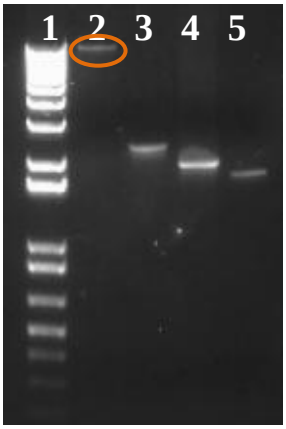


Figure 4.17: Agarose gel of PCR of full length coding regions of *P. trichosuri daf-16a* and *daf-16b* from genomic DNA and cDNA.
The deduced coding region of *P. trichosuri daf-16a and daf-16b* orthologues was amplified by PCR from *P. trichosuri* cDNA and genomic DNA. The 12 kbp product for *Pt daf-16a* is circled in red. Lane Map Lane 1: 1KB Plus MW marker Lane 2: Ptdaf-16a from gDNA (expect >12kbp); Lane 3: Pt daf-16a from cDNA (expect 2265bp); Lane 4: Pt daf-16b from gDNA (expect 1889 bp); Lane 5: Ptdaf-16b from cDNA (expect 1701bp). Pt daf-16a Fwd primer: ATGGTTCATTATTATCATGATGACTT Pt daf-16b Fwd primer: ATGAGTGGATTACCATACTCTCAACA Pt daf-16ab Rev primer: TGATGGTGGTTGT GGAGGCAT

4.9 RESULTS: ANALYSIS OF P. *trichosuri daf-16a* AND *daf-16b* GENE STRUCTURE

A total of 4917 bp of genomic sequence was generated for the *P. trichosuri daf-16a* isoform with a combination of GenomeWalker™ and 3′ RACE, this included 2253 bp

of promoter region, 2267 bp of spliced transcript and 397 bp of 3' UTR. A total of 3200 bp of genomic sequence was generated for the *P. trichosuri daf-16b* isoform with a combination of GenomeWalking and 3' RACE, this included 938 bp of promoter region, 1865 bp of transcript and 397 bp of 3' UTR (Figure 4.19).

The putative polypeptide sequence and stop codon of the open reading frame was determined by sequence alignment with other species and 3′ RACE. The deduced coding region of the *P. trichosuri daf-16* genes were based upon translated protein alignment to *C. elegans* and *S. stercoralis*. SL1 and SL2 PCR did not amplify a RT-PCR product for either *P. trichosuri daf-16a* or *daf-16b*, suggesting that unlike *C. elegans daf-16*, the transcript of the *P. trichosuri daf-16* gene orthologue is not transpliced. The 3′ UTR of *P. trichosuri daf-16*a *and daf-16*b was determined by 3' RACE to be 397 bp. Primers based upon the putative start sites of the two isoforms of this gene and the primer designed to the shared stop site were used to amplify this region from genomic DNA and cDNA (Figure 4.17). The results show that the full length of the *P. trichosuri daf-16a* gene is ~12 kbp; this suggests that *P. trichosuri daf-16a* contains a ~10 kbp intron, which presumably contains the promoter for the *P. trichosuri daf-16b* isoform (Figure 4.18). Massey *et al*, reported that *S. stercoralis fktf-1* (an orthologue to *C. elegans daf-16*) has similar gene structure with a 5 kbp intron (MASSEY *et al.* 2003), *C. elegans daf-16* has a 6.2 kbp intron.

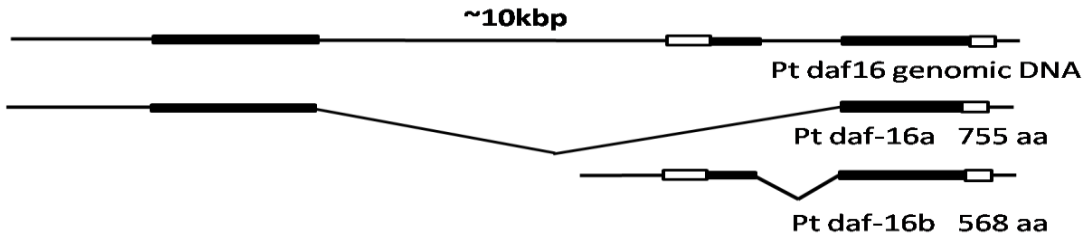
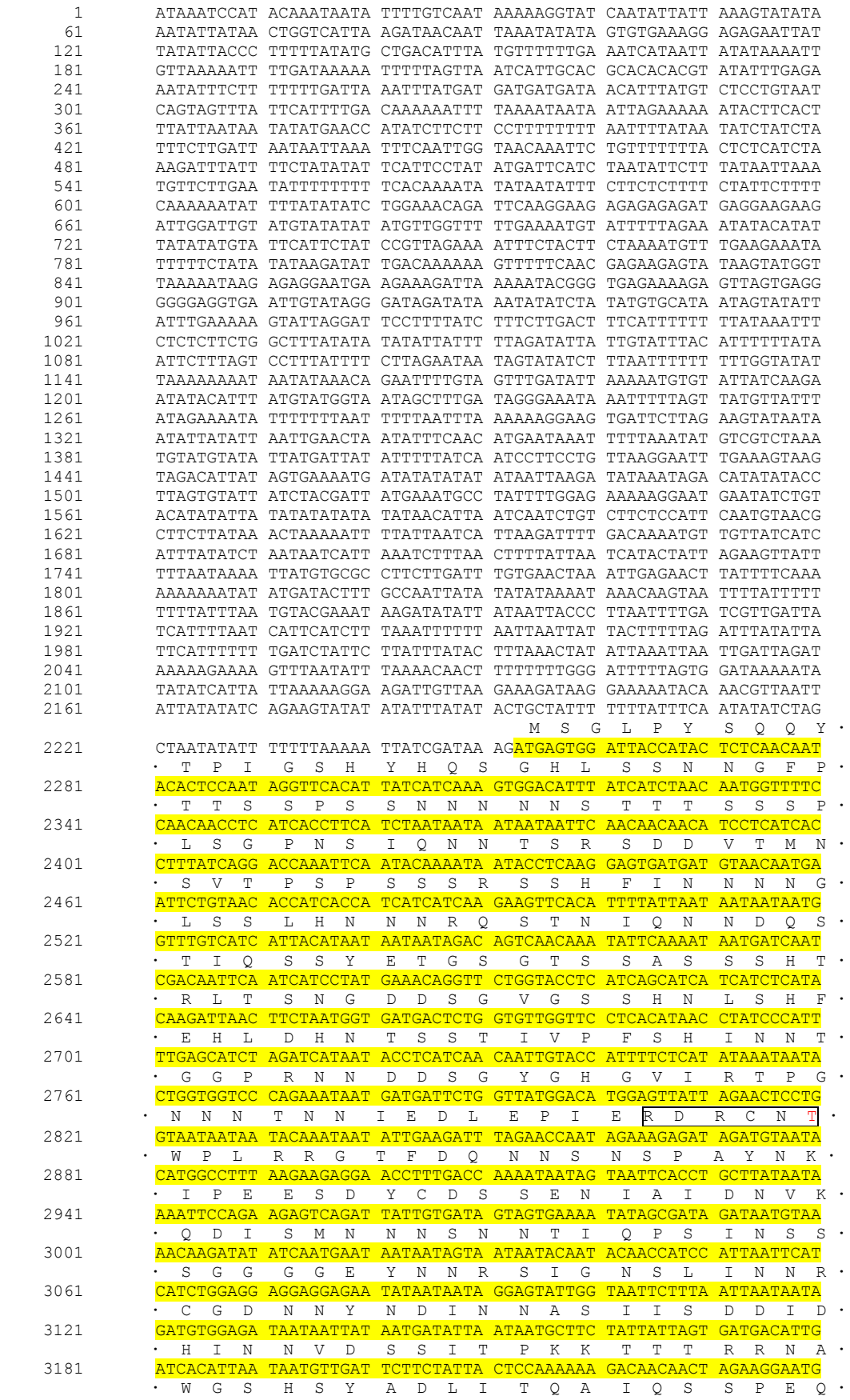


Figure 4.18: Structure of *P. trichosuri daf-16* gene.
Structure of the putative *P. trichosuri daf-16* gene orthologue. Solid black lines indicate coding region, open boxes indicate untranslated region. The top line is the genomic organization of *Pt daf-16* and the bottom two lines illustrate the *Pt daf-16*a and *Pt daf-16*b isoform with the chevrons indicating the spliced intronic regions.

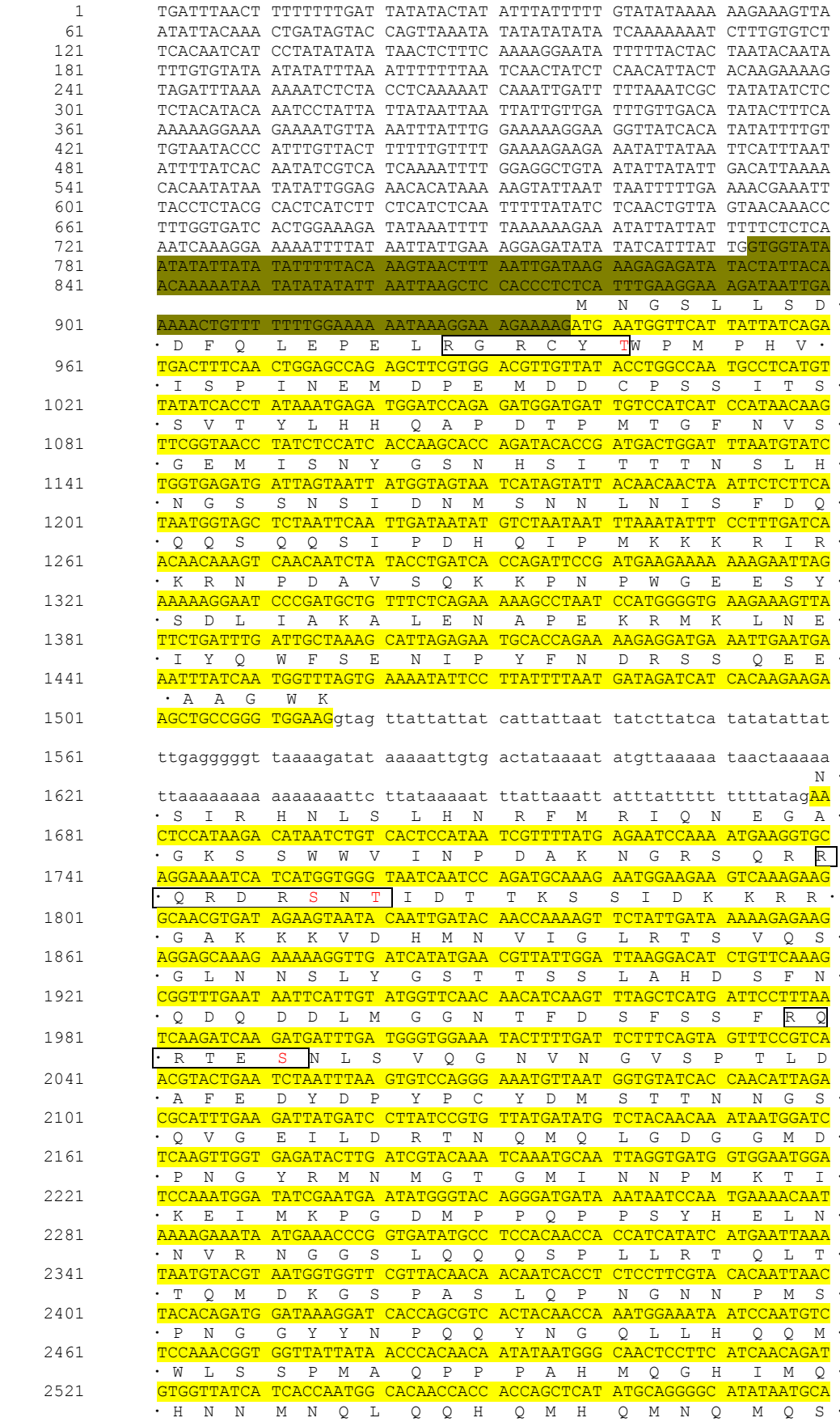
Figure 4.19: Sequence of *P. trichosuri daf-16*a



```
3241        CCTGGGGTAG TCATAGTTAT GCTGATTTAA TTACTCAAGC TATTCAATCT AGTCCAGAAC
          · R  L  T  L  S  Q  V  Y  E  W  M  V  T  N  V  P  F  F  R  D ·
3301        AAAGGTTAAC TTTATCTCAA GTTTATGAAT GGATGGTTAC AAATGTTCCT TTTTTCCGTG
          · K  G  D  S  N  S  S  A  G  W  K  N  S  I  R  H  N  L  S  L ·
3361        ATAAAGGAGA TAGCAATAGT AGTGCAGGAT GGAAAAACTC CATAAGACAT AATCTGTCAC
          · H  N  R  F  M  R  I  Q  N  E  G  A  G  K  S  S  W  W  V  I ·
3421        TCCATAATCG TTTTATGAGA ATCCAAAATG AAGGTGCAGG AAAATCATCA TGGTGGGTAA
          · N  P  D  A  K  N  G  R  S  Q  R [Q  R  D  R  S] N  T  I ·
3481        TCAATCCAGA TGCAAAGAAT GGAAGAAGTC AAAGAAGGCA ACGTGATAGA AGTAATACAA
          · D  T  T  K  S  S  I  D  K  K  R  R  G  A  K  K  K  V  D  H ·
3541        TTGATACAAC CAAAAGTTCT ATTGATAAAA AGAGAAGAGG AGCAAAGAAA AAGGTTGATC
          · M  N  V  I  G  L  R  T  S  V  Q  S  G  L  N  N  S  L  Y  G ·
3601        ATATGAACGT TATTGGATTA AGGACATCTG TTCAAAGCGG TTTGAATAAT TCATTGTATG
          · S  T  T  S  S  L  A  H  D  S  F  N  Q  D  Q  D  D  L  M  G ·
3661        GTTCAACAAC ATCAAGTTTA GCTCATGATT CCTTTAATCA AGATCAAGAT GATTTGATGG
          · G  N  T  F  D  S  F  S  S  F [R  Q  R  T  E  S] N  L  S  V ·
3721        GTGGAAATAC TTTTGATTCT TTCAGTAGTT TCCGTCAACG TACTGAATCT AATTTAAGTG
          · Q  G  N  V  N  G  V  S  P  T  L  D  A  F  E  D  Y  D  P  Y ·
3781        TCCAGGGAAA TGTTAATGGT GTATCACCAA CATTAGACGC ATTTGAAGAT TATGATCCTT
          · P  C  Y  D  M  S  T  T  N  N  G  S  Q  V  G  E  I  L  D  R ·
3841        ATCCGTGTTA TGATATGTCT ACAACAAATA ATGGATCTCA AGTTGGTGAG ATACTTGATC
          · T  N  Q  M  Q  L  G  D  G  G  M  D  P  N  G  Y  R  M  N  M ·
3901        GTACAAATCA AATGCAATTA GGTGATGGTG GAATGGATCC AAATGGATAT CGAATGAATA
          · G  T  G  M  I  N  N  P  M  K  T  I  K  E  I  M  K  P  G  D ·
3961        TGGGTACAGG GATGATAAAT AATCCAATGA AAACAATAAA AGAAATAATG AAACCCGGTG
          · M  P  P  Q  P  P  S  Y  H  E  L  N  N  V  R  N  G  G  S  L ·
4021        ATATGCCTCC ACAACCACCA TCATATCATG AATTAAATAA TGTACGTAAT GGTGGTTCGT
          · Q  Q  Q  S  P  L  L  R  T  Q  L  T  T  Q  M  D  K  G  S  P ·
4081        TACAACAACA ATCACCTCTC CTTCGTACAC AATTAACTAC ACAGATGGAT AAAGGATCAC
          · A  S  L  Q  P  N  G  N  N  P  M  S  P  N  G  G  Y  Y  N  P ·
4141        CAGCGTCACT ACAACCAAAT GGAAATAATC CAATGTCTCC AAACGGTGGT TATTATAACC
          · Q  Q  Y  N  G  Q  L  L  H  Q  Q  M  W  L  S  S  P  M  A  Q ·
4201        CACAACAATA TAATGGGCAA CTCCTTCATC AACAGATGTG GTTATCATCA CCAATGGCAC
          · P  P  P  A  H  M  Q  G  H  I  M  Q  H  N  N  M  N  Q  L  Q ·
4261        AACCACCACC AGCTCATATG CAGGGGCATA TAATGCAACA TAACAATATG AATCAATTAC
          · Q  H  Q  M  H  Q  M  N  Q  M  Q  S  N  N  M  L  S  C  G  A ·
4321        AACAACACCA AATGCATCAA ATGAATCAGA TGCAATCAAA TAATATGTTA AGTTGTGGTG
          · Q  S  N  N  E [L  P  Q  D  L] Q  N  L  N  M  H  E  T  T  Q ·
4381        CCCAATCAAA CAATGAGTTG CCACAAGATC TTCAGAACCT GAATATGCAT GAGACAACAC
          · M  T  E  M  D  F  E  S  I  M  R  H  E  I  S  I  S  N  A  P ·
4441        AGATGACGGA GATGGATTTTGAATCTATAA TGCGTCACGA AATATCTATT AGTAATGCTC
          · I  N  F  D  L  *
4501        CAATCAACTT TGATCTGTAA ATATTATATA TAATTTCTAT GTATATAATA TATTATTTTT
4561        TAAAAAAAAA AACGTTATTG AGAGAGATTC CTAAAGTTGT GACAAAGCTT AATTAATAGA
4621        GAGAAAAAAG ACATCACACA CAAAAGTAAA AGACACTCAC ATCCTATATA TATCTATCTA
4681        TATAATGTAC ACAGACACAT ACAAAAAAAT TATTGACAAA CAATTATCAA TATTATAAAA
4741        AAGATATTTT TGAAAACTTT GATATTGTAA TATAATAATA TTTATACATA CATATATATA
4801        CTCATTCCCA TATATTGATA GCAGCAACAG TATACAAAAA ATATGTTTCA TTTATTTGAC
4861        TATTTGTTAA TATATATTTT TTATAATATT ATAATCCTTT TAAAAAAAAA AAAAAAA
```

Coding region is highlighted in yellow
UTR regions are highlighted in dark gold
Introns are un-highlighted and lower case
Phosphorylation site for nuclear localization RxRxx(S/T) is boxed
FoxO recruitment of cofactors Sir2 LXXLL is boxed

Figure 4.20: Sequence of *P. trichosuri daf-16b*



```
2581        ACATAACAAT ATGAATCAAT TACAACAACA CCAAATGCAT CAAATGAATC AGATGCAATC
            · N  N  M  L  S  C  G  A  Q  S  N  N  E  L  P  Q  D  L  Q  N ·
2641        AAATAATATG TTAAGTTGTG GTGCCCAATC AAACAATGAG TTGCCACAAG ATCTTCAGAA
            · L  N  M  H  E  T  T  Q  M  T  E  M  D  F  E  S  I  M  R  H ·
2701        CCTGAATATG CATGAGACAA CACAGATGAC GGAGATGGAT TTTGAATCTA TAATGCGTCA
            · E  I  S  I  S  N  A  P  I  N  F  D  L  *
2761        CGAAATATCT ATTAGTAATGC TCCAATCAA CTTTGATCTG TAAATATTAT ATATAATTTC
2821        TATGTATATA ATATATTATT TTTTAAAAAA AAAAACGTTA TTGAGAGAGA TTCCTAAAGT
2881        TGTGACAAAG CTTAATTAAT AGAGAGAAAA AAGACATCAC ACACAAAAGT AAAAGACACT
2941        CACATCCTAT ATATATCTAT CTATATAATG TACACAGACA CATACAAAAA AATTATTGAC
3001        AAACAATTAT CAATATTATA AAAAAGATAT TTTTGAAAAC TTTGATATTG TAATATAATA
3061        ATATTTATAC ATACATATAT ATACTCATTC CCATATATTG ATAGCAGCAA CAGTATACAA
3121        AAAATATGTT TCATTTATTT GACTATTTGT TAATATATAT TTTTTATAAT ATTATAATCC
3181        TTTTAAAAAA AAAAAAAAAA
```

Coding region is highlighted in yellow
UTR regions are highlighted in dark gold
Introns are un-highlighted and lower case
PKB Phosphorylation site for nuclear localization RxRxx(S/T) is boxed
FoxO recruitment of cofactors Sir2 LXXLL

4.10 RESULTS: ANALYSIS OF *P. trichosuri* DAF-16A AND DAF-16B DEDUCED PROTEIN STRUCTURE

A local installation of InterProScan (ZDOBNOV and APWEILER 2001) was used to scan the translated sequences against the member databases of InterProScan in order to compare identify protein motif of *C. elegans* and *P. trichosuri* DAF-16. The deduced amino acid sequence of *P. trichosuri* DAF-16A is 755 aa and the deduced amino acid sequence of *P. trichosuri* DAF-16B is 568 aa, the coding regions are highlighted yellow in Figures 4.19 and 4.20. The translated *P. trichosuri* DAF-16A and DAF-16B isoforms share an overall 37.9% and 41.1% similarity with *C. elegans* DAF-16 genes respectively. The translated *P. trichosuri* DAF-16A and DAF-16B isoforms share an overall 81.9% similarity with the closely related *S. stercoralis* DAF-16A isoforms and 90.7% similarity for the *S. stercoralis* DAF-16B isoforms .

The DAF-16 fork head transcription factor is the target of IIS signalling cascade. When the IIS pathway is activated, DAF-16 is phosphorylated by Akt/protein kinase B (Akt/PKB) and is retained in the cytoplasm, where it is inactive. There are four conserved Akt/PKB phosphorylation sites with the motif RxRxx(S/T) which are conserved between *C. elegans, S. stercoralis* and *P. trichosuri*.

There are two stress response pathways, one is IIS dependent and one is independent of the IIS pathway, and both result in DAF-16 nuclear localization (See Section 1.3.4). In *C. elegans*, 14-3-3 scaffolding proteins are bound to phosphorylated DAF-16 to promote its retention in the cytoplasm. There is an IIS independent stress pathway, and when the worm is exposed to environmental stress, JNK-1 phosphorylates DAF-16 (at sites other then the AKT-1/2 sites) and cause it to become localized to the nucleus where it is acetylated by CBP/p300, and it binds to SIR-2.1, a NAD+ -dependent deacetylase. This prevents further acetylation of the transcription factor and promotes the transcription of genes involved in stress response and long life (Berdichevsky, Viswanathan, Horvitz, & Guarente, 2006; van der Heide & Smidt, 2005). The murine "FoxO LXXLL motif" mediates Sirt transcriptional activity (Sirt is the murine orthologue of *C. elegans* SIR-2.1) this LXXLL motif is not well conserved between *C. elegans* and mouse, as specified (NAKAE *et al.* 2006), however, this modified motif is well conserved between the nematodes (Figure 4.21). This suggests that, like *C. elegans*, the *P. trichosuri* DAF-16 transcription factor is also capable of forming an association with SIR-2.1 to promote transcription of stress response genes independently of signalling through the IIS pathway.

```
Mm  FoxO1    (453) NCAPGLLKELLTSDSP
Hs  FOXO1    (456) NCAPGLLKELLTSDSP
Dm  dFOXO    (551) PQSQCLLHRSLNCSCM
CeDAF-16     (464) VASSSALPIDLENLTL
Pt DAF-16    (710) AQSNNELPQDLQNLNM
Ss FKTF-1    (694) AQSGNELPQDLQNLNM
```

Figure 4.21: FoxO LxxLL motif in mouse, human, fly, *C. elegans*, *S. stercoralis* and *P. trichosuri* DAF-16
The murine FoxO LxxLL motif is less conserved in *C. elegans* as identified in (NAKAE *et al.* 2006). Alignment of this region of the polypeptide between *C. elegans*, *P. trichosuri* and *S. stercoralis* shows that the modified motif (LXXDL) is conserved between these species.

P. trichosuri DAF-16A and *S. stercoralis* FKTF-1A share a serine rich N terminal domain, which is greatly expanded compared to *C. elegans* DAF-16A. In *C. elegans* this serine rich region is 37 residues long whereas in *P. trichosuri* and *S. stercoralis* it is 175 and 180 residues respectively. The proportion of serine is increased in this region is 25.4% compared to 10.9% in the rest of the gene. This serine rich region is not found in the DAF-16B isoform of any of these species (Table 4.5 Figure 4.16).

Table 4.5: Protein motifs of *P. trichosuri, S. stercoralis* and *C. elegans* DAF-16 using the suite of analysis programs of InterProScan and Vector NTI.

Analysis of translated protein motifs using InterProScan		
Fork head transcription Factor (HMMSmart analysis)		
*P. trichosuri daf-16*a	at position 329-418 aa	100% similarity 87.6.8% identity to *S. stercoralis*
*C. elegans daf-16*a	at position 140-229 aa	
S. stercoralis fktf-1a	at positon 313-401 aa	
*P. trichosuri daf-16*b	at position 141-230 aa	98.9% similarity 87.8% identity to *S. stercoralis*
*C. elegans daf-16*b	at position 162-251 aa	
S. stercoralis fktf-1b	at position 140-229 aa	
Serine-rich domain		
*P. trichosuri daf-16*a	175 aa long	25.4% serines in N terminal of gene compared to 10.9% in rest of gene.
*C. elegans daf-16*a	37 aa long	
S. stercoaralis fktf-1a	180 aa long	
*P. trichosuri daf-16*b	n/a	
*C. elegans daf-16*b	n/a	
Akt/protein kinase B phosphorylation sites (RxRxx(S/T) start		
*P. trichosuri daf-16*a	4	at location 205 aa, 422 aa, 424 aa, 501aa
*C. elegans daf-16*a	4	at location 49 aa, 235 aa, 237 aa, 309 aa
S. stercoralis fktf-1a	4	at location 193aa, 408aa, 410aa, 487 aa
*P. trichosuri daf-16*b	4	at location 17 aa, 234 aa, 236 aa, 313 aa
*C. elegans daf-16*b	4	at location 14 aa. 255 aa, 257 aa. 329 aa
S. stercoralis fktf-1b	4	at location 17aa, 233 aa, 235 aa, 312 aa
FoxO LxxLL motif (Sirt binding)		
*P. trichosuri daf-16*a	1	at location 716 aa
*C. elegans daf-16*a	1	at location 470 aa
S. stercoralis fktf-1a	1	at location 701 aa
*P. trichosuri daf-16*b	1	at location 528 aa
*C. elegans daf-16*b	1	at location 490 aa
S. stercoralis fktf-1b	1	at location 526 aa

4.10.1 Phylogenetic tree of *P. trichosuri* DAF-16A and DAF-16B to *C. elegans*, *S. stercoralis*, human, mouse and fly FOXO forkhead transcription factors of the IIS pathway.

There are several forkhead transcription factors in the *C. elegans* genome. In order to determine whether the putative *P. trichosuri* DAF-16 genes cloned were more closely related to *C. elegans* DAF-16 rather than other forkhead transcription factors, the entire

P .trichosuri DAF-16 polypeptide sequences were compared to Ce, *C. elegans*; *Ss, S. stercoralis* Dm, *D. melanogaster*; Aca, *A. caninum*; Ace, *A. ceylanicum*; Hs, *H. sapiens*; Sc, *S. cerevisiae*; Mm, *M. musculus*; and several *C. elegans* non-DAF-16 forkhead transcription factors was performed (Figure 4.22). Analysis was performed using the ProML program of the PHYLIP (Phylogeny Inference Package) version 3.68, and indicates that *P. trichosuri* DAF-16A and DAF-16B is most closely related to *S. stercoralis* DAF-16 isoforms and to the *C. elegans* DAF-16 isoforms. The next closely related proteins were the forkhead transcription targets of the insulin family of other species. Table 4.6 gives a text version of the phylogenetic tree, tabulating the evolutionary distances between the sequences and the nodes of the number of mutations per 100 aa necessary to change one sequence to the other.

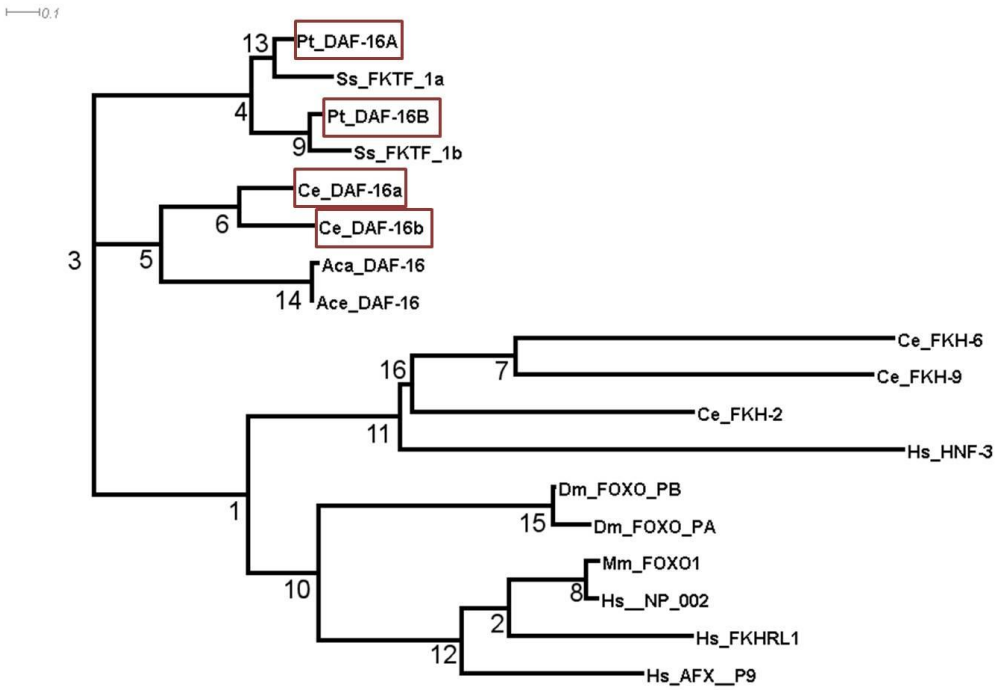


Figure 4.22: Phylogenetic analysis of DAF-16
Phylogenetic guide tree was constructed on full length proteins sequence, using the ProML program version 3.68 of the PHYLIP package (FELSENSTEIN 2005) This program uses Maximum Likelihood in order to create a tree with the highest probability. GenBank or GenPept accession numbers follow two letter species designation. The species used for this analysis were: *Pt, P. trichosuri; Ce, C. elegans; Ss, S. stercoralis;* Aca, *A. caninum*; Ace, *A. ceylanicum* *Hs, H. sapiens; Dm, D. melanogaster; Mm, M. musculus*

Table 4.6: The distances between the nodes of the Phylogenetic guide tree of Insulin/IGF FOXO forkhead transcription factor protein alignments. are given in the following table followed by the upper and lower limits in parenthesis. Maximum Likelihood method was used to estimate distances and give confidence limits.

Between	And	Distance (upper, lower limit)	Confidence Limits
8	Ce DAF-16A(AAB84390)	0.13488 (0.08671, 0.18306)	**
8	3	0.42849 (0.32373, 0.53307)	**
3	2	0.47967 (0.32085, 0.63850)	**
2	1	0.33105 (0.17881, 0.48313)	**
1	10	0.38281 (0.25475, 0.51074)	**
10	11	0.15806 (0.07594, 0.24027)	**
11	12	0.34024 (0.26452, 0.41604)	**
12	Mm FOXO1(AF126056)	0.03976 (0.02160, 0.05776)	**
12	Hs (NP002006)	0.02664 (0.01020, 0.04305)	**
11	Hs FKHRL1(CAC26821)	0.40908 (0.32059, 0.49750)	**
10	Hs AFX (P96177)	0.53113 (0.42927, 0.63296)	**
1	14	0.92061 (0.76011, 1.08114)	**
14	Dm FOXO PA (AAF55012.2)	0.13540 (0.10431, 0.16634)	**
14	Dm FOXO PB (AAS65147.1)	0.00010 (zero, 0.01093)	
2	7	0.47918 (0.27551, 0.68289)	**
7	Ce FKH-9 (AAA81139)	1.05273 (0.79029, 1.31532)	**
7	13	0.42161 (0.20646, 0.63662)	**
13	Hs HNF-3 (AAD51979)	1.14382 (0.89480, 1.39273)	**
13	5	0.17185 (zero, 0.37535)	
5	Ce FKH-2 (AAL02521)	0.87935 (0.63300, 1.12556)	**
5	Ce FKH-6 (AAA80692)	1.12591 (0.85418, 1.39770)	**
3	4	0.47492 (0.36512, 0.58470)	**
4	9	0.15707 (0.11326, 0.20071)	**
9	Ss FKTF 1A (AY281749)	0.00591 (zero, 0.02362)	
9	Pt DAF-16A	0.23433 (0.19684, 0.27182)	**
4	6	0.12311 (0.08168, 0.16459)	**
6	Pt DAF-16B	0.14347 (0.11046, 0.17663)	**
6	Ss FKTF 1B (AY281750)	0.01657 (0.00241, 0.03068)	**
8	Ce DAF-16b (AAB84390)	0.24736 (0.19019, 0.30469)	**

Distance = # of mutations per 100 aa to change one sequence to another
** = Confidence limits significantly positive, $P < 0.01$

4.11 RESULTS: EXPRESSION PATTERNS OF THE PUTATIVE *P. trichosuri daf-2, age-1* AND *daf-16* GENES.

4.11.1 Quantitative Real Time PCR validation of the template and primers

Quantitative PCR (q-PCR) was used to determine expression of each gene in different developmental stages. The developmental stages chosen were free-living adult stage, eggs, newly hatched L1, L1 that have been exposed to conditioned medium for 36 hour, and therefore hypothesized to have initiated the developmental process of becoming infective larvae (L1+CM), mature infective larva (iL3), iL3 that have passed through a patch of possum skin into tissue culture medium (iL3+skin), and parasitic adults. To confirm that the L1+CM were progressing along the developmental path to becoming infective larva, an aliquot was allowed to develop fully rather than being processed for total RNA, and >90% developed to infective larva after 84 hours. The infective larva that were collected after *in vitro* skin penetration were in the process of exiting from infective larva stage as judged by the presence of exsheathed cuticles in the media and infective larva that appeared to have shed the sheath (pictures Appendix 4).

Template for q-PCR was generated by extracting total RNA using TRIzol™, followed by DNAseI treatment as described in Methods Section 2.5.4 and Section 2.5.5 and reverse transcription with Invitrogen Superscript RT as described in Methods Section 2.5.7. Template and primer validation and the q-PCR were as described in Methods (Section 2.6.7).

4.11.2 Relative expression of *P .trichosuri daf-2, age-1* and *daf-16* in various developmental stages normalized to gap3dh

Measurement of relative expression by Quantitative Real Time PCR (q-PCR) revealed that the *P. trichosuri* putative IIS gene orthologues are expressed at all the developmental stages tested.

Pt daf-2 and *Pt age-1* showed high levels of relative expression in the L1 stage, with the greatest up-regulation in the infective larvae stage. Interestingly, there is a down regulation of all the genes in the developmental stage that is in transition from free-living larvae to infective larvae (L1+CM is significantly down regulated compared to either the L1 stage or to the iL3 stage, by Student's t-test, p <0.02), whilst the free-living adults and the parasitic adults show the lowest levels of expression (Figure 4.25).

The *P. trichosuri daf-16* transcripts in general show more differential expression than the *daf-2* and *age-1* genes (Figure 4.23). The free-living and parasitic adult stages show the lowest relative expression. *Pt daf-16a* is most abundant in the egg stage, and both isoforms show high levels of relative expression in the infective larval stage. As observed for *Pt age-1* and *Pt daf-2* there is a decrease in expression of *Pt daf-16a* and *Pt daf-16b* in the larvae exposed to conditioned medium for 36 hours (L1+CM; significant by Student's t-test, p <0.02). These are hypothesised to be in the transitional state of development between the first larval stage and the infective larval stage. This implies expression of all these genes are reduced prior to the increase in expression observed in the iL3 stage.

These *P. trichosuri* IIS pathway gene orthologues share similar expression profiles. In general the lowest expression levels are in the free-living and parasitic adult stages; there is high relative expression in the first larval stages followed by a decrease in expression at the stage of development which is in transition from L1 to infective larvae. The infective larval stage, in general, is the stage with the highest expression levels followed by a drop in expression as the worms exit from the infective larval stage.

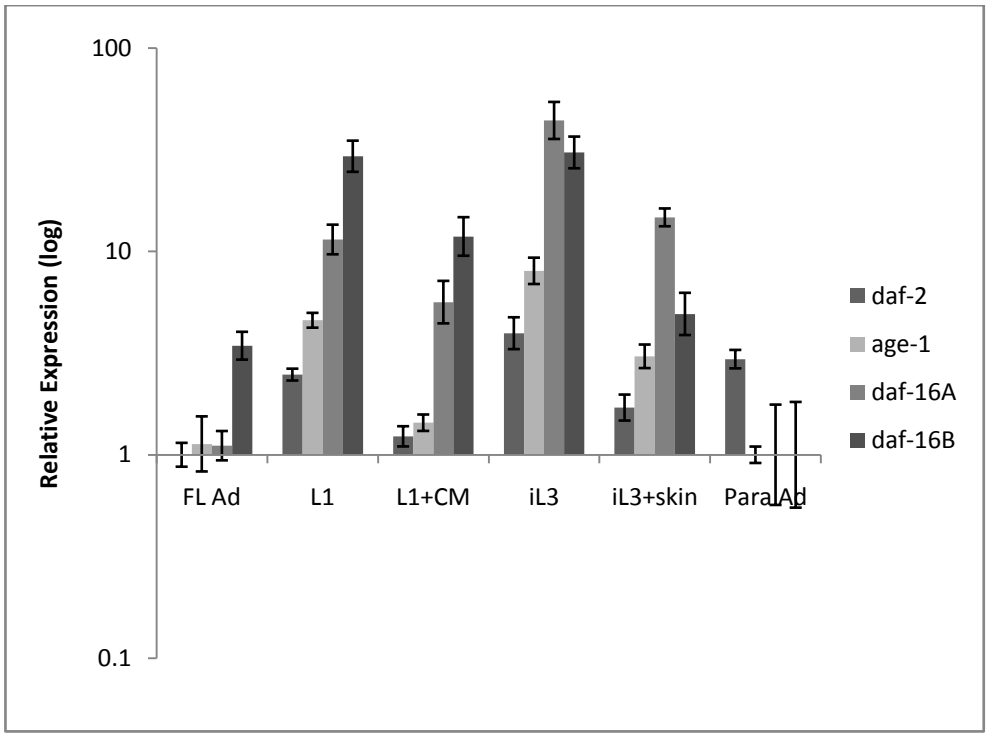


Figure 4.23: Comparative C_T relative expression of *P. trichosuri daf-2, age-1, daf-16a* and *daf-16b* normalized to the endogenous housekeeping gene *gap3dh* at various developmental stages.

Stages of development: free-living adult (FL Ad); embryo (Egg); first larval stage (L1); first larval stage exposed to conditioned medium for 36 hrs (L1+CM); infective larva (iL3); infective larva collected after *in vitro* skin penetration into tissue culture medium (iL3+skin); parasitic adult (Para Ad). Comparative C_T relative expression normalized to *gap3dh* and relative to the ParaAdult stage was employed on an ABI PRISM 7700 Sequencing Detection System (PE Applied Biosystems). Bars are standard deviation of at least 5 replicates. Primers: **daf2 F QPCR** –CTAGCCATACCAAAATCACCAA; **daf2 R QPCR** –ATTGCCTTGTTCA TCGTGATG; **age-1fwd QPCR** –TAAATCCAAGTGGCCCAGAGG; **age-1 rev QPCR** –CGTGGATGTGAACC ACGTTG; **d16A QPCR Fwd** –TTTTTTCCGTGATAAAGGAGATAGC; **d16A QPCR Rev** –GGAGTTTTTCC ATCCTGCACTACTATT; **d16B QPCR Fwd** –ATGATAGATCATCACAAGAAGAA; **d16B QPCR Rev** –TGTCTT ATGGAGTTCTTCCA

4.12 DISCUSSION

Approximately 70% of all *C. elegans* genes are trans-spliced to a 22 nucleotide splice leader sequence (SL1 or SL2), not associated with the gene but rather supplied by a small nuclear ribonucleoprotein particle (snRNP) which is consumed in the process (BLUMENTHAL 2005). The splice leader sequence is quite conserved throughout the nematode phylum and tends to be spliced close to the initiation codon, and so it is thought to play some role in translation initiation, however its true function is unknown (BLUMENTHAL 2005). Of all the genes transpliced in *C. elegans*, approximately 20%

are arranged in clusters of two to eight genes, which are expressed from the same promoter similar to operons in bacteria (GUILIANO and BLAXTER 2006). The first gene of these clusters are transpliced to the SL1 sequence leader and the downstream genes are transpliced to the SL2 sequence leader (GUILIANO and BLAXTER 2006). Many genes arranged into these operons tend to function together, or are needed for either micochondrial function or for gene expression such as transcription, splicing or translation (BLUMENTHAL 2005). A similar trans-splicing reaction and operon organization is found in nematodes throughout the phylum and many other animals such as flatworms, hydra and chordates (BLUMENTHAL 2005). In this study I was able to isolate the 5′ end of both the *Pt daf-2* and *Pt age-1* genes by PCR using the SL1 splice leader primer. This suggests that, like *C. elegans*, these genes in *P. trichosuri* are transpliced to SL1 splice leaders. Whether the gene organization is identical in *P. trichosuri* remains to be determined because, in *C. elegans* the *daf-16a* gene is SL1 transpliced and *C. elegans daf-16b* is not; whereas in *P. trichosuri* I was unable to isolate either *Pt daf-16a* nor *Pt daf-16b* using SL1 or SL2 primer PCR. The fact that SL2 PCR was unable to isolate any of the gene orthologues isolated in this study is perhaps not surprising as SL2 transplicing is a recent evolutionary adaptation of phylum Nematoda which is thought to be particular to Clade V (Rhabditina) nematodes (GUILIANO and BLAXTER 2006). In *C. elegans*, each of the IIS genes of interest are on a different chromosomes (*daf-16* is on chromosome I; *daf-2* is on chromosome III; *age-1* is on chromosome II) and are therefore unlikely to be co-transcribed.

Although trans-splicing has been evolutionarily conserved throughout the phylum Nematoda, there have been some evolutionarily changes in the structure of nematode genes within the phylum, with both gain and loss of introns (CHO *et al.* 2004). Genes that are highly expressed tend to have short introns, to expedite gene expression and the density of introns correlates to generation time, as short lived organisms tend to have less introns (JEFFARES *et al.* 2006). The *P. trichosuri* genes cloned in this study have both fewer and smaller introns (<50bp) compared to their *C. elegans* orthologues. Interestingly, the only two other *P. trichosuri* genes entered into the NCBI (http://www.ncbi.nlm.nih.gov/) database *Pt daf-7* and *Pt hsp-70*, follow a similar trend, each having a single small intron of less than 50 bp located at the 5′ end of the gene. A set of 6 genes is a small number of genes on which to speculate about intron loss in this species relative to *C. elegans*. However if this trend does hold up it highlights the

relative ease of cloning genes from this species versus other parasitic species such as *T. colubriformis*, *P. pacificus* or, *H. contortus,* in which there is an apparent increase in both the number of introns and their length when compared to *C. elegans* (Stewart Bisset, pers. comm.) (DIETERICH *et al.* 2008; LIU *et al.* 2004; PRATT *et al.* 1990).

The putative IIS orthologues cloned from *P. trichosuri* show several protein motifs in common with the *C. elegans* IIS components. For all the putative IIS orthologues cloned from *P. trichosuri*, phylogenetic analysis using the translation of the entire *P. trichosuri* gene sequence of these putative orthologues, revealed that each of the *P. trichosuri* genes are more closely related to the *C. elegans* IIS protein orthologues, and to other nematode putative IIS protein orthologues, than to other members of the *C. elegans* protein family.

Translation of the putative *P. trichosuri* DAF-2 orthologue indicates that it has a conserved motif organization and that there is high similarity in the catalytic region (72.3%) when compared to *C. elegans* DAF-2. For example, both have two ligand binding domains separated by a furin like domain, although the conservation of this domain is somewhat lower (44.6%) than the catalytic domain. There is conservation of four cysteine residues of *Pt* DAF-2, *Ce* DAF-2 and *Bm* DAF-2, which are thought to be involved in the forming interchain disulfide bonds which forms the homodimer holoreceptor (PATEL *et al.* 2008). Using HMMPfam for motif analysis indicates that there are three fibronectin domains in *P. trichosuri* versus one in *C. elegans*. However, a different analysis program: HMMSmart, which has a less stringent motif recognition profile, indicates that *C. elegans* DAF-2 does have 3 fibronectin domains albeit with low e values (Table 4.1). Fibronectin type III domains act as a scaffolding motif to bind ligands and other extracellular proteins. These additional scaffolding binding sites suggest that *P. trichosuri* may interact with different proteins and ligands and therefore perform different roles to *C. elegans* DAF-2. The InterProScan suite of analysis programs did not detect any fibronectin domains for *B. malayi* DAF-2. Another difference that protein analysis of the DAF-2 orthologues has highlighted is the number of IRS-1 motifs. IRS-1motifs are involved in interaction with the p85 domain of PI3′K (WOLKOW *et al.* 2002). There are three IRS-1 sites in *C. elegans* versus one in either *P. trichosuri* or *B. malayi;* however, this single IRS-1 site is spatially conserved between these three species. The different motifs between these species suggest that they may

carry out different protein: protein interactions, and consequently perform different functions.

Translation of the putative *P. trichosuri* AGE-1 orthologue indicates that the same domains are present in both *P. trichosuri* and *C. elegans*. The PI3′K catalytic domain has the highest identity between the two species at 49.5%. Both species have a PI3′K ras binding and accessory domain, a C2 domain and a p85 binding domain.

Cloning of the putative *P. trichosuri daf-16* gene with the use of 5′ RACE indicates that it has two isoforms which share a conserved C terminal but have alternatively spliced N terminal regions and presumably alternative promoters as determined by the differential gene expression. RT-PCR on genomic DNA has indicated that there is a very large (~10kbp) intron in the *Pt daf-16a* gene (Figure 4.17). This intron may contain the promoter region of the *Pt daf-16b* isoform. A similar genomic organization is found in *C. elegans* for the *daf-16a* and *daf-16b* isoforms (OGG *et al.* 1997) and has been reported for *S. stercoralis fktf-1a* and *fktf-1b* (MASSEY *et al.* 2003), suggesting this is an ancient nematode adaptation.

Translation of the putative *P. trichosuri* DAF-16A and DAF-16B orthologues and analysis using InterProScan indicated that these putative proteins have a conserved motif organization with *C. elegans* DAF-16A and DAF-16B and *S. stercoralis* FKTF-1A and FKTF-1B. All have a conserved forkhead transcription factor motif and four Akt/Protein kinase B phosphorylation sites believed to be involved in nuclear localization of the transcription factor. *C. elegans* has a semi-conserved FoxO LxxLL motif, which is thought to be involved in SIR-2.1 binding and nuclear localization during stress response (NAKAE *et al.* 2006). This motif is modified in *C. elegans* to LxxDL (NAKAE *et al.* 2006) and this site is conserved between *Pt* DAF-16, *Ce* DAF-16 and *Ss* FKTF (Figure 4.21) suggesting this may be a nematode specific feature. A point of difference between *P. trichosuri* DAF-16A and *C. elegans* DAF-16A is the length of the serine rich N terminal domain. In *C. elegans* this domain is 37 residues long, whereas in *P. trichosuri*, this domain is greatly extended to 175 residues long. The proportion of serines in this N terminal domain is increased to 25.4% versus 10.9% in the rest of the *P. trichosuri* DAF-16A protein. This extended serine rich domain is also present in the *S. stercoralis* FKTF-1A orthologue (MASSEY *et al.* 2003). The DAF-16B

isoform does not contain this serine rich domain in any of the nematode species for which sequence is available. Putative DAF-16 orthologues have recently been cloned from *A. caninum* and *A. ceylanicum* (hookworms), and similarly to the *P. trichosuri* and *S. stercoralis* DAF-16A orthologues, these hookworm orthologues also have an N-terminal extension which is serine rich, suggesting that this may be a parasite specific feature of DAF-16A orthologues. The hookworms also have 3 potential Akt phosphorylation sites. In contrast to the other nematodes the hookworm DAF-16 orthologues have an alanine-rich, 17 amino acid insert after the forkhead domain. The *Ac*-DAF-16 has the potential to function similarly to *C. elegans* DAF-16 as it has been demonstrated to bind to and drive transcription from a DAF-16 binding element in a cell culture assay (GAO *et al.* 2009).

Measurement of relative expression by Quantitative Real Time PCR (q-PCR) revealed that these genes are differentially expressed throughout the developmental stages tested. Jiang et al (2001) examined *C. elegans*, *daf*-2, *age*-1 and *daf*-16 transcripts levels and showed that there were increased levels of gene expression in the adult or early larval stages (JIANG *et al.* 2001). In contrast, *P. trichosuri* showed that the free-living and parasitic adult stages showed low level of IIS gene expression. Ogg *et al*. (1997) examined *daf-16* expression using *daf-16a*::gfp reporter construct and found expression in late embryos, larvae and dauer larvae (OGG *et al.* 1997). Our data is in agreement with gfp expression data as illustrated by high levels of expression in the egg stage (*Ptdaf-16a* isoform), L1 stage (*Pt daf-16b* isoform) and iL3 stage (both *Pt daf-16a* and *Pt daf-16b* isoforms). In agreement with our results, the expression pattern of metabolic genes including IIS genes were examined by DNA microarray analysis by Jeong et al, and it was found that *Ce age-1* and *Ce daf-16* were both down regulated in transition from L1 to the dauer stage, the pattern of *Ce daf-2* was more randomly expressed during replicates (JEONG *et al.* 2009). Expression levels of *S. stercoralis fktf-1a* and *fktf-1b* genes were analy sed by RT-PCR (which is not a quantitative analysis method), the results were ambiguous but may be interpreted as iL3 having the most abundant transcript levels (Massey et al., 2003), as was observed in this study for *P. trichosuri daf-16a* and *daf-16b*.

The q-PCR analysis revealed these putative *P. trichosuri* IIS genes, share a common expression profile, (with the exception of *Pt daf-16a,* which is greatly up regulated in

the embryo stage, and *Pt daf-2*, which is slightly up regulated at the parasitic adult stage; see Figure 4.25).

IIS has been shown to influence life span in worms flies and mammals (BARBIERI *et al.* 2003; LONGO and FINCH 2003; PARTRIDGE and GEMS 2002). It can be speculated that IIS plays a role in the differential life span displayed between the adult parasitic morph and the free-living adult morph of *P. trichosuri*. If so, one might expect a difference in expression levels of the IIS genes between the parasitic adults and free-living adults. However no significant difference was observed. This suggests that in *P. trichosuri*, IIS does not play a significant role in the differential life span between these two morphs.

Our q-PCR analysis revealed that these genes had greater relative expression during development of the embryo and early larval stages. Development to fully developed infective larva takes approximately 5 days at 20°C. When the first stage larvae are exposed to conditioned medium for 36 hours, (early in development to the infective larval stage – See Chapter 3 and Methods 2.6.7) a more complex pattern of expression is observed: first there is a significant down regulation of all the genes during the transition from free-living L1 larvae to infective larvae. Then, once the worms have completed infective larval development, the expression of all the genes are restored so that, with the exception of *Pt daf-16a*, this is their most highly expressed stage. Upon exit from the infective larval state there is again a decrease in expression of these genes. The decrease in expression during the change in development from free-living to infective larva followed by the greatly increased expression during the infective larvae stage is consistent with a hypothesis that these genes (and IIS signalling) may play a role in maintaining the developmental arrest, or stress resistance of infective larvae.

This hypothesis is presented with the caveat that it is the phosphorylation of the DAF-16 transcription factor rather than its expression level that indicates the activation state of the IIS pathway. However, gene expression levels of the IIS genes may be an indication of the signalling capacity of the pathway. High levels of expression of the IIS pathway genes in the embryo and early larval suggest a strong requirement for these genes in those stages of development, which is not surprising given the role of IIS in development throughout the metazoan phyla. Interestingly, in the stage that is in transition between L1 and infective larvae, the expression levels of each of these genes

are down regulated. In *C. elegans* development, the IIS pathway is down regulated during dauer larvae development, resulting in the dephosphorylation of the DAF-16 transcription factor and its translocated to the nucleus to regulate genes involved in dauer larvae development. In *C. elegans,* down regulation of the IIS pathway at first larval stage leads to dauer larvae development (DILLIN *et al.* 2002; KIMURA *et al.* 1997; 1997). If the dauer hypothesis is valid and IIS plays a similar role in infective larval development, then one can speculate that the differential expression of these genes, as determined by q-PCR, indicates a similar process is occurring in *P. trichosuri*.

Interestingly, in *P. trichosuri* and *S. ratti*, the expression pattern of the *daf*-7 orthologue, which is the activating ligand for the cell surface receptor of the TGF-β signalling pathway (known to control dauer larvae development in *C. elegans*), shows a high level of expression in the infective larval stage and low expression in the L1 stage, this is in complete contrast to the expression levels in *C. elegans* which have high levels of *daf-7* in the L1 stage and low levels in dauer larvae stage (CROOK *et al.* 2005). Viney *et al.* have speculated that the high expression levels of *daf*-7 at the infective larval stage suggests a function in maintenance of the infective larval state rather than a switch from free-living to infective larvae (VINEY *et al.* 2005). The q-PCR presented here are consistent with this hypothesis, but it must be emphasized that, unlike *daf-7* and the TGF-β signalling, transcript levels of the IIS genes are not an indication of signalling.

Expression levels of the insulin signalling genes are at their highest in the infective larval stage, as was the expression levels of *Pt daf-7* the TGF-β ligand, indicating a strong requirement for the signal transduction pathways to be operating whilst in the infective larval state (CROOK *et al.* 2005). In *C. elegans*, when insulin signalling is activated, DAF-16 is phosphorylated and blocked from entry into the nucleus. This allows for normal reproductive development to occur and prevents the activation of dauer larvae formation genes. In essence then, the insulin signalling pathway acts as a brake on a change in development. One can speculate that the *P. trichosuri* gene orthologues of this pathway may be up regulated at the infective larval stage because they may be involved in maintaining the infective larva stage until such time as encountering an appropriate host.

CHAPTER 5

FUNCTIONAL ANALYSIS OF INSULIN/IGF GENE ORTHOLOGUES

5.1 INTRODUCTION

In this chapter, I discuss the functional analysis of *P. trichosuri* IIS *daf* gene orthologues. The study of gene function directly within the organism of interest is the preferable method to determine function, and functional analysis of the *Pt daf* gene orthologues within *P. trichosuri* was attempted by a variety of methods, such as gene knockdown using RNAi, gene knockout by chemical mutagenesis and transformation of *P. trichosuri* with a modified, constitutively activated *Pt*-DAF-16. Although, these initial attempts proved to be unsuccessful, the results of these experiments are presented here. As functional analysis of the gene orthologues within *P. trichosuri* proved to be unsuccessful, rescue of mutant phenotype by complementation in *C. elegans* using the putative *P. trichosuri* orthologues was also performed and the results are presented herein.

RNA interference (RNAi), is a technique used to specifically knock down gene expression, and has proven to be quite efficient in *C. elegans* (FEINBERG and HUNTER 2003; MAY and PLASTERK 2005; TIMMONS *et al.* 2001; WANG and BARR 2005). The basic RNAi mechanism is a two step process (as described in Chapter One): dsRNA may be introduced to an organism by a variety of methods and is cleaved into short interfering RNA (siRNA) fragments of 19-23mers with a 2 bp overhang at their 3'end. This cleavage is performed by the enzyme complex, Dicer. Once cleaved to siRNA the antisense strand then serves as a template and guide for the RNA-induced silencing complex (RISC) to target and cleave the complementary mRNA (LILLEY *et al.* 2007; TOMARI and ZAMORE 2005; WANG and BARR 2005). Most organisms are thought to contain at least part of the RNAi machinery, but there are aspects of the RNAi pathway which differ from organism to organism. For instance, *C. elegans* has an amplification step, which require only a few strands of introduced dsRNA to result in effective silencing of highly expressed genes (TOMARI and ZAMORE 2005). In *C. elegans* RNAi is also heritable, as the dsRNA may be passed on through one or more generations (WANG and BARR 2005). Another interesting feature of RNAi in *C. elegans* is the systemic spreading effect, which allows for the introduction of dsRNA to one site, such as the gut, to result in gene silencing in most tissues; the nervous system being the notable exception to this - nerve cells in *C. elegans* are refractory to RNAi (SIMMER *et al.* 2002; TIMMONS *et al.* 2001).

RNAi is a powerful gene silencing tool that has been attempted in both plant and animal nematode parasites. Moderate success has been achieved with some plant parasites by soaking the J2 juvenile stage with 42 to 1300bp dsRNA transcripts. There is also evidence for RNAi spreading, and surprisingly, RNAi is effective in the nervous system of a variety of plant parasitic nematodes (LILLEY *et al.* 2007). In animal parasites a variety of dsRNA delivery methods have been tried, from ingestion, to soaking, to electroporation. The length of transcript has been varied, in case the target organism lacks the cellular machinery to dice the dsRNA into the 19-23nt size required for the RISC complex. Nevertheless, results of RNAi in mammalian parasites have been variable (GELDHOF *et al.* 2007; KNOX *et al.* 2007).

In order to test the function of genes within *P. trichosuri* by transgenesis, a *P. trichosuri* mutant of the gene of interest is required - one which confers a mutant phenotype. The mutant's phenotype is determined and then rescued by complementation with the wild-type transgene in order to confirm the gene's function. Chemical mutation with ethyl methanesulfonate (EMS) was attempted in *P. trichosuri* to induce gene deletions of IIS genes, and the results presented herein. The complex life cycle of most parasitic nematodes requires the reproductive adult stage to be sequestered within the host, therefore direct genetic manipulation by transgenesis or mutation has been limited to a handful of special cases. As *S. ratti* has a facultative free-living generation, chemical mutagenesis was used to isolate mutants with low levels of resistance to ivermectin (VINEY *et al.* 2002). Transgenesis with a gene that confers a dominant phenotype may also be used to assess function of a gene. Transgenesis with a constitutively activated transcription factor (DAF-16) was attempted in *P. trichosuri* in order to assess the effect of this transgene on development.

If the parasitic gene in question has a *C. elegans* orthologue, then rescue by complementation is possible by microinjecting the parasite gene into the relevant *C. elegans* mutant, and then assessing for recovery of the wild-type phenotype (HASHMI *et al.* 2001). Microinjection of *C. elegans* gonads with foreign DNA results in the formation of a heritable extrachromosomal arrays of the exogenous DNA and has allowed functional analysis of several parasite genes in the *C. elegans* model (BRITTON *et al.* 1999; GRANT 1992; GRANT and VINEY 2001; HASHMI *et al.* 1995). Careful

assessment must be made as differences in temporal patterns and exaggeration of mutant phonotype may occur (BRITTON *et al.* 1999; GRANT 1992).

This chapter aims to test the hypothesis that IIS gene orthologues play a role in the developmental switch from free-living to infective larvae by complementation of *C. elegans* Daf-16 mutants with a *Pt daf-16* transgene.

5.2 RESULTS: FUNCTIONAL ANALYSIS IN *P. trichosuri*

5.2.1 RNAi

In order to determine the function of genes of interest in *P. trichosuri*, RNAi knockdown was attempted. Several methods of dsRNA delivery to the worm were trialled.

5.2.2 Feeding assay

As feeding *C. elegans* with bacteria expressing dsRNA results in a knockdown in expression of the gene of interest; two candidate genes were trialled. The *C. elegans* twitchin' gene (*unc-22),* which is required for the actin/myosin contraction and relaxation cycle and gives an obvious twitching phenotype, and *C. elegans unc-54*, a muscle myosin with a strong, flaccid paralysis RNAi phenotype. Putative *P. trichosuri* gene orthologues to *unc-22* and *unc-54* were chosen as candidate genes to test RNAi by feeding in *P. trichosuri*. Fragments of these putative gene orthologues, *Pt unc-54* (346 bp fragment) and *Pt unc-22* (360 bp fragment) were cloned into the pL4440 dsRNA vector (kindly donated by Andrew Fire) (Appendix 1.14) and ds RNAi feeding assay was performed (as per methods described, Section 2.11.1). Separate experiments were performed for either *Pt unc*-22 or *Pt unc*-54 RNAi by the feeding method. No twitching or paralysis phenotype was observed - data not shown.

To confirm that the bacteria fed to the larvae were transcribing the dsRNA, aliquots of *E. coli* HT115 bacteria, which were transformed with either the empty vector or the vector containing a gene of interest, were first solubilised, sonicated, and then run on an agarose gel, as describe in Method Section 2.11.2. Aliquots were taken both before and after induction with 1mM IPTG. Salts in the solubilization buffer, cause these samples to migrate much higher than the Molecular Weight marker (Figure 5.1); therefore the presence of a band in the induced bacteria, which is not present in the empty vector control nor uninduced lanes is judged to be an indicator of dsRNA expression.

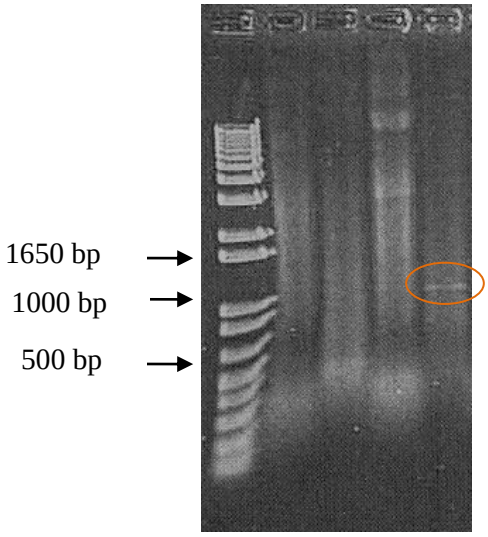


Figure 5.1: Agarose gel of sonicated *E. coli* HT115, transformed with pL4440 vector before and after induction with IPTG.
Lane 1: 1Kb Plus ladder; Lane 2-3, Neg control (empty vector); Lane 4-5 bacteria transformed *with Pt unc-22* in pL4440 dsRNA vector, after induction, Land 5 presence of band suggest expression of putative double stranded *Pt unc-22*. The IPTG induced double stranded *Pt unc-22* transcript is circled in red. Lane 1: 1KB Plus MW marker; Lane 2: pL4440 (empty vector); Lane 3: pL4440 +IPTG; Lane 4: *Pt unc-22* Lane 5: *Pt unc-22* +IPTG.

5.2.3 DAF-16 with mutated phosphorylation sites.

One of the outputs of the IIS pathway is the phosphorylation of the DAF-16 transcription factor. When the insulin pathway is activated, the DAF-16 transcription factor is phosphorylated at AKT-1/2 serine and threonine phosphorylation sites and is sequestered in the cytoplasm and hence inactive. However, when de-phosphorylated,

DAF-16 localizes to the nucleus, where it controls the expression of genes involved in development, stress response and longevity.

Using the molecular techniques described in Methods Section 2.6.4, four *P. trichosuri daf-16* transgenes were created, Figure 5.4 (Appendix 1.10 to 1.13). These consisted of a transgene of each *daf-16* isoform in which the AKT-1 phosphorylation sites were mutated, and transgenes of each of the *daf-16* isoforms without mutated sites (negative controls). If phosphorylation is the primary determinant of DAF-16 localization, the protein encoded by a transgene with mutated phosphorylation sites should be constitutively transported to the nucleus, independent of the signalling state of the IIS pathway. This would test the function of the gene directly in *P. trichosuri* and would determine whether constitutive nuclear localization of DAF-16 results in a change in development. All of these transgenes were epitope tagged with the protein antigen, (HA), in order that expression and the subcellular location could be confirmed by immunohistochemistry (BIGGS *et al.* 1999). The constructs with mutated AKT-1/2 sites were created by M(OE) linkage PCR (AN *et al.* 2005). When sequenced, it was determined that the construct pSSD16A2, contained an amino acid change at position $S_{76} \rightarrow L_{76}$ (most likely a PCR artefact). It is possible that this amino acid change affected the function of this transgene.

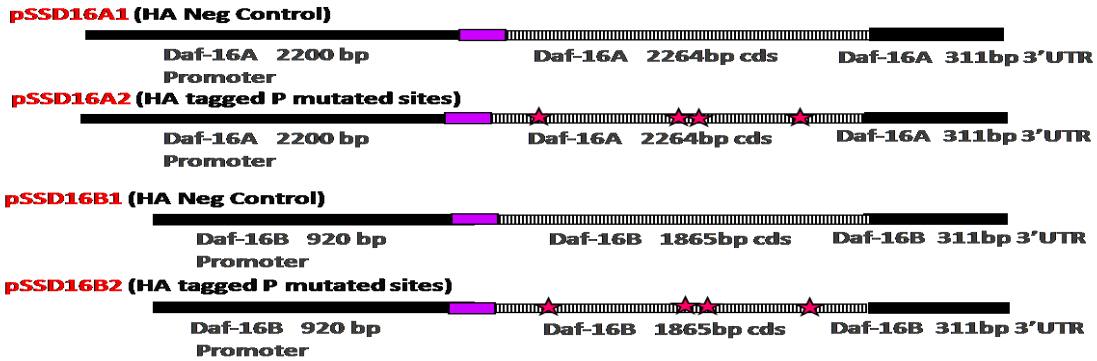


Figure 5.2: *P. trichosuri daf-16* constructs with mutated phosphorylation sites.
Solid black*:* *P. trichosuri* regulatory regions. **Hashed section:** coding region for *daf-16* isoforms. **Red stars:** mutated phosphorylation sites. **Purple section:** HA epitope protein tags for antibody detection by immunohistochemistry.

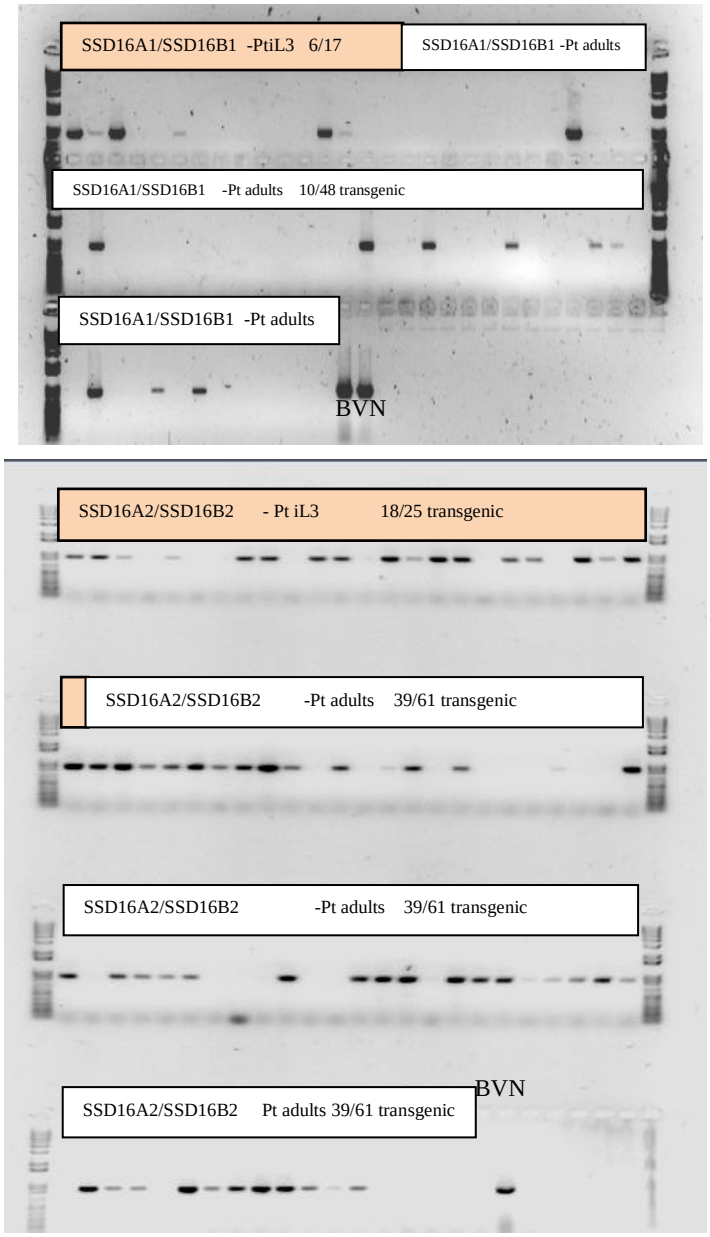


Figure 5.3: Agarose gel of PCR of *P. trichosuri* F_1 microinjected worms, scored for development and presence of transgene.

After 6 days all F_1 worms scored as either infective larva (iL3) or adults and picked into lysis buffer to be processed for PCR template. Light orange box highlights F_1 worms that developed to iL3; White box highlights F_1 worms that developed to free-living adults. B= Bacterial lawn; V= vector template (Positive Control); N= No template control. Image has been colour inverted on QuantityOne gel imaging system Version 4.6.2 [Bio-Rad]. Outer primers: Pt d16BA11: TTAAGTGTCCAGGGAAATGTTAATGGTGT with either pSTBlue SP6: GCAAGGCGATTAA GTTGGGTA or pSTBlue T7 outer: CCATGATTACGCCAAGCTCTAA nested primers: Ptdaf163'Race: GTGATATGCCTCCACAA CCACCAT and either pSTBlue Fwd: ATGAGTATTCAACATTTC CGTGTCG or pSTBlue Rev: TTACCAATGCTTAATCAGTGAGGCA.

Table 5.1: Summary of PCR screen for the presence of transgene in F_1 generation microinjected *P. trichosuri* correlated to their developmental fate.

Injected Construct @ 50ng/mL	Transgenic iL3	Transgenic Adults	Transgenic iL3/total transgenic
SSD16B1 (Neg control)	**5 transgenic** 10 total (+ non GMO)	**13 transgenic** 60 total (+ non GMO)	**5/18 =27.8%**
SSD16B2 (P Mut construct)	**6 transgenic** 23 total (+non GMO)	**19 transgenic** 68 total (+ non GMO)	**6/25 =24.0%**
SSD16A1 (Neg control)	**12 transgenic** 18 total (+non GMO)	**14 transgenic** 33 total (+non GMO)	**12/26 = 46.2%**
SSD16A2 (P Mut construct)	**19 transgenic** 22 total (+non GMO)	**27 transgenic** 29 total (+non GMO)	**19/46 = 41.3%**
SSD16A1 & SSD16B1 (Neg control)	**6 transgenic** 17 total (+non GMO)	**10 transgenic** 48 total (+non GMO)	**6/16 = 37.5%**
SSD16A2 & SSD16B2 (P Mut construct)	**18 transgenic** 25 total (+non GMO)	**39 transgenic** 61 total (+non GMO)	**18/57 = 31.6%**

In order to determine the effect that the transgene had on the developmental fate, all F_1 generation worms were picked from the microinjection plates at day 5 or 6 and scored for development to either free-living adults or infective larvae, then a PCR was performed on each individual worm to determine whether the transgene was present in that worm. Amplification of the transgene was performed using a nested PCR reaction with an internal *Pt daf-16* primer and a vector back bone primer in order to avoid amplification of the endogenous *Pt daf-16* transcript (Figure 5.5). Results were analysed to determine if there was an increase in the proportion of transgenic worms that developed to iL3 containing the construct with the mutated phosphorylation sites versus the transgenic worms containing the non-mutated negative control construct. The results of this PCR suggested that the number of transgenic worms carrying the transgenes with the mutated phosphorylation sites which developed to infective larvae were within +/- 10% of the transgene without mutated phosphorylation sites (Figure 5.5 and Table 5.3). Attempts at immunohistochemistry on the F_1 generation of microinjected worms in order to visualize the HA epitope tags were unsuccessful, and further optimization would be required.

5.3 RESULTS: RESCUE OF MUTANT PHENOTYPE BY COMPLEMENTATION

Rescue of *C. elegans* mutant phenotype by complementation with a *P. trichosuri* transgene was attempted in order to test gene function of the *P. trichosuri daf* gene orthologues. This test rests on the supposition that if a *P. trichosuri* gene orthologue is able to recover the wild-type phenotype of a *C. elegans* mutant of the same gene, then it may perform a similar role in *P. trichosuri*. There are always several assumptions made when testing the function of genes by rescue of mutant phenotype by complementation in a different species. First, that the gene is expressed correctly (both spatially and temporally) and that the resulting protein is processed correctly (modified and folded) and also, that it is able to interact correctly through its various protein domains with potentially several other proteins or co-factors.

Chimeric transgenes of *Pt daf*-2, *Pt age*-1, *Pt daf*-16*a* and *Pt daf*-16*b* were constructed, each consisted of the protein coding region of the *P. trichosuri daf* gene orthologues under the control of the equivalent *C. elegans daf* gene promoter and 3′ UTR regions (Appendix 1.5 to 1.8). These chimeric constructs were microinjected into the gonads of the appropriate *C. elegans* mutant strains. A marker gene expressing green fluorescent protein (gfp) was co-injected with the constructs, except in the case of the *Pt daf-16* chimerics which had an N-terminal linked gfp marker as part of the chimeric construct. Any transgenic offspring were isolated and tested to determine whether the mutant phenotype of the *C. elegans* mutant reverted to the wild-type phenotype.

The promoter and 3′ UTR from the *C. elegans* gene of interest was used to minimize the risk that inclusion of *P. trichosuri* regulatory sequences may introduce differences in spatial and temporal expression patterns and ensure that gene regulation is consistent with *C. elegans* expression patterns. These chimeric constructs should, therefore, directly test the ability of the *P. trichosuri* protein to substitute for the *C. elegans* protein.

Microinjection of the transgene and marker gene into the *C. elegans* mutant strains was as described Methods Section 2.10.1 and performed by Kirsten Grant. Two stable

transformed lines of each of the strain were maintained as described and used for the recovery assays.

Table 5.2: Summary table of *C. elegans* mutant strains used for rescue by complementation.

Strain	Linkage groups and genotypes	Reference
N2 Bristol	Wild-type	(Maupas, 1900)
DR26	*daf*-16 (m26)I	(RIDDLE *et al.* 1981)
CB1370	*daf*-2 (e1370)III	(RIDDLE 1977)
TJ1052	*age*-1 (hx546)II	(JOHNSON *et al.* 1993)
DR1309	*daf*-16 (m26); *daf*-2 (e1370)	(RIDDLE *et al.* 1981)
DR1408	*daf*-16 (m26); *age*-1 (m333)	(RIDDLE *et al.* 1981)

5.3.1 Nuclear Localization

The *C. elegans* DAF-16 transcription factor has four AKT-1/2 serine and threonine phosphorylation sites and additional JNK-1 phosphorylation and SIR-2.1 deactylation sites. When these post-translational motifs are modified (in response to the JNK-1stress pathway or by the IIS) DAF-16 is transported to the nucleus (BAUMEISTER *et al.* 2006; MUKHOPADHYAY *et al.* 2006). Together these pathways acts to regulate gene expression involved in stress response, dauer larvae formation and regulation of life span (see Section 1.5.5). A construct containing the *P. trichosuri daf*-16 coding region in frame with an N terminal linked gfp reporter and under the control of the *C. elegans daf*-16 regulatory regions was created in order to determine whether the expressed *P. trichosuri daf-16* gene orthologues contain the protein motifs which facilitates nuclear localization in response to oxidative stress.

Transgenic worms containing the chimeric *Pt daf-16a* and *Pt daf-16b* gfp fusion genes under the control of the *C. elegans daf-16α* promoter, were incubated with 100mM paraquat for 2 hours and pictures were taken (Figure 5.6). In these constructs the *daf*-16 N terminal linked gfp reporter was used to determine the spatial expression pattern of the *daf*-16 genes and assess whether the *P. trichosuri* DAF-16 protein is able to become nuclear localized in response to oxidative stress.

The top set of panels in Figure 5.6 is the positive control – these worms contain the *C. elegans daf-16a* transgene under the control of *C. elegans daf-16α* regulatory sequences and N terminally linked to gfp. As can be seen, when the *C. elegans daf-16* construct is

microinjected into *C. elegans,* the offspring express the transgene in the expected tissues (neurons, muscles, gut and hypodermis); also as expected, the gfp tagged DAF-16 becomes nuclear localized in response to oxidative chemical stress, as shown by the punctuate expression of the gfp (red arrows). In the middle and bottom right hand panels, the gfp expression in the *C. elegans* mutants transformed with the *Pt daf-16a* and *Pt daf-16b* transgenes and under the control of *C. elegans* regulatory sequences, also become punctuate upon exposure to paraquat, suggesting that like *Ce* DAF-16, the *Pt* DAF-16 protein orthologues also become nuclear localized in response to stress. These data suggest that the *P. trichosuri* DAF-16 protein motifs which control nuclear localization in response to stress are conserved between the *C. elegans* and *P. trichosuri* gene orthologues.

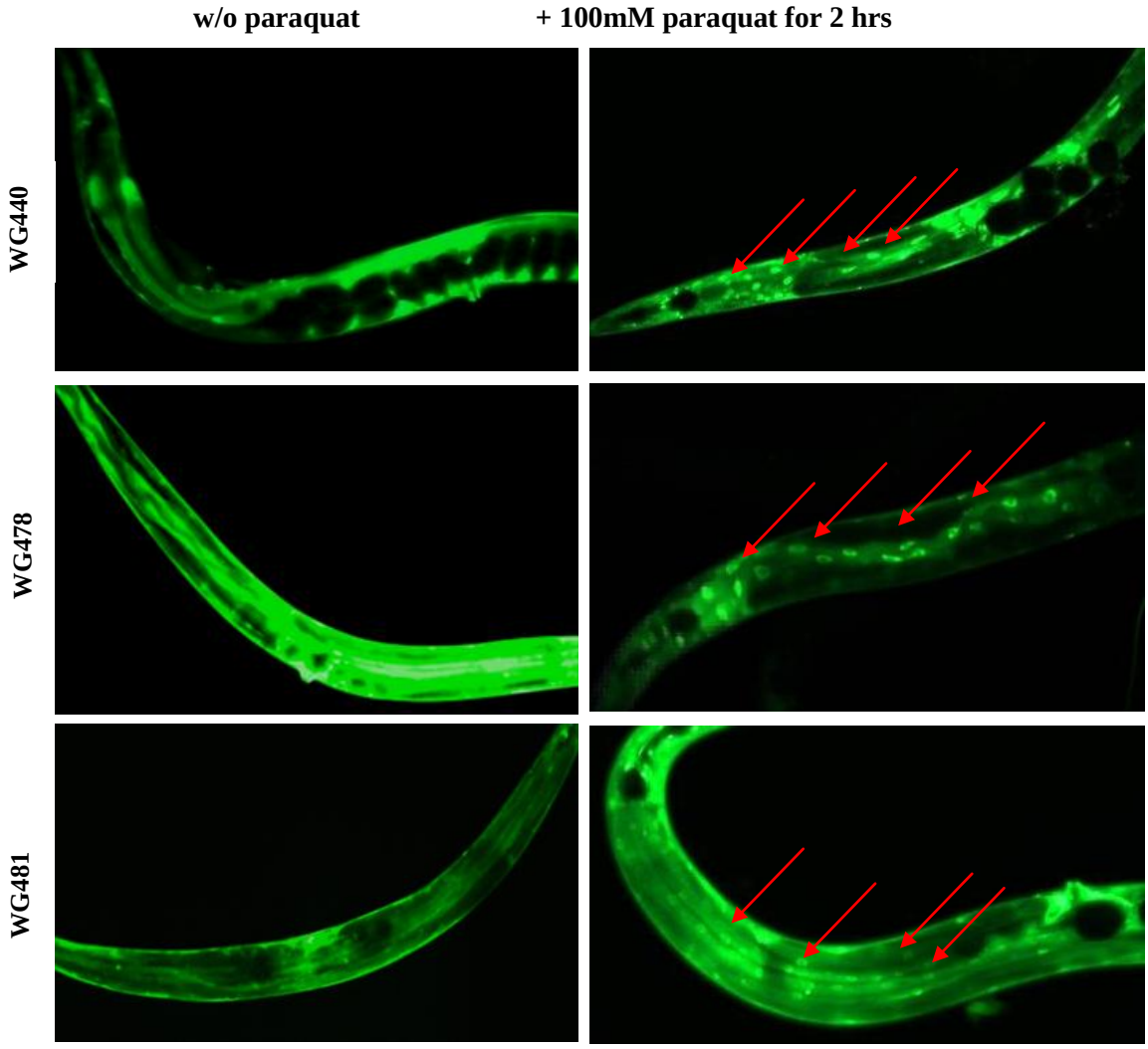


Figure 5.4: *C. elegans* transformed with *Pt daf-16* gfp fusion protein constructs and exposed to the chemical stressor paraquat at 100mM for 2 hours.
Red arrows indicate some of the nuclear localization of gfp linked DAF-16 when exposed to chemical stressor, paraquat. WG440= N2 transformed with *Ce daf-16a* (gfp fusion) transgene; WG478= DR26 mutant transformed with *Pt daf-16a* (gfp fusion) transgene under *Ce daf-16a* regulatory sequence; WG481= N2 transformed with *Pt daf-16b* (gfp fusion) transgene under *Ce daf-16a* regulatory sequence.

5.3.2 Recovery of mutant stress phenotype

P. trichosuri DAF-16, expressed under the control of *C. elegans* regulatory sequences, is nuclear localized in response to stress as would be expected if its function is analogous to *C. elegans* DAF-16. In order to test whether it is able to revert, or rescue the increased stress sensitivity that loss of function *daf-16* mutations confer in *C. elegans,* various strains were exposed to paraquat, an oxidative stress inducer (JEE *et al.* 2005) Figure 5.7. DR26 is a *C. elegans* Daf-16 mutant that is super-sensitive to stress. The transgenic lines WG477 and WG478, (which have the *Pt-daf-16a* transgene in a *C. elegans daf-16*(m26) mutant background) are partially reverted from the stress sensitive phenotype of their *daf-16* mutant parent. To determine whether the rescue by complementation resulted in a greater proportion of mutants with wildtype phenotype Logistic Regression was performed using a Generalized Linear Model (GLM) with number responding out of a total. A bionomial model was fitted using a logit link function (logit= LOGe(number with wildtype phenotype/total – number with wildtype phenotype). Accumulated analysis of deviance indicated that WG477 was significant different from the DR26 parental line (with an approximate F value of <0.001 and a test statistic of 93.63). WG478 was significant different from the DR26 parental line (with an approximate F value of <0.021 and a test statistic of 13.53). WG477 is reverted to a greater extent than WG478, this correlates with the greater degree of transgene expression as estimated by the intensity of gfp expression (Figure 5.8).

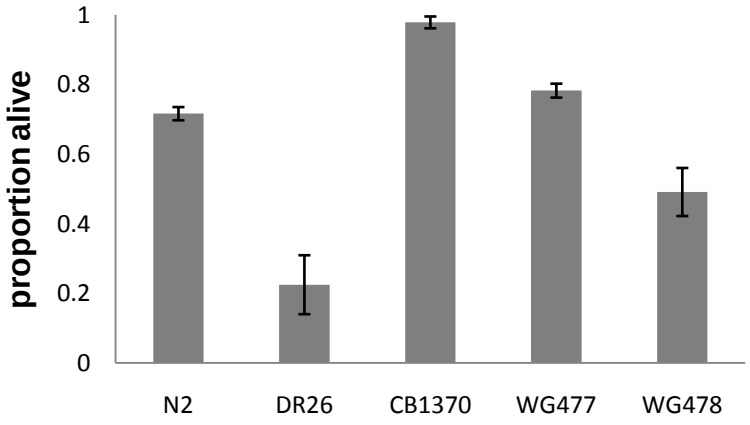


Figure 5.5: Recovery of resistance to chemical stress phenotype by complementation with *Pt daf-16a* chimeric transgene.
L4 or adult strains incubated in 100mM paraquat for 20 hours at 20C. N2= wild-type, DR26= *daf-16* mutant (stress sensitive), CB1370= *daf-2* mutant (stress resistant), WG477= DR26 with *P. trichosuri daf-16*a chimeric construct, WG478= DR26 with *P. trichosuri daf-16*a chimeric construct. The proportion WG477 and WG478 alive are significantly different from the DR26 parental line using Logistic Regression using GLM, the bars show standard deviation.

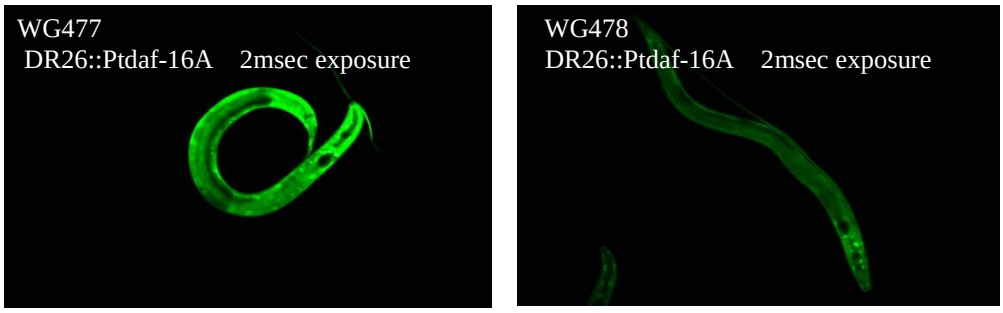


Figure 5.6: Pictures of gfp expression in transgenic lines WG477 and WG478, camera exposure set for same period of time (2msec).
Some lines of transgenic worms have greater expression of the transgene, as illustrated by stronger fluorescence of the fusion protein gfp expression in WG477 compared to WG478. This correlates to the relative amount of recovery by complementation that these lines were able to recover.

In contrast to the DR26 *daf-16*(m26) stress sensitive mutant, the *C. elegans daf-2* mutant (CB1370) has a stress resistant phenotype when compared to the wild-type N2 strain. In order to determine whether the presence of the *Pt daf-2* transgene was able to revert this stress resistant phenotype, the transgenic lines WG473 and WG474, which contain the *Pt-daf-2* chimeric construct in a *C. elegans daf-2* mutant background (strain CB1370) were exposed to paraquat and compared to the controls. If *P. trichosuri* DAF-2 plays a comparable role to *C. elegans* DAF-2 one would expect the stress resistant phenotype of the CB1370 line to be reverted to the sensitivity of the N2, wild-type line (Figure 5.9). To determine whether the rescue by complementation resulted in a greater proportion of mutants with wildtype phenotype Logistic Regression was performed using a Generalized Linear Model (GLM) with number responding out of a total. A bionomial model was fitted using a logit link function (logit= LOGe(number with wildtype phenotype/total – number with wildtype phenotype. Accumulated analysis of deviance indicated that WG473 was not significantly different from the CB1370 parental line (with an approximate F value of 0.426 and a test statistic of 0.84). Accumulated analysis of deviance indicated that WG474 was not significantly different from the CB1370 parental line (with an approximate F value of 0.618 and a test statistic of 0.31).

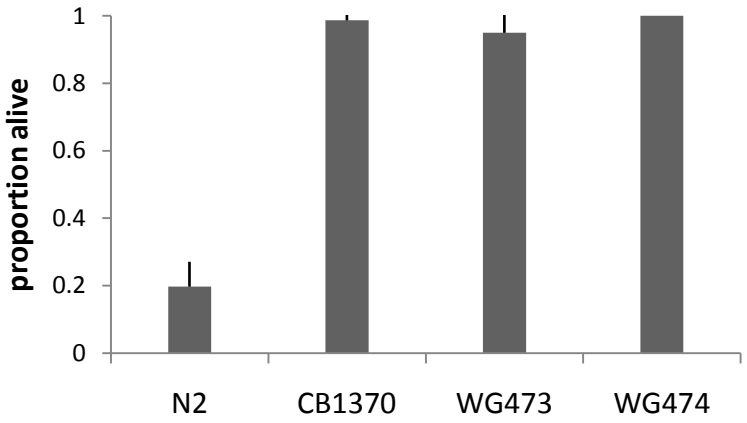


Figure 5.7: Recovery of resistance to chemical stress phenotype by complementation with *Pt daf-2* chimeric transgene.
L4 or adult strains incubated in 100mM paraquat for 20 hours at 20°C. N2= wild-type, DR26= *daf-16* mutant (stress sensitive), CB1370= *daf-2* mutant (stress resistant) WG473= CB1370 with *P. trichosuri daf*-2 chimeric construct + gfp marker (pPD129.51), WG474 = CB1370 with *P. trichosuri daf*-2 chimeric construct + gfp marker (pPD129.51). Bars indicate standard deviation.

5.3.3 Recovery of dauer larvae development

C. elegans conditioned medium contains dauer pheromone, a population signal which influences dauer larvae development (GOLDEN and RIDDLE 1982; GOLDEN and RIDDLE 1984b). The dauer larvae development of several *C. elegans* strains transformed with a *Pt daf-16* transgene were assessed when exposed to *C. elegans* conditioned medium (Figure 5.10). The dauer larvae defective phenotype of a *C. elegans* strain carrying a *daf-16* mutation (DR26) is partially restored to wild-type phenotype when transformed with the *Pt daf-16a* transgene, as illustrated by the significant increase in dauer larvae formation of lines WG477 and WG478. To determine whether the rescue by complementation resulted in a greater proportion of mutants with wildtype phenotype Logistic Regression was performed using a Generalized Linear Model (GLM) with number responding out of a total. A bionomial model was fitted using a logit link function (logit= LOGe(number with wildtype phenotype/total – number with wildtype phenotype). Accumulated analysis of deviance indicated that WG477 was significant different from the DR26 parental line (with an approximate F value of <0.009 and a test statistic of 23.06). Accumulated analysis of deviance indicated that WG478 was significant different from the DR26 parental line (with an approximate F value of

<0.009 and a test statistic of 23.06). This suggests that the *Pt* DAF-16A protein is able to at least partially regulate dauer larvae development in *C. elegans*. However, it is noteworthy, that the rescue of the mutant dauer larvae phenotype by *Pt daf-16a* transgene is weaker than the *Pt daf-16a* stress resistance rescue in the same transgenic lines.

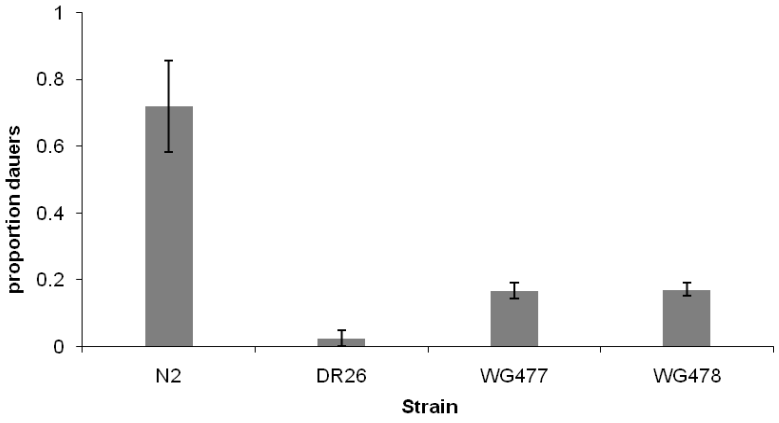


Figure 5.8: Recovery of dauer larvae development phenotype in response to semi-purified C. elegans dauer pheromone by complementation with *Pt daf-16a* chimeric transgene.
L1 larvae were incubated in semi purified *C. elegans* dauer pheromone for 5 days at 20°C. N2- wild-type; DR26= *daf-16* mutant (dauer larvae defective); WG477= DR26 with *Pt daf*-16a chimeric construct; WG478= DR26 with *Pt daf*-16a chimeric construct. WG477 and WG478 are significantly different from the DR26 parental strain using using Logistic Regression using GLM, the bars show standard deviation.

In order to determine whether the *Pt daf*-2 transgene is able to restore wild-type dauer larvae development to a *C. elegans daf*-2 mutant, rescue of the *C. elegans daf-2* allelle e1370 (CB1370) was tested. This is a temperature sensitive (Ts) allele; at 26°C it is dauer constitutive (daf-c), but at 20°C it is dauer defective (daf-d) and insensitive to dauer pheromone. When *C.elegans* CB1370 *daf-2*(e1370), is transformed with the *Pt daf*-2 chimeric transgene, neither the temperature sensitive daf-c phenotype, nor the sensitivity to dauer pheromone were restored (Figure 5.11 and Figure 5.12 respectively). In fact, it was observed that the Ts transformants were less viable at the elevated temperatures (data not shown) the data from Figure 5.12 also suggests that these strains may have become even less sensitive to the dauer pheromone than the mutant parent.

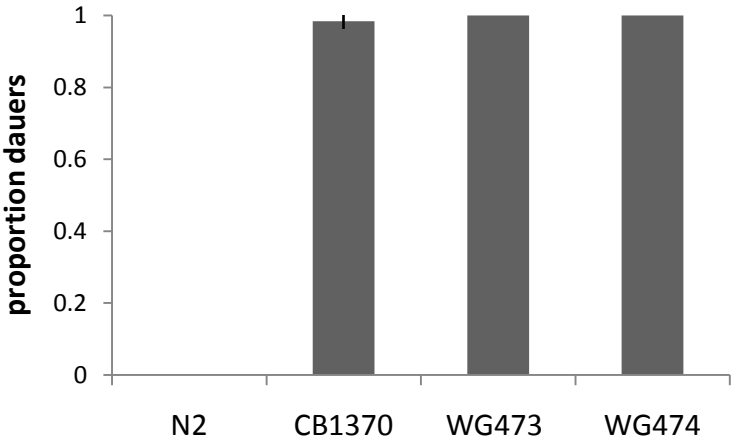


Figure 5.9: Recovery of Ts daf-c dauer larvae development phenotype by complementation with *Pt daf*-2 chimeric transgene.
L1 larvae were incubated for 5 days at 26°C. N2= wild-type; CB1370= *Ce* Daf-2 mutant; WG473= CB1370 with *Pt daf*-2 chimeric transgene + gfp marker (pPD129.51); WG474= CB1370 with *Pt daf*-2 chimeric transgene + gfp marker (pPD129.51).

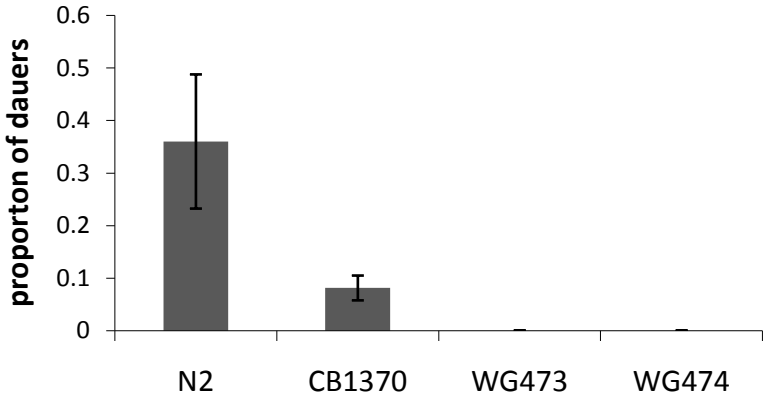


Figure 5.10: Recovery of dauer larvae development phenotype in response to semi-extract *C. elegans* dauer pheromone by complementation with *Pt daf*-2 chimeric transgene.
L1 larvae were incubated in semi extracted *C. elegans* dauer pheromone for 5 days at 20°C. N2= wild-type; CB1370= Ce Daf-2 mutant; WG473= CB1370 with *Pt daf*-2 chimeric transgene + gfp marker (pPD129.51); WG474= CB1370 with *Pt daf*-2 chimeric transgene + gfp marker (pPD129.51).

The *C. elegans* Daf-2 temperature sensitive mutant, CB1370, is a dauer constitutive (daf-c) at elevated temperatures. The double mutant, DR1309 *daf*-16(m26);*daf*-2(e1370) is not a temperature sensitive Daf-c mutant, because the *daf-16*(m26) mutation partially suppresses the Daf-c phenotype of the *daf-2* mutant (GEMS *et al.* 1998). Two lines were created (WG470 and WG530) in which the *Pt daf-16a* transgene was transformed into

the double mutant DR1309. To determine whether the rescue by complementation resulted in a greater proportion of mutants with reverted to single mutant phenotype Logistic Regression was performed using a Generalized Linear Model (GLM) with number responding out of a total. A bionomial model was fitted using a logit link function (logit= LOGe(number with wildtype phenotype/total – number with wildtype phenotype). Accumulated analysis of deviance indicated that WG530 was significant different from the DR1309 parental line (with an approximate F value of <0.010 and a test statistic of 96.36). The WG470 did not recover wildtype phenotype for the DR1309 parental line (approximate F value of 0.125 and a test statistic of 6.52). The recovery of WG530 correlates with the greater amount of transgene expression as estimated by the intensity of gfp expression (Figure 5.14).

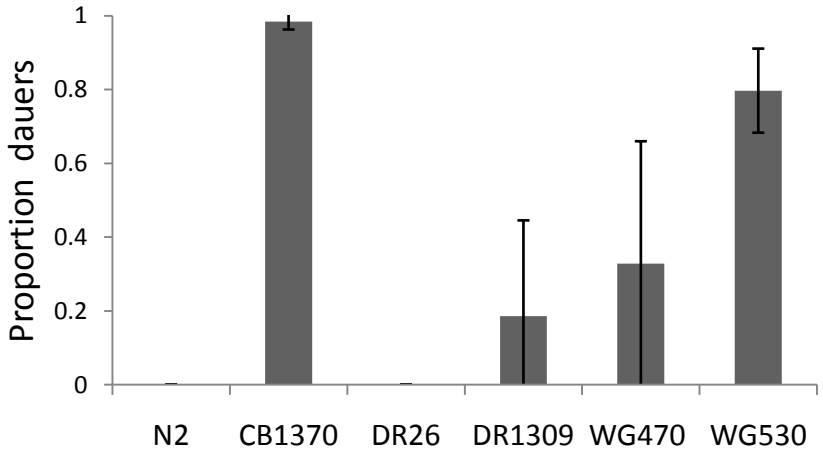


Figure 5.11: Recovery of dauer larvae development of double *daf-16;daf-2* mutant phenotype to the Ts Daf-2 mutant phenotype.
Worms were allowed to lay eggs onto plates which were incubated at 26°C for 5-7 days. N2- wild-type; CB1370= *daf-2* mutant (dauer constitutive), DR26= *daf-16* mutant, DR1309= *daf-2;daf-16* mutant (dauer defective); WG470= DR1309 with *Pt daf-16a* chimeric transgene; WG530= DR1309 with *Pt daf-16a* chimeric transgene. Dauer larvae phenotype recovery of WG530 is significantly greater than DR1309 parental strain by Logistic Regression using a GLM. Bars indicate standard deviation.

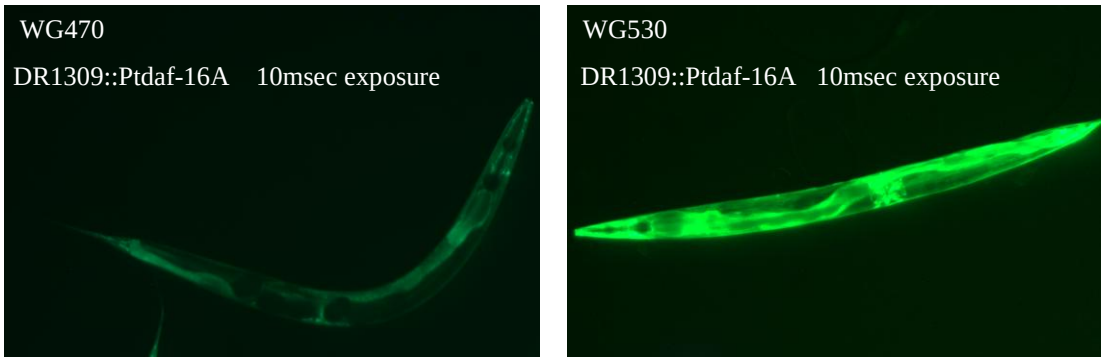


Figure 5.12: Pictures of gfp expression in transgenic lines WG470 and WG530, both exposed for same period of time (10msec).
Some lines of transgenic worms have greater expression of the transgene, as illustrated by stronger fluorescence of the fusion protein gfp expression in WG470 compared to WG530. This correlates to the amount of recovery by complementation that these lines where able to recover.

5.4 RESULTS: PHARMACOLOGICAL INHIBITION OF THE IIS PATHWAY IN *P. trichosuri*

LY294002 is a chemical inhibitor of PI3'K protein (AGE-1) and has been shown to induce dauer larvae formation in *C. elegans* (BABAR *et al.* 1999) and inhibit resumption of iL3 development in the parasite *A. caninum* (BRAND and HAWDON 2004). In order to test the effect on larval development in *P. trichosuri* when the activity of AGE-1 is inhibited, L1 larvae were subjected to various concentrations of LY294002 and scored for the development of infective larva.

P. trichosuri is very sensitive to the pharmacological effects of this chemical inhibitor, and at even moderate concentrations becomes completely arrested at the first or second larval stage for several days, i.e. it appears to be unable to develop to either an infective larvae or into a free-living adult (data not shown). However, at low doses there is a very slight, increase in infective larval development at 0.01mM to 0.05mM LY294002 when compared to the buffer control (Figure 5.13). To determine whether treatment with these concentrations of LY294002 the resulted in a greater proportion of iL3 development Logistic Regression was performed using a Generalized Linear Model (GLM) with number responding out of a total. A bionomial model was fitted using a

logit link function (logit= LOGe(number i L3/total – iL3). Accumulated analysis of deviance indicated that 0.01mM LY294002 was significantly different from the 0mM control (with an approximate F value of <0.002 and a test statistic of 19.93). 0.025mM LY294002 was significantly different from the 0mM control (with an approximate F value of 0.007 and a test statistic of 12.85). 0.05mM LY294002 was significantly different from the 0mM control (with an approximate F value of <0.001 and a test statistic of 30.82).

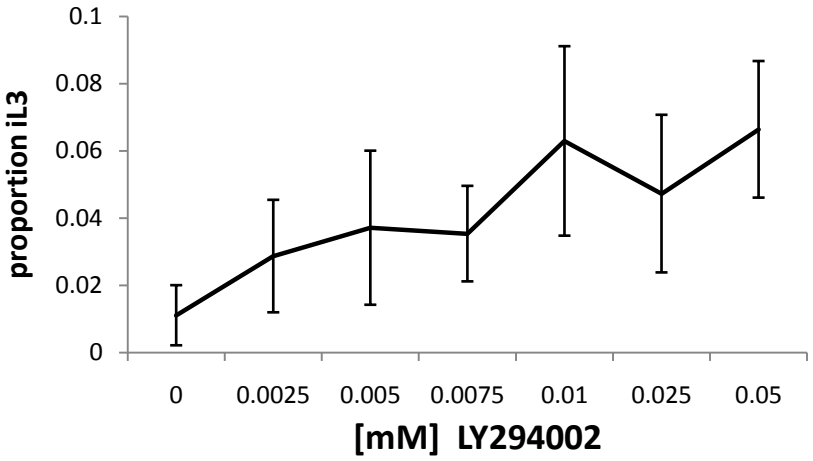


Figure 5.13: The effect of PI3′K chemical inhibitor, LY294002 on *P. trichosuri* larval development.
Proportion of L1 larvae which develop into infective larva in response to various concentrations of LY294002, treatments were incubated at 20°C for 5 days. Significance tested by Student's t-test. Bars indicate standard deviation.

An experiment was performed in which *P. trichosuri* L1 larva were exposed to the PI3'K chemical inhibitor LY294002 (at a concentration of either 0.1mM or 0.75mM), and also an intermediate concentration of the conditioned media (CM). I have shown that conditioned medium contains a biological factor which induces iL3 development; what is not known is what signal transduction pathway the biological signal is processed through. If the IIS's only function is to maintain free-living developmental fate, while the pheromone signal stimulates infective larval development through a non-IIS pathway, than one might expect that chemically inhibiting IIS with LY294002 to arrest free-living development, while presenting the biological signal which induces iL3 development (in the form of conditioned medium) would by-pass the complete developmental arrest observed to result in iL3 development. This was not the case, at

high concentrations (0.75mM) the larva exposed to both LY294002 (which down regulates IIS) and condition media (which contains iL3 development inducing signals) continued to be completely arrested at the L1/L2 stage (Figure 5.16). At 0.1mM LY294002, there is a minor non-significant induction of iL3 development.

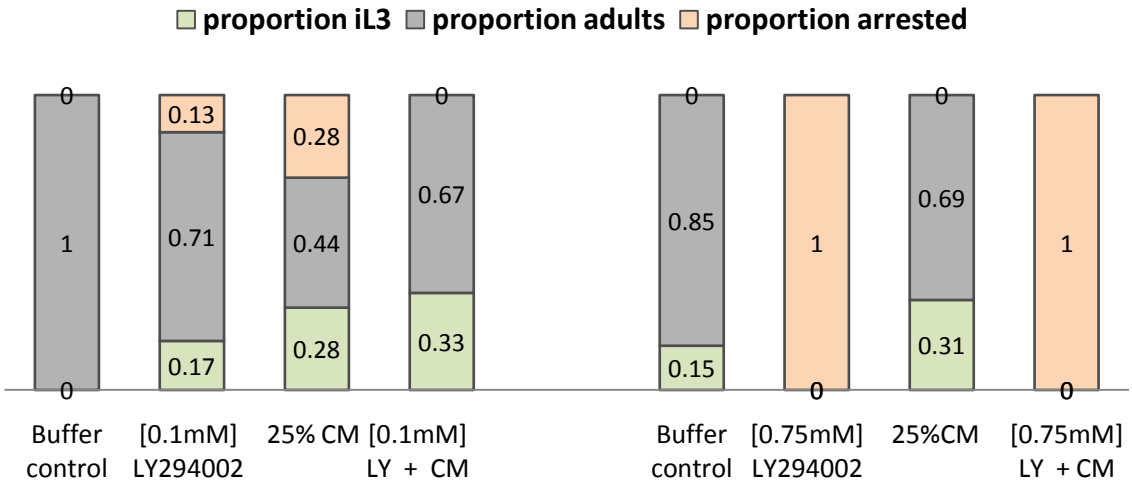


Figure 5.14: Effect of incubation of *P. trichosuri* larvae with PI3′K chemical inhibitor and conditioned medium on iL3 development.
P. trichosuri L1were exposed to either 0.1 mM or 0.75mM of LY294002 and conditioned medium (at a concentration which results in almost 30% iL3 induction) , *E. coli* HB101 (0.35% w/vol) and Gentamicin sulfate (50ug/mL) for 5 days and developmental fate scored. Column one of each set is buffer control (containing media and DMSO which was the solvent for LY294002); column two of each set contains LY294002; column three contains conditioned medium and column four (labelled LY+CM) contains both LY294002 and conditioned medium.

5.5 RESULTS: RECOVERY OF THE *C. elegans* Daf-2 MUTANT LIFE SPAN

C. elegans daf-2(e1370) mutants have a life span that is nearly twice that of wild-type worms (LARSEN *et al.* 1995). In order to determine if the *P. trichosuri daf-16a* transgene is able to rescue the long-lived Daf-2 mutant life span, a life span curve was performed on the transgenic line WG470, which has the *Pt daf-16a* transgene in a *C. elegans* double *daf-16;daf-2* mutant background. The parental strain, DR1309 (*daf-2;daf-16*), showed the expected shortened life span. If *P. trichosuri* DAF-16A is able to revert the double mutant shortened life span to long-lived Daf-2 mutants (such as CB1370 strain) one might speculate that *Pt* DAF-16A has a similar role in influencing

P. trichosuri life span. As can be seen by Figure 5.17, the transgenic worms of line WG470 are not reverted to the Daf-2 life span.

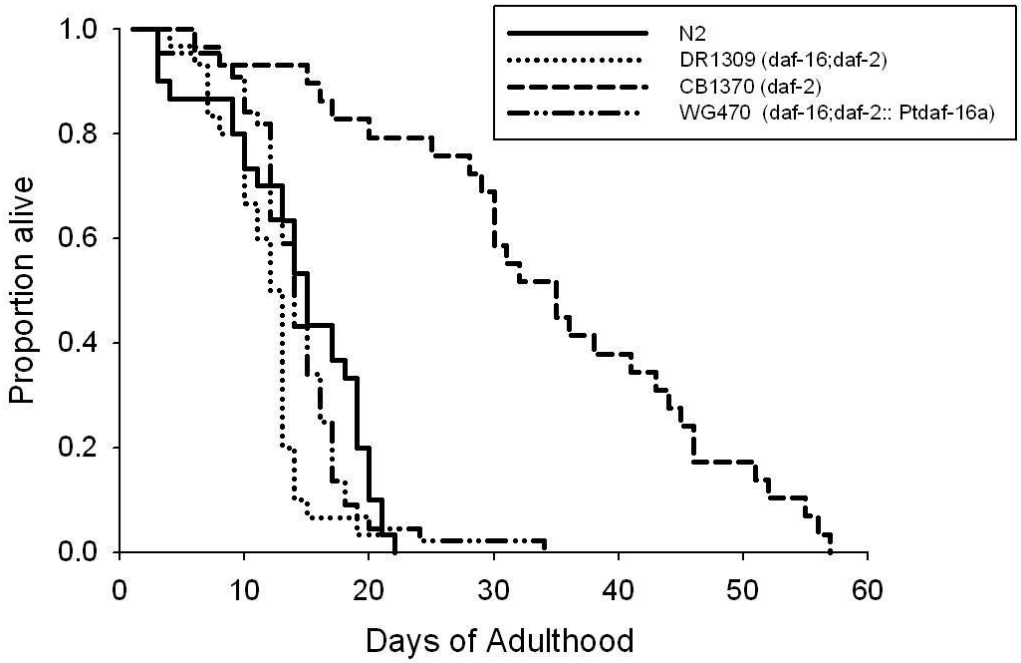


Figure 5.15: Recovery of mutant life span phenotype by complementation with *P. trichosuri daf-16a* transgene.
Adult worms were incubated at 20°C on agar plugs with *E. coli* HB101 as food source and FuDR (at 1.5μM) to prevent reproduction. Worms were assessed daily and the proportion that responded to touch was scored as alive.

5.6 RESULTS: LETHALITY OF *P. trichosuri daf-16b* TRANSGENE

Several attempts were made to generate transgenic lines with the *P. trichosuri daf-16b* transgene (Table 5.6). These transgenes were under the control of *C. elegans* regulatory sequences, the constructs had 1.63 kbp of *C. elegans daf-16* 3′ genomic region to include any regulatory sequences found in the *Ce daf-16* 3′UTR. The promoters used for the constructs were either the *Ce daf-16 α* promoter (α promoter =5.87 kbp upstream of the *C. elegans daf-16a* gene) as supplied in the N terminal gfp fusion vector: pNL209 (kindly gifted from C. Kenyon) (LIBINA *et al.* 2003) or the *Ce daf-16b* promoter (β promoter = 2.09 kbp upstream of the *Ce daf-16b* gene) (LEE *et al.* 2001).

Microinjection of *Pt daf-16b* under the control of either *C. elegans* α or β promoter was attempted into various mutant backgrounds. Only 2 lines were able to be established (WG480 and WG481), these were in a wild-type (N2) background and both these lines consisted of the *Pt daf-16b* construct under the control of the α promoter (Table 5.6). The gfp expression patterns of these lines were as expected for an α promoter i.e.: gfp expression in the neurons, muscles, gut and hypodermis (Figure 5.6). Results suggest that only when native *Ce daf-16a* and *b* are present and the *Pt daf-16b* transgene is under the control of the *C. elegans* α promoter does the *Pt daf-16b* transgene not have a lethal effect.

In order to establish a line with the *Pt daf-16b* transgene in a Daf-16 mutant background, a variety of different parameters were attempted. This included microinjection into a variety of mutant alleles at various microinjection concentrations of the constructs. On several occasions, gfp eggs that did not hatch, or sickly F_1 larvae that did not develop were seen, but no F_2 worms developed. Titering out the concentration of the *Pt daf-16b* transgene under the control of the β promoter to very dilute concentrations was also attempted; this needed to be done in the presence of a gfp co-injection marker (pPD129.51, which gives punctuate nuclear expression). At the lowest concentrations of *Pt daf-16b* transgene with the pPD129.51 co-injection marker, the punctuate gfp expression of marker gene was visualized and lines established, PCR was used to confirm the presence of the *Pt daf-16b* transgene; however, no gfp expression characteristic to the *Pt daf-16b* transgene was observed suggesting that the transgene was not being expressed (Table 5.6).

In an attempt to determine whether the inability to establish a *Pt daf-16b* line was indeed due to a recessive lethal effect of the transgene, RNAi was used to knock down the native *C. elegans daf-16* gene expression in the only two transgenic lines created (which were in a N2 wild-type background: WG480 and WG481). In essence, I attempted to turn the wild-type background into a Daf-16 mutant background using RNAi and thus expose the lethality of the transgene. If the *Pt daf-16b* is recessive lethal, one would expect that the transgenic lines plated onto *Ce daf-16* RNAi plates would not hatch any transgenic worms. In addition, a control experiment was performed to establish that I could use RNAi to completely knock down the transgene expression this was attempted by culturing the transgenic lines WG480 and WG481 on

plates with bacteria expressing *Pt daf-16* dsRNA and detecting for elimination of gfp expression.

These results showed that I was not able to entirely knockdown the gfp expression, using RNAi to the transgene. Transgenic worms were plated onto the RNAi plates and brooded daily onto freshly expressing RNAi plates, they became a fainter green, but continued to have gfp expression in the amphids (head neurons). This suggested that RNAi was not able to knockdown gene expression entirely, even after brooding onto fresh RNAi plates for several generations. In addition, this illustrated that *C. elegans* neurons are indeed refractory to RNAi (Results not shown).

Transgenic worm fed bacteria expressing *Ce daf-16* RNAi, continued to produce gfp eggs and hatching larva, suggesting either that the *Pt daf-16b* transgene is not a recessive lethal or alternatively, that I was not getting full knockdown of the endogenous *C. elegans daf-16* gene. Our previous experiment indicated that RNAi was not able to knock down neuronal expression of the transgene. As Wolkow *et al.*, showed that *daf-16* expression returned specifically to neurons is able to recover dauer larvae formation phenotype (WOLKOW *et al.* 2000), we speculate that we were not able to establish full knock down of the gene and were unable to confirm the hypothesis that the *Pt daf-16b* construct is recessive lethal by this experiment.

Table 5.3: Microinjection summary sheet

Construct									
Name	**Promoter**	Coding region	Strain	Genotype	# attempts	conc.	Co-injected	Phenotype	Transgenic Lines
Pt daf-16B	*Cedaf-16α*	***Pt daf-16b***	N2	wild-type	10	50ng/uL		GFP worms	WG480 WG481
Pt daf-16B	*Cedaf-16α*	***Pt daf-16b***	DR1309	*daf*-16(m26); *daf*-2(e1370)	130	50ng/uL 5 ng/uL		few green eggs or sickly larva in F_1 generation no F_2	
Pt daf-16B	*Cedaf-16α*	***Pt daf-16b***	DR26	*daf*-16(m26)I	50	50ng/uL 5 ng/uL		no GFP worms	
Pt daf-16B	*Cedaf-16α*	***Pt daf-16b***	DR1408	*daf*-16(m26); *age*-1(m333)	20	50ng/uL 5 ng/uL		no GFP worms	
SSD16B3	*Ce daf-16β*	***Pt daf-16b***	N2	wild-type	20	25ng/uL		no GFP worms	
SSD16B3	*Ce daf-16β*	***Pt daf-16b***	CF1038	*daf*-16(mu86)I	20	5 ng/uL		no GFP worms	
SSD16B3	*Ce daf-16 β*	***Pt daf-16b***	DR1408	*daf*-16(m26); *age*-1(m333)	30	100ng/uL		2 green eggs no F_2	
SSD16B3	*Ce daf-16 β*	***Pt daf-16b***	DR26	*daf*-16(m26)I	20	50ng/uL	Ce let 858 dsRED	Red worms	
SSD16B3	*Ce daf-16 β*	***Pt daf-16b***	DR1309	*daf*-16(m26); *daf*-2(e1370)	20	25ng/uL		no GFP worms	
SSD16B3	*Ce daf-16 β*	***Pt daf-16b***	DR26	*daf*-16(m26)I	64	100ng/uL 50ng/uL 25ng/uL		3 unhatched eggs sickly F_1 larva no F_2	
SSD16B3	*Ce daf-16 β*	***Pt daf-16b***	N2	wild-type	90	100ng/uL 25ng/uL 5 ng/uL		few green eggs or sickly larva in F_1 generation no F_2	
SSD16B3	*Ce daf-16 β*	***Pt daf-16b***	DR26	*daf*-16(m26)I	90	50ng/uL 5ng/uL 0.5ng/uL	pPD129.51	sickly F_1 larva no F_2	
SSD16B3	*Ce daf-16 β*	***Pt daf-16b***	DR26	*daf*-16(m26)I	20	50ng/uL	Ce let 858 dsRED	few red eggs no F2	
SSD16B3	*Ce daf-16 β*	***Pt daf-16b***	DR1309	*daf*-16(m26); *daf*-2(e1370)	90	100ng/uL 50ng/uL 5ng/uL	pPD129.51	8 F_1s punctate GFP /dead diffuse	WG533 WG529
SSD16B3	*Ce daf-16 β*	***Pt daf-16b***	DR26	*daf*-16(m26)I	20	0.5 ng/uL	pPD129.51	punctate worms PCR confirmed presence of SSD16B3	WG524 WG525 WG526
SSD16B3	*Ce daf-16 β*	***Pt daf-16b***	N2	wild-type	20	50ng/uL	pPD129.51	no GFP worms (strong autoflourescence)	

5.7 DISCUSSION

5.7.1 Functional analysis of daf gene orthologues in *P. trichosuri*

To determine gene function directly in *P. trichosuri*, manipulation of the expression of the gene is required, followed by assessment of the resulting mutant phenotype. Manipulation of gene expression may be accomplished by 'knocking down' gene expression using RNAi, 'knocking out' gene expression using mutagenesis or 'knocking in' gene expression of a dominant phenotype using transgenesis.

Gene silencing by RNAi is an effective gene knockdown tool used in a variety of species from protozoa to mammals and flies and also in the nematode: *C. elegans* (FIRE *et al.* 1998; HUNTER 2000; HUPPI *et al.* 2005; ULLU *et al.* 2002; ULLU *et al.* 2004). As RNAi works in a variety of organisms including nematodes, it would seem reasonable to assume that it would work effectivly in parasitic nematodes. However, results of RNAi in mammalian parasitic nematodes have been variable to date (GELDHOF *et al.* 2007; KNOX *et al.* 2007; ZAWADZKI *et al.* 2006). Despite testing panels of genes, researchers have found that only a small subset of the genes tested were susceptible to RNAi, and the results difficult to reproduce (GELDHOF *et al.* 2006; ISSA *et al.* 2005; VISSER *et al.* 2006). The effectivness of RNAi depends upon the length of dsRNA being delivered, the delivery method, the larval stage being exposed to dsRNA and the species in question. Whether the entire complement of prerequisite genes required for RNAi exist in parasitic helminths is a question that has been raised, as checking the available genome databases of *H.contortus* and *B. malayi* have failed to yeild many of the known RNAi pathway genes (GELDHOF *et al.* 2007; KNOX *et al.* 2007). Checking the, admittedly small, EST database available for *P. trichosuri* has failed to find hits for genes of the Dicer complex.

A methods of functional gene analysis were attempted in *P. trichosuri* in colaboration with several members of the *P. trichosuri* research team; data from the experiments performed in collaboration with others are presented in Appendix 6, results are discussed below. These include RNAi knockdown by soaking and electrophoration and by microinjection, and gene knock out by chemical mutagenesis.

Different methods of RNAi deleiver were attempted these include feeding bacteria expressing dsRNA, soaking L1 larva in either long transcript or short interferring dsRNA (siRNA), and electroporating either the long or diced siRNA. To assess for gene knockdown in response to these treatments, q-PCR was used. The results were variable, but there is some evidence of a two-fold knockdown with the soaking of siRNA. Literature on RNAi in parasitic nematodes shows that soaking in dsRNA has the greatest number of reported successes (GELDHOF *et al.* 2007). In addition to soaking L1s, microinjection of dsRNA *ama-1*, the RNA polymerase II orthologue, was also attempted in *P. trichosuri*; RNA polymerase II is considered essential for animal health and was used in a *B. malayi* RNAi soaking experiment successfully (ABOOBAKER and BLAXTER 2003). Microinjection of both dsRNA transcripts to the *Pt ama-1* putative orthologue and *Pt rrf-3* genes may have resulted in a RNAi phenotype in *P. trichosuri*: the phenotype observed was the early cessation of egg production, whereas in *C. elegans* microinjection of *Ce ama-1* dsRNA results in embryonic death which is observed as unhatched eggs.

However, it was decided that a two-fold knock down in gene expression and the disparate phenotype observed with the dsRNA microinjection experiment made *P. trichosuri* RNAi too difficult to reproduce for it to be an effective tool with which to study gene function. Therefore, knocking out gene expression of *P. trichosuri* IIS genes by chemical mutagenisis was attempted instead, in order to observe the resulting mutant phenotype and to rescue by complementation using transgenesis.

The parasitic life cycle does not lend itself well to classical genetic manipulation, as parasites only reach maturity within a host. As a result, there have been very few cases of forward genetics in parasites. Two genera of parasitic helminth exhibit facultative free-living life cycle, the *Strongyloides* species, some of which may have up to nine generations, (although, most display only one free-living life cycle), and the *Parastrongyloides* species, which are capable of an unlimited number of free-living generations. Due to the single faculative free-living generation of *S. ratti*, Viney *et al*. were able to use chemical mutagens on the free-living larval stage in which the reproductive tissues start developing (the L4 larval stage). The mutants created were then screened in the next free-living cycle for a resistance to low levels of ivermectin (VINEY *et al.* 2002).

A similar phenotypic mutant screening in *P. trichosuri*, to isolate developmental mutants, would involve exposing the reproductive adults to a mutagen and then screening the F_2 generation in the presence of high concentrations of pheromone and search for non-iL3, or alternatively screen the mutagenized offspring in non-iL3 inducing conditions and search for iL3. Such an assay for detecting IIS developmental mutants would present some difficulties in *P. trichosuri*. There are always low levels of infective larva development within a population therefore finding a Daf-c (putative Daf-2) mutant may present some difficulties; in addition, it is not practical to infect a possum with a low numbers of infective larva. A Daf-d (putative Daf-16) mutant screen might face similar issues as there is always less then 100% response to pheromone. While performing the phenotypic screen just described might very well isolate a Daf-c or Daf-d mutant, the isolated mutant might be any one of several dozen Daf mutants, as opposed to one of the key IIS genes targeted by this project. Therefore, instead of a phenotypic screen, directly screening by PCR for deletion mutants in the *Pt daf-2*, *Pt age-1* or *Pt daf-16* genes was attempted.

P. trichosuri is a facultative parasite which, because of its life cycle, lends itself to this sort of forward genetic screen. The reproductive free-living adults can be exposed to various mutagens, then free-living F_2 and F_3 progeny can be cultured at lower temperatures in order to slow development until PCR (or phenotypic) screening for mutants is complete. If a putative mutant is found, single brother/sisters matings can be performed to generate homozygous mutant lines. *P. trichosuri* were screened for deletions in the IIS *daf* gene orthologues, using poison primer PCR after EMS treatment (EDGLEY *et al.* 2002). Several times small deletion products were amplified, however, when a confirmation PCR was attempted, the small products were not reamplified. The fact that some deletion products were initially detected by PCR suggests that this is a procedure that could potentially be optimized to yeild mutants. Such optimization may involve optimizing the EMS concentrations or to screen less worms per well, as this would increases the proportion of any one genome containing the deletion mutant for PCR detection.

Loss of the IGF-IR (insulin receptor of the IIS pathway) results in an extension of life span and an increased tolerence to oxidative stress in rodents. It was also shown that flies homozygous for mutations in *chico* (which encodes Insulin Receptor Substrate),

show increased SOD (stress resistance gene – superoxide dismutase), increased life span, reduced body size and reduced fecundity (BARBIERI *et al.* 2003; LONGO and FINCH 2003). Signalling through the IIS results in phosphorylation of FOXO transcription factors (forkhead box, subgroup "O" – FOXO) via AKT-1 and AKT-2, and its inactivation by sequestoring in the cytoplasms. Upon dephosphorylation, the FOXO transcription factor becomes nuclear localized, where it promotes transcription of genes involved in stress response, metabolism, longevity and development (LEE *et al.* 2003; MCELWEE *et al.* 2003; MUKHOPADHYAY *et al.* 2006) .

Constitutively activated *P. trichosuri daf-16* transgenes in which the AKT1/2 phosphorylation sites were mutated were constructed and microinjected into *P. trichosuri*. The F_1 generation was assessed to determine whether the constitutively activated transgene resulted in an increased switch to iL3 development. Our findings do not suggest these transgenes have an effect on iL3 development, with the caveat that although I was able to establish clearly the presence of the transgene by PCR, I was unable to confirm expression of the transgene by immunohistochemsitry. Furthermore, there is a chance that the N terminal HA tag or the PCR introduced amino acid change at position $S_{76} \rightarrow L_{76}$ in the *Pt daf-16a* isoform may have interferred with the function of this transgene. This is an unlikely explanation for the failure of the mutated *Pt* DAF-16 to confer a phenotype. Several groups have made *Ce* DAF-16 transgenes with mutated AKT-1/2 phosphorylation sites, in order to determine the effect of constutively activated DAF-16 on development and longevity in *C. elegans*, with contradictory results. Lin *et al*. determined with their mutated construct that, although it was nuclear localized and functional, as demonstrated by its ability to recover the *daf-d* mutant phenotype of *daf-16* null mutants, it did not extend life span nor Daf-c phenotype in a *daf-16*(-);*daf-2*(+) background (LIN *et al.* 2001). In contrast, Lee *et al*., determined that their mutated construct was able to cause a moderate dauer larvae development or larval arrest (LEE *et al.* 2001). The constructs and procedures used by these labs differed. Lin *et al.* had an N terminal GFP tagged construct, and the *daf-16* transgene contained the large intronic region. Lee *et al.*, performed their transformation on *daf-16* RNAi plates until lines were establish, in order to prevent constutive dauer larvae formation and lethality. Lee *et al.* suggested that maintaining lines on *daf-16* RNAi plates may have aided in the selection and maintenance of strains with higher levels of transgene expression, so that not using RNAi may have preferentially selected for low transgene

exrpession in the Lin *et al*. group. In support of this conclusion, Lee *et al*. also noted that there was higher dauer larvae formation in the second generation of worms removed from RNAi plates as the inherited RNAi waned.

More recently, Castelletto *et al.* (2009) used a different approach, they made a *S. stercoralis fktf-1b (daf-16* orthologue) dominant negative construct, which interfered with the function of endogenous FKTF-1b by binding to, but not transcribing, FKTF-1b targets. In these experiments, free-living adult females were microinjected and the transgenic offspring examined, all of whom must develop to infective larva in this obligate parasitic generation. The construct caused morphological changes in some the transgenic worms consistent with depletion of storage granuals of the intestinal cells; more significantly, some transgenic worms seemed to bypass of the larval arrest of the iL3 stage to initiate molt to the free-living forth larval stage (CASTELLETTO *et al.* 2009). The fact that the infective larva had some feature of a second free-living generation worm suggests that FKTF-1b is required for complete iL3 morphorgenesis.

5.7.2 Rescue by complementation in *C. elegans*

Using rescue of phenotype by complementation in *C. elegans* is an established method for testing gene function of parasite genes (BRITTON and MURRAY 2006; BRITTON *et al.* 1999; FENG *et al.* 2002; KAMPKOTTER *et al.* 2003; KWA *et al.* 1995; MASSEY *et al.* 2006) .

The *Ce-daf-2* gene is expressed in the nervous system, intestine, and head neurons. The mutant CB1370 *daf-2* (e1370) has a point mutation in the tyrosine kinase domain ($P_{1465}\rightarrow S_{1465}$), and the phenotype conferred by this mutation is temperature constitutive dauer larvae formation at 25°C. In addition, it is long lived and not supersensitive to pheromone (KIMURA *et al.* 1997). For the *daf-16* gene, the *Ce daf-16a* isoform is expressed in hypodermis, intestine, muscles and neurons, and the *Ce-daf-16b* isoform is expressed in the pharynx and somatic gonad. The mutant DR26 *daf-16* (m26) has a point mutation which affects the splice site of exon 2. The phentoype conferred by this mutation is dauer defective: worms carrying this mutation respond weakly to pheromone, are slightly short-lived, and this allele is able to partially suppress the Daf-2

mutant phenotypes (GEMS *et al.* 1998; LEE *et al.* 2001; OGG *et al.* 1997; RIDDLE *et al.* 1981; VOWELS and THOMAS 1992). *Ce daf-16a* mutants behave as *daf-16* null mutants, and are able to suppress the dauer larvae formation and ageing phenotype of a Daf-2 mutant, and the RNAi phenotype of *Ce daf-16a* is stronger for ageing and dauer larvae formation than that of *Ce daf-16b* RNAi, so that DAF-16A is thought to have the major effect on dauer larvae formation and life span phenotypes. Lee *et al*. (2001) observed that swapping the coding regions of the *daf-16* isoforms, under the control of the other's promoter was able to complement dauer larvae formation and life span, suggesting that the transcriptional regulation rather then the coding sequence regulates biological function (LEE *et al.* 2001).

Table 5.4: *P. trichosuri* rescue of *C.elegans* mutant phenotypes.

Coding region	promoter	*C. elegans* Strain	Genotype	Mutant Phenotype	Degree of rescue
Pt daf-16a	*Ce daf-16α*	DR26	*daf*-16(m26)I	Oxidative stress	strong
Pt daf-16a	*Ce daf-16α*	DR26	*daf*-16(m26)I	Dauer (pheromone)	partial
Pt daf-16a	*Ce daf-16α*	DR1309	*daf*-16(m26); *daf*-2(e1370)	Reverts to Ts daf-c	strong
Pt daf-2	*Ce daf-2*	CB1370	*daf*-2 (e1370)III	Oxidative stress	no
Pt daf-2	*Ce daf-2*	CB1370	*daf*-2 (e1370)III	Ts daf-c	no
Pt daf-2	*Ce daf-2*	CB1370	*daf*-2 (e1370)III	Dauer (pheromone)	no

P. trichosuri DAF-16A and DAF-16B transgenes products are localized to the cytoplasm and upon exposure to oxidative stress they become nuclear localised (Figure 5.6), which suggests that the transgene is expressed and that the motifs involved in nuclear localization and transport are conserved between *C. elegans* DAF-16 and *P. trichosuri* DAF-16. In addition to the nuclear localization in response to stress, *P. trichosuri* DAF-16A is able to recover the stress sensitivity of a *C. elegans daf-16* mutant to wild-type levels for one transgenic line and significantly restore stress resistance to another line (WG477 and WG478 respectively). It is generally accepted that different transgenic lines display differing degrees of mutant recovery, and I

suggest that this different degree of recovery correlates to the expresion level of the transgene, as inferred by the level of transgene gfp expression between the two lines. Recovery of dauer larvae development was also partially restored by the presence of the *P. trichosuri daf-16a* transgene in a Daf-16 mutant background, but to a lesser extent than stress recovery. The presence of the *Pt daf-16a* transgene was also able to restore the temperature sensitive Daf-c phenotype in a *C. elegans daf-16;daf-2* mutant background, which implies it is able to activate genes involved in dauer larvae development as well as stress response.

We observed partial recovery of Daf-16 mutant phenotypes with *Pt daf-16a* isoform only but were unable to establish a line with the *Pt daf-16b* isoform. In contrast to this work, Massey *et al.*, were able to obtain recovery of mutant dauer larvae formation phenotype with the *S. stercoralis fktf-1b* transgene under the control of the *Ce daf-16*α promoter but were unable to get recovery with the *Ss fktf-1a* transgene under the control of the *Ce daf-16α* promoter (MASSEY *et al.* 2006). A similar result has recently been established with a *H. contortus daf-16b* (*Hc daf-16.2*) transgene also under the control of the *Ce daf-16*α promoter (HU *et al.* 2009). These authors hypothesised that the *Ss fktf-1a* or *Hc daf-16.1* did not complement because of the unusually long serine rich 5′ region or alternatively that the less conserved N terminal region of the protein did not allow for the correct interactions with *C. elegans* cofactors (HU *et al.* 2009; MASSEY *et al.* 2006). However, *P. trichosuri* DAF-16A is predicted also to have a similar serine rich 5′ region and our transgene was able to complement mutant stress and to a lesser extend dauer larvae phenotype. Another possiblity, which seems more likely is the regulatory effect of the α promoter and the 3′ UTR. Lee *at al.* showed that both *Ce daf-16a* and *b* were able to recover most mutant phenotype under the other's promoter, suggesting that it is expression pattern rather than the transcription factor isoform which determines the effect of *C. elegans* DAF-16 (LEE *et al.* 2001). Our transgene Pt *daf-16a* under the *Ce* α promoter was able to recover phenotype, but I was unable to establish a line with the *Pt daf-16b* transgene under the β promoter. Massey *et al.* and Hu *et al.* used both *daf-16a* and *b* orthologues under the control of *Ce daf-16*α promoter and an exogenous 3′ UTR (*unc-54*) (MASSEY *et al.* 2006). It is possible that the *Ce* α promoter plays a key role in the recovery of phenotype of these parasite *daf-16* orthologues. It was noted by Lee *et al.* that *C. elegans daf-16* mutants transformed with the *Ce daf-16a* under *Ce daf-16α* promoter were not able to form complete dauer larvae, their pharynx

did not fully remodel (LEE *et al.* 2001). WormBase and NCBI Entrez data on *C. elegans daf-16* show it has an unusually long 3′ UTR which is spliced only in the *Ce daf-16b* transcript, suggesting there are transcript specific regulatory elements within the 3′ UTR and reiterating the importance of the appropriate expression regulation when assessing recovery by complement phenotype.

While I was able to complement Daf-16 mutant phenotype with our *Ptdaf-16a* constructs under the control of the Ce daf-16α promoter I was unable to even establish a transgenic line of the *Pt daf-16b* transgene under the control of either a *Cedaf-16*α or β promoter in a *Ce daf-16*(m26) or *Ce daf-16*(mu86) mutant background (Table 5.6). This suggested that this transgene product may be lethal in the absence of wild-type DAF-16. The only lines I was able to establish with the *Pt daf-16b* transgene were in a wild-type *C. elegans* N2, where the wild-type *daf-16* allele is present.

One of downstream effects of IIS regulation of FOXO3a, in mammalian motorneron cells is the regulation of cell death genes (BARTHELEMY *et al.* 2004), it is possible that ectopic expression of other FOXO transcription factors may trigger this pathway in other organisms including nematodes.

Members of the FOXO forkhead transcription factors which lie downstream of IIS in humans are FOXO1, FOXO3a and FOXO4; *D. melanogastor* has a single orthologue, dFOXO; and the *C. elegans* FOXO transcription factors are the DAF-16 isoforms. A variety of lethal phenotypes have been observed with constutively expressed FOXO transcription factors in non-nematode systems. In mammalian cell culture, activated FOXO is able to cause cell death, cell cycle arrest or senescence. This was observed for a constitutively activated FOXO3a transcription factor, which requires the activation the JNK pathway and triggers cell death in motorneurons (BARTHELEMY *et al.* 2004). In *Drosophila* larva, when a transgene under the contol of an *ActGal4* driver was used to overexpress (or drive expression inappropriately at the third instar stage) of either a wild-type dFOXO or a mutationally activated dFOXO transgene, the result was extreme lethality; the few surviving flies being significantly smaller than the controls (KRAMER *et al.* 2003). In nematodes, as noted above, Lin *et al*. (2001) speculated that the constiutively activated *Ce daf-16b* transgene was lethal, as they were unable to establish any transgenic lines (LIN *et al.* 2001). The Ruvkun lab made a constitutively activated *Ce daf-16a* isoform transgene in 2001, but have not, to our knowledge, ever reported

data on the effects of a constitutively activated *Ce daf-16b* isoform: it is not known whether they chose not to make the constiutively activated B isoform transgene, or had similar difficulties establishing a line.

DAF-2 is the receptor kinase of the IIS pathway. *Pt daf-2* chimeric constructs was transformed into a *C. elegans daf-2* mutant in order to determine if the *P. trichosuri daf-2* gene is able to function and recover *C. elegans daf-2* activity. The *C. elegans daf-2* mutant is stress resistant, long lived and temperature sensitive Daf-c. These mutants are not sensitive to *C. elegans* dauer pheromone at <25ºC but at 25°C they form dauer larvae constitutively, i.e. independently of pheromone signalling. Two transgenic lines were created which contained the *Pt daf-2* transgene (WG473 and WG474). There was no rescue of phenotype in these transgenes for stress resistance upon exposure to oxidative stress, nor rescue of dauer larvae phenotype.

A possible reason for this may be found in the structure and function of the DAF-2 tyrosine receptor. This is a membrane bound protein which has a ligand binding domain exposed on the cell surface and a tryosine kinase domian which activates the signaling cascade on the cytoplasmic cell surface. Given the diversity of the insulin-like family of proteins which exists in *C. elegans* (greater than 39 identified) (PIPER *et al.* 2008), many of which have been shown to activate different downstream responses, it seems likely that there is complex and precise interaction between ligand and receptor. As highlighted in Chapter Four, the ligand binding domain of *P. trichosuri* DAF-2 showed a consensus similarity of 44.6% whereas the the tyrosine kinase domain has a 72.3% consensus similarity with *C. elegans* DAF-2. If the *C. elegans* ligands are unable to bind to the *P. trichosuri* receptor than this may account for the lack of recovery with the *P. trichosuri daf-2* transgene.

AGE-1 is a phosphytidylinositol 3′ kinase (PI3'K). The function of the putative *P. trichosuri* AGE-1 protein was tested directly in *P. trichosuri* with the aid of a PI3'K chemical inhibitor LY294002. In *A. caninum*, inhibition of IIS by LY294002 treatment prevented exit from iL3 (as scored by resumption of pharyngeal pumping and excretion of a post-iL3 associated protein), this suggested that IIS controls exit from infective larval stage (BRAND and HAWDON 2004).

In *P. trichosuri*, our data suggest that even at moderate concentrations of the AGE-1 chemical inhibitor, LY294002 there were strong pharmacological effects upon the larva. No chemical inhibitor (buffer control) results in free-living development, whereas at very low concentrations of LY294002 there is a very slight increase in proportion of iL3 development, however at even moderate concentrations of LY294002, the larvae appear to be completely arrested at the L1 stage (or possibly at pre-L2 or pre-iL3). These larvae remain arrested for several days, before death eventually occurs.

To summarize this section: the *Pt daf-16a* transgene is able to rescue stress response and is also able to partially rescue the dauer larvae development in a *C. elegans daf*-16 mutant, suggesting it may have a similar function in *P. trichosuri*. The *Pt daf-16a* transgene is not able to recover the life span of the *C. elegans* double *daf-16;daf-2* mutant. The *Pt daf-16b* transgene may to be toxic as I was unable to establish a line. The *Pt daf*-2 transgene is not able to recover either the stress response nor the dauer larvae development phenotypes. As discussed above, this may be a result of lack of similarity in the ligand binding domain.

Our research has highlighted several apparent differences in the role that insulin/IGF signalling plays in development between *P. trichosuri* and *C. elegans*. The insulin pathway exists in *P. trichosuri* and our results suggest that some of the functions of the pathway, such as the stress resistance response, are conserved between these species. Results from the rescue by complementation with the *Pt daf-16a* transgene have shown almost a full recovery of the stress phenotype, but the dauer larvae formation phenotype showed weaker recovery from the same lines in a single mutant background. This may be a result of *Pt*-DAF-16 being able to activate the genes involved in oxidative stress resistance, but less effective at interacting with the *C. elegans* transcriptional cofactors involved in the activation of the set of genes involved in dauer larvae development.

An exception to this is illustrated in strain WG530, which has the genotype: transgene consisting of a *Ce daf-16a* promoter and *Pt daf-16a* coding region in a *daf-2;daf-16* double mutant background. In this strain almost complete recovery of the temperature sensitive Daf-c phenotype (Figure 5.12) was observed versus the partial recovery of pheromone induced dauer larvae recovery observed for both transgenic lines in the *daf-2*(+);*daf-16*(-) background (Figure 5.10). A possible explanation for this difference in

levels of recovery may lie in the *C. elegans* mutant alleles used for the transgenesis. The IIS pathway functions cell non-autonomously to regulate development by positive feedback regulation with insulin peptides through the IIS pathway (MURPHY *et al.* 2003), therefore even a partially functional DAF-16 will result in an amplified result. In the *C. elegans daf-2*(+);*daf-16*(-) background, the downstream genes regulated by the *Pt* DAF-16 transcription factor are able to feedback through a working IIS pathway. Essentially making a wild-type worm, where the developmental choice to enter dauer larvae or not exists, and for this particular experiment was determined by *C. elegans* dauer pheromone, which does not induce an 100% effect. However, in the *C. elegans daf-2*(-);*daf-16*(-) background, any downstream genes regulated by the *Pt* DAF-16 transcription factor will feedback into an IIS pathway which is *daf-2*(-), the phenotype of which is ts 100% dauer larvae formation.

Testing the function of the insulin/IGF signalling pathway directly in *P. trichosuri* by inhibiting the function of AGE-1 with the chemical inhibitor has also shown a minor role for insulin signalling in infective larvae development. Incubation of *P. trichosuri* larvae in low concentrations of the inhibitor resulted in low levels of infective larvae development (but still significantly higher than controls), and when the concentration of chemical inhibitor was increased, complete developmental arrest was observed rather than further increase in infective larval development. This suggests that insulin/IGF signalling plays an essential role in free-living development and that, although it may influence infective larvae development, it is not the main control of the infective larvae switch.

The role that insulin/IGF signalling pathway plays in life span extension in *P. trichosuri* is unclear. Our results with the rescue by complementation did not result in recovery of life span phenotype, and activation of the pathway by compounds that induce infective larvae formation did not result in life span extension, suggesting that in *P. trichosuri* different signalling pathways control life span and development.

CHAPTER SIX

CONCLUSIONS AND FUTURE DIRECTIONS

6.1 SUMMARY AND CONCLUSIONS

It has been proposed that the dauer larvae stage of *C. elegans*, and other free-living nematodes, is analogous to the infective larval stage of parasitic helminths because of the many biological features which they have in common. An extension of this hypothesis is that the dauer larvae stage may have served as a stepping stone in the evolution of infective larva and parasitism (HOTEZ *et al.* 1993). The molecular processes which determine dauer larvae development are therefore of particular interest in order to determine if similar processes play a role in infective larval development. The goal of this project was to determine the role that insulin/IGF signalling plays in iL3 development of the possum intestinal parasite, *Parastrongyloides trichosuri*.

The decision to test this hypothesis in *P. trichosuri* was based upon a number of factors. The complex life cycle of parasites and the absolute requirement for a host, in which the reproductive stage is sequestered, makes the study of parasites notoriously difficult. *P. trichosuri* is a facultative parasite which has retained the ability to become either a free-living nematode or a parasite at each generation. In addition, it appeared that one of the signals which influence this developmental decision is population density, because *P. trichosuri* cultures will cycle through several free-living generations until suddenly there is a massive production of iL3. We hypothesized that this was analogous to the dauer larvae developmental decision of *C. elegans* and that *P. trichosuri* would therefore be an appropriate model in which to investigate the dauer hypothesis. Another advantage to using *P. trichosuri* is the ability to maintain the free-living morphs in the laboratory indefinitely for classical genetic manipulation, and then control the switch to the parasitic morph.

6.1 Summary

My research has shown that the biology of the infective stage of development in *P. trichosuri* has some striking similarities to the *C. elegans* dauer larvae stage. Both are developmentally arrested stages which are resistant to heat and oxidative stress; both are resistant to incubation in 1% SDS, and both have increased fat stores. In addition, both the *C. elegans* dauer larva and *P. trichosuri* infective larva undergo pharynx

remodelling and radial constriction; the dauer larva develops a thickened cuticle and a buccal plug while the infective larva develops an enclosed sheath. These modifications of the cuticle are the likely source of their resistance to the environmental stressors mentioned. Of greater significance is the demonstration that the environmental signals which trigger infective larva development in *P. trichosuri* are the same as the signals which trigger dauer larvae development in *C. elegans*. Both species produce a biological factor which serves as a population density indicator and in both species this biological factor is the main developmental signal whose effect is modulated by both temperature, and to a greater degree, food availability.

My research suggests that there is a genetic component to variation in sensitivity to the population cue. The inbred lines, which differ in response to the population cue show different developmental reaction norms in response to the biological factor in conditioned medium. The different morphs of *P. trichosuri* (free-living versus infective larvae) are easily scored, and large numbers of larvae can be generated for statistical robustness - making *P. trichosuri* a good model for studying developmental plasticity. Each of the inbred lines are genetically different from the others; of special interest, inbred line CM20 had a radically different reaction norm profile to the other inbred lines, which strongly suggest that the genetic differences of this line are in a different part of the iL3 development pathway (i.e.: either: detection of the signal, or transduction of the signal, or perhaps a difference in one of the genes regulated by the signalling pathway).

The dauer larvae developmental switch in *C. elegans* is controlled by integrating signalling through IIS and TGF-β via a nuclear hormone pathway. I chose to investigate the role of IIS in the *P. trichosuri* iL3 developmental switch. The IIS signalling transduction genes chosen for analysis were the tyrosine kinase insulin receptor, *daf-2*; the phosphatidylinositol 3′ kinase, *age-1*; and the target of the pathway, the transcription factor *daf-16*. Orthologues to these genes were cloned from *P. trichosuri* and the sequences generated were analyzed bioinformatically using alignment to *C. elegans* and orthologues from other organisms where available. Phylogenetic analysis showed that the gene orthologues cloned from *P. trichosuri*, show a higher similarity to the *C. elegans* IIS gene than to other proteins of the same family groups,

suggesting the correct orthologue had been cloned for further study and characterization.

Expression analysis of the *P. trichosuri* IIS genes using quantitative Real Time-PCR showed a similar expression profile of all the gene orthologues at the developmental stages tested. In *P. trichosuri*, the highest level of IIS gene expression was at the iL3 stage, followed by high levels of expression in the L1 stage (early free-living development) for all the genes. The adult stages of both the free-living adult stage and the parasitic adult stage had relatively low levels of expression. This is in contrast to *S. stercoralis daf-16* isoforms which are apparently not differentially expressed throughout development (MASSEY *et al.* 2003), although these authors based their conclusions on a semi-quantitative rather than quantitative method.

Most intriguingly, there was a decrease in IIS gene expression early in transition from L1 to iL3 (the "L1 + CM" stage) for all the genes, suggesting that the signal transduction pathway may be down regulated at this point. The progression from L1 to iL3 takes a conservative estimation of 100 hours, based upon morphology, and only one time point in this transition was assessed by q-PCR (at 36 hours exposure to conditioned medium). The point during this transition at which the IIS gene expression is down regulated and whether this correlates to the time point at which L1 larvae become committed to iL3 development is an intriguing question. What is shown by the q-PCR data is that the L1 exposed to conditioned medium have a down regulation of the IIS genes early during the developmental switch, followed by elevated IIS gene expression when the iL3 stage has fully developed. It is understood that the activity of the DAF-16 transcription factor is controlled by the post-translational phosphorylation of the protein; however expression levels may be an indication of the signalling capacity of the pathway. If the role of IIS in *P. trichosuri* is to maintain developmental fate - as it appears to do in *C. elegans*, then a decrease in IIS, in order that a change in development may occur, would be an expected expression profile for L1 larvae enroute to iL3. There is a second down regulation in IIS gene expression at the iL3 post-skin penetration stage, when presumably the iL3 have received the signal to resume development toward the parasitic adult stage. Therefore, one interpretation of the data presented here is that developmental change (switching programs or re-activation of development after arrest) requires a transient decrease in IIS component transcription.

The IIS pathway controls many processes. In regard to its possible role in development, genes encoding IIS components may be highly expressed in early development to maintain free-living developmental fate. The IIS genes are again highly expressed during the iL3 stage; one possibility is that this is to maintain the worm in this stage until such a time as it encounters the appropriate host. *daf-7*, the ligand for the TGF-β signalling pathway is also highly expressed at the iL3 stage in several parasite species (BRAND *et al.* 2005; CROOK *et al.* 2005; FREITAS and ARASU 2005; MASSEY *et al.* 2005) and it has been speculated that the TGF-β pathway has evolved in parasites to maintain iL3 developmental fate until a host is encountered (VINEY 2009). An alternative hypothesis for why IIS may be up regulated at the iL3 stage is to regulate stress responses. Infective larvae must be able survive in a stressful environment until they encounter a host. *C. elegans* DAF-16 regulates stress response genes under the control of both IIS dependent and independent signalling (BAUMEISTER *et al.* 2006; BERDICHEVSKY *et al.* 2006; LAMITINA and STRANGE 2005; MUKHOPADHYAY *et al.* 2006; OH *et al.* 2005; OOKUMA *et al.* 2003). I have shown that the iL3 stage (like the dauer larvae stage of *C. elegans*) are resistant to oxidative and heat stress. These data also showed that *Pt daf-16a* transgene is able to rescue stress phenotype of *C. elegans daf-16* mutants by complementation, suggesting *Pt daf-16* may play a similar role in the *P. trichosuri* stress response. Therefore it is possible that the up regulation of expression of IIS genes at the iL3 stage is a because of the increased stress resistance of this stage.

When using pharmacological means to down regulate IIS, with the phosphatidylinositol chemical inhibitor-LY294002, it was found that there was a very sharp threshold of activity. A slight knock down of the signalling pathway, achieved by administering a low concentration of LY294002, resulted in a slight increase in iL3 development; whereas increased concentration of LY294002 resulted in total developmental arrest at the L1 or L2 stage. This suggests IIS signalling is required for free-living development and that a slight decrease in signalling may result in iL3 development. In *C. elegans*, the DAF-16 transcription factor functions as both a positive and negative regulator of genes. During the switch to dauer larvae, IIS is down regulated and DAF-16 is retained in the nucleus where it negatively regulates genes required for reproductive growth and positively regulates gene required for the dauer larvae program. One interpretation of

our data is that pharmacological knock down of a single component of IIS signalling may result in *Pt* DAF-16's retention in the nucleus, where it negatively regulates the genes required for free-living growth, but despite the addition of conditioned medium, the full milieu of regulating events required for a developmental change are not present and therefore the iL3 development program is not stimulated and the larva remains arrested.

This research has shown that the cholesterol requirement for free-living development is conserved between *C. elegans* and *P. trichosuri* and this lends credence to the argument that the role of the signalling transduction pathways upstream of this steroid hormone pathway may also be conserved. Cholesterol, is required for the free-living development of *C. elegans* and *S. papillosus* (OGAWA *et al.* 2009), and is a precursor for Δ4 and Δ7 dafachronic acid: the ligands for the DAF-12 nuclear hormone receptor, which regulates dauer larva formation and developmental timing in *C. elegans* (ANTEBI *et al.* 1998; ANTEBI *et al.* 2000; GERISCH *et al.* 2001; LARSEN *et al.* 1995; MATYASH *et al.* 2004; SNOW and LARSEN 2000). Epistatis analysis (THOMAS *et al.* 1993; VOWELS and THOMAS 1992) suggest this hormonal regulation is downstream of the TGF-β and IIS pathways.

Functional analysis of DAF-2, AGE-1 and the DAF-16 A and B isoforms in *P. trichosuri* was attempted by a variety of methods. RNAi was attempted using various dsRNA delivery techniques; chemical mutagenesis was also attempted to knock out gene function, as was transformation of *P. trichosuri* with constitutively activated form of the DAF-16 transcription factor. Although the presence of the transgene was confirmed, it was not possible to confirm the presence of the translated protein; the results did not indicate a difference in developmental fate due to the presence of the transgene.

Rescue by complementation using *C. elegans* mutants relies on the assumption that a *C. elegans* strain with a mutation of the gene of interest is available, and that the mutant has a phenotype which can be rescued. I was not able to recover phenotype of the *C. elegans* Daf-2 and Age-1 mutants. *C. elegans* has over 39 different insulin ligands, many of which are able to elicit different down stream effects suggesting a precise and complex binding between the ligand and the DAF-2 receptor. No information is

available for putative IIS ligand in any parasitic species, but bioinformatic analysis has shown that between the *C. elegans* and *P. trichosuri* DAF-2 proteins, the area of least conservation is in the ligand binding domains. This implies that the *Pt* DAF-2 ligand(s) are also poorly conserved relative to *C. elegans* ligands and thus may be the reason that the rescue by complementation was not successful. The *P. trichosuri* DAF-16 orthologues become nuclear localized in response to stress, in transgenic *C. elegans*, indicating that the post-translational regulation of these proteins are similar to the *C. elegans* proteins. The Daf-16 stress resistant phenotypes and to a lesser extent dauer larvae development phenotypes were able to be recovered by complementation with *P. trichosuri daf-16*a transgene. This lends credence to the hypothesis that *daf-16* and by extension IIS play similar roles in *C. elegans* and *P. trichosuri.*

*****

Therefore, the results of this research support the hypothesis that the role of the IIS pathway in regulating dauer/infective larva development appears to be at least partially conserved between *C. elegans* and *P. trichosuri*. The data from the rescue by complementation suggest that *P. trichosuri* DAF-16 has a role in both stress response and development. The q-PCR gene expression results indicate that there is a stronger requirement for the IIS genes in the egg and L1 stages. This may indicate IIS plays a role in free-living development at these stages, a conjecture which is supported by the pharmacological results, which indicated that a substantial knock down of IIS at this time results in developmental arrest.

Down regulation of IIS pathway early in *C. elegans* development results in increased dauer larvae formation, and the results of our q-PCR suggest a similar down regulation of IIS occurs in *P. trichosuri* during the switch from L1 to iL3 development, this may indicate that IIS plays a similar role in *P. trichosuri* iL3 development. The up regulation of IIS gene expression during the iL3 stage may suggest a strong requirement for IIS during this stage, but whether this is to facilitate stress resistance or to prevent further development of the iL3 until such time that they receive the appropriate environmental signals is unknown.

6.2 FUTURE DIRECTIONS

This work has raised many questions, the first of which are in regards to the biology of *P. trichosuri*, which has highlighted the interesting developmental plasticity shown in response to the biological factor in the conditioned medium.

Several of the *P. trichosuri* inbred lines created during this project were strikingly diverse in their response to conditioned medium. The transcription profiles of these inbred lines could be examined at various time points during iL3 development using Next Generation Sequencing to determine if there are allelic difference in gene expression or levels of gene expression between the inbred lines. Of particular interest may be the condition medium sensitive Line CM20, which had a distinct reaction norm profile to the condition medium resistant Line CM3. One might expect these two lines to display different transcription profiles. Identifying what these differences are may highlight some of the genes required for infective larvae development. *C. elegans* lines which differ in their response to environmental dauer larvae inducing signals, also display different transcription profiles by microarray analysis (HARVEY *et al.* 2009). Also of interest would be the isolation and characterization of the biological factor or factors within the conditioned medium which trigger the switch to infective larva development to determine how closely related structurally they are to *C. elegans* dauer pheromone derivatives. The active components of *C. elegans* dauer pheromone has been isolated, characterized and synthesized (BUTCHER *et al.* 2007; JEONG *et al.* 2005).

This work has shown that there is a down regulation of IIS gene expression early in the transition from L1 to iL3. What is not yet known is when *P. trichosuri* L1 become committed to the iL3 developmental fate upon exposure to conditioned medium. In other *Strongyloides* species this has been shown to be early in development, before the first moult (MINATO *et al.* 2008; NWAORGU 1983; VINEY 1996). In *P. trichosuri* the exact timing of the developmental switch could be pinpointed very precisely using highly synchronous populations of L1 and exposing them to conditioned medium for specific periods of time and then removing them to see if they revert of free-living development, thus determining when the developmental switch occurs. By using larvae exposed to conditioned medium from these same time points in order to examine IIS

gene expression with q-PCR it may be possible to determine whether down regulation of the IIS pathway correlates to the timing of the switch in iL3 development.

The results of the q-PCR has shown a second dip in expression of IIS genes when iL3 are presumably initiating exit from iL3 stage, therefore it would be of interest to determine if IIS plays a role in *P. trichosuri* iL3 recovery. Exit from the *A. caninum* iL3 stage - as determined by the resumption of feeding and the production of post iL3 associated proteins, can be triggered by incubation in foetal calf serum and S-methyl-glutathione (MOSER *et al.* 2005). It was found that inhibiting the IIS pathway with the application of the AGE-1 chemical inhibitor LY294002 also inhibited this resumption of feeding, suggesting IIS is required for *A. caninum* iL3 recovery (BRAND and HAWDON 2004). To determine if IIS is required for *P. trichosuri* iL3 recovery, it should first be determined whether *P. trichosuri* iL3 could be stimulated to resume development *in vitro* as was performed in *A. caninum*, and then whether treatment with the AGE-1 chemical inhibitor, LY294002 inhibits this resumption; if inhibition occurs this may suggest that IIS plays a role in *P. trichosuri* iL3 activation.

There are many *P. trichosuri* projects which would benefit from having the genome sequence available. The expression profile of the IIS genes indicate they are up regulated at the egg, L1 and iL3 stages of development; it would be of interest to determine whether the role of IIS is primarily to maintain development or whether it is to promote stress response at the iL3 stage. One way to do this may be to compare the expression profiles of *P. trichosuri* DAF-16 regulated stress response genes between these stages. In *C. elegans*, DAF-16 directly controls several stress response genes such as: the Mn-superoxide dismutase gene (*sod-3*) (HONDA and HONDA 1999), the metallothionein gene (*mtl-1*) (BARSYTE *et al.* 2001), and heat shock protein (*hsp-16.2*) (WALKER *et al.* 2001). With the caveat that we do not know whether these gene orthologues are directly controlled by DAF-16 in *P. trichosuri* it may be possible to examine the relative expression of these stress response genes by q-PCR at the different developmental stages. If results show that while IIS is up regulated in both the L1 stage and the iL3 stage, but that up regulation of stress response genes is seen only in the iL3 stage this may suggest that IIS is controlling a stress response program in iL3. With *P. trichosuri* genome sequence available it would be possible to determine

bioinformatically whether the promoter regions of these stress response genes contain conserved DAF-16 DNA binding elements (OH *et al.* 2006).

This project has highlighted several technical matters which should be resolved. First is the issue of whether the *Pt daf-16b* transgene is toxic. Transgenesis was achieved with a *H. contortus* or *S. stercoralis daf-16b* transgene orthologue and resulted in recovery of phenotype (HU *et al.* 2009; MASSEY *et al.* 2006), the main differences between our experiments and the *H. controtus* and *S. stercoralis* experiments is that the *Hc daf-16.2* and *Sc daf-fktf-1b* transgenes were under the control of the *Ce daf-16*α promoter and also that these groups used a different Daf-16 mutant allele. Different background alleles and the use of the *Ce daf-16*α promoter are two transgenesis parameters which may be attempted with *Pt daf-16b* again.

Another question which should be resolved is whether the constitutively activated *Pt daf-16* transgenes were expressed in transgenic *P. trichosuri*. *P. trichosuri daf-16* transgenes were created in which the AKT1/2 phosphorylation sites were mutated, these were then microinjected into *P. trichosuri* and the F_1 generation was assessed for both the presence of the transgene and a change in developmental fate from free-living to iL3. A PCR detection approach was chosen because the immunohistochemistry to detect the HA tagged transgene product proved unsuccessful. What perhaps should be done is to determine, not just the presence of the transgene but resolve whether it is expressed. Expression of the transgene product may be resolved by using whole worm lysate on a Western and using a HA tag antibody to detect the translated protein.

Functional analysis of genes directly in *P. trichosuri* using RNAi should be re-attempted. Recent literature has suggested that parasites may lack some of the key requirements for RNAi processing (namely RDE-4, which is required for interaction with Dicer) (KNOX *et al.* 2007) these authors have speculated that this may suggest the RNAi effects sometimes observed have been the result of contaminating short interfering dsRNA (siRNA) products (GELDHOF *et al.* 2007; KNOX *et al.* 2007). Our results suggested electroporation with siRNA may have resulted in knockdown of gene expression and that microinjection with *in vitro* transcribed dsRNA in combination with *Pt rrf-3* may have resulted in a phenotype. It would be intriguing to determine the

RNAi effect on *P. trichosuri* gene function when microinjecting siRNA of a gene of interest in combination with siRNA of *Pt rrf-3*.

A *Pt daf-16a* transgene is able to complement dauer defective phenotypes in *C. elegans daf-16* mutants, and the transcriptional profile suggests there is differential expression of *Pt* IIS genes at the iL3 developmental switch. It can be speculated that direct targets of the *Pt* DAF-16 transcription factor may have a role in iL3 development or recovery and therefore direct the expression of key components of parasitism. Direct targets of the *P. trichosuri* DAF-16 transcription factor may be determined by chromatin immunoprecipitation (ChIP). ChIP assays require an antibody to the *Pt* DAF-16 transcription factor. Genomic DNA is first cross-linked to protein transcription factors and sheared, then the *Pt* DAF-16 affinity antibody is used to pull down the sheared promoter fragments that have *Pt* DAF-16 bound to them. These fragments are then cloned and sequenced. GenomeWalking maybe used to determine downstream targets, although this project would be greatly enhanced if the *P. trichosuri* genome sequence was available. ChIP has been proven successful in determining DAF-16 targets in *C. elegans*; a greater quantity of direct DAF-16 targets were isolated by this process than by microarray or bioinformatics analysis (Oh *et al.* 2006). A similar process could be used in *P. trichosuri* to compare transcriptional targets of L1 in transition to iL3, the iL3 stage, and larvae exiting the iL3 stage. If IIS is the signalling transduction pathway that controls *P. trichosuri* iL3 entry or exit, ChIP analysis would provide direct DAF-16 targets.

Although, more work is required, this work demonstrates the potential that *P. trichosuri* has as a model in which to study evolution of parasitism and genetic regulation of development.

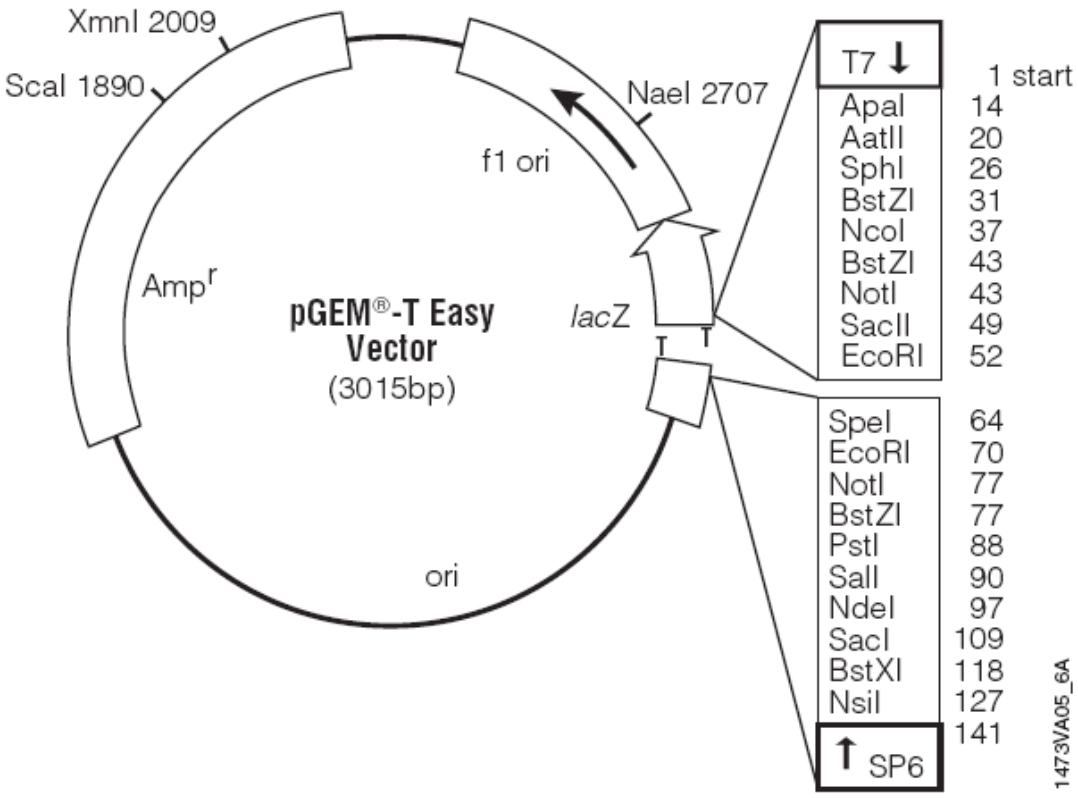
APPENDICES

APPENDIX 1: Plasmid maps and vector construction

APPENDIX 1.1

pGEM® –T Easy Vector (3015bp)

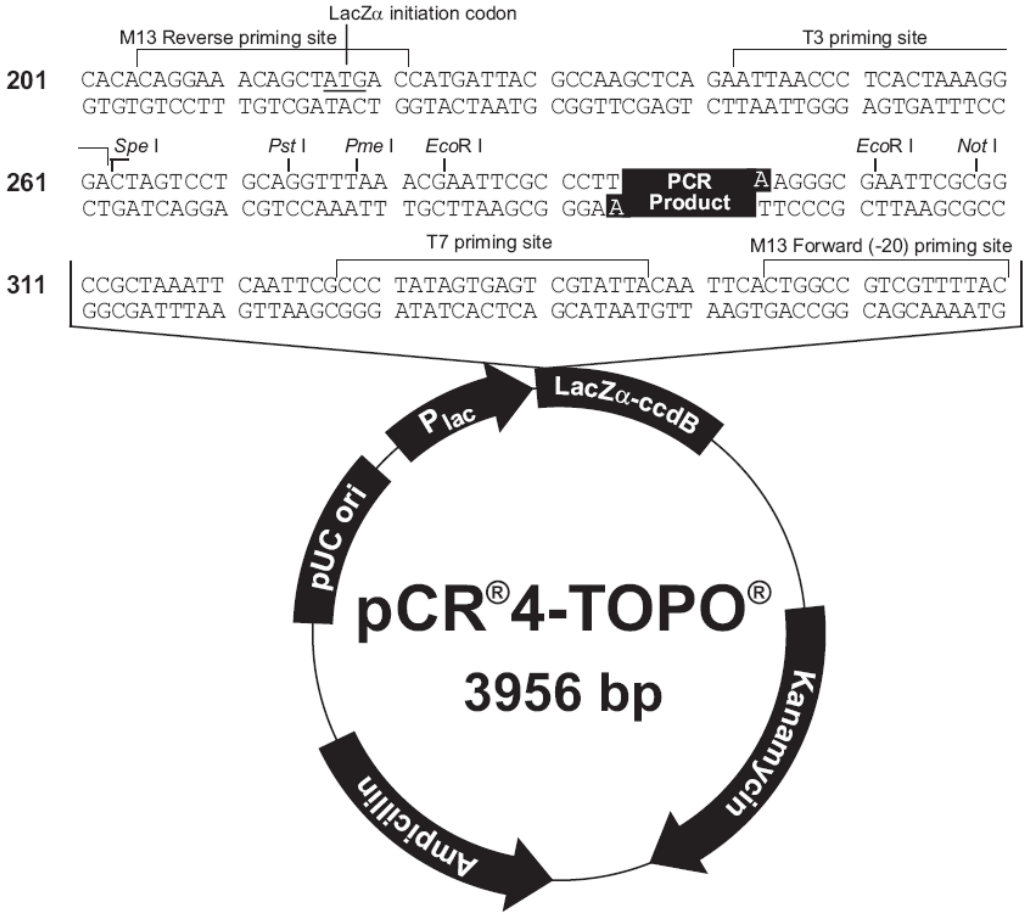
This vector was used for all TA cloning of PCR products generated from the GenomeWalker™ procedures (Promega).



APPENDIX 1.2

pCR®4-TOPO® vector (3956bp)

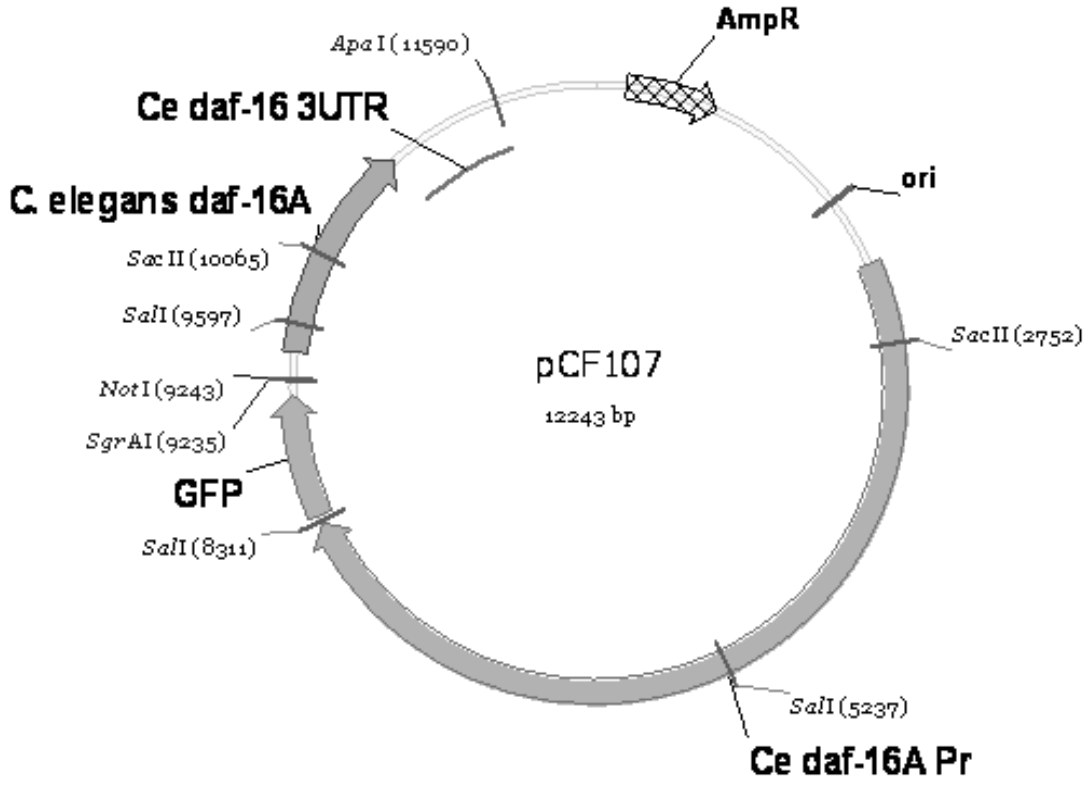
This vector was used for cloning PCR products used in vector construction (Invitrogen).



APPENDIX 1.3

pNL209 aka: pCF107 (12243 bp)

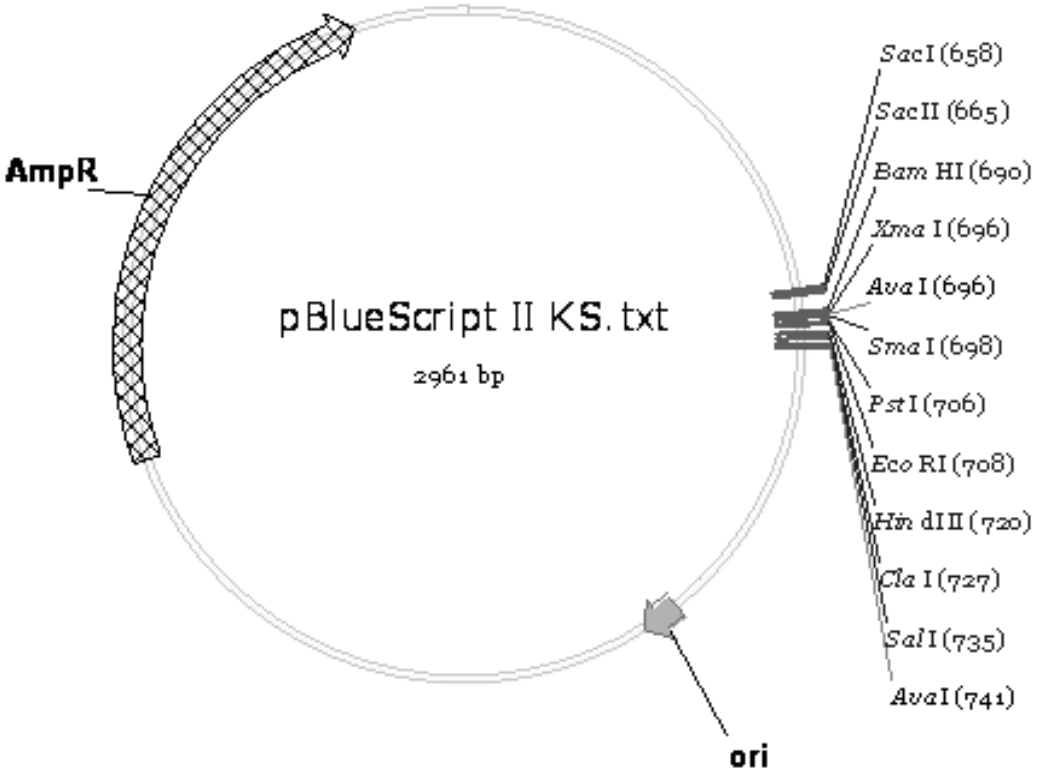
This vector was kindly supplied by Nataliya Libina. It consists of 5559 bp of the *C. elegans daf*-16α promoter, and an N- terminal GFP fusion tag linked to the *C. elegans daf-16a* gene.



APPENDIX 1.4

pBlueScript II KS (2961 bp)

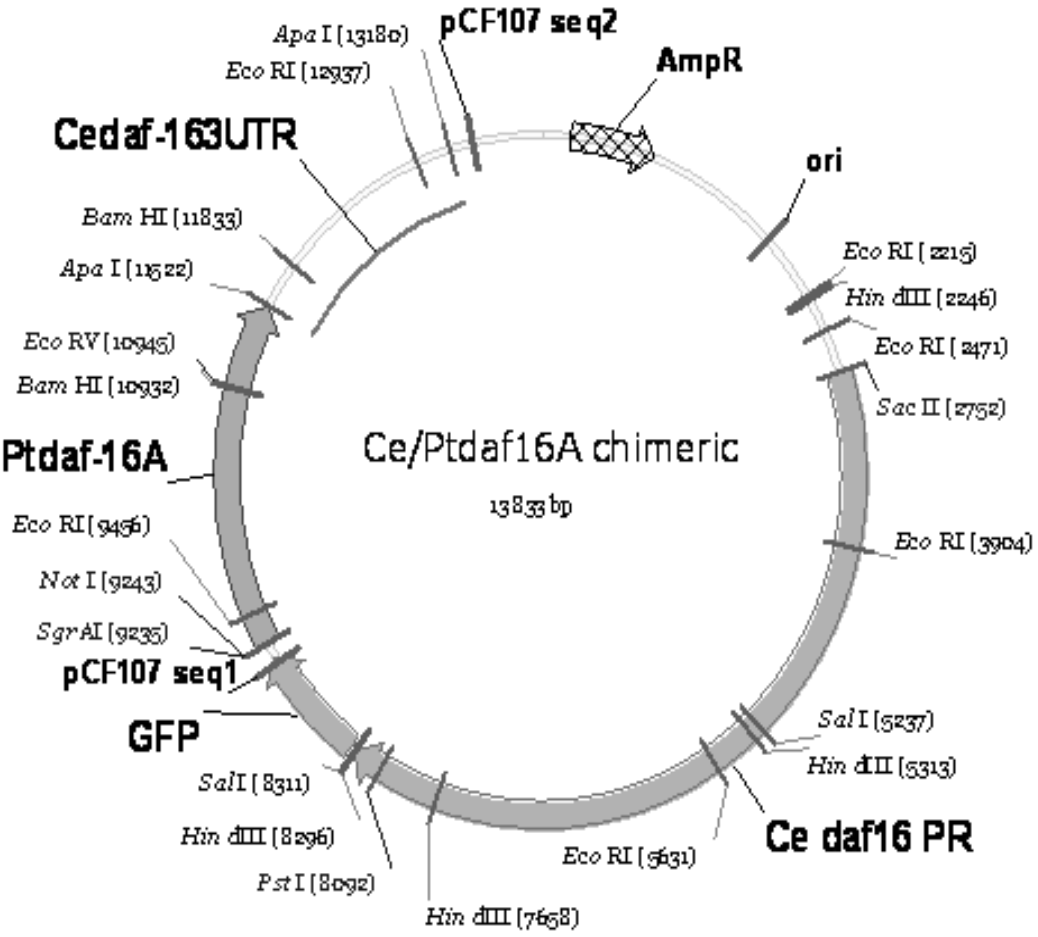
This vector was used for the cloning of the *Pt age-1* chimeric construct and *Pt daf-2* chimeric construct (Strategene).



APPENDIX 1.5

pPt daf-16a chimeric (13833bp)

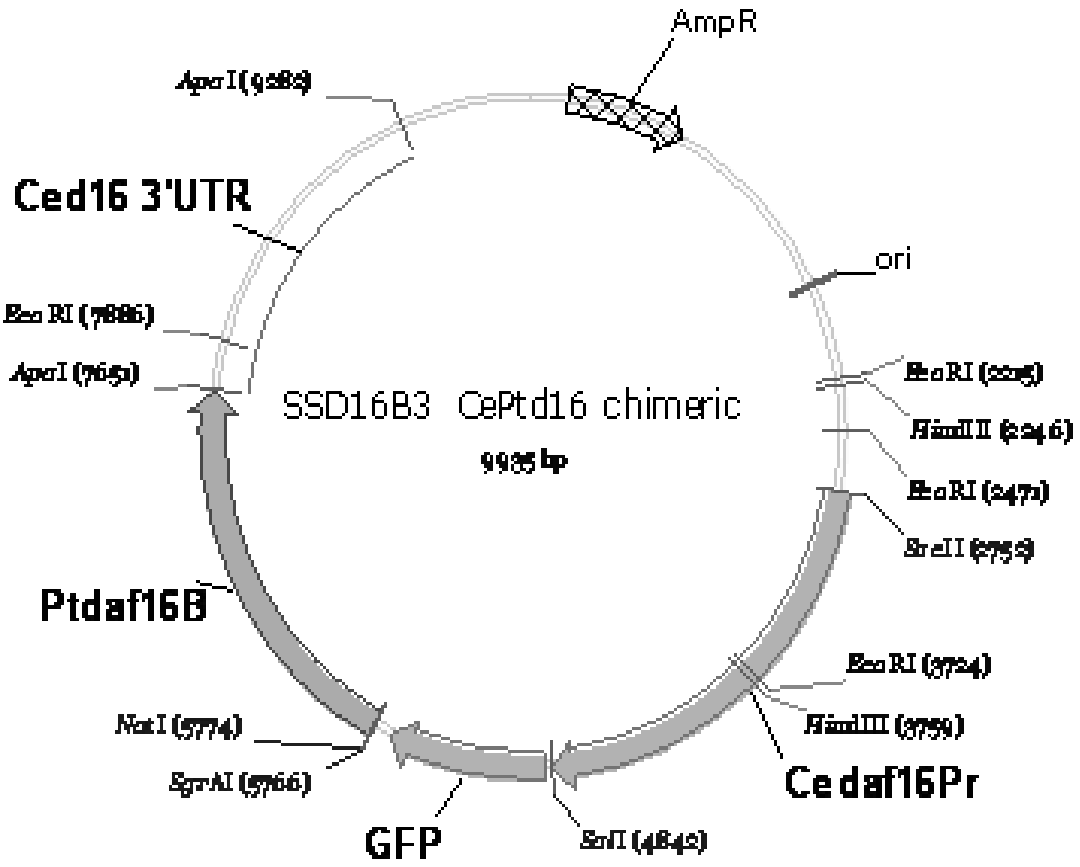
This vector was modified from pNL209, which was kindly supplied by Nataliya Libina. *Pt daf-16a* consists of 5559 bp of the *C. elegans daf*-16α promoter, and an N- terminal GFP fusion linked tag. The *P. trichosuri daf-16a* gene (2264 bp) was cloned into the SgrAI and ApaI restriction sites, and 1653bp of the *C. elegans daf-16* 3′ UTR was cloned into the ApaI in a subsequent cloning step.



APPENDIX 1.6

pSSD16B3 aka *Pt daf-16b* chimeric (9935bp)

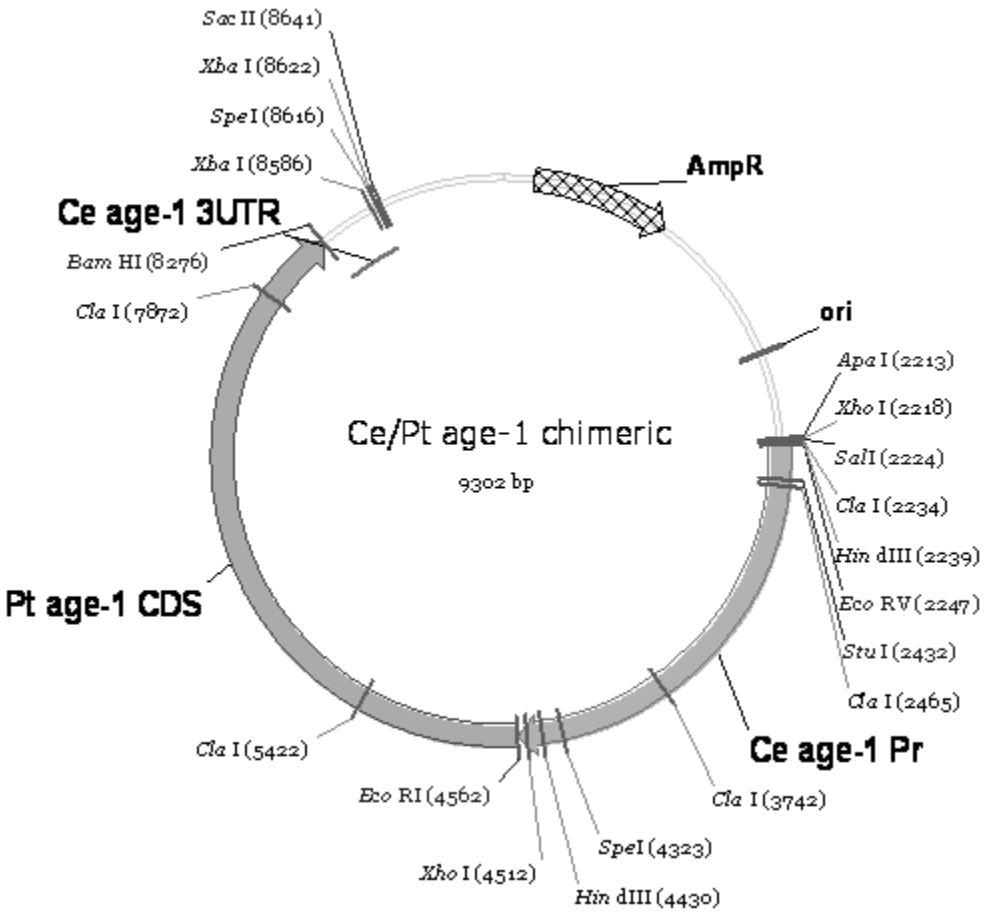
This vector was modified from pNL209, which was kindly supplied by Nataliya Libina. SSD16B3 consists of 1885 bp of the *P. trichosuri daf*-16b gene cloned into the SgrAI and ApaI restriction sites, in a subsequent cloning step 1653bp of the *C. elegans daf*-16 3′UTR was cloned into the ApaI site, this was followed by cloning 2093 bp of the *C. elegans daf*-16β promoter into the SacII and SalI sites, upstream of the N- terminal GFP fusion linked tag.



APPENDIX 1.7

pPt age-1 chimeric (9302 bp)

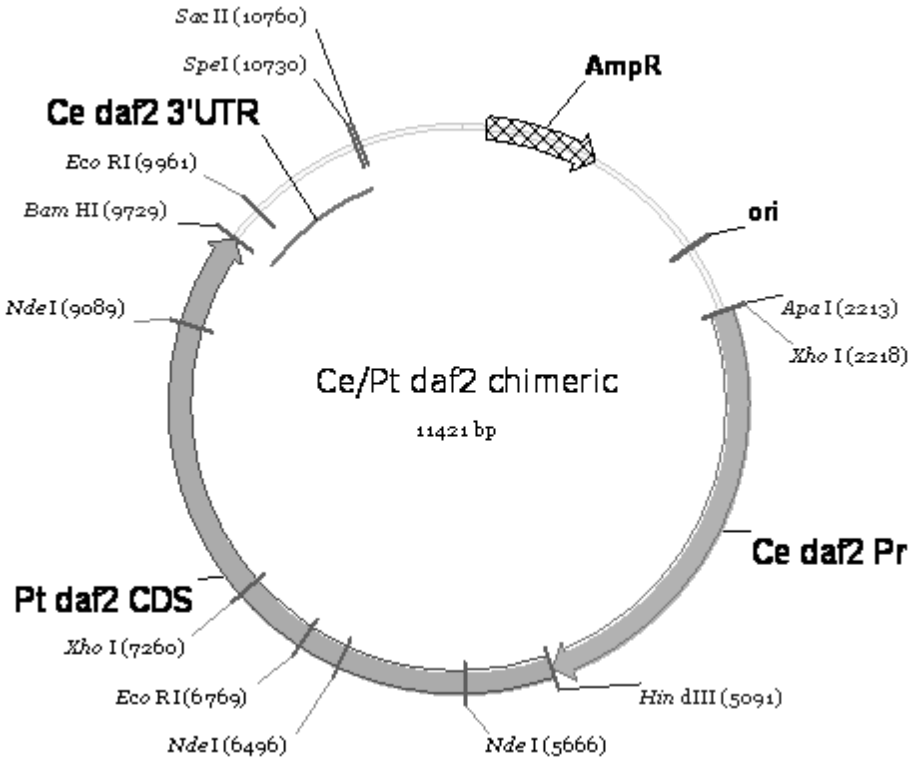
This vector was modified on pBlueScript II KS (Strategene). The 340 bp genomic region of the *C. elegans age*-1 3′ UTR was cloned into the BamHI and SpeI sites. In a subsequent cloning step 3714 bp of *P. trichosuri age*-1 gene was cloned into the EcoRI and BamHI site. In a subsequent cloning step 2315 bp the *C. elegans age*-1 promoter region was cloned into the EcoRV and EcoRI sites.



APPENDIX 1.8

pPt daf-2 chimeric (1142 bp)

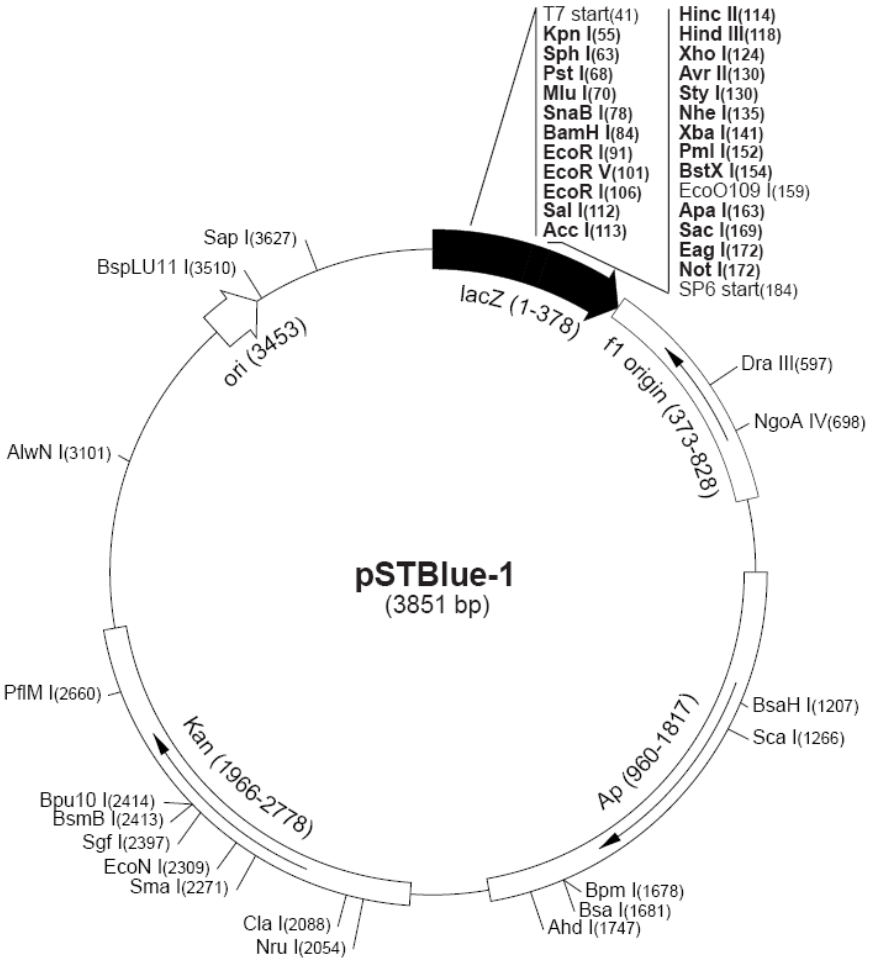
This vector was modified on pBlueScript II KS (Strategene). The 1001 bp genomic region of the *C. elegans daf*-2 3′ UTR was cloned into the BamHI and SpeI sites. In a subsequent cloning step 4638 bp of *P. trichosuri daf*-2 gene was cloned into the HindIII and BamHI site. In a subsequent cloning step 2873 bp the *C. elegans daf*-2 promoter region was cloned into the XhoI and HindIII sites.



APPENDIX 1.9

pSTBlue-1 (3851 bp)

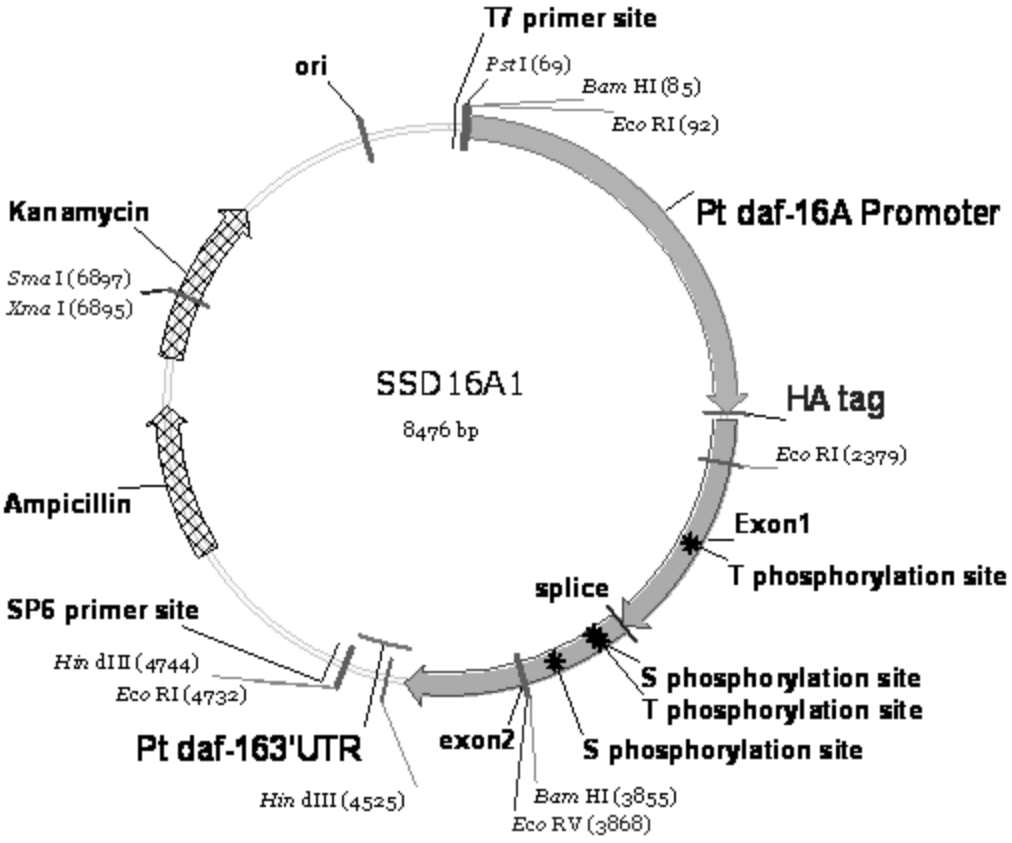
This vector (Novagen) was used in the cloning procedure of the modified overlap extension PCR (M)OE-PCR, in the creation of SSD16A1, SSD16A2, SSD16B1 and SSD16B2 (Section 2.6.4).



APPENDIX 1.10

pSSD16A1 (8476 bp)

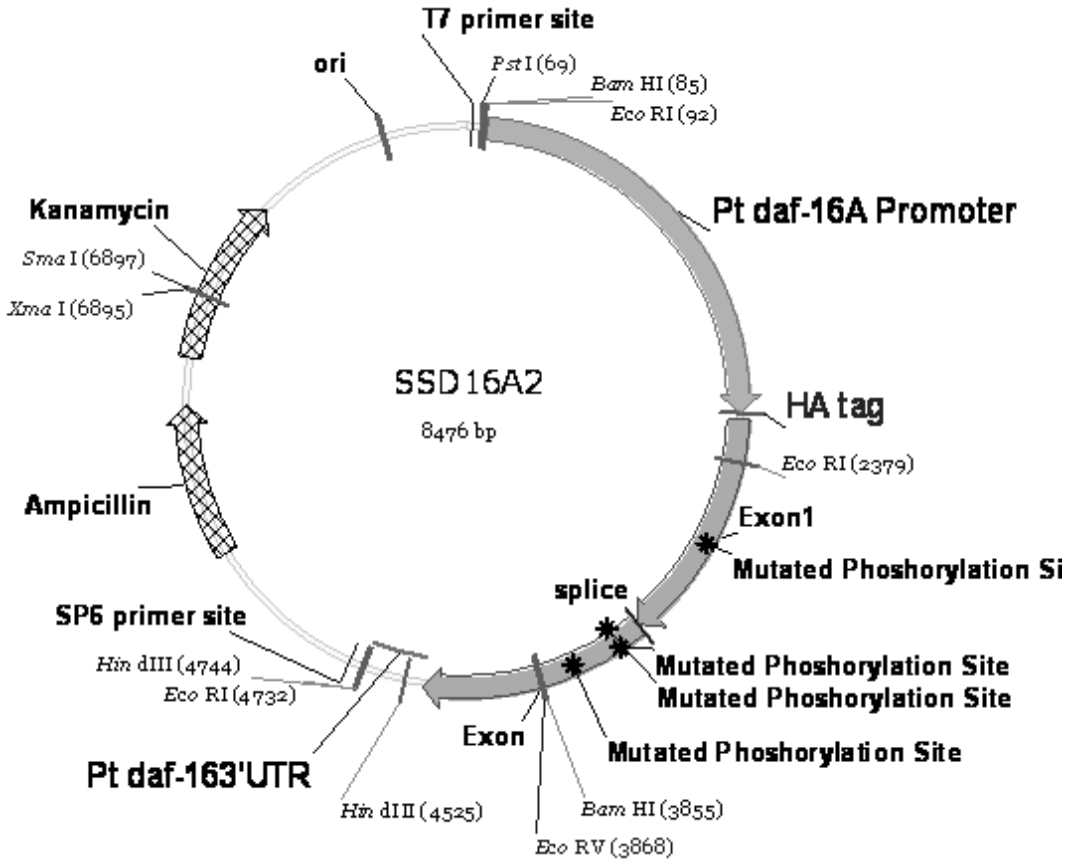
This vector was based on pSTBue-1, it consists of 2039 bp of the *P. trichosuri daf-16a* promoter, an N-terminal HA epitope, 2264 bp *P. trichosuri daf-16a* gene and 289 bp of *P. trichosuri* 3′ UTR. Cloning of this construct, was as described in Section 2.6.4.



APPENDIX 1.11

pSSD16A2 (8476 bp)

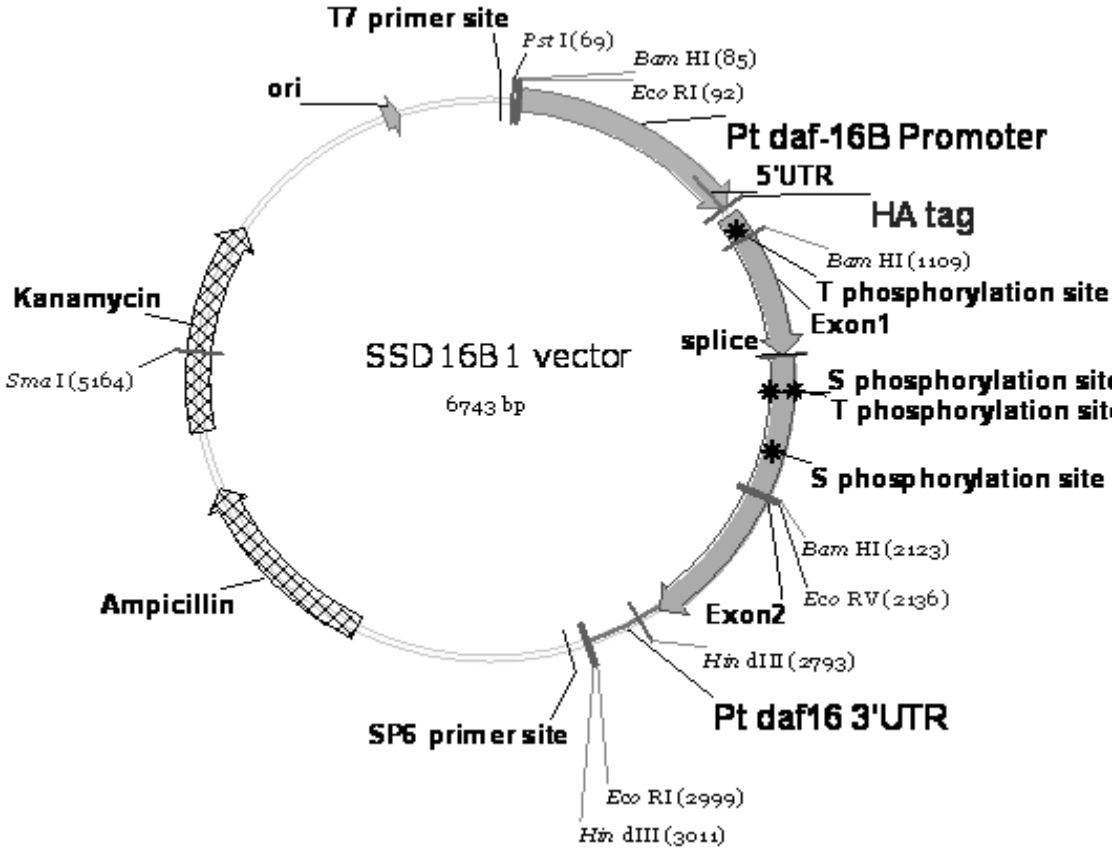
This vector was based on pSTBue-1, it consists of 2039 bp of the *P. trichosuri daf*-16A promoter, an N-terminal HA epitope, 2264 bp *P. trichosuri daf*-16A gene with mutated phosphorylation sites generated by (M)OE-PCR and 289 bp of *P. trichosuri* 3′ UTR. Cloning of this construct, was as described in Section 2.6.4.



APPENDIX 1.12

pSSD16B1 (6743 bp)

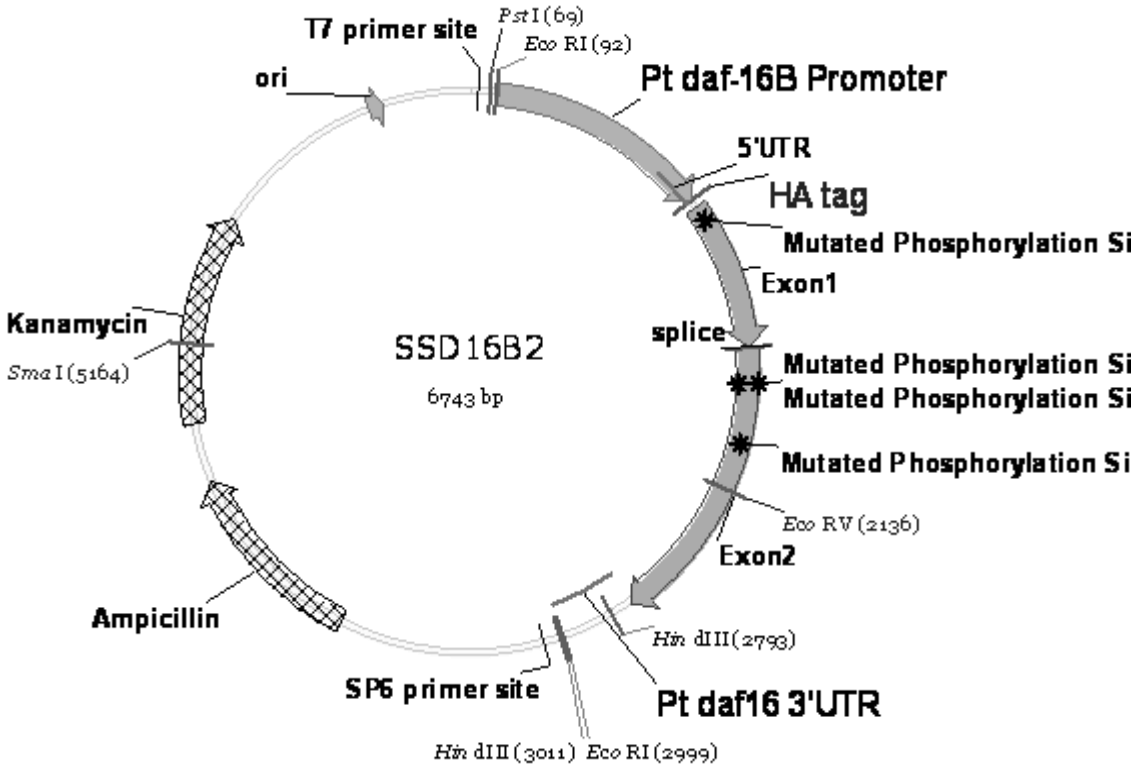
This vector was based on pSTBue-1, it consists of 871 bp of the *P. trichosuri daf*-16B promoter, an N-terminal HA epitope, 1703 bp *P. trichosuri daf*-16B gene and 289 bp of *P. trichosuri* 3' UTR. Cloning of this construct, was as described in Section 2.6.4.



APPENDIX 1.13

pSSD16B2 (6743 bp)

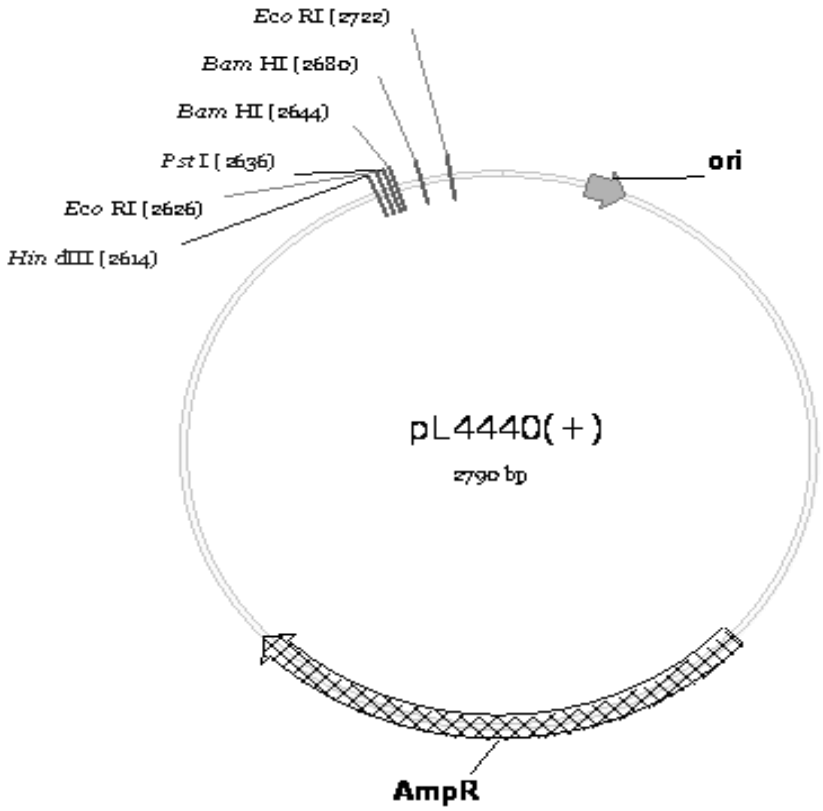
This vector was based on pSTBue-1, it consists of 871 bp of the *P. trichosuri daf*-16B promoter, an N-terminal HA epitope, 1703 bp *P. trichosuri daf*-16B gene with mutated phosphorylation sites generated by (M)OE-PCR and 289 bp of *P. trichosuri* 3' UTR. Cloning of this construct, was as described in Section 2.6.4.



APPENDIX 1.14

pL4440 (2790 bp)

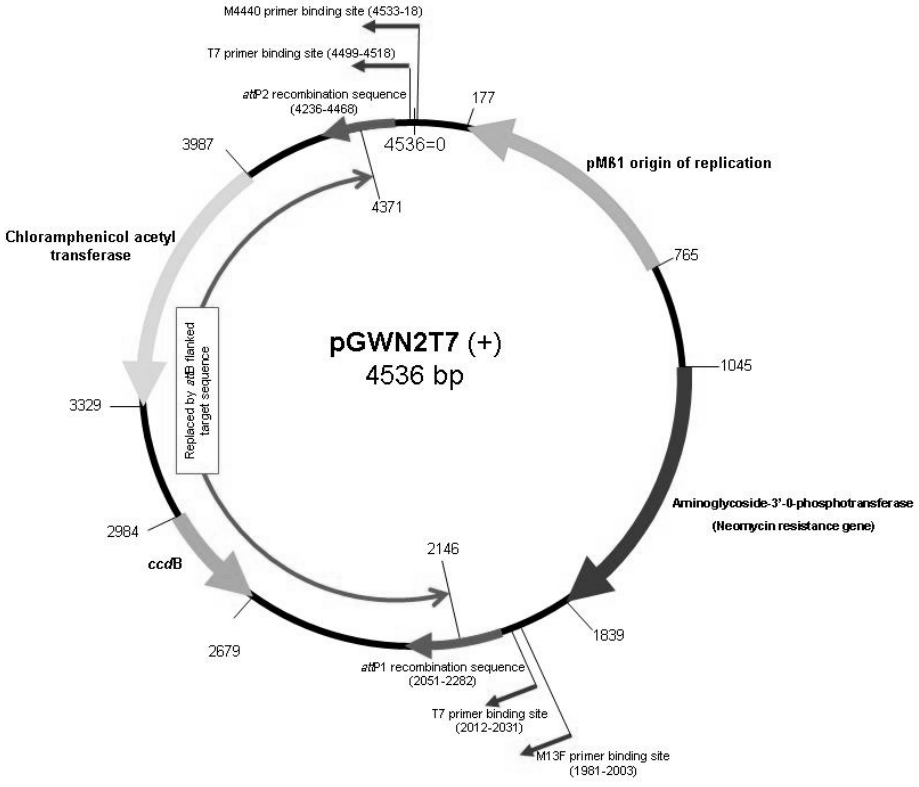
This vector was kindly supplied by Andrew Fire. It consists of two inverted repeat of the T7 promoter separated by a multiple cloning site. This vector was used for RNAi feeding assay of *C. elegans* and *P. trichosuri*



APPENDIX 1.15

pGWN2T7(+) (4536 bp)

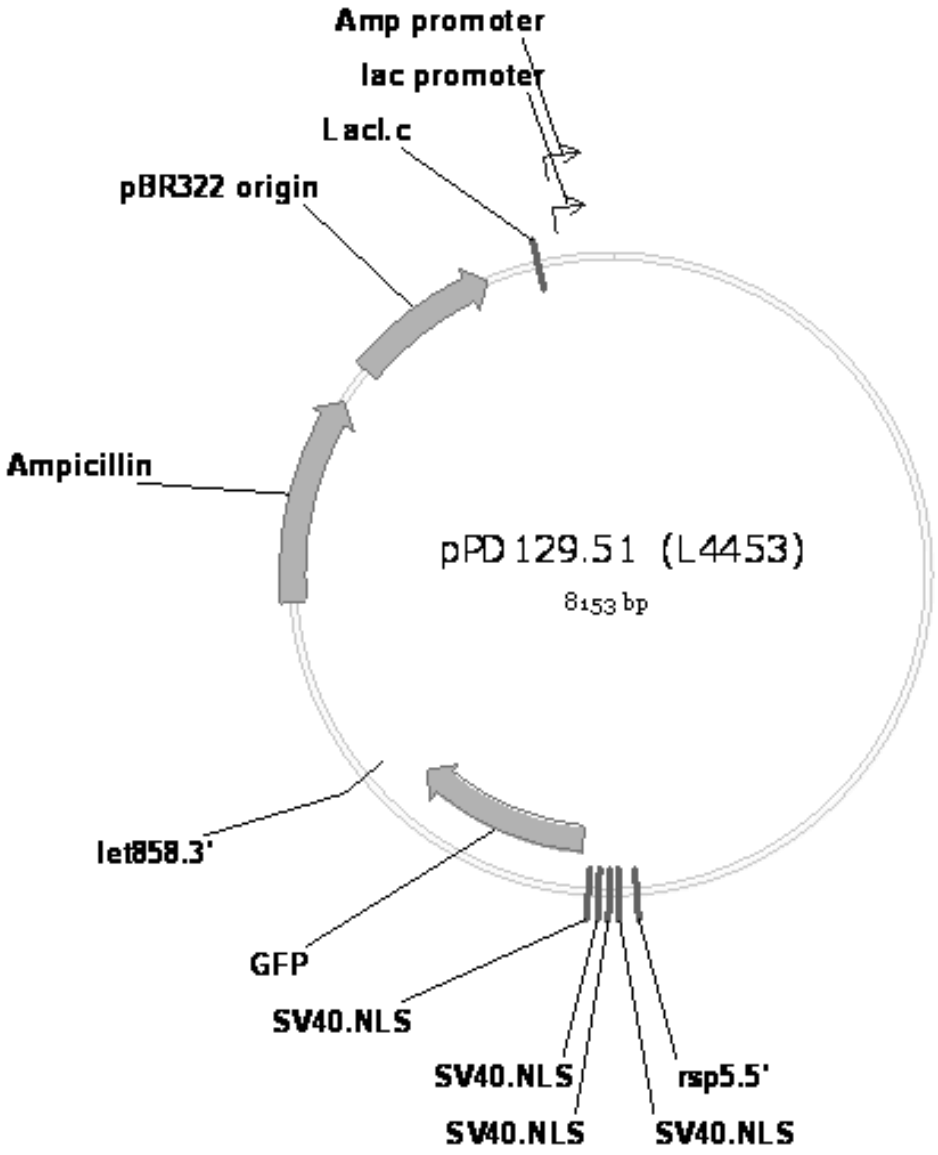
This vector was kindly supplied by Andrew Dubowsky. It consists of two flanking T7 primer binding sites for in vitro transcription of recombinant clones constructed through the Gateway® cloning system (inserting the PCR products amplified with the GENattB1/GENattB2 primers from your "GEN-target specific" PCR amplified product).



APPENDIX 1.16

pPD129.51 (8153 bp)

This vector was kindly supplied by Andrew Fire. Used for worm expression green fluorescent protein under the control of rsp5.5 promoter.



APPENDIX 2: SOLUTIONS AND MEDIA

Lauria-Bertani Medium (LB) per litre

Tryptone	10 g
Yeast Extract	5 g
NaCl	5 g
NaOH(1N)	1 mL

Per 1 liter of water. Autoclave. For solid medium 15g agar was added

(Low Peptone) NGM Agar per litre (Brenner, 1974)

NaCl	3g
Peptone	0.25g
Agar	17g
distilled water	975mL

Autoclave. Then cool to 55^{o}C, sterilely add in order:

Cholesterol (5mg/mL in Ethanol)	1mL
1M $CaCl_2$	1mL
1M $MgSO_4$	1mL
1M KH_2PO_4 (pH 6.0)	25mL

Note: Low Peptone NGM liquid broth as above without the agar

NGM Agar (Brenner, 1974)

NaCl	3g
Peptone	2.5g
Agar	17g
Water	975mL

Autoclave. Then cool to 55^{o}C, sterilely add in order:

Cholesterol (5mg/mL in Ethanol)	1mL
1M $CaCl_2$	1mL
1M $MgSO_4$	1mL
1M KH_2PO_4 (pH 6.0)	25mL

Note: NGM liquid broth (as above without the agar)

Low peptone NGM Agar (No Cholesterol)

NaCl	3g
Peptone	0.25g
Bacto Agar	17g
Water	975mL

Autoclave. Then cool to 55^{o}C, sterilely add in order:

1M $CaCl_2$	1mL
1M $MgSO_4$	1mL
1M KH_2PO_4 (pH 6.0)	25mL

Faecal extract

The possum faecal extract consists of pooled fresh faeces suspended at 10% w/vol in water: 200 µL aliquots were dispensed into microcentrifuge tubes, dried in a SpeediVac and stored at -20^{o}C until being rehydrated.

Solubilization Buffer

300mM NaCl
50mM PO4, pH8
10mM immidazole
0.5% Tween-20

Trehalose electroporation buffer

272 mM trehalose
7 mM KH_2PO_4 (pH 6)
1 mM $MgSO_4$

TE Buffer

10mM Tris-HCl, pH 8.0
0.1mM EDTA, pH8.0

Worm Axenization Solution:

4N NaOH	2mL
8% Na Hypochlorite	3mL

C. elegans Freezing Solution:

1M NaCl	20mL
1M KH_2PO_4 (pH 6.0)	10mL
100% glycerol	60mL
and water up to 200mL	

Worm lysis solution

1 mL of lysis solution consists of 985 µL DirectPCR(Tail) [Viagen Biotech Inc Cat# 501-K] and 15 µL of Proteinase K [Roche Cat # 03115 828 001].

Worm Immunohistochemistry

β-mercaptoethanol Buffer (BMB)

125mM tris-HCl
1% triton X-100
pH=6.9. Store at RTP

Collagenase Buffer

1mM $CaCL_2$
100mM Tris-HCl
pH = 7.5. Store at RTP.

Collagenase Mix

Add 120 U/mL collagenase IV to collagenase buffer immediately prior to use.

e.g. collagenase IV with 231 units/mg, need 8.7mg to give 2000U, dilute in 16.7mL.

Antibody Diluent

0.1% BSA
0.5% triton X-100
0.05% sodium azide
Made in PBS. Store at 4°C

APPENDIX 3: Primer Sequences

These primers were used for all PCR-based cloning or analyses

Name	Sequence	Function
P.t. age-1 Fwd1	GTGTAACATTATTTGAGATTCAAAC	original clone
P.t. age-1 Rev1	GCTTTTAAATAGTTCAAATCTTCC	original clone
Pt a1 down GSP1	CATTGACCGGTTATTGTGTCGCAAC	GenomeWalker
Pt a1 down GSP2	GGTTTACCAGAATGCTCTACACAGG	GenomeWalker
Pt a1 up GSP1	CCATCCTAATGTTTCTGTTTCTGC	GenomeWalker
Pt a1 up GSP2	GCAAATGATAATTGTGCAGCAGGAG	GenomeWalker
Pt a1 down GSP3	CTCCTCCTTGGCCAATATTGCTTGATC	GenomeWalker
Pt a1 up GSP3	CCAACAGCTACACCAATATCCTGTTC	GenomeWalker
Pt a1 up GSP4	CTAGGTTGATGAAGTGCTAATATTGGTG	GenomeWalker
Pt a1 GSP5	TCCACCACCACTTCCACCAAGACCAA	GenomeWalker
Pt a1 GSP6	TTGAACAACGAAGCCAACAATGGTGTGTA	GenomeWalker
Pt a1 GSP7	TGAATCCACCACCACTTCCACCAAGACCA	GenomeWalker
Pt a1 GSP8	GAAGCCAACAATGGTGTGTATTCTTTCAATCC	GenomeWalker
Pt age1 5'R SP1	GCCCATTTCCACTACAGCATCGA	RACE primer
Pt age1 5'R SP2	CCAACAGCTACACCAATATCCTGT	RACE primer
Pt age1 5'R SP3	CACTAGGTTGATGAAGTGCT	RACE primer
Pt a1 seq1	CAACCATTTGTTAATATGCACGTT	confirm Sequence
Pt a1 seq2	CACCTTCTGTTAATGCCGTT	confirm Sequence
age-1fwd QPCR	TAAATCCAAGTGGCCCAGAGG	Q-PCR
age-1 rev QPCR	CGTGGATGTGAACCACGTTG	Q-PCR
Pt a1 XhoI Fwd	CCGCTCGAGCGTACAAAATTGCTTTCAGGTGT	Vector construction
Pt a1 PstI Pr Rev	AACTGCAGTTCTTTGACATTATCATACAATGT	Vector construction
CeA1 Pr Fwd-EcoRV	cgggatatcCGGTCGGTGTTTGCGTACTT	Vector construction
CeA1 Pr Rev-EcoRI	cggaattcCTGCGAGATACGTCGGGAGT	Vector construction
PtA1 X1 Fwd-EcoRI	cggaattcATGGATGGTAAGTAAATATTATATA	Vector construction
PtA1 X2 Rev-BamHI	cgggatccTTACATATGTTTAACTGAATGGAA	Vector construction
CeA1 3UTR Fwd-BamHI	cgggatccAACCTCTGTTATCTAATAATATAAC	Vector construction
CeA1 3UTR Rev-SpeI	ggactagtGTTCAGCCAAGAAATCATGGAA	Vector construction
Pt Age-1 cds	ATGGATGGTTCTTCATCAAAAT	confirm Sequence
Pt A1 Pr seq	GCACAATGTTGATCAGCGCA	confirm Sequence
CeA1 Pr Fwd-EcoRV 2 nested	cgggatatcCGGTCGGTGTTTGCGTACTTT	Vector construction
CeA1 Pr Rev-EcoRI	cggaattcCTGCGAGATACGTCGGGAGTTCCGTCC	Vector construction

CeA1 Pr Fwd-EcoRV 3 nested	cgggatatcCGTTTGCGTATGAAGCATCCAA	Vector construction
Pt age1 mut A	GACGTGACGATTTTGTCGAT	Mutagenesis
Pt age1 mut B	CAAAGCGAAGGCATGAAGAA	Mutagenesis
Pt age1 mut C	GGTACAGTACTTACCAAAGCAA	Mutagenesis
Pt age1 mut D	GTGTCAAAGGCACAATGTTGAT	Mutagenesis
Pt age1 mut E	CTCTCTTCTGTGATGTCATTTCCCAT	Mutagenesis
Pt age1 mut F	GATAATTGTGCAGCAGGAGGT	Mutagenesis
Pt age1 mut G	GAATACACACCATTGTTGGCT	Mutagenesis
Pt age1 mut H	GTCTTGGTGGAAGTGGTGGT	Mutagenesis
Pt age1 mut I	GCTTCCAAACATTATGTCTAAA	Mutagenesis
Pt age1 mut J	CCATTAACGGCTAACATAACAA	Mutagenesis
Pt daf2 degen F3	GGACAAGGATCWTTTGGAATGGT	Degenerate primer
Pt daf2 degen R2	ACAACTCCRTAAGACCADACRT	Degenerate primer
Pt daf2 GSP1	CAGGCTGTCCTTCACTAACAACACC	GenomeWalker
Pt daf2 GSP2	CTGCTGGCGTAGCACTATCTCTAACAG	GenomeWalker
Pt daf2 GSP3	GCTGCCAGAAATTGCCTTGTTCATCG	GenomeWalker
Pt daf2 GSP4	CCAACAGGAAAACGTCTTATTCCTGTA	GenomeWalker
daf2 GSP5	CAAGACCATTAGGATTTGTCGGATTATTCCAC	GenomeWalker
daf2 GSP6	TTGCAACAGGATTGCTTGTTATTGCCCTAACA	GenomeWalker
Pt d2 GSP7	TTCAGGAATACTTGGTTCTGCTAACACT	GenomeWalker
Pt d2 GSP8	ACACTAAATCACCCTCCATAACAGTGCATTTCTGA	GenomeWalker
Pt d2 5'R SP1	CATCCTCCCACACAAAGATCAT	RACE primer
Pt d2 5'R SP2	GGGCCTTTCTCAACTCCAGTTCCTA	RACE primer
Pt d2 5'R SP3	CATCTAGTTTTTGAGGAGTCTTCCA	RACE primer
Pt d2 5'R SP4	CCTGGTTCTGCTAACACTAAAT	RACE primer
Pt d2 5'R SP5	CCCTCCATAACAGTACATTTCTGA	RACE primer
Pt d2 5'R SP6	CCCCAGCACAAATGGTATCATT	RACE primer
Pt d2 3'R SP1	TGTTAGTGAAGGACAGCCTGTTCTGG	RACE primer
Pt d2 3'R SP2	TGGGCAGCACAAATTGCTGAT	RACE primer
Pt d2 3'R SP3	CAAGACCACAAGGATGTGCTGAC	RACE primer
daf2 F QPCR	CTAGCCATACCAAAATCACCAA	QPCR
daf2 R QPCR	ATTGCCTTGTTCATCGTGATG	QPCR
Pt D2 seq1	GATACATTTGGGAGACACCTT	confirm Sequence
daf2 seq2	GGAACTGGAGTTGAGAAAGGC	confirm Sequence
Pt d2 seq 3	ATGATTTATGCTATGCTTATTAATTT	confirm Sequence
Pt d2 fwd nest	CTTTCTTCCTCTAATGCAGCTA	confirm Sequence
Pt d2 rev nest	CCTATATGCCTTTTTTAAATACCA	confirm Sequence
Ced2Pr Rev - HindIII	cccaagcttTCTCGTCATCGTTCTGTCTG	Vector construction
Ced2Pr Fwd-XhoI	ccgctcgagGCACTATTTGTGTGTGTGAGT	Vector construction

PtD2 X1 Fwd-HindIII	cccaagcttGCAAAACTAATGATTTATGCTATG	Vector construction
PtD2 X2 Rev-BamHI	cgggatccCTATTTTTCATCAATTTCATCTG	Vector construction
CeD23UTR Fwd-BamHI	cgggatccAACCCCCAAAAAATCCCGCC	Vector construction
CeD23UTR Rev-SpeI	ggactagtGGAGAGAGAGAGGGGGAATT	Vector construction
Pt daf2 mut A	CCGGCTTCCTATATGAATGA	Mutagenesis
Pt daf2 mut B	CCATTTGTGCTGGGGTTTCAA	Mutagenesis
Pt daf2 mut C	CGATACACAATGCTTTGGAT	Mutagenesis
Pt daf2 mut D	GGGAGACACCTTATTGTAAG	Mutagenesis
Pt daf2 mut E	CATAATCCGTCGTCTGTTGGTT	Mutagenesis
Ce ama-1 GENF	GAGGAGTCGCAATGCGTACAATGCGGATTTCGATG	RNAi
Ce ama-1 GENR	GCTCTTCTGGAATGCCCAGTCTCGGCAGTCTTTACA	RNAi
Pt RNApol2 fwd	GAGGAGTCGCAATGGATTTTGATGGAGAYGARATGAAY	RNAi
Pt RNApol2 rev	GCTCTTCTGGAATGCTGAATATATCCWGTTTCNGCNGT	RNAi
Pt rrf3 GSP1	GCTCCATGATCACGCATTTGAGAGTT	RNAi
Pt rrf3 GSP2	GTTTACCATCATAACTTCTGGAGCCATA	RNAi
Pt Dgen daf16a Fwd1	ATGTCTGGACATCCNTAYWSNCAR	Degenerate primer
Pt Dgen daf16a Fwd2	CAAGTTTATGARTGGATGGTN	Degenerate primer
Pt Dgen daf16 Rev1	ATCAAAATTDATNGGNGCRTTNSW	Degenerate primer
Pt Dgen daf16b Fwd1	ATGACTTCWATTGTNWSNGAYGAY	Degenerate primer
Pt daf16a degenF3	GGACATCCATATTCWCAACARTAYAC	Degenerate primer
Pt daf16a degenF4	ACTAATAATTCWGGAAAYAAYCAR	Degenerate primer
Pt daf16b degenF3	TGTTATACATGGCCAATGCCNCAY	Degenerate primer
Pt daf16b degenF4	ATGGATCCAGAAATTGAYGAYTGY	Degenerate primer
Pt d16 down GSP1	CAAAATGAAGGTGCAGGAAAATCATCATGG	GenomeWalker
Pt d16 down GSP2	GTAGTGCAGGATGGAAAAACTCCATAAG	GenomeWalker
Pt d16 down GSP3	GGTGCCCAATCAAACAATGAGTTGCCA	GenomeWalker
Pt d16 down GSP4	GAGACAACACAGATGACGGAGATGGA	GenomeWalker
Pt d16up GSP1	CTATCACGTTGCCTTCTTTGACTTC	GenomeWalker
Pt d16 up GSP2	CCATGATGATTTTCCTGCACCTTC	GenomeWalker
Pt d16 up GSP3	TGTGAGGAACCAACACCAGAGTCATCACC	GenomeWalker
Pt d16 up GSP4	GGTGATGAGGTTGTTGGAAAACCATTGTTAG	GenomeWalker
Pt d16 up GSP5	GGTCAAAGGTTCCTCTTCTTAAAGGCCATG	GenomeWalker
Pt d16 up GSP6	CCAGGAGTTCTAATAACTCCATGTCCATAACC	GenomeWalker
Pt d16 GSP7	TGGGCAACTCCTTCATCAACAGATGTGGT	GenomeWalker
Pt d16 GSP8	TGTTGAGAGTATGGTAATCCACTCATCT	GenomeWalker

Pt d16 GSP9	AGTTCACAAATCAAGAAGGCGCACATA	GenomeWalker
Pt d16GSP11	CCACGAAGCTCTGGCTCCAGTTGAAAGTCATCTG	GenomeWalker
Pt d16GSP10	CTGGTGCTTGGTGATGGAGATAGGTTACCGAACT	GenomeWalker
Pt d16 GSP12	GGATTTGTATGTAGAGAGATATATAGCGAT	GenomeWalker
Pt d16 GSP13	CTACTTTTCTTGTAGTAATGTTGAGATAGTTG	GenomeWalker
Pt d16 GSP14	GGAATCCCGATGCTGTTTCTCAGAAA	GenomeWalker
Pt d16 GSP15	CATTAGAGAATGCACCAGAAAAGAGGATGAAA	GenomeWalker
daf16 SP1 5R	CCATTCCACCATCACCTAATTGC	RACE primer
daf16 SP2 5R	GCATCTGGATTGATTACCCACC	RACE primer
daf16 SP3 5R	CCCACCATGATGATTTTCCTGCAC	RACE primer
daf16 SP5 3R	ACCCGGTGATATGCCTCCACAA	RACE primer
daf16 SP6 3R	GTGATATGCCTCCACAACCACCAT	RACE primer
3-d16A QPCR Fwd	TTTTTTCCGTGATAAAGGAGATAGC	Q-PCR
3-d16A QPCR Rev	GGAGTTTTTCCATCCTGCACTACTATT	Q-PCR
2-d16B QPCR Fwd	ATGATAGATCATCACAAGAAGAA	Q-PCR
2-d16B QPCR Rev	TGTCTTATGGAGTTCTTCCA	Q-PCR
daf-16 fwd sub	GAGTGGATTACCATACTCTCAA	confirm Sequence
daf-16 rev sub	CTATCTCCTTTATCACGGAAA	confirm Sequence
daf-16a fwd X1	ATGGTTCATTATTATCATGATGACTT	confirm Sequence
daf-16b Fwd X1	ATGAGTGGATTACCATACTCTCAACA	confirm Sequence
Pt d16ab Rev1	TGATGGTGGTTGTGGAGGCAT	confirm Sequence
Pt d16A Fwd1	CATCTGGAGGAGGAGGAGAA	confirm Sequence
Pt d16A Fwd2	GAAGGAATGCCTGGGGTAGT	confirm Sequence
Pt d16B Fwd1	GAATCCCGATGCTGTTTCTCA	confirm Sequence
Pt d16B Fwd2	GAGAATGCACCAGAAAAGAGGA	confirm Sequence
Ced16b Pr Fwd SacII	actactaccgcggCCCGATGAAGATACCTAGAGA	Vector construction
Ce d16B Pr -SalI R	ttatcgtcgacTCTGGAAGCTGTGCTCCTCCGA	Vector construction
Pt d16B ATG-SgrAI	gctccaccggtggcggccgcATGAATGGTTCATTATTATCAGAC	Vector construction
Pt d16A ATG-SgrAI	gctccaccggtggcggccgcATGAGTGGATTACCATACTCTCA	Vector construction
Pt d16AB Rev ApaI	gctcagggcccTTACAGATCAAAGTTGATTGGAGC	Vector construction
Ce d16 3UTR ApaI fwd	atagggcccATTCTCTTCATTTGTTTCCCCTG	Vector construction
Ce d16 3UTR ApaI rev	atagggcccTTCAAATTTGATTTTTATTAAATCATC	Vector construction
Pt d16 3UTR X1	CTGTTGCTGCTATCAATATATGGGA	Vector construction
Pt d16 3UTR X2	GGATGTGAGTGTCTTTTACT	Vector construction
Pt daf16 mut A	GCCAGAGCTTCGTGGACGTT	Mutagenesis
Pt daf16 mut B	GAAGGTGCAGGAAAATCATC	Mutagenesis
Pt daf16 mut C	CCATCTCCGTCATCTGTGTTGT	Mutagenesis
Pt daf16 mut D	GGCCAATGCCTCATGTTATAT	Mutagenesis

Pt daf16 mut E	GATTGGGCACCACAACTTAACA	Mutagenesis
Ptd16RNAi Fwd GW2T7	GAGGAGTCGCAATGGGACATCTGTTCAAAGCGGTT	RNAi of Ptdafb
Ptd16RNAi Rev GW2T7	GCTCTTCTGGAATGCGGTGGTTGTGCCATTGGTGAT	RNAi of Ptdafb
Ced16RNAi Fwd GS2T7	GAGGAGTCGCAATGCCTGAAGAAGATGCTGACCTA	RNAi of Ce d16
Ced16RNAi Rev GW2T7	GCTCTTCTGGAATGGCTGGAGAAACACGAGACGA	RNAi of Ce d16
Ptd16B2-PstI	ttatcctgcagACAAACTGATAGTACCAGTTAAATA	Site specific mutagenesis
Ptd16 A2-PstI	ttatcctgcagCATTGCACGCACACACGTATATTTGA	Site specific mutagenesis
Ptd16 A3-Pst	ttatcCTGCAGGTGAAAGGAGAGAATTATTATATTACCCTTT	Site specific mutagenesis
Ptd16B3-Pst	ttatcctgcagTTGATTATATACTATATTTATTTTTGTAT	Site specific mutagenesis
Ptd16 A4-HA	GGCGTAATCGGGCACATCGTAGGGGTACATCTTTATCGATAATTTTTAAAAAAATA	Site specific mutagenesis
Ptd16B4-HA	GGCGTAATCGGGCACATCGTAGGGGTACATCTTTTCTTTCCTTTATTTTTTCCAAA	Site specific mutagenesis
Ptd16 A5-HA	ATGTACCCCTACGATGTGCCCGATTACGCCATGAGTGGATTACCATACTCTCAACAATACA	Site specific mutagenesis
Ptd16B5-HA	ATGTACCCCTACGATGTGCCCGATTACGCCATGAATGGTTCATTATTATCAGATGACTTTCA	Site specific mutagenesis
Ptd16 A6	GTTCCTCTTCTTAAAGGCCATGCATTACATCTATCT	Site specific mutagenesis
Pt16 B6	CCAATGCCTCATGTTATATCACCTATAAATGA	Site specific mutagenesis
Ptd16 A7	GGCCTTTAAGAAGAGGAACCTTTGACCA	Site specific mutagenesis
Ptd16 B7	TGATATAACATGAGGCATTGGCCAGGCATAACAACGTCCA	Site specific mutagenesis
Ptd16 BA8	ATAGAACTTTTGGTTGTATCAATTGCATTACCTCTATCACGTT	Site specific mutagenesis
Ptd16 BA9	TTGATACAACCAAAAGTTCTATTGATAAAAAG	Site specific mutagenesis
Ptd16 BA10	ACATTTCCCTGGACACTTAAATTAGCTTCAGTACGTTGA	Site specific mutagenesis
Ptd16 BA11	TTAAGTGTCCAGGGAAATGTTAATGGTGT	Site specific mutagenesis
Ptd16BA12- NotI	atcttgcggccgcTGTTGCTGCTATCAATATATGGGAATGA	Site specific mutagenesis
Ptd16BA13- NotI	atcttgcggccgcGGGAATGAGTATATATATGTATGTAT	Site specific mutagenesis
Ptd16BA14	CAATATCAAAGTTTTCAAAAATATCTT	Site specific mutagenesis
pCF107 seq-1	GTCCACACAATCTGCCCTTT	Sequencing Vector
pCF107 seq-2	CATTCAGGCTACGCAACTGTT	Sequencing Vector
pSTBlue Fwd	ATGAGTATTCAACATTTCCGTGTCG	Sequencing Vector
pSTBlue Rev	TTACCAATGCTTAATCAGTGAGGCA	Sequencing Vector

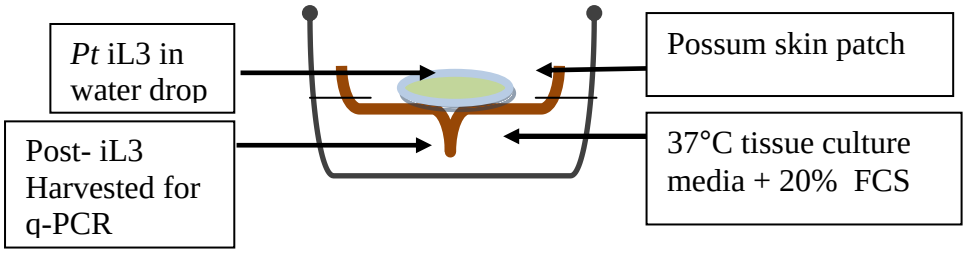
FirelacZ rev	GCCAGGGTTTTCCCAGTCACGA	Sequencing Vector
Firegfp rev	CGTGTCTTGTAGTTCCCGTCAT	Sequencing Vector
pSTBlue SP6 outer	GCAAGGCGATTAAGTTGGGTA	Sequencing Vector
pSTBlue T7 outer	CCATGATTACGCCAAGCTCTAA	Sequencing Vector
SL1 primer	GTTTAATTACCCAAGTTTGAG	Splice leader primer
SL2 primer	GGTTTTAACCCAGTTACTCAAG	Splice leader primer
Histone fwd P1	CACCCTTTACAGTCATTAATATATAATTTA	Q-PCR validation
Histone rev P2	GTAACAAAATTAAAAACTCTGGTG	Q-PCR validation
tre1016e fwd	ATGCGCTTTAACATCTATTGCAC	Q-PCR validation
tre1016e rev	GGAGGTTGTGATCTATTAAGAT	Q-PCR validation
Pt gap3dh fwd	TGAGGACCAAGATGGGTGAC	Q-PCR ref. gene
Pt gap3dh rev	ACGACGAGAGAAACTATGAGAC	Q-PCR ref. gene

APPENDIX 4: L1+CM and Post iL3 developmental samples for q-PCR.

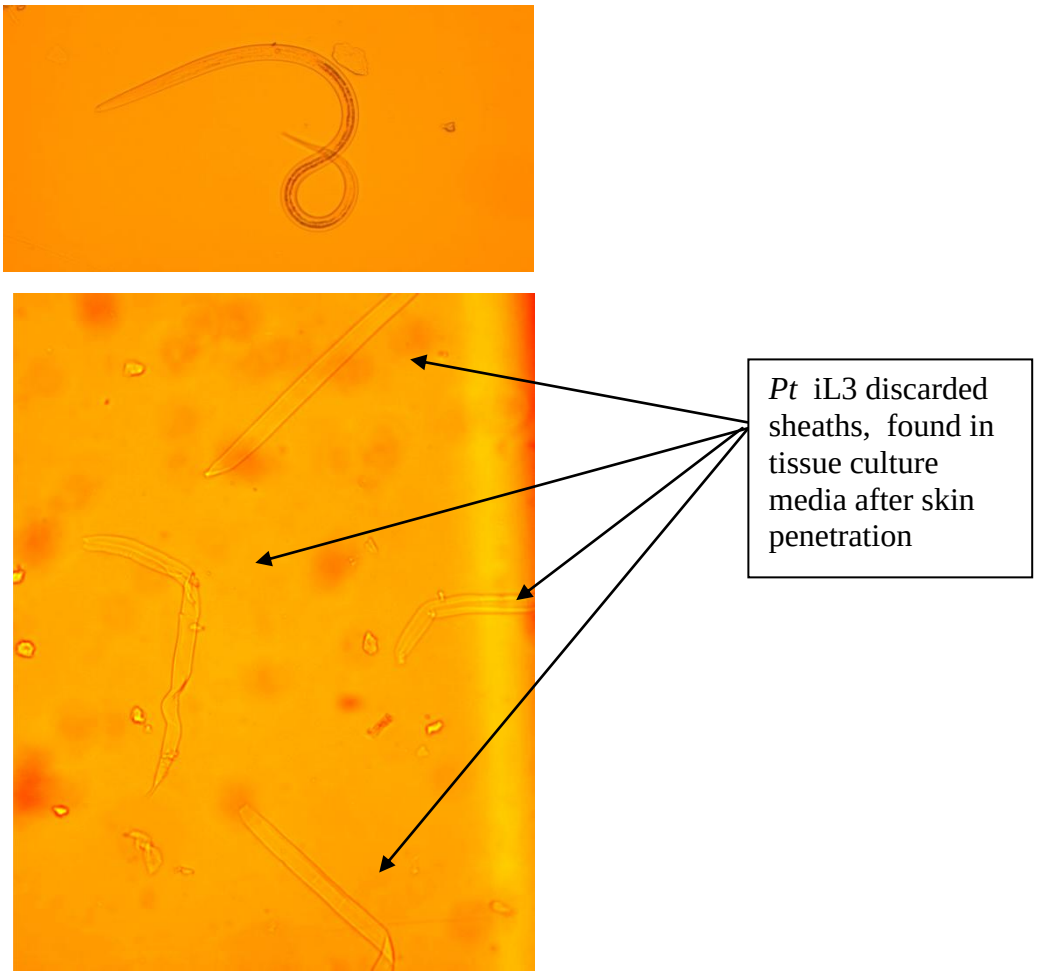
To confirm that the L1+CM larvae were progressing along the developmental path to becoming infective larva, an aliquot of the culture was allowed to develop fully rather than being processed for total RNA and >90% developed full to infective larva after 84 hours.

A. Set up of apparatus for collecting post-iL3 diagramed below. B. Pictures of *P. trichosuri* infective larva harvested after skin penetration. Note the discarded sheaths from the bottom reservoir of apparatus suggesting a possible resumption of development.

A. Set up of apparatus for collecting post-iL3

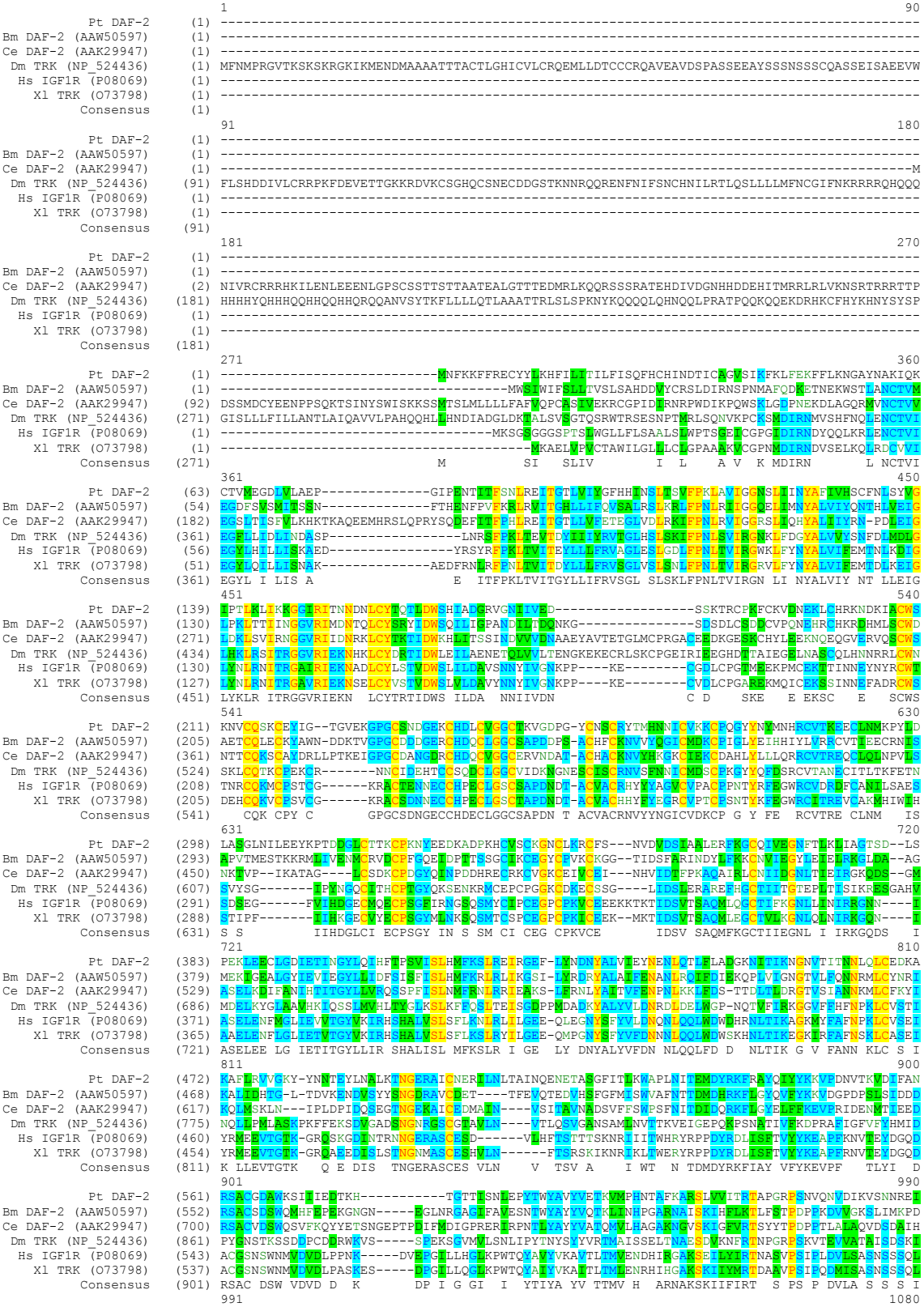


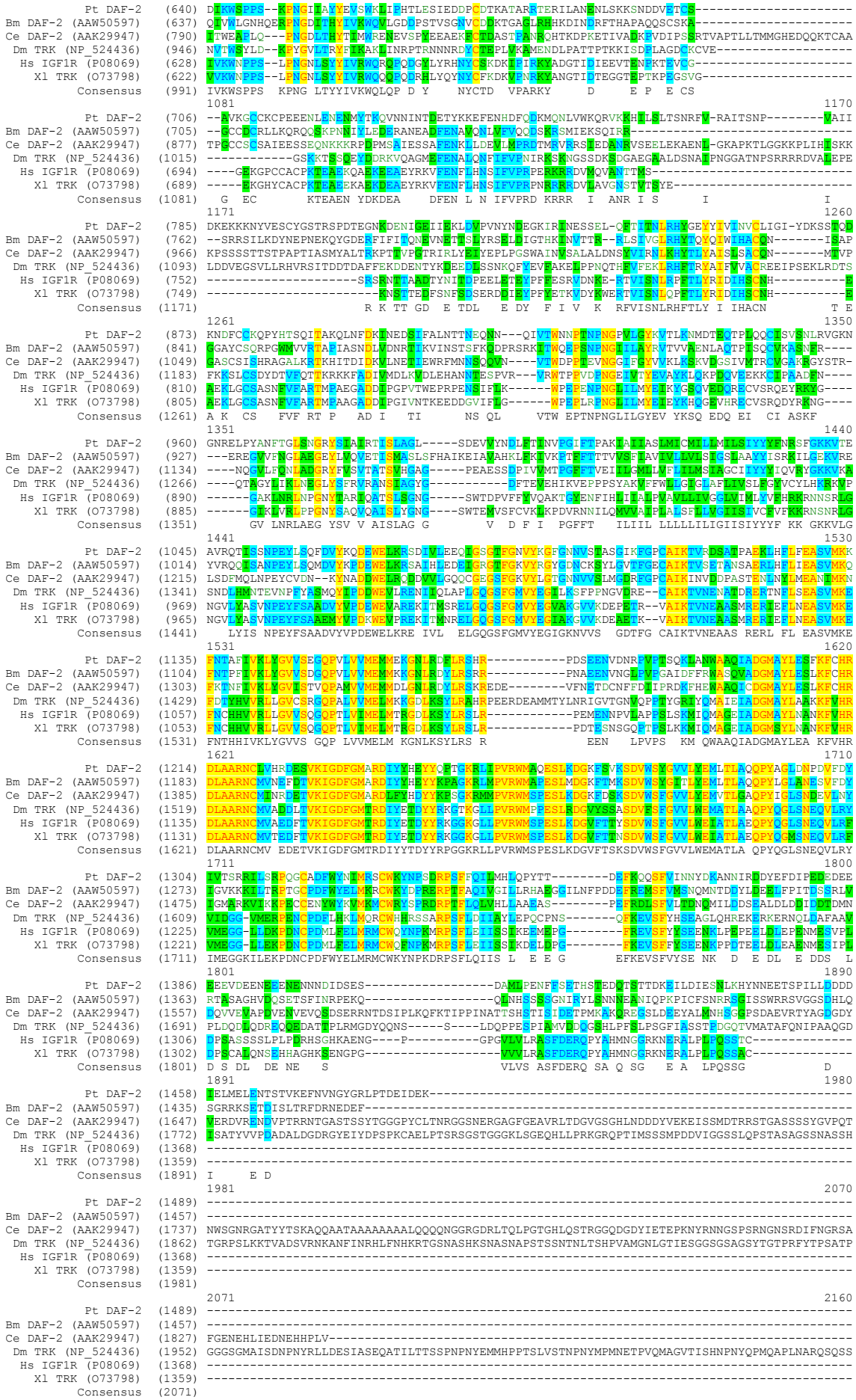
B. Pictures of post skin penetration *P. trichosuri* iL3 collected from reservoir of 37°C tissue culture media (RPMI) + 20% FCS.



APPENDIX 5 : Alignment of translated sequence of *P. trichosuri* DAF-2

P. trichosuri (Pt); *B. malayi*, (Bm); *C. elegans* (Ce); *D. melanogaster*, (Dm); *H. sapiens* (Hs); and *X. laevis* (Xl).





```
                          2161                                                                                2250
           Pt DAF-2  (1489) ----------------------------------------------------------------------------------------
Bm DAF-2 (AAW50597)  (1457) ----------------------------------------------------------------------------------------
Ce DAF-2 (AAK29947)  (1844) ----------------------------------------------------------------------------------------
 Dm TRK (NP_524436)  (2042) SDEDNEQEEDDEDEDDDVDDEHVEHIKMERMPLSRPRQRALPSKTQPPRSRSVSQTRKSPTNPNSGIGATGAGNRSNLLKENWLRPASTP
  Hs IGF1R (P08069)  (1368) ----------------------------------------------------------------------------------------
    Xl TRK (O73798)  (1359) ----------------------------------------------------------------------------------------
          Consensus  (2161)
                          2251     2263
           Pt DAF-2  (1489) -------------
Bm DAF-2 (AAW50597)  (1457) -------------
Ce DAF-2 (AAK29947)  (1844) -------------
 Dm TRK (NP_524436)  (2132) RPPPPNGFIGREA
  Hs IGF1R (P08069)  (1368) -------------
    Xl TRK (O73798)  (1359) -------------
          Consensus  (2251)
```

APPENDIX 6: FUNCTIONAL ANALYSIS IN P. trichosuri and performed by other members of the Molecular Parasitology team.

Table Appendix 6.i q-PCR analysis of RNAi gene knockdown in *P. trichosuri*

Test hsp-70	Δ ct of hsp-70 RNAi (Normalized to *act-1*)	Δ ct of negative (exogenous gene) RNAi control (Normalized to *act-1*)	Change in gene expression by RNAi	
hsp-70 long/soaked	-6.9	-7.4	up	
hsp-70 diced/soaked	-6.5	-1.5	down	by 5 Ct units
hsp-70 long/electro	-4.5	no result	no result	
hsp-70 diced/electro	-5.1	no result	no result	
hsp-70 feeding	no result	-2.2	no result	
hsp-70 long/soaked	-4.5	-5.6	up	
hsp-70 diced/soaked	-5.5	-3.6	down	by 1.9 Ct units
hsp-70 long/electro	-4	-3.2	slightly down	
hsp-70 diced/electro	-3.8	-2.8	slightly down	
hsp-70 feeding	-2	-1	slightly down	

Chemical mutagenesis EMS/poison primers

In order to determine gene function in *P. trichosuri*, knock out of genes by chemical mutagenesis with ethyl methanesulfonate (EMS) was performed. Mutagenized worms were screened by poison primers PCR to detect rare deletion products within large numbers of wild-type products, as described in Methods (Section 2.13.1 and Section 2.13.2) (EDGLEY *et al.* 2002). Screening of chemically mutagenized *P. trichosuri* for deletions in the *Pt daf-2*, *Pt age-1* or *Pt daf-16* genes was performed on approximately 1.40 x10^5 F_1 worms. Mutagenesis and worm culturing was performed by Matt Crook and poison primer screening was performed by Marlene Richter and Tina Englbrecht. Initial poison primer screening resulted in several putative deletion mutants (Figure 1). However, re-screening by PCR for conformation of the putative deletion mutant bands were unsuccessful, suggesting the original deletion bands may have been PCR artefacts.

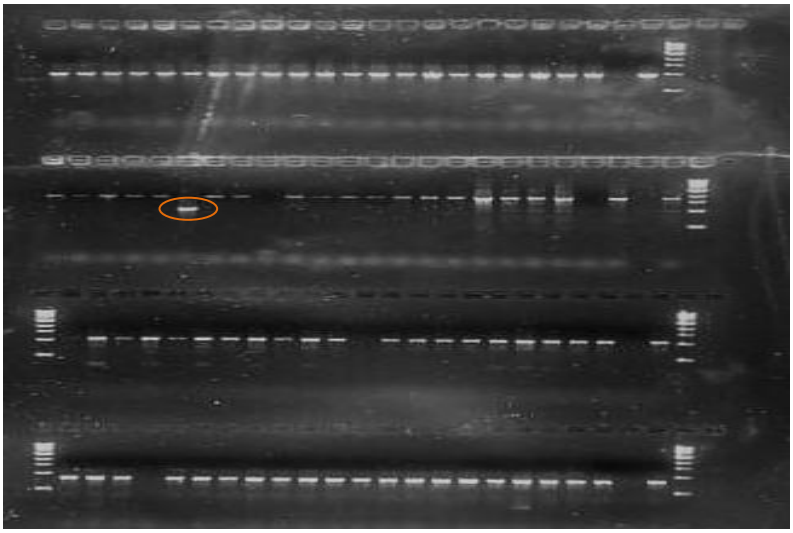


Figure Appendix 6.i : Chemical mutagenesis of *P. trichosuri*, poison primer PCR screen of F_1 generation worms, pooled across columns and rows of a 96 well plate.
P. trichosuri L4/young Adults were treated with [30mM] EMS mutagen, the F_1 generation eggs were cultured in 96 well agar plates at 20°C for 2 days in order to lay F_2 generation eggs, the F_1 generations adults were washed off plate and lysed overnight as described previously, 5 µL of the lysate was pooled across rows and columns and used as template in poison primer-PCR shown above, a potential Pt daf-16 deletion mutant is circled in red. Primers: Pt daf-16MutD: GGCCAATGCCTCATGTTATAT Pt daf-16MutE: GATTGGGCACCACAACTTAACA

Soaking and electroporation of *P. trichosuri* L1 in dsRNA.

As RNAi by feeding did not result in an obvious phenotype, it was decided that a more quantitative approach would be attempted in order to assess whether knockdown in gene expression occurred after RNAi treatment. This was done using quantitative Real Time PCR methods (q-PCR). Five methods of dsRNA delivery were tested, these were: feeding *P. trichosuri* larvae bacteria expressing dsRNA, soaking *P. trichosuri* L1 larva in *in vitro* transcribed dsRNA, soaking the L1 larva in *in vitro* transcribed dsRNA which was diced to 21-23 bp, electroporating *P. trichosuri* L1 larva with *in vitro* transcribed dsRNA and electroporating L1 larva with *in vitro* transcribed dsRNA diced to 21-23 bp. In order to test gene knock down by q-PCR, a gene fragment for *in vitro* transcription isa required as are q-PCR primers, which were designed across an intron. The gene, *Pt hsp-70* was the only resource available at the time that fit these criteria; *Pt hsp-70* was therefore the gene candidate chosen for this experiment (NEWTON-HOWES *et al.* 2006). A 637 bp gene fragment of *Pt hsp-70* and q-PCR primers were supplied by Jan Newton-Howes, and she also performed the q-PCR and analysis. *In vitro* transcription of

dsRNA, RNAi treatment of worms and q-PCR template preparation were performed by me. See appendix 5 for data.

An initial q-PCR was performed with technical difficulties and repeated. When repeated, one treatment resulted in down regulation of gene expression (soaking of diced dsRNA). This was down regulated by 5 Ct units in the first experiment and 1.9 Ct units in the second experiment, suggesting that there might be a knock down in expression with *P. trichosuri* soaked in diced dsRNA.

Microinjection

In order to determine if the RNAi pathway exists in *P. trichosuri, in vitro* transcribed dsRNA of the RNA polymerase II gene orthologue (*Pt ama-1*) was injected into the gonads of adult females: if the RNAi pathway is functional in *P. trichosuri*, this should result in an embryonic lethal phenotype. This is considered the best candidate and delivery method to establish whether RNAi functions in an organism (Hunter C., pers comm. (ABOOBAKER and BLAXTER 2003)). *C. elegans ama-1* encodes the large subunit of RNA polymerase II and is required for embryonic development (BIRD and RIDDLE 1989). A 1.2 kbp fragment of *C. elegans ama*-1 gene was also cloned for *in vitro* transcription and microinjection into *C. elegans* as proof of technique and for microinjection into *P. trichosuri* as an exogenous negative control.

In *C. elegans* knockdown of *rrf*-3 gene, which is an RNA directed RNA polymerase (RdRP), increases the strength of RNAi phenotypes (SIMMER *et al.* 2002). It is thought that RRF-3 and the RdRPs of the RNAi pathway compete for components and subunits, so that when RRF-3 is knocked down the RdRPs of the RNAi pathway become more efficient (GRISHOK 2005). The putative *Pt ama-1* and *Pt rrf*-3 orthologues were cloned from *P. trichosuri* cDNA with degenerate primers designed on the basis of nematode/fly/mouse protein alignments, and the resulting RT-PCR fragments were cloned into the Neo^R double T7 plasmid: pGW2T7L4440 (a Gateway derivative of pL4440; Appendix 1.15). The sequences were analysed using BLASTx to confirm similarity to the *C. elegans* gene.

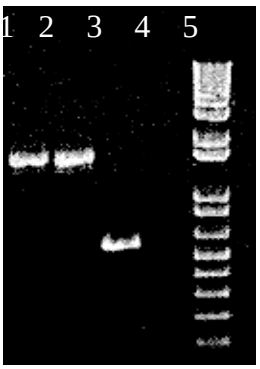


Figure Appendix 6.ii: Gene fragments used for *in vitro* transcribed RNAi
Lane 1: *C. elegans ama*-1 gene fragment; Lane 2: *P. trichosuri ama-I* putative orthologue to *ama-1;* Lane 3: *P. trichosuri rrf-3* gene orthologue fragment; Lane 4: No template control, lane 5: 1KB Plus MW Marker. Primers: Ce ama-1 fwd: GAGG AGTCGCAATGCGTACAATGCGGATTTCGATG Ce ama-1 rev: GCTCTTCTGGAATGCCCAGTCTCGGCAGTCTTTACA Pt ama-1 fwd: GAGGAGTCGCAATGGATTTTGAT GGAGAYGARATGAAY Pt ama-1 rev: GCTCTT CTGGAATGCTGAATATATCCWGTTTCNGCNGT Pt rrf-3 fwd: GCTCCATGATCACGCATTTGAGAGTT Pt rrf-3 rev: GTTTACCATCATAACTTCTGGAGCCATA

In vitro transcription and MegaClear™ clean up of dsRNA products were as described in the Methods Section 2.11.3 (Figure 2). Microinjection of *C. elegans* and *P. trichosuri* was performed as described in the Methods section 2.11.6. Kirsten Grant performed *C. elegans* and *P. trichosuri* microinjections and the larvae counts for this experiment.

Table Appendix 6.ii: Summary of *P. trichosuri* RNAi by microinjection

Worm / number	**dsRNA injected**	**F_1 on 12 hr Injection plate**	**F_1 on 24 hr Injection plate**	**F_1 on 36 hr Transfer plate**	**F_1 on 48 hr Transfer plate**
C. elegans n=17	*Ce ama-1*	47 larvae 278 eggs	39 larvae 235 eggs	1 larvae 365 eggs	0 larvae 365 eggs
C. elegans n=10	Neg control	TNTC 72 eggs	394 larvae 441 eggs	394 larvae 441 eggs	TNTC 34 eggs
C. elegans n= 18	*Pt ama-1* (exogenous control)	365 larvae 24 eggs	318 larvae 472 eggs	318 larvae 472 eggs	TNTC 41 eggs
Worm / number	**dsRNA injected**	**F_1 on 12 hr Injection plate**	**F_1 on 24 hr Injection plate**	**F_1 on 36 hr Transfer plate**	
P. trichosuri n=4*	*Pt ama-1*	77 larvae 30 eggs	17 larvae 10 eggs	23 larvae 13 eggs	
P. trichosuri n=4*	*Ce ama-1* (exogenous control)	56 larvae 23 eggs	40 larvae 5 eggs	32 larvae 13 eggs	
P. trichosuri n=4*	*Pt ama-1/Pt rrf-3*	112 larvae 10 eggs	10 larvae 3 eggs	0 larvae 0 eggs	
P. trichosuri n=4*	Neg control	173 larvae 104 eggs	100 larvae 4 eggs	110 larvae 104 eggs	

TNTC= Too numerous to count.
* 10 -12 worms were injected but 4 worms survived the microinjection process

Microinjected worms were plated individually, after 12 hours these were transferred to new 'transfer plates'. The F_1 larvae on the injection plates were counted and removed at 12 hours and any newly hatched larvae were counted at 24 hours. On the transfer plates, F_1 larvae were counted and removed, along with the microinjected parent, at 36 hours. Plates were re-incubated and any newly hatched larvae were counted at 48 hours. Microinjection of *C. elegans* with *Ce ama-1 in vitro* transcribed dsRNA resulted in large numbers of embryonic deaths as expected, this is illustrated by the number of eggs that remained unhatched on the 48 hour plate (Table 2 - highlighted in green). Both the un-injected *C. elegans* (negative control) and *C. elegans* injected with *Pt. ama-1* dsRNA (exogenous negative control) had comparable numbers of hatching larvae. Un-injected *P. trichosuri* (negative control), produced eggs over the entire 36 hour period. Microinjection of *P. trichosuri* resulted in reduced fecundity as shown by the microinjection of *P. trichosuri* with *Ce ama-1* (exogenous control). *P. trichosuri* injected with *Pt ama-1* alone also resulted in reduced numbers of larva but comparable to the exogenous control. Interestingly, the worms co-microinjected with both *Pt ama-1/Pt rrf-3* stopped laying eggs by 36 hours (Table 2 – highlighted in light orange).

REFERENCES

1995 *Methods in Cell Biology*. Academic Press, San Diego.

ABOOBAKER, A. A., and M. L. BLAXTER, 2003 Use of RNA interference to investigate gene function in the human filarial nematode parasite Brugia malayi. Mol Biochem Parasitol **129:** 41-51.

ALCEDO, J., and C. KENYON, 2004 Regulation of C. elegans longevity by specific gustatory and olfactory neurons. Neuron **41:** 45-55.

ALTSCHUL, S. F., W. GISH, W. MILLER, E. W. MYERS and D. J. LIPMAN, 1990 Basic local alignment search tool. J Mol Biol **215:** 403-410.

AN, Y., J. JI, W. WU, A. LV, R. HUANG *et al.*, 2005 A rapid and efficient method for multiple-site mutagenesis with a modified overlap extension PCR. Appl Microbiol Biotechnol **68:** 774-778.

ANTEBI, A., J. G. CULOTTI and E. M. HEDGECOCK, 1998 daf-12 regulates developmental age and the dauer alternative in Caenorhabditis elegans. Development **125:** 1191-1205.

ANTEBI, A., W. H. YEH, D. TAIT, E. M. HEDGECOCK and D. L. RIDDLE, 2000 daf-12 encodes a nuclear receptor that regulates the dauer diapause and developmental age in C. elegans. Genes Dev **14:** 1512-1527.

APFELD, J., and C. KENYON, 1998 Cell nonautonomy of C elegans daf-2 function in the regulation of diapause and life span. Cell **95:** 199-210.

APFELD, J., and C. KENYON, 1999 Regulation of lifespan by sensory perception in Caenorhabditis elegans. Nature **402:** 804-809.

ARIZONO, N., 1976a Studies on the free-living generations of *Strongyloides planiceps* Rogers, 1943. I. Effects of quantitiy of food and population density on the developmental types. Japaneses Journal of Parasitology **25:** 274-282.

ARIZONO, N., 1976b Studies on the free-living generations of *Strongyloides planiceps*, 1943 II. Effect of temperature on the developmental types. Japaneses Journal of Parasitology **25:** 328-335.

ASHTON, F. T., V. M. BHOPALE, D. HOLT, G. SMITH and G. A. SCHAD, 1998 Developmental switching in the parasitic nematode Strongyloides stercoralis is controlled by the ASF and ASI amphidial neurons. J Parasitol **84:** 691-695.

ASHTON, F. T., and G. A. SCHAD, 1996 Amphids in strongyloides stercoralis and other parasitic nematodes. Parasitol Today **12:** 187-194.

BABAR, P., C. ADAMSON, G. A. WALKER, D. W. WALKER and G. J. LITHGOW, 1999 P13-kinase inhibition induces dauer formation, thermotolerance and longevity in C. elegans. Neurobiol Aging **20:** 513-519.

BARBIERI, M., M. BONAFE, C. FRANCESCHI and G. PAOLISSO, 2003 Insulin/IGF-I-signaling pathway: an evolutionarily conserved mechanism of longevity from yeast to humans. Am J Physiol Endocrinol Metab **285:** E1064-1071.

BARGMANN, C. I., and H. R. HORVITZ, 1991a Chemosensory neurons with overlapping functions direct chemotaxis to multiple chemicals in C. elegans. Neuron **7:** 729-742.

BARGMANN, C. I., and H. R. HORVITZ, 1991b Control of larval development by chemosensory neurons in Caenorhabditis elegans. Science **251:** 1243-1246.

BARSYTE, D., D. A. LOVEJOY and G. J. LITHGOW, 2001 Longevity and heavy metal resistance in daf-2 and age-1 long-lived mutants of Caenorhabditis elegans. Faseb J **15:** 627-634.

BARTHELEMY, C., C. E. HENDERSON and B. PETTMANN, 2004 Foxo3a induces motoneuron death through the Fas pathway in cooperation with JNK. BMC Neurosci **5:** 48.

BAUMEISTER, R., E. SCHAFFITZEL and M. HERTWECK, 2006 Endocrine signaling in Caenorhabditis elegans controls stress response and longevity. J Endocrinol **190:** 191-202.

BEALL, M. J., and E. J. PEARCE, 2002 Transforming growth factor-beta and insulin-like signalling pathways in parasitic helminths. Int J Parasitol **32:** 399-404.

BELLINO, F. L., 2006 Advances in endocrinology of aging research, 2005-2006. Exp Gerontol **41:** 1228-1233.

BERDICHEVSKY, A., M. VISWANATHAN, H. R. HORVITZ and L. GUARENTE, 2006 C. elegans SIR-2.1 interacts with 14-3-3 proteins to activate DAF-16 and extend life span. Cell **125:** 1165-1177.

BERNSTEIN, E., A. A. CAUDY, S. M. HAMMOND and G. J. HANNON, 2001 Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature **409:** 363-366.

BIGGS, W. H., 3RD, J. MEISENHELDER, T. HUNTER, W. K. CAVENEE and K. C. ARDEN, 1999 Protein kinase B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor FKHR1. Proc Natl Acad Sci U S A **96:** 7421-7426.

BIRD, D. M., C. H. OPPERMAN, S. J. JONES and D. L. BAILLIE, 1999 THE CAENORHABDITIS ELEGANS GENOME: A Guide in The Post Genomics Age. Annu Rev Phytopathol **37:** 247-265.

BIRD, D. M., and D. L. RIDDLE, 1989 Molecular cloning and sequencing of ama-1, the gene encoding the largest subunit of Caenorhabditis elegans RNA polymerase II. Mol Cell Biol **9:** 4119-4130.

BLAXTER, M., 1998 Caenorhabditis elegans is a nematode. Science **282:** 2041-2046.

BLAXTER, M. L., P. DE LEY, J. R. GAREY, L. X. LIU, P. SCHELDEMAN *et al.*, 1998 A molecular evolutionary framework for the phylum Nematoda. Nature **392:** 71-75.

BLUMENTHAL, T., 2005 Trans-splicing and operons, pp. The C. elegans Research Community in *WormBook*, edited by L. GIRARD.

BOYER, H. W., and D. ROULLAND-DUSSOIX, 1969 A complementation analysis of the restriction and modification of DNA in Escherichia coli. J Mol Biol **41:** 459-472.

BRAECKMAN, B. P., K. HOUTHOOFD and J. R. VANFLETEREN, 2001 Insulin-like signaling, metabolism, stress resistance and aging in Caenorhabditis elegans. Mech Ageing Dev **122:** 673-693.

BRAND, A., and J. M. HAWDON, 2004 Phosphoinositide-3-OH-kinase inhibitor LY294002 prevents activation of Ancylostoma caninum and Ancylostoma ceylanicum third-stage infective larvae. Int J Parasitol **34:** 909-914.

BRAND, A. M., G. VARGHESE, W. MAJEWSKI and J. M. HAWDON, 2005 Identification of a DAF-7 ortholog from the hookworm Ancylostoma caninum. Int J Parasitol **35:** 1489-1498.

BRENNER, S., 1974 The genetics of Caenorhabditis elegans. Genetics **77:** 71-94.

BRITTON, C., and L. MURRAY, 2006 Using Caenorhabditis elegans for functional analysis of genes of parasitic nematodes. Int J Parasitol **36:** 651-659.

BRITTON, C., D. L. REDMOND, D. P. KNOX, J. H. MCKERROW and J. D. BARRY, 1999 Identification of promoter elements of parasite nematode genes in transgenic Caenorhabditis elegans. Mol Biochem Parasitol **103:** 171-181.

BROOKS, D. R., and R. E. ISAAC, 2002 Functional genomics of parasitic worms: the dawn of a new era. Parasitol Int **51:** 319-325.

BURGLIN, T. R., E. LOBOS and M. L. BLAXTER, 1998 Caenorhabditis elegans as a model for parasitic nematodes. Int J Parasitol **28:** 395-411.

BUTCHER, R. A., M. FUJITA, F. C. SCHROEDER and J. CLARDY, 2007 Small-molecule pheromones that control dauer development in Caenorhabditis elegans. Nat Chem Biol **3:** 420-422.

CASADABAN, M. J., and S. N. COHEN, 1980 Analysis of gene control signals by DNA fusion and cloning in Escherichia coli. J Mol Biol **138:** 179-207.

CASSADA, R. C., and R. L. RUSSELL, 1975 The dauerlarva, a post-embryonic developmental variant of the nematode Caenorhabditis elegans. Dev Biol **46:** 326-342.

CASTELLETTO, M. L., H. C. MASSEY, JR. and J. B. LOK, 2009 Morphogenesis of Strongyloides stercoralis infective larvae requires the DAF-16 ortholog FKTF-1. PLoS Pathog **5:** e1000370.

CHO, S., S. W. JIN, A. COHEN and R. E. ELLIS, 2004 A phylogeny of caenorhabditis reveals frequent loss of introns during nematode evolution. Genome Res **14:** 1207-1220.

COWAN, P. E., M. J. RALSTON, D. D. HEATH and W. N. GRANT, 2006 Infection of naive, free-living brushtail possums (Trichosurus vulpecula) with the nematode parasite Parastrongyloides trichosuri and its subsequent spread. Int J Parasitol **36:** 287-293.

CROLL, N. A., 1972 Feeding and lipid synthesis of Ancylostoma tubaeforme preinfective larvae. Parasitology **64:** 369-378.

CROOK, M., F. J. THOMPSON, W. N. GRANT and M. E. VINEY, 2005 daf-7 and the development of Strongyloides ratti and Parastrongyloides trichosuri. Mol Biochem Parasitol **139:** 213-223.

DIETERICH, C., S. W. CLIFTON, L. N. SCHUSTER, A. CHINWALLA, K. DELEHAUNTY *et al.*, 2008 The Pristionchus pacificus genome provides a unique perspective on nematode lifestyle and parasitism. Nat Genet **40:** 1193-1198.

DILLIN, A., D. K. CRAWFORD and C. KENYON, 2002 Timing requirements for insulin/IGF-1 signaling in C. elegans. Science **298:** 830-834.

DORRIS, M., M. E. VINEY and M. L. BLAXTER, 2002 Molecular phylogenetic analysis of the genus Strongyloides and related nematodes. Int J Parasitol **32:** 1507-1517.

EDGLEY, M., A. D'SOUZA, G. MOULDER, S. MCKAY, B. SHEN *et al.*, 2002 Improved detection of small deletions in complex pools of DNA. Nucleic Acids Res **30:** e52.

FANELLI, E., M. DI VITO, J. T. JONES and C. DE GIORGI, 2005 Analysis of chitin synthase function in a plant parasitic nematode, Meloidogyne artiellia, using RNAi. Gene **349:** 87-95.

FEINBERG, E. H., and C. P. HUNTER, 2003 Transport of dsRNA into cells by the transmembrane protein SID-1. Science **301:** 1545-1547.

FELSENSTEIN, 2005 PHYLIP (Phylogeny Inference Package) version 3.6, pp. Phylogeny Inference Package, edited by F. J, Seatle.

FENG, X. P., J. HAYASHI, R. N. BEECH and R. K. PRICHARD, 2002 Study of the nematode putative GABA type-A receptor subunits: evidence for modulation by ivermectin. J Neurochem **83:** 870-878.

FENTON, A. A. H. P. J., 2002 Optimal infection strategies: should macroparasites hedge their bets? Oikos **96:** 92-101.

FIELENBACH, N., and A. ANTEBI, 2008 C. elegans dauer formation and the molecular basis of plasticity. Genes Dev **22:** 2149-2165.

FIRE, A., S. XU, M. K. MONTGOMERY, S. A. KOSTAS, S. E. DRIVER *et al.*, 1998 Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature **391:** 806-811.

FREITAS, T. C., and P. ARASU, 2005 Cloning and characterisation of genes encoding two transforming growth factor-beta-like ligands from the hookworm, Ancylostoma caninum. Int J Parasitol **35:** 1477-1487.

GAO, X., D. FRANK and J. M. HAWDON, 2009 Molecular cloning and DNA binding characterization of DAF-16 orthologs from Ancylostoma hookworms. Int J Parasitol **39:** 407-415.

GARDNER, M. P., D. GEMS and M. E. VINEY, 2004 Aging in a very short-lived nematode. Exp Gerontol **39:** 1267-1276.

GARDNER, M. P., D. GEMS and M. E. VINEY, 2006 Extraordinary plasticity in aging in Strongyloides ratti implies a gene-regulatory mechanism of lifespan evolution. Aging Cell **5:** 315-323.

GEARY, T. G., and D. P. THOMPSON, 2001 Caenorhabditis elegans: how good a model for veterinary parasites? Vet Parasitol **101:** 371-386.

GELDHOF, P., L. MURRAY, A. COUTHIER, J. S. GILLEARD, G. MCLAUCHLAN *et al.*, 2006 Testing the efficacy of RNA interference in Haemonchus contortus. Int J Parasitol **36:** 801-810.

GELDHOF, P., A. VISSER, D. CLARK, G. SAUNDERS, C. BRITTON *et al.*, 2007 RNA interference in parasitic helminths: current situation, potential pitfalls and future prospects. Parasitology **134:** 609-619.

GEMS, D., A. J. SUTTON, M. L. SUNDERMEYER, P. S. ALBERT, K. V. KING *et al.*, 1998 Two pleiotropic classes of daf-2 mutation affect larval arrest, adult behavior, reproduction and longevity in Caenorhabditis elegans. Genetics **150:** 129-155.

GENGYO-ANDO, K., and S. MITANI, 2000 Characterization of mutations induced by ethyl methanesulfonate, UV, and trimethylpsoralen in the nematode Caenorhabditis elegans. Biochem Biophys Res Commun **269:** 64-69.

GERISCH, B., C. WEITZEL, C. KOBER-EISERMANN, V. ROTTIERS and A. ANTEBI, 2001 A hormonal signaling pathway influencing C. elegans metabolism, reproductive development, and life span. Dev Cell **1:** 841-851.

GOLDEN, J. W., and D. L. RIDDLE, 1982 A pheromone influences larval development in the nematode Caenorhabditis elegans. Science **218:** 578-580.

GOLDEN, J. W., and D. L. RIDDLE, 1984a A *Caenorhabditis elegans* dauer-inducing pheromone and an antagonistic component of the food supply. Journal of Chemical Ecology **Vol. 10:** 1265- 1280.

GOLDEN, J. W., and D. L. RIDDLE, 1984b The Caenorhabditis elegans dauer larva: developmental effects of pheromone, food, and temperature. Dev Biol **102:** 368-378.

GOLDEN, J. W., and D. L. RIDDLE, 1984c A pheromone-induced developmental switch in Caenorhabditis elegans: Temperature-sensitive mutants reveal a wild-type temperature-dependent process. Proc Natl Acad Sci U S A **81:** 819-823.

GOLDEN, J. W., and D. L. RIDDLE, 1985 A gene affecting production of the Caenorhabditis elegans dauer-inducing pheromone. Mol Gen Genet **198:** 534-536.

GOTTLIEB, S., and G. RUVKUN, 1994 daf-2, daf-16 and daf-23: genetically interacting genes controlling Dauer formation in Caenorhabditis elegans. Genetics **137:** 107-120.

GRANT, S. G., J. JESSEE, F. R. BLOOM and D. HANAHAN, 1990 Differential plasmid rescue from transgenic mouse DNAs into Escherichia coli methylation-restriction mutants. Proc Natl Acad Sci U S A **87:** 4645-4649.

GRANT, W. N., 1992 Transformation of Caenorhabditis elegans with genes from parasitic nematodes. Parasitol Today **8:** 344-346.

GRANT, W. N., S. J. SKINNER, J. NEWTON-HOWES, K. GRANT, G. SHUTTLEWORTH *et al.*, 2006a Heritable transgenesis of Parastrongyloides trichosuri: a nematode parasite of mammals. Int J Parasitol **36:** 475-483.

GRANT, W. N., S. STASIUK, J. NEWTON-HOWES, M. RALSTON, S. A. BISSET *et al.*, 2006b Parastrongyloides trichosuri, a nematode parasite of mammals that is uniquely suited to genetic analysis. Int J Parasitol **36:** 453-466.

GRANT, W. N., and M. E. VINEY, 2001 Post-genomic nematode parasitology. Int J Parasitol **31:** 879-888.

GREENER, A., 1990 E. coli SURE tm strain: Clone 'unclonable' DNA, pp. 5-6 in *Strategies in molecular biology*.

GREENER, A. J. B., 1993 Strategene develops the Highest-efficiency chemically competent cells available, pp. 57 in *Strategegies in molecular biology*.

GRISHOK, A., 2005 RNAi mechanisms in Caenorhabditis elegans. FEBS Lett **579:** 5932-5939.

GUILIANO, D. B., and M. L. BLAXTER, 2006 Operon conservation and the evolution of trans-splicing in the phylum Nematoda. PLoS Genet **2:** e198.

GUO, S., and K. J. KEMPHUES, 1995 par-1, a gene required for establishing polarity in C. elegans embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. Cell **81:** 611-620.

HARVEY, S. C., G. L. BARKER, A. SHORTO and M. E. VINEY, 2009 Natural variation in gene expression in the early development of dauer larvae of Caenorhabditis elegans. BMC Genomics **10:** 325.

HARVEY, S. C., A. W. GEMMILL, A. F. READ and M. E. VINEY, 2000 The control of morph development in the parasitic nematode Strongyloides ratti. Proc R Soc Lond B Biol Sci **267:** 2057-2063.

HASHMI, S., P. LING, G. HASHMI, M. REED, R. GAUGLER *et al.*, 1995 Genetic transformation of nematodes using arrays of micromechanical piercing structures. Biotechniques **19:** 766-770.

HASHMI, S., W. TAWE and S. LUSTIGMAN, 2001 Caenorhabditis elegans and the study of gene function in parasites. Trends Parasitol **17:** 387-393.

HILL, R. L., JR., and E. L. ROBERSON, 1985 Differences in lipid granulation as the basis for a morphologic differentiation between third-stage larvae of Uncinaria stenocephala and Ancylostoma caninum. J Parasitol **71:** 745-750.

HONDA, Y., and S. HONDA, 1999 The daf-2 gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in Caenorhabditis elegans. Faseb J **13:** 1385-1393.

HOTEZ, P., J. HAWDON and G. A. SCHAD, 1993 Hookworm larval infectivity, arrest and amphiparatenesis: the Caenorhabditis elegans Daf-c paradigm. Parasitol Today **9:** 23-26.

HSIN, H., and C. KENYON, 1999 Signals from the reproductive system regulate the lifespan of C. elegans. Nature **399:** 362-366.

HSU, A. L., C. T. MURPHY and C. KENYON, 2003 Regulation of aging and age-related disease by DAF-16 and heat-shock factor. Science **300:** 1142-1145.

HU, M., J. B. LOK, N. RANJIT, H. C. MASSEY, JR., P. W. STERNBERG *et al.*, 2009 Structural and functional characterisation of the fork head transcription factor-

encoding gene, Hc-daf-16, from the parasitic nematode Haemonchus contortus (Strongylida). Int J Parasitol.

HUNTER, C. P., 2000 Gene silencing: shrinking the black box of RNAi. Curr Biol **10:** R137-140.

HUPPI, K., S. E. MARTIN and N. J. CAPLEN, 2005 Defining and assaying RNAi in mammalian cells. Mol Cell **17:** 1-10.

HUSSEIN, A. S., K. KICHENIN and M. E. SELKIRK, 2002 Suppression of secreted acetylcholinesterase expression in Nippostrongylus brasiliensis by RNA interference. Mol Biochem Parasitol **122:** 91-94.

HUTVAGNER, G., and P. D. ZAMORE, 2002 RNAi: nature abhors a double-strand. Curr Opin Genet Dev **12:** 225-232.

ISSA, Z., W. N. GRANT, S. STASIUK and C. B. SHOEMAKER, 2005 Development of methods for RNA interference in the sheep gastrointestinal parasite, Trichostrongylus colubriformis. Int J Parasitol **35:** 935-940.

JEE, C., L. VANOAICA, J. LEE, B. J. PARK and J. AHNN, 2005 Thioredoxin is related to life span regulation and oxidative stress response in Caenorhabditis elegans. Genes Cells **10:** 1203-1210.

JEFFARES, D. C., T. MOURIER and D. PENNY, 2006 The biology of intron gain and loss. Trends Genet **22:** 16-22.

JEONG, P. Y., M. JUNG, Y. H. YIM, H. KIM, M. PARK *et al.*, 2005 Chemical structure and biological activity of the Caenorhabditis elegans dauer-inducing pheromone. Nature **433:** 541-545.

JEONG, P. Y., M. S. KWON, H. J. JOO and Y. K. PAIK, 2009 Molecular time-course and the metabolic basis of entry into dauer in Caenorhabditis elegans. PLoS One **4:** e4162.

JIANG, M., J. RYU, M. KIRALY, K. DUKE, V. REINKE *et al.*, 2001 Genome-wide analysis of developmental and sex-regulated gene expression profiles in Caenorhabditis elegans. Proc Natl Acad Sci U S A **98:** 218-223.

JOHNSON, T. E., P. M. TEDESCO and G. J. LITHGOW, 1993 Comparing mutants, selective breeding, and transgenics in the dissection of aging processes of Caenorhabditis elegans. Genetica **91:** 65-77.

JORGENSEN, E. M., and S. E. MANGO, 2002 The art and design of genetic screens: caenorhabditis elegans. Nat Rev Genet **3:** 356-369.

JUNIO, A. B., X. LI, H. C. MASSEY, JR., T. J. NOLAN, S. TODD LAMITINA *et al.*, 2008 Strongyloides stercoralis: cell- and tissue-specific transgene expression and co-transformation with vector constructs incorporating a common multifunctional 3' UTR. Exp Parasitol **118:** 253-265.

KALINNA, B. H., and P. J. BRINDLEY, 2007 Manipulating the manipulators: advances in parasitic helminth transgenesis and RNAi. Trends Parasitol **23:** 197-204.

KAMPKOTTER, A., T. E. VOLKMANN, S. H. DE CASTRO, B. LEIERS, L. O. KLOTZ *et al.*, 2003 Functional analysis of the glutathione S-transferase 3 from Onchocerca volvulus (Ov-GST-3): a parasite GST confers increased resistance to oxidative stress in Caenorhabditis elegans. J Mol Biol **325:** 25-37.

KAWANO, T., N. KATAOKA, S. ABE, M. OHTANI, Y. HONDA *et al.*, 2005 Lifespan extending activity of substances secreted by the nematode Caenorhabditis elegans that include the dauer-inducing pheromone. Biosci Biotechnol Biochem **69:** 2479-2481.

KENNEDY, C. R., 1976 *Ecological Aspects of Parasitology*. North-Holland Publishing Company - Amsterdam, New York.

KIMURA, K. D., H. A. TISSENBAUM, Y. LIU and G. RUVKUN, 1997 daf-2, an insulin receptor-like gene that regulates longevity and diapause in Caenorhabditis elegans. Science **277:** 942-946.

KNOX, D. P., P. GELDHOF, A. VISSER and C. BRITTON, 2007 RNA interference in parasitic nematodes of animals: a reality check? Trends Parasitol **23:** 105-107.

KOTZE, A. C., and N. H. BAGNALL, 2006 RNA interference in Haemonchus contortus: suppression of beta-tubulin gene expression in L3, L4 and adult worms in vitro. Mol Biochem Parasitol **145:** 101-110.

KRAMER, J. M., J. T. DAVIDGE, J. M. LOCKYER and B. E. STAVELEY, 2003 Expression of Drosophila FOXO regulates growth and can phenocopy starvation. BMC Dev Biol **3:** 5.

KWA, M. S., J. G. VEENSTRA, M. VAN DIJK and M. H. ROOS, 1995 Beta-tubulin genes from the parasitic nematode Haemonchus contortus modulate drug resistance in Caenorhabditis elegans. J Mol Biol **246:** 500-510.

LAMITINA, S. T., and K. STRANGE, 2005 Transcriptional targets of DAF-16 insulin signaling pathway protect C. elegans from extreme hypertonic stress. Am J Physiol Cell Physiol **288:** C467-474.

LARSEN, P. L., P. S. ALBERT and D. L. RIDDLE, 1995 Genes that regulate both development and longevity in Caenorhabditis elegans. Genetics **139:** 1567-1583.

LEE, R. Y., J. HENCH and G. RUVKUN, 2001 Regulation of C. elegans DAF-16 and its human ortholog FKHRL1 by the daf-2 insulin-like signaling pathway. Curr Biol **11:** 1950-1957.

LEE, S. S., S. KENNEDY, A. C. TOLONEN and G. RUVKUN, 2003 DAF-16 target genes that control C. elegans life-span and metabolism. Science **300:** 644-647.

LI, W., S. G. KENNEDY and G. RUVKUN, 2003 daf-28 encodes a C. elegans insulin superfamily member that is regulated by environmental cues and acts in the DAF-2 signaling pathway. Genes Dev **17:** 844-858.

LI, X., H. C. MASSEY, JR., T. J. NOLAN, G. A. SCHAD, K. KRAUS *et al.*, 2006 Successful transgenesis of the parasitic nematode Strongyloides stercoralis requires endogenous non-coding control elements. Int J Parasitol **36:** 671-679.

LIBINA, N., J. BERMAN, R. and C. KENYON, 2003 Tissue-specific activities of C elegans DAF-16 in the regulation of lifespan. Cell **115:** 489-502.

LILLEY, C. J., M. BAKHETIA, W. L. CHARLTON and P. E. URWIN, 2007 Recent progress in the development of RNA interference for plant parasitic nematodes. Molecular Plant Pathology **8:** 701-711.

LIN, K., H. HSIN, N. LIBINA and C. KENYON, 2001 Regulation of the Caenorhabditis elegans longevity protein DAF-16 by insulin/IGF-1 and germline signaling. Nature genetics **28:** 139-145.

LIU, L. X., J. M. SPOERKE, E. L. MULLIGAN, J. CHEN, B. REARDON *et al.*, 1999 High-throughput isolation of Caenorhabditis elegans deletion mutants. Genome Res **9:** 859-867.

LIU, T., K. K. ZIMMERMAN and G. I. PATTERSON, 2004 Regulation of signaling genes by TGFbeta during entry into dauer diapause in C. elegans. BMC Dev Biol **4:** 11.

LONGO, V. D., and C. E. FINCH, 2003 Evolutionary medicine: from dwarf model systems to healthy centenarians? Science **299:** 1342-1346.

LUDEWIG, A. H., C. KOBER-EISERMANN, C. WEITZEL, A. BETHKE, K. NEUBERT *et al.*, 2004 A novel nuclear receptor/coregulator complex controls C. elegans lipid metabolism, larval development, and aging. Genes Dev **18:** 2120-2133.

LUSTIGMAN, S., J. ZHANG, J. LIU, Y. OKSOV and S. HASHMI, 2004 RNA interference targeting cathepsin L and Z-like cysteine proteases of Onchocerca volvulus

confirmed their essential function during L3 molting. Mol Biochem Parasitol **138:** 165-170.

MACKERRAS, M. J., 1959 Strongyloides and Parastrongyloides (Nematoda: Rhabdiasoidea) in Australian Marsupials. Aust. J. of Zoology **7:** 87-104.

MASSEY, H., C., M. NISHI, K. CHAUDHARY, N. PAKPOUR and J. LOK, B., 2003 Structure and developmental expression of Strongyloides stercoralis fktf-1, a proposed ortholog of daf-16 in Caenorhabditis elegans. International journal for parasitology **33:** 1537-1544.

MASSEY, H. C., M. L. CASTELLETTO, V. M. BHOPALE, G. A. SCHAD and J. B. LOK, 2005 Sst-tgh-1 from Strongyloides stercoralis encodes a proposed ortholog of daf-7 in Caenorhabditis elegans. Mol Biochem Parasitol **142:** 116-120.

MASSEY, H. C., JR., M. K. BHOPALE, X. LI, M. CASTELLETTO and J. B. LOK, 2006 The fork head transcription factor FKTF-1b from Strongyloides stercoralis restores DAF-16 developmental function to mutant Caenorhabditis elegans. Int J Parasitol **36:** 347-352.

MATYASH, V., E. V. ENTCHEV, F. MENDE, M. WILSCH-BRAUNINGER, C. THIELE *et al.*, 2004 Sterol-derived hormone(s) controls entry into diapause in Caenorhabditis elegans by consecutive activation of DAF-12 and DAF-16. PLoS Biol **2:** e280.

MAY, R. C., and R. H. PLASTERK, 2005 RNA interference spreading in C. elegans. Methods Enzymol **392:** 308-315.

MCCULLOCH, D., and D. GEMS, 2003 Body size, insulin/IGF signaling and aging in the nematode Caenorhabditis elegans. Experimental gerontology **38:** 129-136.

MCELWEE, J., K. BUBB and J. H. THOMAS, 2003 Transcriptional outputs of the Caenorhabditis elegans forkhead protein DAF-16. Aging Cell **2:** 111-121.

MCELWEE, J. J., E. SCHUSTER, E. BLANC, J. THORNTON and D. GEMS, 2006 Diapause-associated metabolic traits reiterated in long-lived daf-2 mutants in the nematode Caenorhabditis elegans. Mech Ageing Dev **127:** 458-472.

MELLO, C. C., J. M. KRAMER, D. STINCHCOMB and V. AMBROS, 1991 Efficient gene transfer in C.elegans: extrachromosomal maintenance and integration of transforming sequences. Embo J **10:** 3959-3970.

MINATO, K., E. KIMURA, Y. SHINTOKU and S. UGA, 2008 Effect of temperature on the development of free-living stages of Strongyloides ratti. Parasitol Res **102:** 315-319.

MINEMATSU, T., T. MIMORI, M. TANAKA and I. TADA, 1989 The effect of fatty acids on the developmental direction of Strongyloides ratti first-stage larvae. J Helminthol **63:** 102-106.

MITREVA, M., J. P. MCCARTER, J. MARTIN, M. DANTE, T. WYLIE *et al.*, 2004 Comparative genomics of gene expression in the parasitic and free-living nematodes Strongyloides stercoralis and Caenorhabditis elegans. Genome Res **14:** 209-220.

MONCOL, D. J., and A. C. TRIANTAPHYLLOU, 1978 Stronglyoides ransomi: factors influencing the in vitro development of the free-living generation. J Parasitol **64:** 220-225.

MOORE, T. A., S. RAMACHANDRAN, A. A. GAM, F. A. NEVA, W. LU *et al.*, 1996 Identification of novel sequences and codon usage in Strongyloides stercoralis. Mol Biochem Parasitol **79:** 243-248.

MORGAN, D. O., 1928 Parastrongyloides winchesi gen. et sp. nov. A remarkable new nematode parasite of the Mole and the Shrew. Journal of Helminthology **6:** 79-86.

MORRIS, J. Z., H. A. TISSENBAUM and G. RUVKUN, 1996 A phosphatidylinositol-3-OH kinase family member regulating longevity and diapause in Caenorhabditis elegans. Nature **382:** 536-539.

MOSER, J. M., T. FREITAS, P. ARASU and G. GIBSON, 2005 Gene expression profiles associated with the transition to parasitism in Ancylostoma caninum larvae. Mol Biochem Parasitol **143:** 39-48.

MOTOLA, D. L., C. L. CUMMINS, V. ROTTIERS, K. K. SHARMA, T. LI *et al.*, 2006 Identification of ligands for DAF-12 that govern dauer formation and reproduction in C. elegans. Cell **124:** 1209-1223.

MUKHOPADHYAY, A., S. W. OH and H. A. TISSENBAUM, 2006 Worming pathways to and from DAF-16/FOXO. Exp Gerontol **41:** 928-934.

MURPHY, C., T., S. MCCARROLL, A., C. BARGMANN, I., A. FRASER, R. KAMATH, S. *et al.*, 2003 Genes that act downstream of DAF-16 to influence the lifespan of Caenorhabditis elegans. Nature **424:** 277-283.

NAKAE, J., Y. CAO, H. DAITOKU, A. FUKAMIZU, W. OGAWA *et al.*, 2006 The LXXLL motif of murine forkhead transcription factor FoxO1 mediates Sirt1-dependent transcriptional activity. J Clin Invest **116:** 2473-2483.

NEWTON-HOWES, J., D. D. HEATH, C. B. SHOEMAKER and W. N. GRANT, 2006 Characterisation and expression of an Hsp70 gene from Parastrongyloides trichosuri. Int J Parasitol **36:** 467-474.

NOLAN, T. J., L. M. AIKENS and G. A. SCHAD, 1988 Cryopreservation of first-stage and infective third-stage larvae of Strongyloides stercoralis. J Parasitol **74:** 387-391.

NWAORGU, O. C., 1983 The development of the free-living stages of Strongyloides papillosus. I. Effect of temperature on the development of the heterogonic and homogonic nematodes in faecal culture. Vet Parasitol **13:** 213-223.

OGAWA, A., A. STREIT, A. ANTEBI and R. J. SOMMER, 2009 A conserved endocrine mechanism controls the formation of dauer and infective larvae in nematodes. Curr Biol **19:** 67-71.

OGG, S., S. PARADIS, S. GOTTLIEB, G. I. PATTERSON, L. LEE *et al.*, 1997 The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans. Nature **389:** 994-999.

OH, S. W., A. MUKHOPADHYAY, B. L. DIXIT, T. RAHA, M. R. GREEN *et al.*, 2006 Identification of direct DAF-16 targets controlling longevity, metabolism and diapause by chromatin immunoprecipitation. Nat Genet **38:** 251-257.

OH, S. W., A. MUKHOPADHYAY, N. SVRZIKAPA, F. JIANG, R. J. DAVIS *et al.*, 2005 JNK regulates lifespan in Caenorhabditis elegans by modulating nuclear translocation of forkhead transcription factor/DAF-16. Proc Natl Acad Sci U S A **102:** 4494-4499.

OOKUMA, S., M. FUKUDA and E. NISHIDA, 2003 Identification of a DAF-16 transcriptional target gene, scl-1, that regulates longevity and stress resistance in Caenorhabditis elegans. Current biology **13:** 427-431.

PARTRIDGE, L., and D. GEMS, 2002 Mechanisms of ageing: public or private? Nat Rev Genet **3:** 165-175.

PARTRIDGE, L., D. GEMS and D. J. WITHERS, 2005 Sex and death: what is the connection? Cell **120:** 461-472.

PATEL, D. S., A. GARZA-GARCIA, M. NANJI, J. J. MCELWEE, D. ACKERMAN *et al.*, 2008 Clustering of genetically defined allele classes in the Caenorhabditis elegans DAF-2 insulin/IGF-1 receptor. Genetics **178:** 931-946.

PIERCE, S. B., M. COSTA, R. WISOTZKEY, S. DEVADHAR, S. A. HOMBURGER *et al.*, 2001 Regulation of DAF-2 receptor signaling by human insulin and ins-1, a member

of the unusually large and diverse C. elegans insulin gene family. Genes Dev **15:** 672-686.

PIGLIUCCI, M., 2005 Evolution of phenotypic plasticity: where are we going now? Trends Ecol Evol **20:** 481-486.

PIGLIUCCI, M., 2008 Is evolvability evolvable? Nat Rev Genet **9:** 75-82.

PIPER, M. D., C. SELMAN, J. J. MCELWEE and L. PARTRIDGE, 2008 Separating cause from effect: how does insulin/IGF signalling control lifespan in worms, flies and mice? J Intern Med **263:** 179-191.

PRATT, D., G. N. COX, M. J. MILHAUSEN and R. J. BOISVENUE, 1990 A developmentally regulated cysteine protease gene family in Haemonchus contortus. Mol Biochem Parasitol **43:** 181-191.

RALSTON, M. J., M. STANKIEWICZ and D. D. HEATH, 2001 Anthelmintics for the control of nematode infections in the brushtail possum (Trichosurus vulpecula). N Z Vet J **49:** 73-77.

RIDDLE, D. L., 1977 C. elegan daf-2 mutant CB1370 pp. 101-120 in *Stadler Genetics Symposium*.

RIDDLE, D. L., 1987 Post-embryonic development in Caenorhabditis elegans. Int J Parasitol **17:** 223-231.

RIDDLE, D. L., M. M. SWANSON and P. S. ALBERT, 1981 Interacting genes in nematode dauer larva formation. Nature **290:** 668-671.

RIDDLE, D. L., T. BLUMENTHAL, B.J. MEYER AND J.R. PRIESS., 1997 *C. elegans II*. Cold Spring Harbor laboratory Press, New York.

RINCON, M., R. MUZUMDAR, G. ATZMON and N. BARZILAI, 2004 The paradox of the insulin/IGF-1 signaling pathway in longevity. Mech Ageing Dev **125:** 397-403.

RINCON, M., E. RUDIN and N. BARZILAI, 2005 The insulin/IGF-1 signaling in mammals and its relevance to human longevity. Exp Gerontol **40:** 873-877.

SANGER, F., S. NICKLEN and A. R. COULSON, 1977 DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A **74:** 5463-5467.

SCHWARZ, D. S., G. HUTVAGNER, T. DU, Z. XU, N. ARONIN *et al.*, 2003 Asymmetry in the assembly of the RNAi enzyme complex. Cell **115:** 199-208.

SHIWAKU, K., Y. CHIGUSA, T. KADOSAKA and K. KANEKO, 1988 Factors influencing development of free-living generations of Strongyloides stercoralis. Parasitology **97 (Pt 1):** 129-138.

SIGRIST, C. B., and R. J. SOMMER, 1999 Vulva formation in Pristionchus pacificus relies on continuous gonadal induction. Dev Genes Evol **209:** 451-459.

SIMMER, F., M. TIJSTERMAN, S. PARRISH, S. P. KOUSHIKA, M. L. NONET *et al.*, 2002 Loss of the putative RNA-directed RNA polymerase RRF-3 makes C. elegans hypersensitive to RNAi. Curr Biol **12:** 1317-1319.

SKORPING, A., A. F. READ and A. KEYMER, 1991 Life history covariation in intestinal nematodes of mammals. OIKOS **60:** 365-372.

SNOW, M. I., and P. L. LARSEN, 2000 Structure and expression of daf-12: a nuclear hormone receptor with three isoforms that are involved in development and aging in Caenorhabditis elegans. Biochim Biophys Acta **1494:** 104-116.

SOMMER, R. J., and P. W. STERNBERG, 1996 Evolution of nematode vulval fate patterning. Dev Biol **173:** 396-407.

STANKIEWICZ, M., 1996 observations on the biology of free-living stages of *Parastrongyloides trichosuri* (Nematoda, Rhabditoidae). Acta Parasitologica **41:** 38-42.

STEARNS, S. C., 1989 The Evolutionary Significance of Phenotypic Plasticity. BioScience **39:** 436-445.

STREIT, A., 2008 Reproduction in Strongyloides (Nematoda): a life between sex and parthenogenesis. Parasitology **135:** 285-294.

STUDIER, F. W., A. H. ROSENBERG, J. J. DUNN and J. W. DUBENDORFF, 1990 Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol **185:** 60-89.

SWANSON, M. M., and D. L. RIDDLE, 1981 Critical periods in the development of the Caenorhabditis elegans dauer larva. Dev Biol **84:** 27-40.

TATAR, M., A. BARTKE and A. ANTEBI, 2003 The endocrine regulation of aging by insulin-like signals. Science **299:** 1346-1351.

TAYLOR, K. A., and P. P. WEINSTEIN, 1990 The effect of oxygen and carbon dioxide on the development of the free-living stages of Strongyloides ratti in axenic culture. J Parasitol **76:** 545-551.

THOMAS, J. H., D. A. BIRNBY and J. J. VOWELS, 1993 Evidence for parallel processing of sensory information controlling dauer formation in Caenorhabditis elegans. Genetics **134:** 1105-1117.

THOMAS, J. H., and T. INOUE, 1998 Methuselah meets diabetes. Bioessays **20:** 113-115.

TIJSTERMAN, M., R. C. MAY, F. SIMMER, K. L. OKIHARA and R. H. PLASTERK, 2004 Genes required for systemic RNA interference in Caenorhabditis elegans. Curr Biol **14:** 111-116.

TIMMONS, L., D. L. COURT and A. FIRE, 2001 Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in Caenorhabditis elegans. Gene **263:** 103-112.

TISSENBAUM, H. A., J. HAWDON, M. PERREGAUX, P. HOTEZ, L. GUARENTE *et al.*, 2000 A common muscarinic pathway for diapause recovery in the distantly related nematode species Caenorhabditis elegans and Ancylostoma caninum. Proc Natl Acad Sci U S A **97:** 460-465.

TISSENBAUM, H. A., and G. RUVKUN, 1998 An insulin-like signaling pathway affects both longevity and reproduction in Caenorhabditis elegans. Genetics **148:** 703-717.

TOMARI, Y., and P. D. ZAMORE, 2005 Perspective: machines for RNAi. Genes Dev **19:** 517-529.

TSUJI, N., and K. FUJISAKI, 1994 Development in vitro of free-living infective larvae to the parasitic stage of Strongyloides venezuelensis by temperature shift. Parasitology **109 (Pt 5):** 643-648.

ULLU, E., A. DJIKENG, H. SHI and C. TSCHUDI, 2002 RNA interference: advances and questions. Philos Trans R Soc Lond B Biol Sci **357:** 65-70.

ULLU, E., C. TSCHUDI and T. CHAKRABORTY, 2004 RNA interference in protozoan parasites. Cell Microbiol **6:** 509-519.

URWIN, P. E., C. J. LILLEY and H. J. ATKINSON, 2002 Ingestion of double-stranded RNA by preparasitic juvenile cyst nematodes leads to RNA interference. Mol Plant Microbe Interact **15:** 747-752.

VAN DER HEIDE, L. P., and M. P. SMIDT, 2005 Regulation of FoxO activity by CBP/p300-mediated acetylation. Trends Biochem Sci **30:** 81-86.

VIA, S., 1993 Adaptive phenotypic plasticity: target or by-product of selection in a variable environment? Am Nat **142:** 352-365.

VINEY, M. E., 1996 Developmental switching in the parasitic nematode Strongyloides ratti. Proc Biol Sci **263:** 201-208.

VINEY, M. E., 2009 How did parasitic worms evolve? Bioessays **31:** 496-499.

VINEY, M. E., and N. R. FRANKS, 2004 Is dauer pheromone of Caenorhabditis elegans really a pheromone? Naturwissenschaften **91:** 123-124.

VINEY, M. E., M. P. GARDNER and J. A. JACKSON, 2003 Variation in Caenorhabditis elegans dauer larva formation. Dev Growth Differ **45:** 389-396.

VINEY, M. E., L. D. GREEN, J. A. BROOKS and W. N. GRANT, 2002 Chemical mutagenesis of the parasitic nematode Strongyloides ratti to isolate ivermectin resistant mutants. Int J Parasitol **32:** 1677-1682.

VINEY, M. E., and J. B. LOK, 2007 Strongyloides spp. , pp. in *WormBook The C. elegans Research Community*, edited by J. HODGKIN and P. ANDERSON. http://www.wormbook.org. .

VINEY, M. E., F. J. THOMPSON and M. CROOK, 2005 Tgf-beta and the evolution of nematode parasitism. Int J Parasitol.

VISSER, A., P. GELDHOF, V. DE MAERE, D. P. KNOX, J. VERCRUYSSE *et al.*, 2006 Efficacy and specificity of RNA interference in larval life-stages of Ostertagia ostertagi. Parasitology **133:** 777-783.

VOWELS, J. J., and J. H. THOMAS, 1992 Genetic analysis of chemosensory control of dauer formation in Caenorhabditis elegans. Genetics **130:** 105-123.

WALKER, G. A., T. M. WHITE, G. MCCOLL, N. L. JENKINS, S. BABICH *et al.*, 2001 Heat shock protein accumulation is upregulated in a long-lived mutant of Caenorhabditis elegans. J Gerontol A Biol Sci Med Sci **56:** B281-287.

WANG, J., and M. M. BARR, 2005 RNA interference in Caenorhabditis elegans. Methods Enzymol **392:** 36-55.

WANG, Z., X. E. ZHOU, D. L. MOTOLA, X. GAO, K. SUINO-POWELL *et al.*, 2009 Identification of the nuclear receptor DAF-12 as a therapeutic target in parasitic nematodes. Proc Natl Acad Sci U S A **106:** 9138-9143.

WEST-EBERHARD, M. J., 2005 Developmental plasticity and the origin of species differences. Proc Natl Acad Sci U S A **102 Suppl 1:** 6543-6549.

WINSTON, W. M., C. MOLODOWITCH and C. P. HUNTER, 2002 Systemic RNAi in C. elegans requires the putative transmembrane protein SID-1. Science **295:** 2456-2459.

WOLKOW, C. A., K. D. KIMURA, M. S. LEE and G. RUVKUN, 2000 Regulation of C. elegans life-span by insulinlike signaling in the nervous system. Science **290:** 147-150.

WOLKOW, C. A., M. J. MUNOZ, D. L. RIDDLE and G. RUVKUN, 2002 Insulin receptor substrate and p55 orthologous adaptor proteins function in the Caenorhabditis elegans daf-2/insulin-like signaling pathway. J Biol Chem **277:** 49591-49597.

YAMADA, M., S. MATSUDA, M. NAKAZAWA and N. ARIZONO, 1991 Species-specific differences in heterogonic development of serially transferred free-living generations of Strongyloides planiceps and Strongyloides stercoralis. J Parasitol **77:** 592-594.

ZAMORE, P. D., T. TUSCHL, P. A. SHARP and D. P. BARTEL, 2000 RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell **101:** 25-33.

ZAWADZKI, J. L., P. J. PRESIDENTE, E. N. MEEUSEN and M. J. DE VEER, 2006 RNAi in Haemonchus contortus: a potential method for target validation. Trends Parasitol **22:** 495-499.

ZDOBNOV, E. M., and R. APWEILER, 2001 InterProScan--an integration platform for the signature-recognition methods in InterPro. Bioinformatics **17:** 847-848.

ZHENG, M., D. MESSERSCHMIDT, B. JUNGBLUT and R. J. SOMMER, 2005 Conservation and diversification of Wnt signaling function during the evolution of nematode vulva development. Nat Genet **37:** 300-304.