GTP-Cyclohydrolase function in parasitic nematode development

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3　Investigation of function by using a GTP-Cyclohydrolase inhibitor *in vitro*

Declaration

The work presented in this thesis is my own work, unless otherwise stated, and has not been submitted for any other degree.

_____ Rachael Baker

Acknowledgements

> "At rest, however, at the centre of everything is the sun."
>
> Nicolaus Copernicus, 1543.

Abstract

Parasitic nematodes of grazing livestock represent an increasing economic and welfare problem for British agriculture. By investigating specific life-cycle stages of these parasites, it may be possible to identify key molecules or pathways that are required for the survival of the worms, and thus exploit these for future control strategies. It has been shown previously that the third larval stages (L_3) of the ovine parasitic nematode *Teladorsagia circumcincta* produce high levels of transcript for the enzyme GTP-Cyclohydrolase relative to later developmental stages. As the rate-limiting factor in the production of tetrahydrobiopterin, GTP-Cyclohydrolase is required for a number of different biochemical pathways, including those involved in the production of serotonin and melanin. As the L_3 do not feed, it can be hypothesised that, if finite resources are being used in the production of transcript encoding this enzyme, then it may be important for survival.

In this thesis, a number of approaches were taken to explore the function of GTP-Cyclohydrolase in the life-cycle development of *T. circumcincta*. The closely related parasite, *Dictyocaulus viviparus*, was used as a model organism to explore the role of GTP-Cyclohydrolase and serotonin production with regards to larval arrest, or hypobiosis. This process occurs readily under experimental conditions in *D. viviparus*, which is not possible with *T. circumcincta*. Quantitative PCR was used to examine GTP-Cyclohydrolase transcript levels in two different strains of *D. viviparus*, one that enters larval arrest when exposed to cold conditions and one that does not. No differences were observed between the two strains suggesting that GTP-Cyclohydrolase was unlikely to be involved in hypobiosis. The model nematode, *Caenorhabditis elegans*, was used to perform functional complementation experiments to assess the role of GTP-Cyclohydrolase in the cuticle, as it has been shown previously that *C. elegans* GTP-Cyclohydrolase mutants have a 'leaky cuticle' and are killed by lower doses of anthelmintics and bleach than the wild-type worms. The *T. circumcincta* gene for GTP-Cyclohydrolase was able to restore cuticular integrity of *C. elegans* GTP-Cyclohydrolase-deletion mutants, suggesting that the role played by the protein in both species is similar. *In vitro* inhibition

experiments using a chemical inhibitor of GTP-Cyclohydrolase showed that *T. circumcincta* larval development was disrupted in the presence of the inhibitor. It was also shown that *T. circumcincta* L_3 that were exposed to sunlight produced melanin, suggesting that the levels of GTP-Cyclohydrolase observed in the pre-parasitic stages of *T. circumcincta* may be required for the synthesis of melanin. Together, these data suggest that GTP-Cyclohydrolase is required by the pre-parasitic stages to survive on pasture. Ultraviolet radiation has been shown previously to be harmful to *T. circumcincta* L_3, so if the melanin production provides protection from this, then it would be crucial for the survival of the pre-parasitic stages.

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1   Introduction

1.1   Nematodes

The phylum Nematoda is divided into two classes, the predominantly marine Adenophorea and the predominantly terrestrial Secernentea. Research has shown that the ancestor for the Secernentea may be an adenophore, casting doubt about previously held theories of the evolution of the phylum, whereby parasitic nematodes evolved from free-living species in a single event (Blaxter *et al.*, 1998). Within the Secernentea the evolutionary relationships remain disputed (Parkinson *et al.*, 2004), but the Clades are comprised of a mixture of parasitic and free-living species. In Clade V, the nematodes are subdivided into the Strongylida (mainly parasites of vertebrates), the Rhabditida (mainly free-living in soil and includes the model organism, *Caenorhabditis elegans*) and the Diplogasterida. The superfamily Trichostrongyloidea within the order Strongylida (Chilton *et al.*, 2006; Durette-Desset *et al.*, 1999) contains many of the species that cause health problems for sheep and other ruminants, and includes *Haemonchus contortus*, *Teladorsagia circumcincta* and *Trichostrongylus colubriformis* (see Maggenti, 1982).

1.1.1   Parasitic nematodes

Parasitic nematodes of grazing livestock are one of the most serious problems facing agriculture today, with a significant economic impact and an increasing issue for welfare. It is estimated that gastrointestinal nematode infections of sheep alone cost the UK industry £84 million per year (Nieuwhof and Bishop, 2007), although the costs of subclinical disease are difficult to quantify. In 2007, the global market in anti-parasitic treatments was worth over $US5 billion (£2.5 billion) and is estimated to have increased at a rate of half a billion U.S. dollars a year for the 3 years prior to that (Wood Mackenzie, 2008). In 1995, it was estimated that in Australia the annual cost of the major livestock parasites was $AUS684 million (£300 million). Of this, the single largest category of expenditure was on control of sheep worms, with an estimated annual cost of $AUS222 million (approximately £97 million at 1995 rates) (Mcleod, 1995). Of these very large costs, only about one third was estimated to be due to labour and drug costs involved in controlling the parasites; two thirds of the

cost was estimated to be due to production losses. The greatest proportion of these production losses was estimated to be due to production losses and clinical disease in growing lambs, in which parasitic gastroenteritis is a major welfare problem (Stear et al., 2007).

1.2 *Teladorsagia circumcincta*

Teladorsagia (Ostertagia) circumcincta, the 'brown stomach worm', is the most prevalent parasitic nematode of sheep and goats in the UK (Bartley *et al*., 2003). Infections can result in severe weight loss and diarrhoea. Damage is caused not only by the presence of the adult worms on the mucosal surface, but also by larvae developing in and emerging from gastric glands in which they have been developing (Taylor *et al*., 2007). Subclinical infections suppress appetite (Greer *et al*., 2008); this, combined with a loss of plasma protein into the gastrointestinal tract and of epithelial cell function, results in reduced metabolism of protein (McKellar, 1993). Even with relatively low levels of infection, lambs take longer to reach their target weight and have poor body condition (Taylor *et al*., 2007).

Immunity to gastrointestinal nematodes in sheep is acquired and relies on prolonged and/or repeated exposure to the parasites. Lambs exposed to *T. circumcincta* over their first year of life develop immunity gradually, excluding the third stage larvae (L_3), retarding development of adult worms and affecting egg output as they age and have exposure to the worms (Seaton *et al*., 1989). Worms that remain in the abomasa of a previously infected sheep are smaller (for example, at 10 days post-challenge, worms are approximately 1 mm longer in sheep that have not been previously infected) and are less fecund than those that develop in parasite naïve animals (Halliday *et al*., 2007; Stear *et al*., 1995). A reduction in total serum IgA levels occurs in ewes around lambing time which correlates with greater numbers of worms producing higher numbers of eggs which contaminate pasture and infect lambs (Jeffcoate *et al*., 1992). This peri-parturient relaxation of immunity, lasts for approximately 6-8 weeks after lambing and has been linked to the competing

demands for ewe nutrition in lactation and the immune response (Houdijk *et al*., 2001).

1.2.1 Life-cycle

The life-cycle of *T. circumcincta* is direct (Figure 1), with eggs passing out of the sheep in faeces. Eggs can develop into first stage larvae (L_1) within 24 h; these feed on bacteria in the faeces. Second stage larvae (L_2) also feed, but when the moult to third stage larvae (L_3) occurs (approximately 10-14 days after the moult to L_2 under optimal conditions of temperature and moisture), the L_2 cuticle is retained as a sheath for the L_3, providing protection from adverse environmental conditions, but also preventing feeding (Keith et al., 1990). It has been shown that the L_3 are more resistant to extremes of temperature and humidity compared to the eggs, L_1 and L_2 stages (O'Connor *et al*., 2006). *T. circumcincta* L_3 are able to survive at lower temperatures than other, closely related nematodes such as *H. contortus* and *T. colubriformis*, with L_3 able to survive for up to 13 weeks at -10 °C (Pandey *et al*., 1993). *T. circumcincta* L_3 seem to be less well equipped for surviving at higher temperatures though; one study in Western Australia showed that no *T. circumcincta* L_3 were able to survive between December and February when soil temperatures reached 28 °C (Callinan, 1978), although brief periods of high temperatures (i.e. 90 min at 45 °C), as might be experienced within a faecal pellet on a very sunny afternoon, did not cause larval death (Walker *et al*., 2007). This ensures that *T. circumcincta* L_3 are suited for survival in the temperate regions which they populate, where cooler winters are more common than hot summers.

Humidity is crucial for L_3 survival, but is also an important factor that determines L_3 migration out of the faecal pellet; with more rapid migration occurring under wet conditions (Young, 1983). *T. circumcincta* L_3 appear to be more resistant to desiccation than other trichostrongyle nematode species such as *H. contortus*, which may help to facilitate survival (Pandey *et al*., 1993). It is hypothesised that the coiled posture of infective L_3 helps to prevent desiccation by reducing the surface area that is exposed to air (Wharton, 1982; Womersley, 1978). In studies of *T. colubriformis*, the proportion of coiled L_3 increased from 20 % to 60 % over a 30 min period when

the L_3 were placed on a slide in 10 µl water that evaporated slowly. The proportion of coiled L_3 that were suspended in the same volume but protected from evaporation by a coverslip did not change over time (Wharton, 1981). This suggests that the initiation of coiling was in response to water evaporation. To persist in the environment, alongside temperature and humidity fluctuations, L_3 must also be capable of surviving changes in levels of ultraviolet (UV) radiation. This has been hypothesised as the cause of the observed rapid reduction in infective L_3 present on pasture in the spring in the UK, when UV levels increase more quickly than temperature (van Dijk *et al.*, 2009). Experiments performed at Weybridge in the south of England, showed that *T. circumcincta* L_3 levels peaked 4-6 weeks after the faeces were deposited on the pasture; levels were then maintained at a reduced but steady level over winter, followed by a sharp decline in the spring (Gibson and Everett, 1972). This pattern of pasture contamination occurred irrespective of the month that the faeces were deposited. It has also been observed that the reduction in trichostrongyle L_3 on pasture is correlated with the absence of larvae found within the faecal material; this has led to the hypothesis that the L_3 can survive for longer periods in faeces than when exposed on grass (Gibson and Everett, 1967). While faeces may provide limited protection for parasitic nematode larvae from temperature fluctuations, protection from UV is likely to be much greater. *T. circumcincta* L_3 have been shown to be more susceptible to UV radiation than *H. contortus* L_3; experiments in Bristol, in the south of England, showed that over six days when ensheathed L_3 were exposed to constant levels of UV, death occurred in *T. circumcincta* L_3 twice as quickly as in *H. contortus* (van Dijk *et al.*, 2009). This effect was also observed when L3 were exposed to natural sunlight; on the day with the highest level of UV radiation, 43 % of the *H. contortus* L_3 survived, but none of the *T. circumcincta* L_3 survived.

Trichostrongylid L_3 migrate up herbage under moist conditions and are ingested. They exsheath in the ruminoreticulum, after which, another two moults occur in the abomasal glands, before the parasites emerge onto the surface of the abomasum and become sexually mature approximately 3 weeks post-infection (p.i.; Soulsby, 1982). In the Northern hemisphere, if L_3 of abomasal nematodes are ingested by sheep after

October, the larvae may undergo hypobiosis (larval arrest) and remain as early L_4 in the abomasum for up to 6 months before resuming development (Makovcová *et al.*, 2009). In order to detect environmentally-induced hypobiotic larvae, tracer lambs have been used that have been kept under worm-free conditions to eliminate any confounding effects of immunity (Capitini *et al.*, 1990). In studies in Bohemia in the west of the Czech Republic, after a four-week exposure to naturally infected pasture and a two week housing period to allow development of parasites, 52 % of tracer lambs were found to have arrested *T. circumcincta* larvae in their abomasa. The highest proportion of arrested larvae was observed in lambs in December with 88 % of the *T. circumcincta* worms present at necropsy found to be hypobiotic (Langrová *et al.*, 2008). As the proportion of larvae that were hypobiotic differed depending on the month in which the tracer lambs were exposed to infection, the rates of arrest could be due to changes in climatic conditions rather than host immunity, although age of the host is likely to play a role even without prior exposure to the parasite. While the processes surrounding larval arrest in *T. circumcincta* are still not fully understood, it is hypothesised that there is a role for both environmentally induced hypobiosis and immune-mediated arrest. Research by Smith (2007a) showed that lambs that were trickle infected with *T. circumcincta* L_3 for six weeks, treated with anthelmintic, and then re-challenged with 50,000 L_3, had 98 % of the worms present at necropsy ten days later in the arrested L_4 stage as opposed to 2 % in the naïve controls that were challenged with 50,000 L_3 at the same time. These experiments also highlighted that resumption of development could occur within 19 days of the initial challenge, implying that larval arrest is not always long-lasting, and can be more rapid if animals are immune suppressed. Immunity to *T. circumcincta* develops between four and eight weeks post challenge, with increasing numbers of arrested larvae found with increasing lengths of exposure to the parasites (Seaton *et al.*, 1989). One of the complicating factors in the study of hypobiosis under experimental conditions is that the process of repeatedly passaging larvae through donor animals has been shown to reduce the chances of hypobiosis occurring (Armour *et al.*, 1967), and few studies are performed on field strains under field conditions.

The L3 exsheath in the ruminoreticulum and then complete development into sexually mature adults in the abomasum after moulting to L4 and L5.

Life-cycle of *Teladorsagia circumcincta*

Females release eggs that are passed out in faeces and develop into L1

L3 migrate up blades of grass in moist conditions where they are ingested by sheep

L1 develop to L2 and then to L3 stages in the faecal pat

Figure 1. The life-cycle of *T. circumcincta*. Images courtesy of Dave McBean (Moredun Research Institute) and Nick Sangster (University of Sydney).

1.3 Treatments and anthelmintic resistance

Prior to the 1940's, a wide variety of toxic products had been used to treat ruminants for internal parasites; for example, arsenic, oil of turpentine and tin. These were used with varying degrees of success and toxicity (McKellar and Jackson, 2004). The first anthelmintic that had a specific target parasite species was phenothiazine in 1940 (Gordon, 1945); but it was not until the discovery of the benzimidazoles (BZ) in the 1960's and levamisole in the 1970's that agents with a broad spectrum of activity and a much lower therapeutic dose than phenothiazine were available. BZs act by binding to nematode β-tubulin, preventing microtubule formation, which leads to cell lysis (Prichard, 1990), and levamisole acts on neuromuscular junctions as a cholinergic agonist (McKellar and Jackson, 2004). The introduction of the first avermectin/ miblemycin anthelmintic, ivermectin, in 1981, was a key step in the control of parasites, as it had broad spectrum activity against endo- and ecto-parasites

and a high degree of potency (Geary, 2005). Ivermectin is hypothesised to affect gamma amino butyric acid (GABA) and glutamate-gated chloride channels, which interferes with nematode neuronal transmission (Feng *et al*., 2002). An important recent development has been the introduction of two new classes of anthelmintics, the amino-acetonitrile derivatives (AADs), for example Monepantel, which act on nicotinic acetylcholine receptors unique to nematodes (Kaminsky *et al*., 2008) and the spiroindoles which block cation channels in muscle cell membranes of nematodes (Little *et al*., 2011).

Anthelmintic resistance in nematodes is well documented. The first case of phenothiazine resistance was reported by Drudge (1957) – and, since then, resistance has been identified to all but the most recently released commercially available compounds. Resistance in single populations to multiple classes is also well established in small ruminants in the UK (Sargison *et al*., 2001) and worldwide. In the UK, a number of cases of multiple resistance to benzimidazole, levamisole and macrocyclic lactone anthelmintics have been reported, resulting in the suspension of sheep farming on some farms until the parasites can be removed from the environment (Bartley *et al*., 2004; Cheng *et al*., 2003; Sargison *et al*., 2004). While the current rate of reported multiple anthelmintic resistance in UK sheep flocks remains low (less than 0.01 %), it is likely that the actual number of flocks affected is much higher (Sargison *et al*., 2007). At an individual animal level, it was demonstrated that treatment with an ineffective anthelmintic can result in an average reduction in carcass weight of 2.8 kg after 112 days with monthly treatments compared to those treated with an effective anthelmintic (Sutherland *et al*., 2010). While there are no reports of field resistance to the AAD class of anthelmintic, it has been artificially selected in the laboratory so field resistance will, most probably, occur with time (Kaminsky *et al*., 2008).

There are a number of genetic features within parasitic nematodes that increase the likelihood of anthelmintic resistance developing. High levels of nucleotide sequence diversity and large effective population sizes give nematode populations high levels of genetic polymorphism (Blouin *et al*., 1992; Gilleard and Beech, 2007). The rate

of gene flow between populations for some species is known to be high, implying that the movement of hosts is an important factor in the diversity of nematode populations – this has a positive effect on the ability to develop anthelmintic resistance (Blouin *et al*., 1995). The combination of these factors means that anthelmintic resistance may develop rapidly following the introduction of new classes of drugs. Combination therapy using a number of different classes of drugs is likely to remain the best option in the short term for quarantine drenching new animals to help prevent the introduction of resistant individuals from other farms (Dobson *et al*., 2011; Leathwick and Hosking, 2009).

A number of surveys of anthelmintic resistance in sheep flocks have been undertaken in countries around the world in an attempt to ascertain the effectiveness of current anthelmintics (Cernanska *et al*., 2006; Chartier *et al*., 1998; Farias *et al*., 1997; Pedreira *et al*., 2006). Bartley *et al.* (2003) showed that in 90 Scottish farms surveyed, 64 % displayed signs of benzimidazole resistance. The same survey reported that *T. circumcincta* was the only species to be found on all farms sampled, with other species ranging in prevalence from 29 % (*Oesophagostomum venulosum)* to 73 % (*H. contortus*). The prevalence of resistant *T. circumcincta* is further confirmed by another report (Bartley *et al*., 2004) where, on two farms with anthelmintic resistance to all three classes of anthelmintic, the only species found post-treatment was *T. circumcincta*. In New Zealand, the first reported case of nematode resistance to both abamectin and moxidectin in sheep was in 2004 (Hughes *et al*., 2004). In this case, mixed species worm burdens were detected; however *T. circumcincta* was the predominant resistant species present.

It has been hypothesised that the kinetics of expulsion of ivermectin may differ between species of parasitic nematode (Gill and Lacey, 1998). *In vitro* tests that exposed L_3 to avermectin suggested that drug effects on motility were more important in *H. contortus* and *T. colubriformis,* while other effects such as those on pharyngeal pumping seem to be more significant in *T. circumcincta* (Gill and Lacey, 1998). If the methods of metabolism and excretion of anthelmintics or other xenobiotics vary between parasite species, then it is possible that the way in which

resistance develops could be different and could help explain observed variation in the predominance of resistant species.

One feature of anthelmintic resistance is that it does not appear to be reversible, even in long-term absence of the selecting anthelmintic or class of anthelmintic (Dash, 1986; Hall *et al*., 1982; Martin *et al*., 1988). For example, with regards to BZ resistance, which has been linked to the substitution of a phenylalanine with a tyrosine at residue 200 on the β–tubulin isotype 1 nematode gene in some species, there has been no reversion to sensitivity observed in field populations of *H. contortus* and *T. colubriformis* after the removal of selection pressure (Roos *et al*., 1995; Wolstenholme *et al*., 2004). In other words, if a sheep flock with BZ resistant nematodes is not treated with BZs for a number of years, levels of resistance, as determined by the allelic frequency, would be expected to remain the same after the treatment break as they were immediately post-treatment. The phenylalanine to tyrosine amino acid substitution is in a conserved gene, whereby there is 99 % homology between the peptide sequence in the β–tubulin of *T. circumcincta* and *H. contortus* (Elard *et al*., 1996). Work by Elard *et al.* (2000) has shown that despite the conserved nature of β–tubulin, there appears to be no fitness cost to the individual worms that are genotypically resistant. If there is no fitness cost to being resistant to BZ drugs, then this reduces the probability of reversion to susceptibility occurring naturally as there is no selection pressure to do so (Leignel *et al*., 2010).

1.4 Potential alternatives for parasite control

A number of alternative parasite control methods have been investigated *in vitro* and *in vivo*, with varying degrees of success. Alternative control measures include novel methods of parasite management, genetic selection of nematode resistant sheep, and immunological, biological and nutritional treatments.

1.4.1 Management measures

Maintaining genes for susceptibility to anthelmintics in a parasite population is paramount for extending the effectiveness of presently available drug classes. One

mechanism for maintaining sensitivity to anthelmintics is to reduce the selective pressure on the parasites by reducing the proportion of the population exposed to the drug. At any one time, there is a proportion of the parasite population that is not exposed to anthelmintic (whilst on pasture or present inside animals that are not treated), these parasites are said to be *in refugia*. Some management systems aim to increase the proportion of the total parasite population *in refugia* by leaving a percentage of a sheep flock untreated. One strategy for this is the FAMACHA system (named after its creator, FAffa MAlan CHArt), developed in South Africa for the control of haemonchosis. This system compares the colour of the mucous membranes of the conjunctiva of sheep and goats to a colour chart, which indicates the level of anaemia, and therefore the level of blood loss due to the parasite - and identifies those animals which are most in need of treatment for *H. contortus* infections (Mahieu *et al*., 2007; Vatta *et al*., 2001; 2002). This allows animals that are not showing clinical signs of the parasitic infection to remain untreated and to contaminate pasture with parasites that are more likely to be anthelmintic-susceptible. This system is effective and variations are being developed that could use information such as body weight gain (Besier, 2008; Greer *et al*., 2009) or milk production (Hoste *et al*., 2002a; 2002b) for the control of teladorsagiosis.

1.4.2 Genetic selection

It has been demonstrated that various breeds of sheep respond differently to nematode infections (Miller and Horohov, 2006). More 'primitive' or traditional breeds such as the Shetland or Manx Loaghtans have lower faecal egg counts under the same conditions than those breeds, such as the Southdowns, that have been bred more for commercial traits (Golding and Small, 2009). By selecting appropriate genetic lines of animals, it may be possible to produce animals that have an improved ability to regulate their gastrointestinal helminths, and as a result, offer a relatively high/better level of flock immunity. A number of animal lines have been developed for experimental purposes, and their genetic profiles have been studied, although, as yet, there has been no common single "resistance-associated" gene found amongst them (Dominik, 2005). For example, in cattle with *Ostertagia* and *Cooperia* infections, it has been shown that with selective breeding, calves that were grazed on

contaminated pasture for 120 days fell into three categories: one group that showed no rise in egg per gram (EPG) values; a second group that demonstrated rises in EPG values for only two months post infection and a third group that maintained high EPG values throughout the experiment (Sonstegard and Gasbarre, 2001). Through selection for the first two categories, researchers have successfully reduced, over four generations, the proportion of the herd that were immunologically non-responsive to parasites (group three). The theoretical basis for genetic selection is a sound one, but it must be remembered that there is a potential for selection of negative effects on performance if solely selecting for resistance to parasites.

Studies on Soay sheep which live on St Kilda have shown that under normal evolutionary conditions, individuals with high levels of anti-nuclear antibodies (antibodies that bind mammalian nuclear and cytoplasmic antigens that are used as a measure of autoimmunity) lived longer, but produced fewer offspring each year than those sheep with lower levels of antibodies. This led to the same total number of offspring being produced over the lifetime of both groups of individuals (Graham *et al*., 2010). The extra resources required for mounting an elevated antibody response could result in a diversion from the requirements for reproduction, a trade-off that could be reproduced with the selection for resistance to parasites. It is also worth remembering that the choice of measure is critical. Selecting for individuals with low faecal egg counts may present a false picture as animals may require a large physiological effort to maintain this response (Greer *et al*., 2008). A better approach might be to identify those animals that do not mount such a strong immune response, but are able to cope with the levels of parasitism that they are exposed to. These animals are able to perform and grow normally despite helminth challenge and are 'resilient' rather than 'resistant' to parasite infection.

1.4.3 Immunological methods

There has been a large amount of research in developing vaccines against gastrointestinal nematodes. This has met with mixed results. This may be due in part to the seemingly slow development of an appropriate immune response to natural nematode exposure that does not provide the host with sufficient protection

from disease (Miller and Horohov, 2006); in cattle with *O. ostertagi* infection, re-exposure over more than one season is thought to be required before full protection develops (Mitreva *et al*., 2007). One area of relative success is vaccination with antigens derived from the gut surface of adult *H. contortus*. Some antigens such as the aminopeptidase H11 and H-gal-GP (a galactose-containing glycoprotein complex) have provided promising results in native form (Kabagambe *et al*., 2000; Knox and Smith, 2001; Smith, 2007b). These antigens have proven effective in lambs vaccinated with native H-gal-GP in the context of QuilA adjuvant, with over 90 % reduction in faecal egg counts and 72 % mean reduction in total worm numbers (Smith *et al*., 1994). Because the immunity induced is thought to be against "hidden" antigens, there is no stimulus by natural parasite exposure and, therefore, repeated vaccination may be required. One of the reasons for success with *H. contortus* may be that it is a blood-feeder, meaning that antigen specific antibodies circulating in the blood stream of the host are consumed directly by the worm. For non blood-feeding nematodes, such as *T. circumcincta*, it may be necessary for antigens to stimulate a more local mucosal response (Claerebout *et al*., 2003). There have been particular problems in developing recombinant vaccines against parasitic nematodes, in part, due to the complex way in which the native antigens are folded and glycosylated (Murray *et al*., 2007; Smith and Zarlenga, 2006).

1.4.4 Targeting specific components of the nematode life-cycle

One other area of active research for nematode control targets is to examine the life-cycle of the parasites and determine if there are specific processes that could be inhibited or targeted. One phase of the life-cycle that may be of particular relevance is the change from the free-living L_3 stage to the parasitic L_4 stage. At this point in the life-cycle, the parasites undergo major physiological changes and this has been shown to be associated with an alteration in transcription of a large number of genes (Nisbet *et al*., 2004). A number of different molecular techniques have been used to study the transcriptional differences between free-living and parasitic stages of nematodes. One method is to use complementary DNA (cDNA) microarrays, as used on the canine hookworm *Ancylostoma caninum* (see Moser *et al*., 2005). In this case, 113 genes were found to be differentially expressed between the infective L_3

and larvae that had been stimulated with dog serum *in vitro* to mimic the conditions of the early stages of parasitism within the host.

Nisbet *et al.* (2008) used suppression subtractive hybridisation (SSH) to identify expressed sequence tags (ESTs) that were differentially expressed between L_3 and L_4 stages of *T. circumcincta*. These experiments involved the synthesis of cDNA from L_3 and L_4 messenger RNA (mRNA) and the ligation of specific adapters to each cDNA pool. A sample of L_3 cDNA was then hybridised to an excess of L_4 cDNA to remove any common cDNA sequences (Figure 2). The reverse process was also performed, so that only those cDNA sequences that were differentially expressed were amplified by polymerase chain reaction (PCR) using primers specific for the adapter sequences. This study found that, of the 361 unique sequences from the L_3-specific EST dataset, 85 % had significant homology to known genes from other nematode species such that putative functions could be ascribed to 62 % of the 'L_3-specific' EST dataset. The stand-out result from this experiment was that 33 % of the 769 cDNA sequences derived from the 'L_3-specific' EST dataset represented the enzyme, guanosine-5'-triphosphate-cyclohydrolase (GTP-Cyclohydrolase). Other studies have also identified GTP-Cyclohydrolase as an abundant transcript in infective L_3 of other parasitic nematode species; *O. ostertagi* (see Moore *et al*., 2000), *H. contortus* (see Hoekstra *et al*., 2000) and *A. caninum* (see Moser *et al.,* 2005).
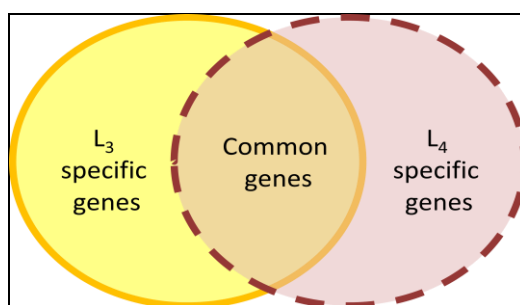
Figure 2. Schematic representation of the suppressive subtractive hybridisation technique, whereby genes common to both the L_3 and L_4 stage were removed, so that only those relatively abundant in each life-cycle stage were examined (Nisbet *et al.* 2008).

As L_3 do not feed, it can be hypothesised that the production of large quantities of transcript for GTP-Cyclohydrolase might be critical for larval survival, as finite levels of resources are likely to be available for its production. It is also possible that the transcript is being produced in advance of the transition to parasitism, at which point a sudden change in environmental conditions require a different array of proteins. If GTP-Cyclohydrolase, or another enzyme required downstream in the associated pathways, is indeed critical for the nematodes to develop in the host, then it could provide a route to the discovery of novel drug targets for use in ruminants.

It has been shown that immune sheep are capable of expelling *T. colubriformis* within 2 h of infection (Wagland *et al*., 1996). If this rapid expulsion could be induced by a drug then it may be possible to target the nematodes before they become pathogenic. The focus of most new anthelmintic discovery has been through the screening of potential compounds for effects *in vitro* and then developing potential candidates further (Prichard and Geary, 2008). This method of discovery also needs to be complemented by research into the basic biology of parasitic nematodes (as discussed in Geary *et al*., 2004), to further understand key mechanisms of parasite development and survival. Research into GTP-Cyclohydrolase is an example of how understanding the basic biology of why the L_3 require such high levels of transcript could lead to insights for the development of novel therapeutics.

1.5 GTP-Cyclohydrolase

The enzyme GTP-Cyclohydrolase (EC 3.5.4.16) catalyses the initial and rate-limiting steps in the synthesis of tetrahydrobiopterin (BH_4), which is an essential cofactor in metabolic pathways in eukaryotic and prokaryotic organisms (Cameron *et al*., 2008; Higgins and Gross, 2011). There have been many studies to identify the structure and function of GTP-Cyclohydrolase (Duch and Smith, 1991; McIntosh *et al*., 2008; Nar *et al*., 1995a; Nar *et al*., 1995b). A comparative study of a 93 amino acid region of the GTP-Cyclohydrolase sequences of rainbow trout, chicken, three different fungi species, a cellular slime mould, a phytoflagellate and a plant showed that there

was a very high degree of sequence conservation amongst the species. This ranged from 50 % identity between bacteria and vertebrates to 95 % amongst the various vertebrate GTP-Cyclohydrolase sequences (Maier *et al*., 1995). In particular, 12 amino acids, believed to contribute to the GTP-binding pocket, were found to be 100 % identical amongst all the species studied. The majority of work on the characterisation of the structure of GTP-Cyclohydrolase has been performed using the enzyme derived from *Escherichia coli*, however the high degree of homology amongst the enzymes from various species has enabled much of the knowledge to be extrapolated from this work (Burg and Brown, 1966). The C-terminal sequence is particularly highly conserved among species and, in particular, the final 120 residues are 60 % identical between human and *E. coli* GTP-Cyclohydrolase. It has also been shown that those residues involved in pterin binding and catalysis are highly conserved (Thöny *et al*., 2000). For these reasons, it is generally accepted that the tertiary and quarternary structure observed in *E. coli* will be very similar to that seen in GTP-Cyclohydrolase derived from other species.
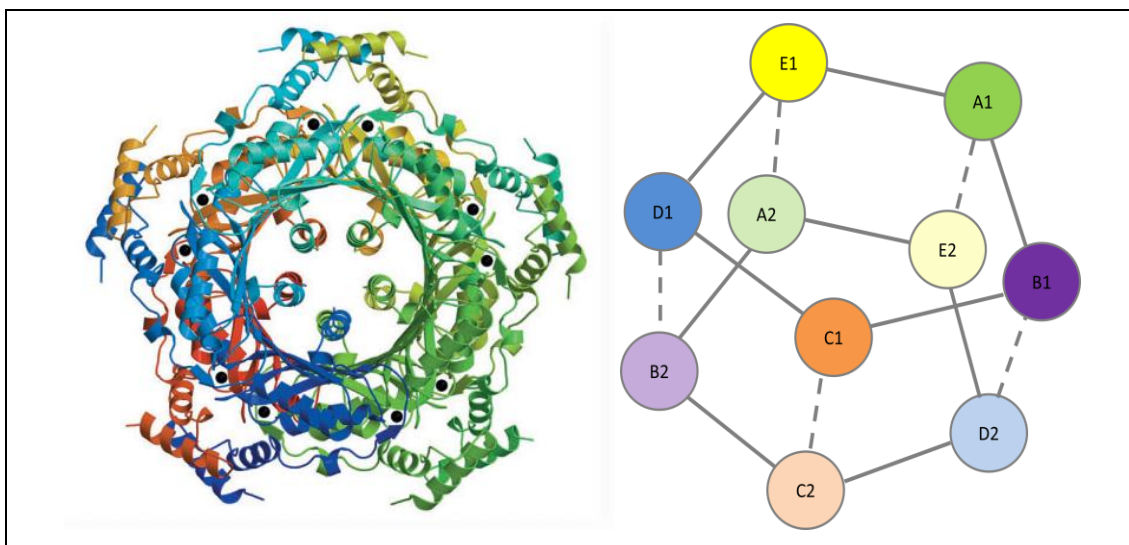
Figure 3. The 3D structure of GTP-Cyclohydrolase in *Thermus thermophilus* with a simplified schematic. Each subunit is represented by a different colour, with five subunits making up each horizontal pentamer. Black balls represent the active sites (Tanaka *et al*., 2005).

GTP-Cyclohydrolase is hypothesised to be a decamer (Figure 3), made up of two five-fold symmetrical pentamers, which attach to each other on the horizontal plane (Tanaka *et al*., 2005). Within each pentamer, the N-terminal β-strand of one monomer forms a hydrogen bond ladder with the C-terminal β-strand of the next.

The central cavity of the molecule is made up of C-terminal helices from each of the monomers. There are hydrogen bonds and salt bridges throughout the enzyme that provide stability and the helices in the centre are stabilised via a hydrophobic cluster that they enclose (Nar *et al*., 1995a). The active site in GTP-Cyclohydrolase is located at the interface of three different monomers, two adjoining monomers in the first pentamer and one from the other pentamer (Nar *et al*., 1995b). Due to the symmetry within the enzyme, there are 10 equivalent active sites. The high degree of conservation in sequence between species is mainly focused at the C-terminus; the N-terminus of the enzyme forms the outer-most surface and is, therefore, exposed to different conditions depending on the organism, which may account to some degree for the variability within the N-terminal sequence.
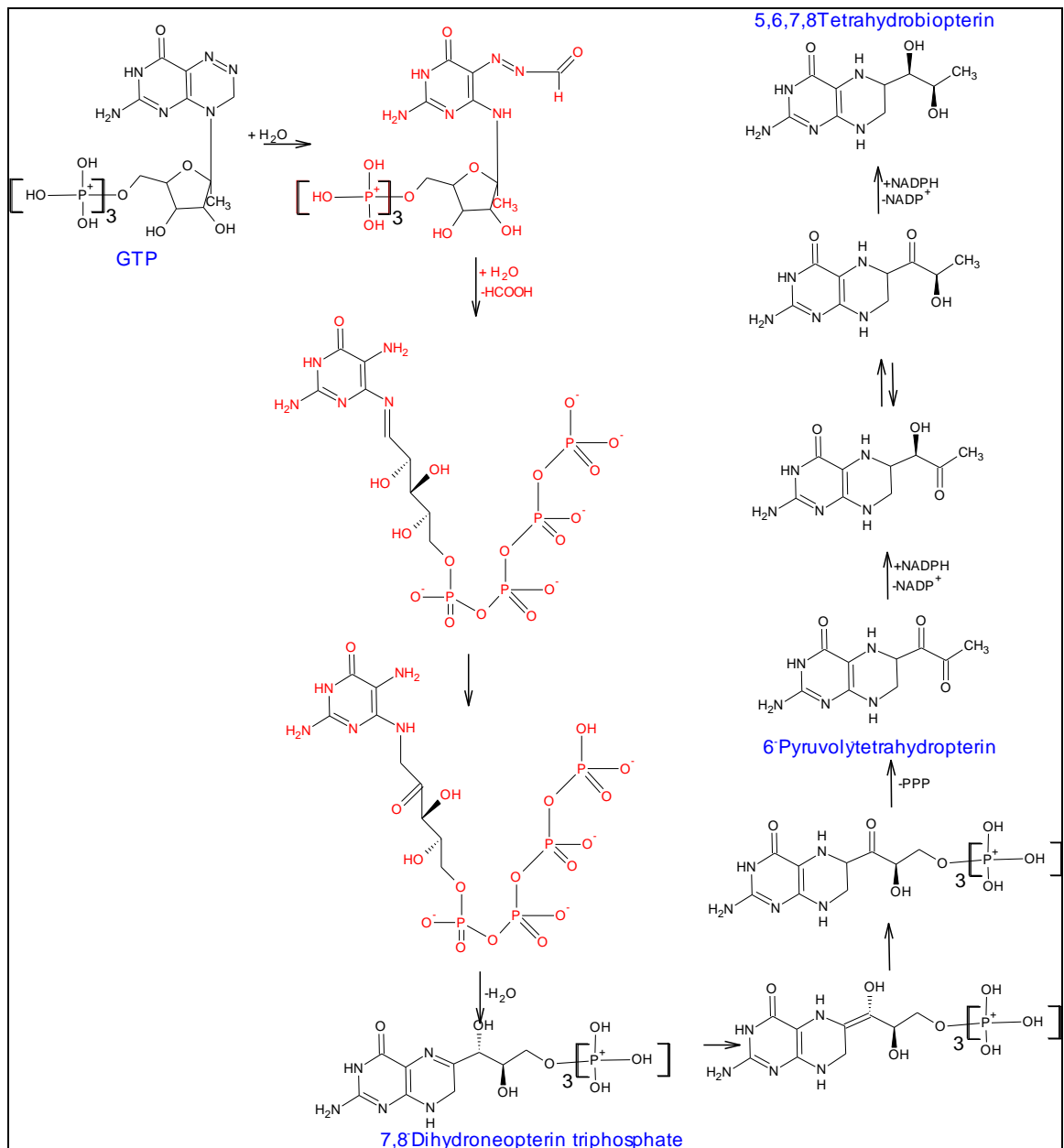
Figure 4. The biosynthetic pathway from GTP to BH$_4$. The processes in red are those that involve GTP-Cyclohydrolase function. Adapted from Thöny *et. al.* (2000), Duch and Smith (1991) and Nar *et. al.* (1995b).

The reaction pathway that converts Guanosine triphosphate (GTP) to BH$_4$ is one that is well characterised (Burg and Brown, 1966; Duch and Smith, 1991; Nar *et al.*, 1995b; Thöny *et al.*, 2000) and is shown in Figure 4. GTP-Cyclohydrolase catalyses the conversion of GTP to 7,8-Dihydroneopterin triphosphate. The conversion of this to 6-Pyruvolytetrahydropterin is then catalysed by 6-pyruvoyl-tetrahydropterin synthase (EC 4.2.3.12), and the conversion of 6-Pyruvolytetrahydropterin to

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tetrahydrobiopterin (BH_4) is catalysed by sepiapterin reductase (EC 1.1.1.153). BH_4 is an essential cofactor for a number of varied pathways within organisms; these include the aromatic amino acid hydroxylase reactions that convert phenylalanine to tyrosine, tyrosine to DOPA and tryptophan to serotonin as shown in Figure 5. BH_4 has also been shown to stimulate cell proliferation in human and rat cells (Thöny *et al.*, 2000) Much of this research has focussed on human diseases that have been linked to altered levels of BH_4, such as Alzheimer's and Parkinson's (Nar *et al.*, 1995a).

Tryptophan

Tryptophan hydroxylase $\Rightarrow$ $\Leftarrow$ Tetrahydrobiopterin (BH$_4$)

5-hydroxytryptophan

Serotonin

Phenylalanine

Phenylalanine hydroxylase $\Rightarrow$ $\Leftarrow$ Tetrahydrobiopterin (BH$_4$)

Tyrosine

Tyrosine hydroxylase $\Rightarrow$ $\Leftarrow$ Tetrahydrobiopterin (BH$_4$)

L-DOPA

Dopaquinone

2-Carboxy-2-3dihydro-5,6-dihydroxinidole

Dopachrome

5,6-Dihydroxyindole

Indole-5,6-quinone   5,6-dihydroxyindole-2-carboxylic acid

Melanin

Figure 5. Two of the biological pathways requiring tetrahydrobiopterin within eukaryotic organisms.

1.6 Nematode biogenic amines

With regards to possible control mechanisms for parasitic nematodes such as *T. circumcincta*, the biogenic amines, particularly serotonin, provide an interesting avenue of research. Unlike mammals, nematodes do not have an autonomic nervous system to regulate key processes within the individual. Instead, processes such as muscle contraction, locomotion, egg-laying and pharyngeal pumping are regulated by the biogenic amines such as serotonin and dopamine in combination with other

peptides such as the FMRFamide-related peptides (FaRPs) and *flp* genes (Hunter White *et al*., 2007; Komuniecki *et al*., 2004; Loer and Kenyon, 1993; Ranganathan *et al*., 2000; Waggoner *et al*., 1998; Waggoner *et al*., 2000). A number of specific mutant phenotypes have been identified that can be rescued with serotonin in the model nematode *C. elegans*, including the *cat-1, cat-4*[1] and *bas-1* mutants that are deficient in serotonin resulting in males being defective in specific mating behaviours. This behaviour could be restored by the addition of exogenous serotonin for all three mutants, and for the *cat-1* and *cat-4* mutants, behaviour could also be restored by the immediate precursor to serotonin, 5-hydroxytryptophan (Loer and Kenyon, 1993). Waggoner *et al*. (1998) showed that serotonin released from neurons controls the transition between the inactive and active egg-laying states in *C. elegans*. In egg-laying deficient mutants (*egl-4*, *egl-11, egl-21, egl-24* and *egl-30*), the frequency of the active egg-laying phase was reduced, but when it did occur, egg-laying appears to occur as in the wild-type. This pattern of behaviour can be reproduced when serotonergic motor neurons are laser ablated (Waggoner *et al*., 1998). While other studies have shown that entirely serotonin deficient individuals can lay eggs (Sulston *et al*., 1975), serotonin appears to play a role in egg-laying and the serotonin deficient mutants can have function partially restored with exogenous serotonin.

Further evidence for the role of serotonin in nematode physiological processes has been shown using the dauer stage of the *C. elegans* life-cycle. Under normal conditions, the nematode will develop from egg to L_1, L_2, L_3, L_4 and then adult stages in approximately three days (Brenner, 1974). If, however, there is a shortage of food, or a high concentration of larvae, individual larvae may enter a second life-cycle pathway known as the dauer pathway, at which point the larvae remain as third stage dauer larvae for many weeks. Comparisons have been drawn between the dauer stage of *C. elegans* and the L_3 stage of parasitic nematodes, as both exist for many months before resuming development after receiving an external cue. *C. elegans* individuals with loss of function mutations in the tryptophan hydroxylase

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[1] *cat-4* is the gene name for GTP-Cyclohydrolase in nematodes. For research in humans it is known as *gch1*.

gene (*tph-1*) are viable but do not produce tryptophan hydroxylase, the catalyst for the tryptophan to serotonin reaction, which requires BH_4 as a cofactor. Approximately 20 – 30 % of these mutants will enter dauer (or partial dauer) stage, even if there is an abundance of food and no over-crowding, compared to wild-type animals (Sze *et al.*, 2000). This process can be rescued with the addition of external serotonin. If the comparison between *C. elegans* dauer larvae and parasitic nematode L_3 is valid, then the high levels of GTP-Cyclohydrolase seen in the *T. circumcincta* L_3 could be linked to serotonin synthesis and the downstream hypobiotic events within the host, described earlier in Section 1.2.1.

The serotonin agonist, PAPP (*p*-amino-phenethyl-*m*-trifluoromethylphenyl piperazine), has been compared as an anthelmintic with commercially available anthelmintics for anti-nematode effects (Hunter White *et al.* 2007). In these studies, gerbils were infected with *T. circumcincta*, *T. colubriformis* and *H. contortus* L_3 and then treated with one of three anthelmintics (pyrantel, levamisole or ivermectin) or with PAPP. PAPP was effective at reducing numbers of *T. circumcincta* and *H. contortus* (although not *T. colubriformis*) in the gerbils, when administered orally at 100 mg / kg. PAPP was significantly more effective than pyrantel, as effective as levamisole but less effective than ivermectin, at inhibiting migration of the L_3 of all three nematode species, in an *in vitro* assay. Serotonin agonists could, therefore, potentially be used to kill immature larvae within the ruminant before they develop into later developmental stages. The results of this study highlight that there is potential in studying biogenic amines and their related pathways in parasitic nematodes and that these molecules could be valid targets for novel anthelmintics (as discussed in Komuniecki *et al.*, 2004).

A greater understanding of the basic biology surrounding parasitic nematode development and GTP-Cyclohydrolase in particular may therefore help provide insights into potential novel drug targets. As *T. circumcincta* produces large quantities of GTP-Cyclohydrolase transcript at the L_3 stage, it may be important for function at this stage. Until all of the biochemical pathways requiring GTP-

Cyclohydrolase have been explored, the role of the enzyme in the development of parasitic nematodes will remain uncertain. With this in mind, the role of GTP-Cyclohydrolase in *T. circumcincta* larvae will be studied in this thesis.

1.7 Thesis aims

There are a number of avenues of research that will complement each other, enabling a greater understanding of nematode GTP-Cyclohydrolase function. These include:

a) Determining the role of GTP-Cyclohydrolase in hypobiosis. This analysis will include comparison of the developmental transcription pattern of GTP-Cyclohydrolase in *T. circumcincta* and *Dictyocaulus viviparus* and in hypobiotic and non-hypobiotic strains of the latter nematode species.

b) An investigation of GTP-Cyclohydrolase enzyme function and characterisation in *T. circumcincta*. This will involve *in vitro* inhibition, antibody production for immunolocalisation and enzyme analysis across the life-cycle.

c) Studies of GTP-Cyclohydrolase function in the model nematode *C. elegans* to examine the role of GTP-Cyclohydrolase in cuticular integrity.

2 GTP-Cyclohydrolase and development in *Teladorsagia circumcincta* and *Dictyocaulus viviparus*[2]

2.1 Introduction

2.1.1 Developmental arrest and hypobiosis

Comparisons have been drawn between the infective third larval stages (iL$_3$) of parasitic nematodes and arrested dauer larvae of *Caenorhabditis elegans* (Hotez *et al*., 1993). Both are thought to be long-lived, non-feeding stages that remain in a static state until the next developmental cue (i.e. food availability in the case of *C. elegans* and ingestion by the host in the case of the parasitic nematodes). These cues signal further development to the next larval stage (Viney *et al*., 2005). In addition to the pause in development by iL$_3$, in some parasitic nematode life-cycles, later, intra-host stages can also enter arrest (or inhibition) in the host (Gibbs, 1993). Cues for developmental arrest (Armour and Bruce, 1974) within the host include environmental stress prior to ingestion, and/or host immune responses (Smith, 2007a). Hypobiosis (a specific form of arrest in response to environmental conditions) is thought to provide an opportunity for larvae to delay development in the host until external conditions improve for the next generation of environmental stages (Gibbs, 1986). Hypobiosis has been well documented in the abomasal nematode of cattle, *Ostertagia ostertagi* (see Frank *et al*., 1988) and the bovine lungworm, *Dictyocaulus viviparus* (see Strube *et al*., 2008) whereas, for other nematode species, reports are more anecdotal or region-specific (Gatongi *et al*., 1998; Prociv and Luke, 2007). Exposure of iL$_3$ to falling or low temperatures, changes in humidity and photoperiod have all been demonstrated to play a role in hypobiosis (Fernandez *et al*., 1999; Lutzelschwab *et al*., 2005). In some situations, it has been demonstrated that a combination of these factors is required; for example,

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where falling, fluctuating temperatures interact with decreasing day length to induce hypobiosis in *O. ostertagi* (see Armour, 1978). The triggers for resumption of parasite development in the host are less well documented. In some cases, emergence from hypobiosis of *O. ostertagi* can happen continuously at a steady rate irrespective of the time of initial infection (Michel *et al*., 1976a; Michel *et al*., 1976b). In contrast, in other studies on the same parasitic nematode, the emergence event appeared to be more synchronous and leads to clinical disease known as Type II ostertagiosis (Armour and Bruce, 1974).

2.1.2 Control of development in model species

Hypobiosis in certain isolates of *D. viviparus* L_5 can be induced by chilling iL_3 at 4 °C for 8 weeks prior to experimental infection (Oakley, 1979), whereas the ability to bring about experimentally-induced hypobiosis in *Teladorsagia circumcincta* or *O. ostertagi* L_4 is less reliable, with parasite isolates that have been repeatedly passaged through animals and stored at 4 ℃ losing the ability to undergo hypobiosis (Armour *et al*., 1967).

Adult *D. viviparus* live in the mainstem bronchi and proximal trachea of cattle causing coughing, weight loss, and sometimes, death. The life-cycle of *D. viviparus* is similar to that of other trichostrongylid nematodes and is shown in Figure 6. Hypobiosis in *D. viviparus* can be induced artificially in the laboratory by chilling L_3 at 4 °C for a number of weeks prior to infection, a feature that makes *D. viviparus* an exemplar species for studying the process and the pathways involved in hypobiosis. For calves to maintain strong immunity to *D. viviparus*, they must be regularly exposed to L_3 challenge, one of the reasons why the *D. viviparus* irradiated larval vaccine (Bovillis® Huskvac, Intervet) may be most effective in endemic areas (Schnieder *et al*., 1993). The need for specific antigenic challenge to help maintain immunity may provide a reason for the synchronous resumption of development of larvae in spring (Michel and Shand, 1955), as emergence could occur at the point at which the antigen-specific immune responses have diminished through a lack of exposure during housing.

Adult lungworm live in the mainstem bronchi and proximal trachea

The L_4 moult to L_5 in the bronchioles. If larvae of certain isolates are chilled before ingestion, hypobiosis can occur at this stage

Once inside the mesenteric lymph, the L_3 moult to L_4 and travel via the lymph to the lungs

L_3 penetrate the intestinal mucosa after ingestion

Females produce eggs that contain fully-developed L_1

L_1 hatch and are coughed up the trachea, where they are swallowed and passed out in the faeces

L_1 develop into L_3 in the faecal pat

The L_3 migrate onto grass, or are dispersed by fungi

Figure 6. The life-cycle of *D. viviparus*.

In contrast to what is known about the role of environmental cues for hypobiosis, the molecular and biochemical mechanisms underlying this phenomenon in parasitic nematodes are poorly understood (Nisbet *et al.*, 2004). In *C. elegans*, serotonin is crucial for maintaining the correct life-cycle pathway in different environmental conditions. A 'normal' life-cycle pathway occurs when there are abundant food resources and no overcrowding of worms. The dauer pathway occurs if resources are scarce, as shown in Figure 7.
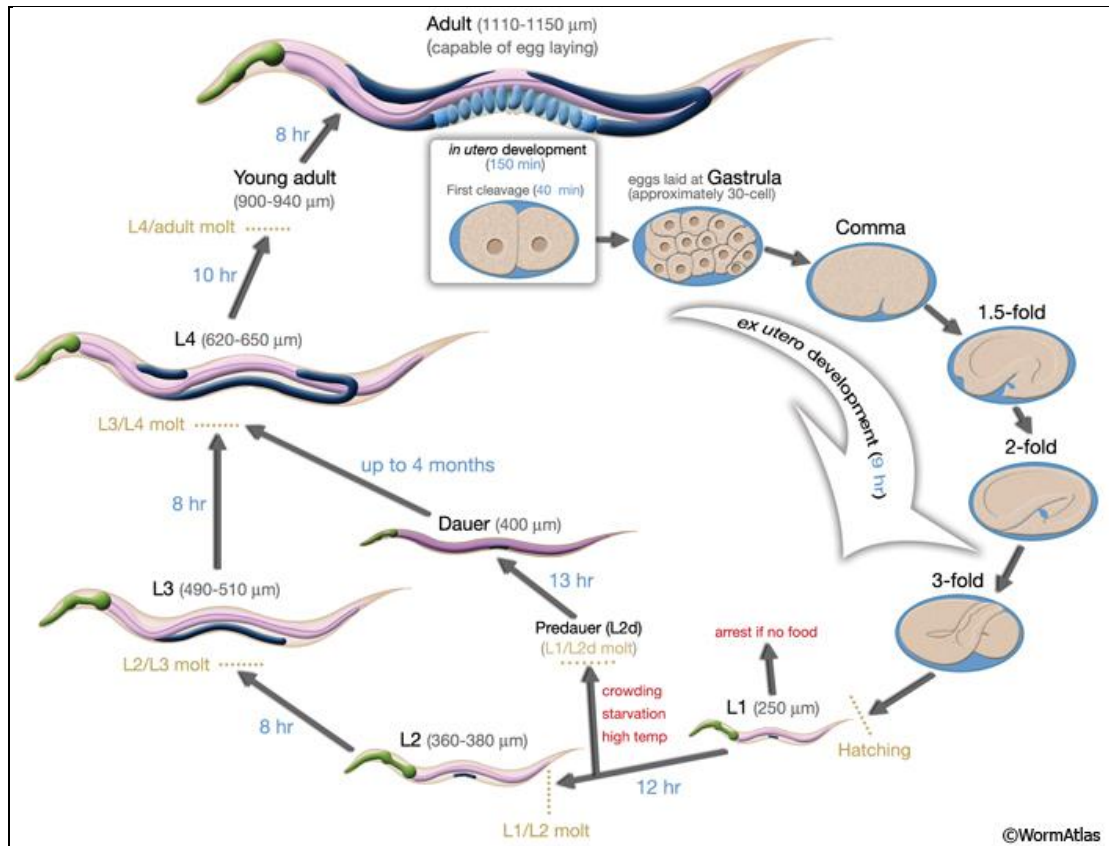
Figure 7. The life-cycle of *C. elegans*. Under optimum conditions, the worms develop through the outer circle of stages in the times shown in blue. If food is scarce or there is overcrowding, then larvae will enter the dauer pathway until conditions improve. Reproduced with permission from Wormatlas.org (Altun and Hall, 2009).

C. elegans individuals with a deletion of the tryptophan hydroxylase gene (*tph-1*) are viable, but do not produce tryptophan hydroxylase, an enzyme required for the synthesis of serotonin from tryptophan (Sze *et al.*, 2000). Of these mutants, 10 - 15 % enter dauer stage when there is an abundance of food, with a further 10 - 15 %

forming partial dauer larvae, i.e. worms in which there is incomplete expression of all classical dauer characteristics and a tendency for a more transient dauer state. This process can be reversed with the addition of external serotonin. Compared to the wild-type, 92 % of L_2 and L_3 *tph-1* mutants accumulate larger fat reserves, preparing individuals for entry into the dauer state, even if they do not become dauer larvae (Sze *et al*., 2000). As BH_4 is an essential cofactor of TPH-1, it follows that there is a potential role for GTP-Cyclohydrolase in the control of nematode developmental events. *C. elegans* dauer larvae also produce higher levels of *cat-4* mRNA compared to other larval stages (Jeong *et al*., 2009). The high levels of *cat-4* transcript and, therefore, potentially high levels of GTP-Cyclohydrolase protein are unlikely to be involved in producing serotonin while the larvae remain as dauer, since producing large quantities of serotonin would trigger the exit from the dauer state (Moussaif and Sze, 2009). It is possible, therefore, that GTP-Cyclohydrolase is accumulated either as transcript or protein in advance for transition out of the dauer state. Such accumulations have been observed to occur in *C. elegans* with other proteins; for example, two of the five most abundant dauer SAGE (Serial Analysis of Gene Expression) tags have been shown to be G-protein-coupled receptors hypothesised to be the key mono-receptors involved in triggering dauer exit (Jones *et al*., 2001).

The objective of the work presented in this chapter was to compare the transcription of the gene encoding GTP-Cyclohydrolase in two different *D. viviparus* isolates, one which is capable of entering environmentally-induced hypobiosis and one which is not. This was done to test the hypothesis that the high levels of transcript encoding GTP-Cyclohydrolase in iL3 of trichostrongylid nematodes have a role in developmental regulation through regulation of serotonin synthesis. Current procedures for the naming of parasitic nematode genes are based on their homology with genes in *C. elegans*, in which genes are named for their mutant phenotype (Beech *et al*., 2010); as such, in this thesis the GTP-Cyclohydrolase gene will be referred to as *cat-4* with the appropriate three letter species prefix. An analysis of the transcript levels of the gene encoding GTP-Cyclohydrolase across different life-cycle stages of *T. circumcincta* was also undertaken as transcript data for *Tci-cat-4* had

only been described previously in the iL$_3$ and L$_4$ stages. Alongside this, transcript levels of the gene encoding phenylalanine hydroxylase (*pah-1*) in *T. circumcincta* were investigated. As shown in Figure 5, phenylalanine hydroxylase is one of the enzymes that requires BH$_4$ as a cofactor in the melanisation pathway, so the effects downstream in the pathway could be assessed.

2.1.3 Quantitative PCR

Real-time reverse transcription polymerase chain reaction (RT-PCR) has become one of the most common methods of analysing gene transcript levels in a wide variety of organisms and has been used to address various hypotheses (Vandesompele *et al*., 2002). First, experiments can be undertaken to determine if an organism is present, for example, testing lettuce for the presence of noroviruses (Baert *et al*., 2008) or detecting Colorado Tick Fever by screening human blood for the presence of viral mRNA (Lambert *et al*., 2007). These experiments produce a positive or negative result, based on the number of cycles needed to amplify a specific gene. The other main use of real-time RT-PCR is in the quantification (either relative or absolute) of gene transcript within different tissues or at different time points, known as quantitative PCR or qPCR (Karlsson *et al*., 2007; Whitten *et al*., 2007). It is standard practice when performing qPCR to use internal reference genes (or housekeeping genes) with stable transcription levels when exposed to the experimental treatment. These are genes whose products are synthesised in all cell types within the organism. Traditionally, it was hypothesised that these genes did not vary in their transcript levels between cell types or over time, but several studies have shown that this is often not the case, and in fact, specific transcript levels can vary quite dramatically (Thellin *et al*., 1999). To minimise errors caused by using inappropriate reference genes, a research group from Ghent University has developed a computer program (geNorm) that enables analysis of a number of reference genes and the selection of the best possible cohort for a particular experiment (Vandesompele *et al*., 2002). This programme works on the principle that the most appropriate and stable reference genes will stay at a constant ratio to each other, irrespective of the environment or stage of development. By using this ratio rather than the measure of a single gene transcript, the process of real-time RT-PCR analysis should be more

accurate. The geNorm program has been used to examine transcript levels in a number of different parasite species, including *O. ostertagi*, and the results highlighted that several previously used reference genes were not appropriate (van Zeveren *et al.*, 2007). These studies also demonstrated that the stability of a housekeeping gene in one nematode species does not imply stability in another; for example, one of the genes with the most reliable levels of transcript used in a study on the canine hookworm *A. caninum* (see Trivedi and Arasu, 2005) was the least stable of the genes tested for *O. ostertagi* (see van Zeveren *et al.*, 2007).

A previous study of *D. viviparus* transcript levels using the geNorm software enabled accurate normalisation to be performed for quantitative real-time PCR (qPCR) (Strube *et al.*, 2008). Once this had been done, a seven-fold difference in transcript levels of protein disulfide isomerise 2 *(pdi-2)* was observed between the L_1 and egg stages. This difference was not observed when *pdi-2* transcript levels were normalised to a single housekeeping gene, highlighting the importance of accurate normalisation. To date, no analysis of the appropriate reference genes for use in qPCR studies of *T. circumcincta* has been published. This was undertaken as part of the work presented here. Once this was completed, analysis of transcript levels of *cat-4* in both *D. viviparus* and *T. circumcincta* across different life-cycle stages was performed, alongside a comparison between two different isolates of *D. viviparus* that had been shown previously to differ in their ability to undergo temperature-associated larval arrest.

2.2 Materials and methods

2.2.1 Parasite Material

2.2.1.1 *D. viviparus* material

Parasite material was collected from both the *D. viviparus* field isolate HannoverDv2000 (Strube *et al.*, 2008), which can undergo hypobiosis, and the Intervet vaccine isolate that does not appear to undergo hypobiosis. L_1 were extracted from the faeces of *D. viviparus* infected calves and stored in tap water for 14 days at room temperature to allow development to L_3 (Baermann, 1917) or were stored immediately at -80 °C in 5.5 M guanidinium isothiocyanate. This latter method of storage was also used for all other life-cycle stages. To assess the effects of cold-conditioning on transcript levels of *Dvi-cat-4*, L_3 of both HannoverDv2000 and Intervet vaccine isolates were chilled for 8 weeks at 4 °C and designated as L_3i (hypobiosis induced L_3). L_4 were collected via lung perfusion at post-mortem from infected calves at 7 days post infection. After a final moult in the host, L_4 develop into pre-adult stages (see Figure 6) that are designated herein as L_5 for easier terminology. Non-hypobiotic L_5 of the HannoverDv2000 field isolate, as well as the Intervet vaccine isolate, were collected at 15 days post infection by perfusion. Hypobiotic L_5 (HannoverDv2000 field isolate) were collected at 27 days after infection with cold-conditioned L_3i. Larvae were confirmed as hypobiotic if they were less than 5 mm in length at this time point, as under normal conditions, adult worms should be greater than 5 mm. Adult worms were collected 28 days post infection after infection with L_3 of the HannoverDV2000 isolate. In addition to the material collected for developmental gene transcript analysis, to assess *Dvi-cat-4* (the gene encoding GTP-Cyclohydrolase in *D. viviparus*) transcription levels during the free-living stages, samples were taken daily during culture of L_1 to L_3 from both the HannoverDv2000 field isolate and the Intervet vaccine isolate.

2.2.1.2 *T. circumcincta* material

To collect eggs and L_1, several grams of faeces were taken *per rectum* from a male sheep infected with MTci2 (an anthelmintic-susceptible laboratory isolate from Moredun Research Institute), mixed with tap water to create a liquid suspension and

homogenized using a stomacher (800, Seward). The suspension was washed through a column of stainless steel sieves (Fisher Scientific) with mesh sizes of 1 mm; 212 µm; 63 µm and 38 µm, respectively. The retentate containing the eggs was collected from the 38 µm sieve. This was decanted into a 15 ml Beckmann polyallomer centrifuge tube and centrifuged at 203 $\times g$ for 2 min. The supernatant was removed with a vacuum line and the faecal pellet re-suspended in 10 ml of saturated sodium chloride solution (NaCl; Ministry of Agriculture, 1986) before centrifugation for a further 2 min at 203 $\times g$. Due to the relative density of the saturated NaCl solution, the eggs rose to the surface. The centrifuge tubes were clamped just below the meniscus using artery forceps and the contents of the upper portion poured over a 38 µm sieve where they were washed thoroughly with tap water to remove any residual NaCl. Eggs were snap frozen and stored in liquid nitrogen or incubated in a Petri dish for 18-24 h at 25ºC to allow development to L_1 before snap freezing.

For the collection of L_3, a male sheep infected with MTci2 was harnessed to collect faecal output daily. The faeces were cultured for 10 days at 22 °C in a tray covered with a polythene bag that had been punctured to allow aeration. The tray was then flooded with tepid tap water (at approximately 22 °C) for 4 h. The faecal material was poured through a 1 mm sieve and the filtrate collected. The filtrate was sedimented overnight and the volume reduced by aspiration. The L_3 (contained within the sediment) were Baermannised and snap frozen in liquid nitrogen. One harvest of L_3 was stored at 4 °C for 10 weeks with 50, 000 L_3 removed and stored at two weekly intervals in liquid nitrogen.

To collect parasitic stages, nine month-old helminth-free lambs were infected with 200,000 and 50,000 MTci2 respectively and euthanased at 7 and 28 days post infection to recover L_4 and adults. The abomasum was removed through a midline incision and transferred to a clean bucket to be opened lengthwise and washed with 1 L warm saline (0.85 % NaCl). The contents were sedimented and the volume adjusted so that there was twice the volume of fluid supernatant as sediment. Next, 9 g of technical grade agar (Sigma) were dissolved in 500 ml water and heated to

approximately 56 °C. Once this had cooled to approximately 45 °C, it was mixed with the digesta. This mix was poured over a 1 mm mesh with a detachable lid underneath. The lid was removed after the mixture had set and the mixture was transferred to a large filter funnel containing warm physiological saline (0.85 %). After incubation of 4 h at 39 °C the worms had migrated out of the agar, into the saline. The worms on the surface of the agar were harvested using forceps and the worms that had sunk to the base of the funnel were recovered using a tap at the base to ensure only a small volume of saline was collected. The worms were then sedimented in a smaller container and the volume reduced further using a vacuum line before the worms were snap frozen and stored in liquid nitrogen.

2.2.2 Complementary DNA and genomic DNA production

To produce complementary DNA (cDNA) for quantitative PCR, parasite material was homogenised using the TissueRuptor (Qiagen) (*D. viviparus*) or with a mortar and pestle in liquid nitrogen (*T. circumcincta*), and mRNA extracted using the Illustra™ QuickPrep Micro mRNA Purification Kit (GE Healthcare) according to the manufacturer's instructions. The quantity of the mRNA was confirmed using a spectrophotometer (Nanodrop$^®$ ND-1000 UV-Vis Spectrophotometer) and then 300 ng mRNA used to synthesise single stranded cDNA using Sprint™ Powerscript™ PrePrimed Single Shots with Oligo (dT)$_{18}$ Primers (Clontech). The cDNA was diluted 1:20 with molecular biology grade water before use and stored at -20 °C. For each life-cycle stage analysed for both species, separate mRNA extractions and cDNA syntheses were performed on separate pools of parasite material, providing biological replicates for each stage. Genomic DNA (gDNA) was prepared from L$_3$ of both species using the DNeasy blood and tissue kit (Qiagen) as per manufacturer's guidelines and stored at 4 °C.

2.2.3 Sequencing *Dvi-cat-4*

RNA was extracted from *D. viviparus* iL_3 and a pool of mixed life-cycle stages. Parasites that had been stored in liquid nitrogen were crushed in liquid nitrogen using a mortar and pestle. Trizol® reagent (Invitrogen) was used to extract total RNA according to the manufacturer's protocol. The concentration of RNA was measured using a spectrophotometer and the level of integrity assessed visually by agarose gel electrophoresis (Section 8.1.1). Total RNA was reverse transcribed to cDNA using the SuperScript Reverse Transcriptase II kit (Invitrogen). Amplification of the partial *Dvi-cat-4* gene was achieved using PCR with degenerate primers designed from the conserved regions of gene sequences for a number of different nematodes (as shown in Figure 8) and L_3 cDNA as a template. The sequences for the degenerate primers were: 5'-GTGARGACGTVAATMGKCAAGG-3' and 5'-CCACGCATAACCATRCACAT-3'. Reaction conditions were as follows: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min followed by a final 72 °C extension for 10 min. PCR products were visualized by gel electrophoresis on 1 % agarose gels with GelRed (Cambridge Bioscience). An amplicon of the appropriate size was purified using the Qiaquick PCR purification kit (Qiagen), ligated into pGEM-T vector (Promega) and transformed into JM109 competent *E.coli* cells (Promega). The transformed plasmids were incubated for 1 h at 37 ° C in a shaking incubator in the presence of SOC medium and then spread on Luria-Bertani (LB) medium plates containing 100 μg / ml ampicillin. The plates also contained 0.5 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside; Promega) and 80 μg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside; Promega) to enable alpha complementation to be used as a screening tool for the plasmids containing a DNA insert. Colony PCR was performed on white colonies using T7 and SP6 primers, with positive colonies selected for culture. Plasmids were amplified in 10 ml LB medium with 100 μg / ml ampicillin at 37 ° C for 16 h in a shaking incubator. Plasmids were purified using the Wizard plus SV kit (Promega) and sequenced (Eurofins MWG). Using the resulting sequences, gene-specific oligonucleotide primers were designed and rapid amplification of cDNA ends (RACE) performed to extend the sequence of the gene to the 3' and 5' ends. RACE-ready cDNA was synthesised using the SMART RACE cDNA Amplification Kit (Clontech) and

Touchdown PCR performed using appropriate gene-specific primers (Figure 9) and conditions detailed by the manufacturers. This process was repeated until full-length sequence was produced, with internal primers (Figure 9) used to confirm positive colonies once the initial sequencing results had been verified. A final PCR was performed to confirm that the full length sequence existed in nature using cDNA and primers that incorporated the initiation and termination codons. Assembly of the sequences was performed using SeqMan (DNASTAR). Neighbour-joining analysis of the amino acid sequences of GTP-Cyclohydrolase from a number of different species was performed using ClustalX, bootstrapped 1000 times and viewed using Treeview (Larkin *et al*., 2007).

```
P. penetrans      --------------------CCTCTCGTCTGACAGCTCCAGCGAGGACTGCGATCAGAAGCTTATTGCTTTATCCAAGAAGACCTCAAACTTG-GACAAAATGACGGCCGCCTATTCATC
T. spiralis       --------------------CTTTCATCTGACAGCTCCAGCGAGGATTGCGATCACAAG-ATATCGCTTTTTCTAAGAAGACCTCAAACTTG-GACAAAATGACGGCCGCCTATTCATC
O. ostertagi      ATGGCAAGTGAAAGCGGTTTCCTTTCATCTGACAGCTCCAGCGAGGATTGCGATCAGAAGATTATCGCTTTTTCTAAAAAGACCTCAAACTTG-GACAAAATGACGGCCGCCTATTCATC
T. circumcincta   ATGGCTAGCGAAAGCGGTTTCCTTTCGTCTGACAG------CGAGGACGTGCGATCAGAAGATTATCGCCGAACTCAAATACACTTCAAACTTG-GATAAAATGACGGCCGCGTATTCATC
H. contortus      --GGCTACTGAAAGCGGTTTTATCTCGTCCGACAGCGCAAGCGAGGATGCCGAGCATAAGACTATTGCTGAATTCAAGAAGACATCAAACTTGTGTGAAAGAATGGCTGACGCCTACTCTTC
                            .* **.** *****       ******   *** ** ****** **    ::    **.:* ** ********* ** *.****.* *.*** ** **:**

P. penetrans      GATTATATCCCATGTTGGTGAAGACGTCAATCGTCAAGGACTTCTGAAAACTCCCGACCGGGCTGCCAAAGCAATGCTGTACTTCACAAAAGGCTACGAACAACAGCTTGACGATATTTT
T. spiralis       AATTATATCCCATGTTGGTGAAGACGTCAATCGTCAAGGACTTCTGAAAACTCCCGACAGGGCTGCCAAAGCAATGCTGTACTTCACAAAAGGCTACGAACAACAACTTGACGATATTCT
O. ostertagi      AATTATATCCCATGTTGGTGAAGACGTCAATCGTCAAGGACTTCTGAAAACTCCCGACAGGGCCGCCAAAGCAATGCTGTACTTCACCAAAGGCTACGAACAACAACTTGACGATATTTT
T. circumcincta   AATAATATCCCATGTTGGTGAAGACGTGAATCGTCAAGGCTTCTGAAAACTCCTGAGAGGGCTGCCAAAGCAATGCTGTACTTCACAAAAGGCTACGAACAACAACTTGACGATATCCT
H. contortus      AATTATCTCTCATGTTGGTGAGGACGTAAATAGGCAAGGACTCTTGAAAACCCCTGAAAGGGCTGCGAAAGCGATGTTGTATTTTACGAAAGGATACGAGCAACAATTAGATGATATTTT
                  .**:**.** *********** ***** ***.* *****.**  ******* ** ** .* *** ** *****.*** **** ** ** *****.*****.*****. *:** ***** *

P. penetrans      GAACGATGCTGTGTTCGACGAGGATCACGATGAAATGGTTATCGTAAAAGATATTGAGATGTTCTCACTATGTGAACATCATCTAGTGCCATTCAATGGAAAGGTTCACATTGGATATAT
T. spiralis       GAACGATGCTGTGTTTGACGAGGATCACGATGAAATGGTTATCGTAAAAGATATTGAGATGTTCTCACTATGTGAACATCATCTAGTGCCATTCAATGGAAAGGTTCACATTGGGTATAT
O. ostertagi      GAACGATGCCGTGTTTGACGAGAATCACGATGAAATGGTTATCGTGAGAGATATCGAAATGTTCTCACTATGTGAACATCATCTAGTGCCATTCAATGGAAAGGTTCACATTGGGTACAT
T. circumcincta   GAACGAAGCTGTGTTTGACGAGAATCACGATGAAATGGTTATCGTGAAAGATATTGAAATGTTCTCTCTATGTGAGCATCATCTAGTGCCATTCAATGGAAAGGTTCACATTGGGTATAT
H. contortus      GAATGAAGCGGTCTTCGATGAGGATCACGATGAAATGGTCATTGTGAAGGACATAGAAATGTTTTCGCTATGCGAACATCACCTTGTGCCGTTTAATGGAAGGGTGCACATCGGGTATCT
                  *** **:** ** ** ** ***.***************** ** **.*..** ** **.***** ** ***** **.***** **:***** ** ******* ***.***** **.** .*

P. penetrans      ACCAAACAAAAAAGTTCTAGGCCTCTCAAAACTGGCTCGAATTGTTGAGATGTTCAGCCGACGACTCCAAGTACAAGAACGACTAACGAAGCAAATAGCGACGGCAATGGTGCAAGCGGT
T. spiralis       ACCGAACAAAAAAGTTCTAGGCCTCTCAAAACTGGCTCGAATTGTTGAGATGTTCAGCCGACGACTTCAAGTACAAGAACGACTAACAAAGCAAATAGCGACAGCAATGGTGCAAGCGGT
O. ostertagi      ACCGAACAAAAAAGTTCTAGGCCTCTCAAAACTGGCTCGAATTGTTGAGATGTTCAGCCGACGACTTCAAGTACAAGAACGACTAACAAAGCAAATAGCGACAGCAATGGTGCAAGCGGT
T. circumcincta   ACCGAACAAAAAAGTCCTAGGCCTTTCAAAACTGGCTCGAATTGTTGAGATGTTCAGCCGACGACTTCAAGTACAAGAACGACTAACAAAGCAAATAGCGACGGCAATGGTGCAAGCGGT
H. contortus      GCCCAACAAAAAAGTTCTGGGTCTCTCAAAATTGGCTCGAATTGTCGAGATGTTTAGTCGACGACTTCAAGTACAAGAACGGTTAACTAAGCAGATAGCGACAGCAATGGTTCAGGCAGT
                  .** ********** **.** **.** ** ****** ************* ** ******** *************.* **** ***** .********.******** ** **.**

P. penetrans      TCATCCTGCCGGTGTTGCAGTCGTCATTGAGGCAAGTCACATGTGCATGGTTATGCGAGGAGTCCAGAAAATCAATGCTACCACCTCCACATCCTGCATGCTTGGAGTATTTAGGGAAGA
T. spiralis       TCAACCTGCCGGTGTCGCAGTTGTCATTGAGGCGAGCCACATGTGTATGGTTATGCGTGGAGTCCAGAAAATCAATGCTACCACCTCCACGTCCTGCATGCTCGGAGTATTTAGGGACGA
O. ostertagi      TCAACCTGCCGGTGTCGCAGTTGTCATTGAGGCGAGCCACATGTGTATGGTTATGCGTGGAGTTCAGAAAATCAATGCTACCACCTCCACATCCTGCATGCTCGGAGTATTTAGGGACGA
T. circumcincta   TCAGCCTGCCGGTGTTGCAGTTGTGATAGAGGCAAGTCACATGTGCATGGTTATGCGTGGAGTTCAGAAGATCAATGCCACCACCTCCACATCCTGCATGCTTGGAGTGTTTAGGGAAGA
H. contortus      TCAACCTGCTGGTGTTGCAGTAGTTATTGAAGCAAGCCACATGTGCATGGTTATGCGTGGAGTTCAGAAAATAAATGCGACGACGTCCACCTCCTGTATGCTCGGAGTGTTTAGGGATGA
                  *** ***** ***** ***** ***** ** **:**.** ******* ***** ***********:***** *****.**.***** ** ** ***** ***** ***** *****.*******

P. penetrans      CCCCAAGACAAGAGAGGAATTCTTGAATCTCATTAACAAAAAGTGA
T. spiralis       TCCAAAAACGAGAGAAGAATTCTTGAATCTCATTCACAA---GTGA
O. ostertagi      TCCAAAAACGAGAGAAGAATTCCTGAATCTCATTCACAA---GTGA
T. circumcincta   CCCAAAAACCAGAGAAGAATTCTTGAATCTCATCAACAAAAAGTGA
H. contortus      TCCAAAGACGAGAGAAGAATTTTTGAATCTCATCAACAAGAAGTGA
                   **.**.** *****.***** ** ********** .****   ****
```

Figure 8. Alignments of the *cat-4* mRNA sequences from five different parasitic nematodes used to generate degenerate primers using GTP-Cyclohydrolase sequences from *Pratylenchus penetrans* (accession number not assigned), *Trichinella spriralis* (XP_003380282), *Ostertagia ostertagi* (O61573), *Teladorsagia circumcincta* (FR726162) and *Haemonchus contortus* (AW670739). High sequence homology enabled primers with low degeneracy scores to be designed. The yellow highlighted region shows the location of the oligonucleotide primers.

```
TTTAATTACCCAAGTTTGAGAATATCGATGACTACTGAGAGTGGCTTTGTTTCATCTGACAGCGCA
AGTGAGGAAACTAACGATCAGAAGGCTATCACTCAGCTGAAAAAGAAAACCTCAAATTTGGAAAAT
ATGGTGTCAGCATATTCATCAATTATTTCTCATGTTGGTGAAGATGTGAATCGACAAGGTCTYCTA
AAAACACCAGAAAGAGCAGCAAAAGCGATGTTGTATTTCACGAAAGGATACGAACAACAGCTAGAC
GAGATTTTGAACGAAGCCGTTTTTGATGAAGATCACGATGAAATGGTAATTGTAAAAGACATCGAA
ATGTTCTCGTTATGTGAACATCATTTAGTACCATTCAATGGCAAAGTTCACATTGGATACATTCCA
AATAAAAAAGTGCTCGGATTGTCTAAACTCGCTCGAATAGTGGAGATGTTCAGTCGACGACTACAA
GTACAAGAACGCTTAACAAAGCAAATTGCAACTGCAATGTTRCAAGCTGTACAGCCAGTTGGTGTT
GCAGTTGTTATTGAAGCGAGTCACATGTGTATGGTGATGAGAGGTGTTCAAAAAATTAACGCTACA
ACATCAACGTCATGTATGCTCGGTGTATTCAGAGATGATCCAAAAACTCGCGAAGAATTTTTAAAT
CTTATCAACAAAAAATAACCACATAACATTGTGTTGTTGTTGTTGTTGTTGTTGTTTTTTTTCCTT
CCTGGAGGCATATACGAATGCAACTATTTGTGTTGCATGTATGTATATGAAAATAAGGAACGTTTC
CAATATTATATGAATATTTACGCAGGGACGTCACTTTCTCGAAAGTTTTTTTTTTGAGGAAGAGA
GTTTCCGGCTTATTTTTTTCCCTCATGTCACTCAATAGCTGCTTTTAGGAGGTTTAATTCGATCTT
TTTTTTTTTCCTTTTACTTACGCATAAACAAACATAAGTACTTTAATCTGTATGTTTATATTTTAA
ACATTTCCATCAATACAGTATTTTTAGAATTTTTTCCTCACATGTTTATCATTTTTTTTATTGAT
TGAGGACAATGAGTGAACAGTTTCAAATTGTTGCCGTAATTTTAATTGCAATTATTGTATTGCTAT
GATTAAGATAAATAATAAAATTGTTTTTAACAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
```

```
3' RACE Forward Primer    CACCAGAAAGAGCAGCAAAAGCGATGTTGTA
5' RACE Reverse Primer 1  CCTCTCATCACCATACACATGTGACTCGCTTC
5' RACE Reverse primer 2  CGGCTTCGTTCAAAATCTCGTCTAGCTGTTG
Internal Forward Primer   GATGAAGATCACGATGAAATGG
Internal Reverse Primer   TTGCAGTTGCAATTTGCTTT
```

Figure 9. The location of oligonucleotide primers used to generate full-length sequence of the *Dvi-cat-4* gene and internal oligonucleotide primers used for PCR amplification of clones. The untranslated regions are shown in grey, the initiation and termination codons are underlined and the oligonucleotide primers are in colour.

2.2.4 Generation of plasmid standards for qPCR

PCR was carried out using cDNA from *D. viviparus* and *T. circumcincta* L$_3$ as a template with specific oligonucleotide primers for each of the genes of interest (*Dvi-cat-4*, *Tci-cat-4* and *Tci-pah-1*) and the reference genes (*β-tubulin, elongation factor-1α, 60S Ribosomal Protein* and *gapdh*) for each species to generate amplicons of 200-450 basepairs (bp; primer sequences shown in Table 1). PCR was carried out using the Advantage 2 Polymerase system (Clontech) to ensure proofreading activity, with primers designed to span multiple exons (where genomic structure was known) performed under the conditions 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s and 68 °C for 90 s, with a final extension step of 10 min at 68 °C. These amplicons were purified using a PCR purification kit (Qiagen), cloned using the TOPO TA cloning kit (Invitrogen) and sequenced (Eurofins MWG). A spectrophotometer was used to determine the concentration of DNA in preparations of purified plasmids from these clones and, from this value, the number of copies calculated using the formula:

$$number\ of\ copies = \frac{quantity\ (ng) \times 6.022 \times 10^{23}}{length\ (bp) \times 1 \times 10^{9} \times 650}$$

This is based on the assumption that the average mass of a purine or pyrimidine residue of DNA is 650 Daltons, therefore, 1 M bp equals 650 g. Avogadro's number (6.022×10^{23}) can then be used to calculate the number of molecules per Mole. 1×10^{9} converts the mass from grams into nanograms.

Plasmids were diluted to 1×10^{9} copies / μl and a ten-fold serial dilution generated with concentrations ranging from 1×10^{9} to 1×10^{0} copies / μl.

Table 1. Oligonucleotide primer sequences used to generate plasmid standards for qPCR

Gene	Forward primer sequence		Reverse primer sequence		Predicted product size (bp)	
	5'	3'	5'	3'	cDNA	gDNA
Tci β-tubulin	TTCCATTCCCTCGTCTTCAC		AGCCATTTTCAATCCACGAG		320	376
Tci gapdh	TCGGAATTATCGAAGGGTTG		CCATGAAATGAGCTTGACGA		437	515
Tci 60S	AAACAATGGGTCGCCGGA		GCAGCAACAACGGTGCT		240	240
Tci ef-1α	TGAGCGTAAGGAAGGAAATG		CGTATAACCAGCAGCGATTT		428	486
Tci-cat-4	CGTCAAGGGCTTCTGAAAAC		GGTGGTGGCATTGATCTTCT		414	1725[3]
Tci-pah-1	TTGGAGCATCAGACGAAGTG		TGAGCTGCAGCACTGACTTT		436	436
Dvi-cat-4	GGGGAACGATCAGAAGGCTATC		TTGCAGTTGCAATTTGCTTT		425	1421
Dvi β-tubulin	TGGTTCGAAGTTTTGGGAAG		ACATCCTTCAGCCTCTTTACG		334	1042
Dvi ef-1α	TCATGTCGACTCAGGCAA		TGACCTGGGGCATCAATT		243	553

[3] When these primers were designed, no introns had been located within the *Tci-cat-4* gene (see Section 2.3.6) and as such the predicted gDNA size was 414 bp.

2.2.5 qPCR

PCR was performed, using genomic DNA of both parasite species as template, to generate sequences for each of the genes of interest (both *cat-4* genes, *Tci-pah-1* and all of the reference genes) using the same primers and conditions as for the plasmid standard generation (Table 1). From these sequences, oligonucleotide primers and Minor Groove Binding (MGB) hydrolysis probes for qPCR were designed using Primer Express software (Applied Biosystems) and purchased from Invitrogen and Applied Biosystems; these are shown in Table 2. Probes were designed across intron / exon junctions where possible to ensure amplification signals from cDNA only and were labelled with the fluorescent dye HEX or FAM at the 3' end and a non-fluorescent quencher at the 5' end. Standard curves were generated using plasmids containing cDNA representing the reference genes or genes of interest, diluted 1/10 over a range of 10^1-10^7 copies / µl. Negative controls were identical but with no template added. For each life-cycle stage described in Section 2.2.1, there were at least three biological replicates (n=3 for all *D. viviparus* samples, for *T. circumcincta* samples: n=5 for eggs and adults, n=6 for L1 and L3, n=7 for L4), each sample was run in duplicate on the same plate and each plate was replicated at least twice to provide technical replicates. For the L_3 that had been chilled for 10 weeks, there was only one biological replicate at each time-point.

2.2.5.1 *D. viviparus* qPCR

Each singleplex qPCR reaction consisted of 10.76 µl water, 12.5 µl Brilliant II mastermix (Stratagene), 0.15 µl of the relevant forward and reverse primers (50 µM each), 0.06 µl probe (10 µM), 0.38 µl reference dye (ROX) and 1 µl template. The qPCR was performed using the MX 3005p multiplex qPCR system (Stratagene) using cycling conditions of 10 min at 95 °C, followed by 40 cycles of 20 s at 95 °C, 20 s at 55 °C and 30 s at 72 °C. The levels of *Dvi-cat-4* transcript in the different life-cycle stages were evaluated using qbase software, normalizing all results to the two reference genes β-tubulin and *ef-1α* (Hellemans *et al*., 2007).

Table 2. Primer pairs and probes for quantitative PCR for both *D. viviparus* (D.v) and *T. circumcincta* (T.c). Intron / exon boundaries are identified by the underlined bases.

Species	Gene	Accession Number	Forward Primer 5' 3'	Reverse Primer 5' 3'	Hydrolysis Probe 5' 3'	Amplicon length (bp)
D. viviparus	β-tubulin	EU152298	TGAATCAGATCTTCAACTTGAAAGGA	TTCCAGGCTCGAGATCAACAA	GAAGCACAT<u>GG</u>TGGC	88
D. viviparus	*ef-1α*	EU152294	GGATTGACAAAAGAACCATTGAAA	AAAGAGCGATATCAATGGTAATACCA	CAAGA<u>GA</u>TGGGTAAGG	135
D. viviparus	*cat-4*	FR726161	TCACGATGAAATGGTAATTG	GCGTTCTTGTACTTGTAG	TGGCAAA<u>GT</u>TCACATTG	178
T. circumcincta	β-tubulin	Z69258	GCTTCAACTGTCGCTGAGCTTA	TCACAAGCAGCCATCATGTTC	AGCAAATGTT<u>C</u>GATGCC	65
T. circumcincta	*ef-1α*	FR726165	TCGTCTTCCACTTCAGGATGTCT	CGCCCGACAGGGACAGT	CAAGATCG<u>GA</u>GGTATTG	60
T. circumcincta	60S	FR726163	CAAGGACATCATCCACGATCCT	GGATCGCGGAAAGCAATG	ACGTGGTGCCCCTC	62
T. circumcincta	gapdh	CAA03897	TTGAGAAACCAGCTAGCATGGA	CGCACCCTCCGAAGCA	ACATTAAGAAGG<u>TC</u>ATCAAGT	62
T. circumcincta	*cat-4*	FR726162	GTTCAGCCGACGACTTCAAGT	CACCATTGCCGTCGCTATTT	CAAGAACGACTAACAAAG	61
T. circumcincta	*pah-1*	Not assigned	TGCCAGTGCCAAAAACAAAC	CTTGGAAAGAACGTGGAATCG	CAGGCTTGGGCGTCT	59

2.2.5.2   *T. circumcincta* qPCR

Each multiplex qPCR reaction (β-tubulin and *ef-1α* samples run together; *pah-1* and *cat-4* run together) consisted of 9.7 µl water, 12.5 µl universal gene expression mastermix (Applied Biosystems), 0.15 µl of each of the two forward and reverse primers (50 µM each), 0.1 µl of each of the two probes (50 µM each) and 2 µl template cDNA. The qPCR was performed using the ABI 7000 system (Applied Biosystems) using cycling conditions of 2 min at 50 °C (Uracil-DNA Glycosylase incubation, which helps to prevent PCR contamination by preventing the re-amplification of any previously amplified PCR products), 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The sensitivity of the reactions in multiplex was assessed by repeating assays in singleplex and the efficiencies were similar. The limit of accurate quantification was found to be 100 copies for all genes, so levels recorded below this threshold were excluded from the analysis. When standards had been verified, the levels of *Tci-cat-4* and *Tci-pah-1* transcript in the different life-cycle stages were evaluated using qbase software, normalising all results to two reference genes (Hellemans *et al*., 2007).

2.2.5.3   Statistical analysis

For each sample under investigation, the biological samples were analysed individually using the qbase software, generating mean transcript levels with standard errors generated from the technical replicates. Transcript levels were then expressed as values relative to the lowest transcript level in the egg samples. Although the data did not fail a statistical test for non-normality at the scale of the biological replicates, it was appropriate to log-transform these means since the associated standard errors indicated a high probability of non-normality at the technical replicate stratum. Weights were then assigned to each of the means in inverse proportion to the square of the associated standard error, the assigned weights varying over two orders of magnitude within the dataset.

In this form of analysis, as the standard error increases, the weight decreases and, therefore, the influence of the biological replicate on the statistical model decreases. A weighted ANOVA was fitted using Minitab (version 15 software). Using pooled estimates of the variability, comparisons between different means were made using t-tests in Microsoft Excel 2007 with p-values corrected for multiple testing using the False Discovery Rate method (Benjamini and Hochberg, 1995). Confidence intervals were calculated at the 95 % level.

2.2.6 *T. circumcincta cat-4* gene sequences derived from individual worms

L_3 were examined microscopically and live, motile L_3 were picked out individually in 1 µl PBS from a laboratory culture of the MTci2 isolate and placed in a 96-well plate containing 30 µl of worm lysis buffer (50 mM KCl, 10 mM Tris pH 8, 2.5 mM $MgCl_2$, 0.45 % Nonidet P-40, 0.45 % Tween-20, 0.01 % gelatin and 200 µg/ml Proteinase K) (Kwa *et al*., 1995). The plate was frozen for 30 min at -80 °C and incubated overnight at 56 °C to allow worm lysis to occur. Overnight lysates were heated at 95 °C for 15 min to inactivate the Proteinase K. One µl of each lysate was used as template for a series of PCR reactions containing 15 µl 2x Biomix (Bioline), 5 µl of each of the forward and reverse *cat-4* gene-specific primers at 2 µM

(forward: 5'- ATGGCTAGCGAAAGCGGTTTCC-3'

reverse: 5'-TCACTTTTTGTTGATGAGATTCAAGAATTCTTCT-3')

and 4 µl water. Cycling conditions were 94 °C for 5 min, followed by 40 cycles of 30 s at 94 °C, 30 s at 58 °C and 90 s at 72 °C, followed by a final 10 min extension at 72 °C. PCR products were analysed as detailed above, excised using a clean scalpel, purified using the Qiaquick gel extraction kit (Qiagen), ligated into pGEM-T vector (Promega) and three individual clones representative of each worm were sequenced (Eurofins MWG).

2.3 Results

2.3.1 Comparative sequence analysis of *cat-4* in *T. circumcincta* and *D. viviparus*

Sequences representing the coding sequences (CDS) of *cat-4* of *D. viviparus* (651 bp, 217 aa) and *T. circumcincta* (639 bp, 213 aa) have been deposited on the European Bioinformatics Institute (EBI) database with Accession Numbers; FR726161 and FR726162, respectively. Analyses of the translated, inferred amino acid sequences (CBX89809 and CBX89810) with the orthologous sequences available for *O. ostertagi* and *C. elegans* (Figure 10) showed that the 12 amino acids thought to form the GTP binding pocket (Moore *et al*., 2000) were conserved in the sequences of GTP-Cyclohydrolase from *D. viviparus* and *T. circumcincta*. There was 89 % amino acid identity between the GTP-Cyclohydrolase of *T. circumcincta* and that of *D. viviparus*, and 82 % aa identity between each parasite species and the *C. elegans* protein over 200 residues. Phylogenetic analysis indicated a high level of relatedness between the nematode proteins (Figure 11) and that their GTP-Cyclohydrolase was more distant in relationship to these enzymes in bacteria or mammalian species. This analysis confirmed that the sequences isolated here were not derived from bacteria present in the gut of the nematodes, or from the sheep host.

2.3.2 Reference gene evaluation

Analysis was carried out on four potential reference genes over the whole life-cycle of *T. circumcincta*. The M-values generated from the *T. circumcincta* samples are shown in Table 3. More stable genes have lower M-values; stability being measured as variation in the expression ratio between different genes, so ideal reference genes would have a constant expression ratio in all experimental conditions.

The least stable genes were excluded sequentially and the M-values re-calculated. The most stable genes for these samples were shown to be *ef-1α* (elongation factor 1α) and *β-tubulin,* with a calculated M-value of 1.481 (Figure 12). These genes were used to normalise the transcription data derived from the genes of interest in all subsequent qPCR analyses for *T. circumcincta*. These were also used for *D.*

viviparus as these have been shown previously to be the most stable reference genes in the latter species (Strube *et al*., 2008).

```
Teladorsagia circumcincta    ---MASESGFLSSDS--EDGDQK--IIAELK------YTSNLDKMTAAYSSIISHVGEDVNRQGLLKTPERAAKAMLYFTKGYEQQ
Ostertagia ostertagi         ---MASESGFLSSDSSSEDCDQK--IIAFSK------KTSNLDKMTAAYSSIISHVGEDVNRQGLLKTPDRAAKAMLYFTKGYEQQ
Dictyocaulus viviparus       ---MTTESGFVSSDSASEETNDQKAITQLKK------KTSNLENMVSAYSSIISHVGEDVNRQGLLKTPERAAKAMLYFTKGYEQQ
Caenorhabditis elegans       MSRIENESGFLSSDAASVGSEDD--KVEMKKRNGTIPKEDHLKSMCNAYQSIIQHVGEDINRQGLLKTPERAAKAMMAFTKGYDDQ
                                :  .****:***:       ::.        *       .:*..*  **.***.****:*********:******: *****::*


Teladorsagia circumcincta    LDDILNEAVFDENHDEMVIVKDIEMFSLCEHHLVPFNGKVHIGYIPNKKVLGLSKLARIVEMFSRRLQVQERLTKQIATAMVQAVQ
Ostertagia ostertagi         LDDILNDAVFDENHDEMVIVRDIEMFSLCEHHLVPFNGKVHIGYIPNKKVLGLSKLARIVEMFSRRLQVQERLTKQIATAMVQAVQ
Dictyocaulus viviparus       LDEILNEAVFDEDHDEMVIVKDIEMFSLCEHHLVPFNGKVHIGYIPNKKVLGLSKLARIVEMFSRRLQVQERLTKQIATAMLQAVQ
Caenorhabditis elegans       LDELLNEAVFDEDHDEMVIVKDIEMFSLCEHHLVPFMGKVHIGYIPNKKVLGLSKLARIVEMFSRRLQVQERLTKQIATAMVQAVQ
                             **:.:**.:****.*****.*******:.*************** ****************************************:.****


Teladorsagia circumcincta    PAGVAVVIEASHMCMVMRGVQKINATTSTSCMLGVFREDPKTREEFLNLINKK
Ostertagia ostertagi         PAGVAVVIEASHMCMVMRGVQKINATTSTSCMLGVFRDDPKTREEFLNLIHK-
Dictyocaulus viviparus       PVGVAVVIEASHMCMVMRGVQKINATTSTSCMLGVFRDDPKTREEFLNLINKK
Caenorhabditis elegans       PSGVAVVIEASHMCMVMRGVQKINASTTTSCMLGVFRDDPKTREEFLNLINKR
                             * ************************:*:**********:*************:*
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Figure 10. ClustalW alignment of the inferred translated sequence of the *cat-4* mRNAs of four nematode species showing high levels of sequence homology between *Teladorsagia circumcincta* (FR726162), *Ostertagia ostertagi* (O61573), *Dictyocaulus viviparus* (FR726161) and *Caenorhabditis elegans* (NP_505710). The grey shaded box highlights the residues thought to form the GTP binding pocket (Moore *et al.*, 2000), these residues are highly conserved across all the sequences. Blue indicates acidic residues, pink indicates basic residues, green indicates hydroxyl, amine and basic residues and red indicates small and hydrophobic residues. "*" identifies the residues in that column that are identical in all sequences, ":" identifies the conserved substitutions and "." identifies the semi-conserved substitutions.
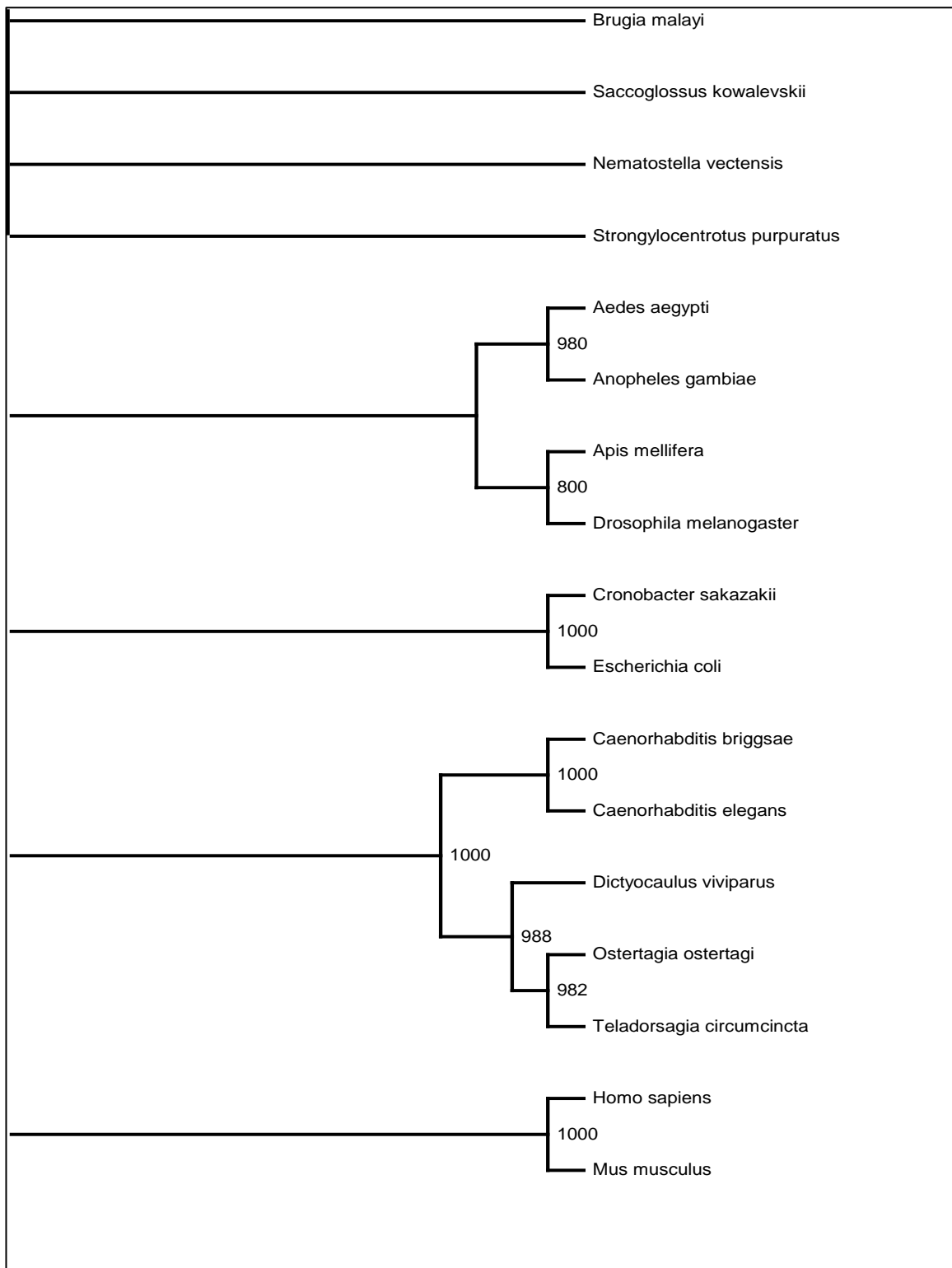
Figure 11. Neighbour joining analysis of the amino acid sequences of GTP-Cyclohydrolase from a number of different species. Alignments were generated using ClustalX, bootstrapped 1000 times and viewed using Treeview. The sequences for *T. circumcincta* and *D. viviparus* align closely with those of other nematodes, separately from the bacterial and mammalian sequences. *T. circumcincta* (CBX89810), *D. viviparus* (CBX89809), *C. elegans* (NP_505710), *Caenorhabditis briggsae* (XP_002637140), *O. ostertagi* (O61573), *Nematotella vectensis* (XP_001638883), *Strongylocentrotus purpuratus* (XP_788725), *Saccoglossus kowalevskii* (NP_001161562), *Brugia malayi* (XP_001901663), *Apis mellifera* (XP_393086), *Mus musculus* (BAE29744), *Homo sapiens* (AAB23164), *Drosophila melanogaster* (ACU32630), *Anopheles gambiae* (XP_001688869), *Aedes aegypti* (XP_001661181), *E. coli* (ZP_06937418), *Cronobacter sakazakii* (YP_001437189).
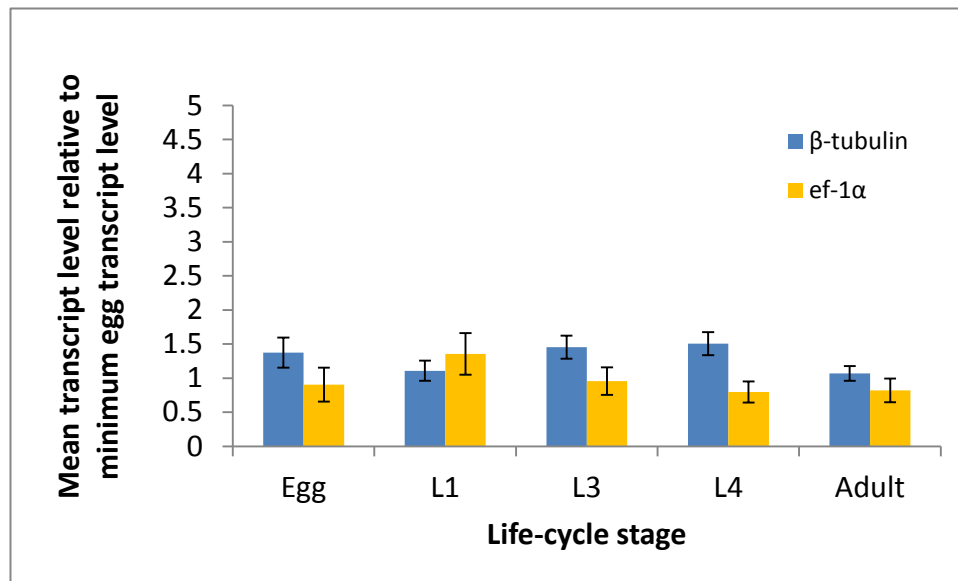
Figure 12. The mean transcript (± standard error) levels of the two most stable reference genes (*ef-1α* and *β-tubulin)* across the different life-cycle stages studied in *T. circumcincta*. The ratio of the expression remains close to 1 across all the samples indicating a high level of stability.

Table 3. M-values of reference gene stability calculated by the geNorm software (Vandesompele *et al.*, 2002) identifying that *ef-1α* and β-tubulin are the most stable reference genes for these cDNA samples from *T. circumcincta*. The more stable a gene is, the lower the M-value. As the least stable genes are excluded in stages, the M-values are re-calculated showing the increasing stability of the combination being used. As the data are normalised against the reference genes it is important for them to remain consistently expressed across all of the tissue samples used.

β-tubulin	*ef-1α*	60S	gapdh	
2.840	2.675	4.002	3.59	60S excluded
2.258	2.310		3.087	gapdh excluded
1.481	1.481			

2.3.3 Stage-specific gene expression of *cat-4* in *D. viviparus*

Initial examination showed that the highest mean levels of *Dvi-cat-4* transcript were present during the transition between L_1 and L_3 (Figure 13), with the peak mean transcript levels observed at day 3-4 after hatching. This was similar in both timing and magnitude in the field isolate and the vaccine isolate, with the only statistically significant differences in the mean between isolates occurring at days 5 ($p<0.001$, higher transcript levels in the field isolate), 10 ($p=0.03$, higher transcript levels in the vaccine isolate) and in the L_5 males and mixed samples ($p<0.001$ for both, with higher levels of transcript in the field isolate). Analysis of the field isolate samples

showed there were no statistically significant differences between the means for hypobiotic and non-hypobiotic L_5 samples (p-values ranged from 0.89 to 0.95, even before adjusting for multiple testing). In the adult stage and the L_5 stage in the vaccine isolate, *Dvi-cat-4* mean transcription levels in males were statistically significantly lower ($p<0.001$) than those measured in female worms. Males from the field isolate had lower observed mean transcription levels than females, but to a less marked, and statistically non-significant, extent. This discrepancy between the sexes of the two strains explains the differences seen between the two isolates at the L_5 stage. Transcription of the gene in eggs within females could not account for this difference because the adult females were lysed using osmotic pressure to remove eggs before storage, and L_5 females would not contain eggs. Within the field isolate there were significant differences observed between each of the pre-parasitic larval stages (day $1-14$) and egg, L_3i, L_4, L_5 and adult stages.
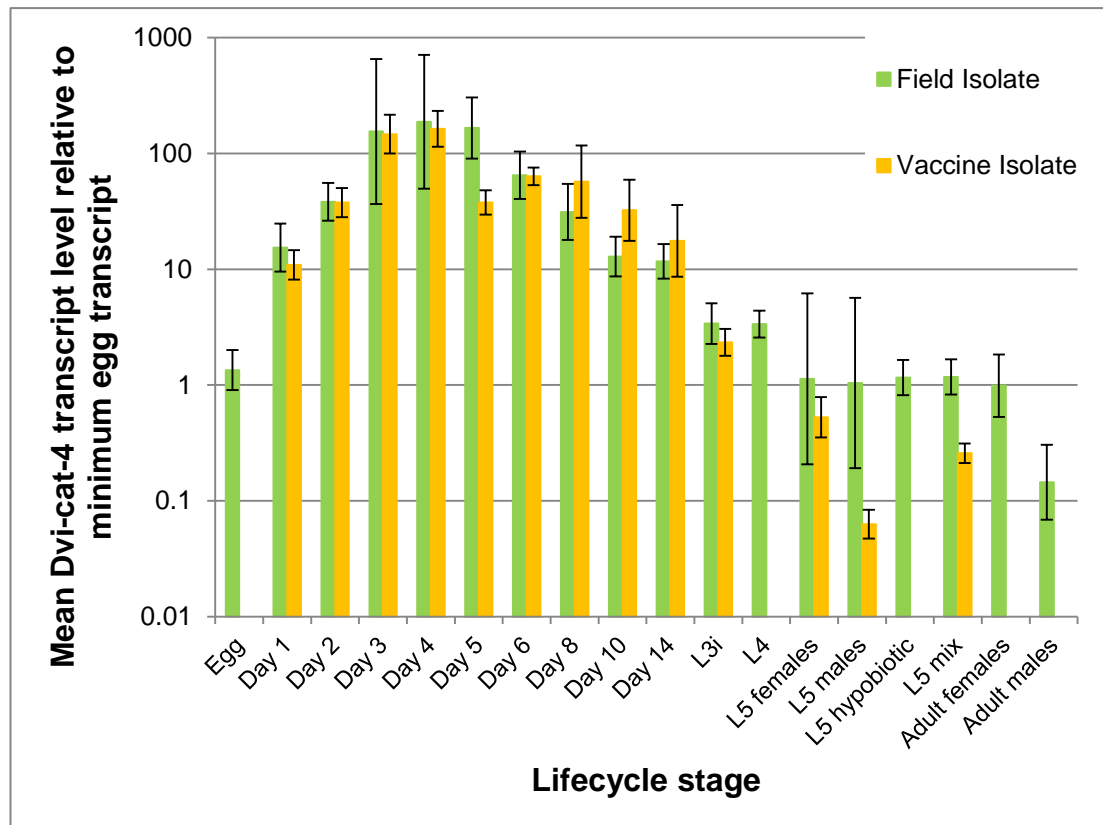
Figure 13. Mean transcript levels of *Dvi-cat-4* during the transition of *D. viviparus* from first stage larvae (Day 1) and third stage larvae (Day 10) and the other life-cycle stages, represented on a logarithmic scale. Data shown are for the field isolate (green bars) and the vaccine isolate (orange bars), which does not enter hypobiosis. Results are normalised to reference genes ef-1α and β-tubulin and shown relative to the transcript levels in the egg sample with the lowest transcript. Each data point represents the mean of three biological replicates, run in duplicate with at least two repetitions of the experiment. Error bars show the 95 % confidence interval of the mean.

2.3.4 Developmental transcription pattern of *cat-4* in *T. circumcincta*

The transcript levels of *Tci-cat-4* across the different life-cycle stages of *T. circumcincta* are shown in Figure 14. As with *D. viviparus*, the highest mean levels of transcript were seen in the pre-parasitic stages (L_1 and L_3). There were significantly higher levels of transcript in the L_1 compared with eggs and adult stages ($p =< 0.001$ and $p = 0.019$, respectively). The L_3 also had significantly higher levels of transcript compared with eggs and adults ($p =< 0.001$ and $p = 0.010$, respectively).
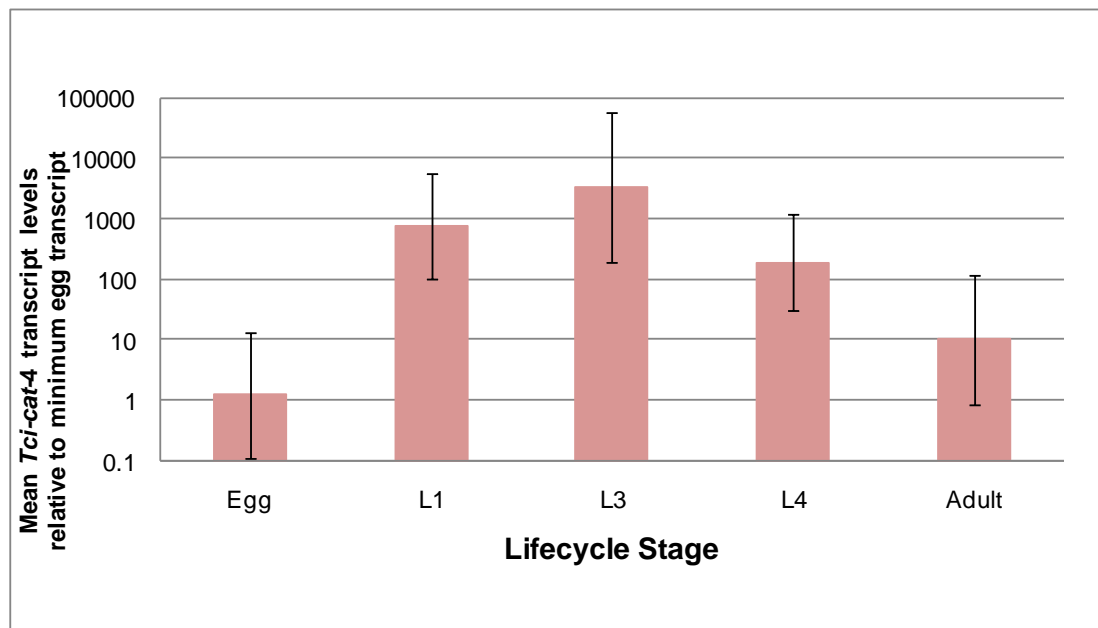
Figure 14. Mean transcript levels of *Tci-cat-4* in the different life-cycle stages of *T. circumcincta* on a logarithmic scale. Results are normalised to reference genes *ef-1α* and β-tubulin and shown relative to the transcript levels in the egg sample with the lowest transcript. Each data point represents the mean of the biological replicates, run in duplicate with at least 2 repetitions of the experiment. Error bars show the 95 % confidence interval of the mean.

A preliminary analysis of *T. circumcincta* larvae that were chilled for ten weeks showed that the *Tci-cat-4* transcript level did not remain constant over this period of time, gradually increasing to a peak at eight weeks, before reducing again at ten weeks (data not shown). These data were derived from a single experimental repetition, and, as such, must be treated with caution. A further experiment

attempting to reproduce this experiment on a larger scale was performed but the samples were lost in a centrifuge malfunction.

2.3.5 Developmental transcription pattern of *pah-1* in *T. circumcincta*

The transcript levels of *Tci-pah-1* across different life-cycle stages of *T. circumcincta* are shown in Table 4. The qPCR efficiencies for this gene were 138 % on average, which implied that there were some inhibitory processes occurring within the *pah-1* qPCR reactions. This means that analysis of this dataset should be assessed as a preliminary finding, and explains why the confidence intervals are similar to the means. From the data generated it would appear that there was a trend for higher levels of *pah-1* in L_1 and L_3 stages compared to the other stages.

Table 4. Mean transcript levels of *Tci-pah-1* in different life-cycle stages of *T. circumcincta*. Results are normalised to reference genes *ef-1α* and β-tubulin and shown relative to the transcript levels in the egg sample. Each data point represents the mean of the biological replicates, run in duplicate with at least two repetitions of the experiment.

Life-cycle stage	Mean *Tci-pah-1* transcript	95 % Confidence interval
Egg	2.66	2.04
L1	117.80	117.80
L3	70.39	70.39
L4	13.96	13.92
Adult	0.10	0.01

2.3.6 *T. circumcincta* genomic organisation of *cat-4* gene

There were differences in genomic structure of *Tci-cat-4* both between, and within, individual *T. circumcincta* worms. Figure 15 shows an image of the PCR products generated from a number of different adult worms. Most individuals possessed a gene of 2106 bp; however, smaller PCR products (~ 640 bp) were amplified from other individuals in addition to the larger amplicons. The smaller PCR product was amplified exclusively in some individuals. PCR products from one of each of the

three different patterns were cloned (i.e. pattern 1: only the large product, pattern 2: the smaller product only, and pattern 3: both products) and three clones for each pattern selected for sequencing. Larger PCR products were found to represent the entire *Tci-cat-4* gene, which contained 6 exons, with 5 introns. The latter varied in size from 89 to 698 bp (Figure 16). The smaller PCR products were found to include the coding sequence and no introns were observed. As no bands were visible in the negative controls, and RNA was presumably destroyed during the genomic DNA extraction protocol, these products are unlikely to have been mRNA or cDNA artefacts. The alignment of the derived amino acid sequences is shown in Figure 17. Alignment demonstrates the high level of conservation in the residues thought to contribute to GTP binding and binding to a pteridine ring structure. Even when PCR was performed using genomic DNA from individual worms, there was evidence of multiple, slightly different, *Tci-cat-4* gene copies. For example, sequences 1a-c (Figure 17) represent the inferred amino acid sequences from 'intron-containing' *Tci-cat-4* sequences cloned from an individual worm, and contain amino acid substitutions at residues 119 and 196. In addition, the *Tci-cat-4* genes with no introns, for example 1d-f (Figure 17), are different from each other and from the intron-containing sequences at residues 13, 166 and 187, which indicated that there exist multiple, slightly different copies of the gene within an individual worm.

Figure 15. Agarose gel image showing the PCR products of *Tci-cat-4* generated from genomic DNA of individual worms. Lanes A-P represent individual worms, lane Q a negative control. The size of the 1 kb plus ladder (Invitrogen) is shown in bp.

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Figure 16. The genomic structure of *C. elegans Ce-cat-4* and *T. circumcincta Tci-cat-4* showing the relative sizes of the exons and introns

```
1a         MASESGFLSSDSEDGDQKIIAELKYTSNLDKMTAAYSSIISHVGEDVNRQGLLKTPERAAKAMLYFTKGYEQQLDDILNEAVFDENHDEIVIVKDIEMFSLCEHHLVPFNGKVHIGYILN 120
1b         MASESGFLSSDSEDGDQKIIAELKYTSNLDKMTAAYSSIISHVGEDVNRQGLLKTPERAAKAMLYFTKGYEQQLDDILNEAVFDENHDEIVIVKDIEMFSLCEHHLVPFNGKVHIGYILN 120
1c         MASESGFLSSDSEDGDQKIIAELKYTSNLDKMTAAYSSIISHVGEDVNRQGLLKTPERAAKAMLYFTKGYEQQLDDILNEAVFDENHDEIVIVKDIEMFSLCEHHLVPFNGKVHIGYIPN 120
1d         MASESGFLSSDSEDGDQKIIAELKYTSNLDKMTAAYSSIISHVGEDVNRQGLLKTPERAAKAMLYFTKGYEQQLDDILNEAVFDENHDEIVIVKDIEMFSLCEHHLVPFNGKVHIGYIPN 120
1e         MASESGFLSSDSEDGDQKIIAELKYTSNLDKMTAAYSSIISHVGEDVNRQGLLKTPERAAKAMLYFTKGYEQQLDDILNEAVFDENHDEIVIVKDIEMFSLCEHHLVPFNGKVHIGYIPN 120
1f         MASESGFLSSDSGDGDQKIIAELKYTSNLDKMTAAYSSIISHVGEDVNRQGLLKTPERAAKAMLYFTKGYEQQLDDILNEAVFDENHDEIVIVKDIEMFSLCEHHLVPFNGKVHIGYIPN 120
2a         MASESGFLSSDSEDGDQKIIAELKYTSNLDKMTAAYSSIISHVGEDVNRQGLLKTPERAAKAMLYFTKGYEQQLDDILNEAVFDENHDEIVIVKDIEMFSLCEHHLVPFNGKVHIGYIPN 120
2b         MASESGFLSSDSEDGDQKIIAELKYTSNLDKMTAAYSSIISHVGEDVNRQGLLKTPERAAKAMLYFTKGYEQQLDDILNEAVFDENHDEIVIVKDIEMFSLCEHHLVPFNGKVHIGYIPN 120
2c         MASESGFLSSDSEDGDQKIIAELKYTSNLDKMTAAYSSIISHVGEDVNRQGLLKTPERAAKAMLYFTKGYEQQLDDILNEAVFDENHDEIVIVKDIEMFSLCEHHLVPFNGKVHIGYIPN 120
3a         MASESGFLSSDSEDGDQKIIAEPKYTSNLDKMTAAYSSIISHVGEDVNRQGLLKTPERAAKAMLYFTKGYEQQLDDILNEAVFDENHDEIVIVKDIEMFSLCEHHLVPFNGKVHIGYIPN 120
3b         MASESGFLSSDSEDGDQKIIAEPKYTSNLDKMTAAYSSIISHVGEDVNRQGLLKTPERAAKAMLYFTKGYEQQLDDILNEAVFDENHDEIVIVKDIEMFSLCEHHLVPFNGKVHIGYIPN 120
3c         MASESGFLSSDSEDGDQKIIAEPKYTSNLDKMTAAYSSIISHVGEDVNRQGLLKTPERAAKAMLYFTKGYEQQLDDILNEAVFDENHDEIVIVKDIEMFSLCEHHLVPFNGKVHIGYIPN 120
reference  MASESGFLSSDSEDGDQKIIAELKYTSNLDKMTAAYSSIISHVGEDVNRQGLLKTPERAAKAMLYFTKGYEQQLDDILNEAVFDENHDEMVIVKDIEMFSLCEHHLVPFNGKVHIGYIPN 120
           * * * * * * * * * *   * * * * * * * *   * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *  :* * * * * * * * * * *****************  *


1a         KKVLGLSKLARIVEMFSRRLQVQERLTKQIALAMVHAVQPTGVAVVIEASHMCMVMRGVQKINATTSTSCMLGVFRDDPKTREEFLNLINKK 212
1b         KKVLGLSKLARIVEMFSRRLQVQERLTKQIALAMVHAVQPTGVAVVIEASHMCMVMRGVQKINATTSTSCMLGVFRDDPKTREEFLNLINKK 212
1c         KKVLGLSKLARIVEMFSRRLQVQERLTKQIALAMVHAVQPTGVAVVIEASHMCMVMRGVQKINATTSTSCMLGVFKDDPKTREEFLNLINKK 212
1d         KKVLGLSKLARIVEMFSRRLQVQERLTKQIALAMVHAVQPTGVAVVIEASHMCMVMRGVQKINATTSTSCMLGVFKDDPKTREEFLNLINKK 212
1e         KKVLGLSKLARIVEMFSRRLQVQERLTKQIALAMVHAVQPTGVAVVIEASHMCMVMRGVQKINATTSTSCMLGVFKDDPKTREEFLNLINKK 212
1f         KKVLGLSKLARIVEMFSRRLQVQERLTKQIALAMVHAVQPTGVAVVIEASHMCMVMRGVQKINATTPTSCMLGVFKDDPKTREEFLNLINKK 212
2a         KKVLGLSKLARIVEMFSRRLQVQERLTKQIALAMVHAVQPTGVAVVIEASHMCMVMRGVQKINATTSTSCMLGVFRDDPKTREEFLNLINKK 212
2b         KKVLGLSKLARIVEMFSRRLQVQERLTKQIALAMVHAVQPTGVAVVIEASHMCMVMRGVQKINATTSTFCMLGVFRDDPKTREEFLNLINKK 212
2c         KKVLGLSKLARIVEMFSRRLQVQERLTKQIALAMVHAVQPTGVAVVIEASHMCMVMRGVQKINATTSTSCMLGVFRDDPKTREEFLNLINKK 212
3a         KKVLGLSKLARIVEMFSRRLQVQERLTKQIALAMVHAVQPTGVAVVIEASHMCMAMRGVQKINATTSTSCMLGVFKDDPKTREEFLNLINKK 212
3b         KKVLGLSKLARIVEMFSRRLQVQERLTKQIALAMVHAVQPTGVAVVIEASHMCMAMRGVQKINATTSTSCMLGVFKDDPKTREEFLNLINKK 212
3c         KKVLGLSKLARIVEMFSRRLQVQERLTKQIALAMVHAVQPTGVAVVIEASHMCMVMRGVQKINATTSTSCMLGVFKDDPKTREEFLNLINKK 212
reference  KKVLGLSKLARIVEMFSRRLQVQERLTKQIATAMVQAVQPAGVAVVIEASHMCMVMRGVQKINATTSTSCMLGVFREDPKTREEFLNLINKK 212
           * * * * *  * * * * * * * * * * * * * * * * * *  * * * * * * *  * * * : * * * * : * * * * * * : *** **  * * * * * * * * * * *  * *   * * * * * * : * :  * * * * * * * * * * * * * * * * *
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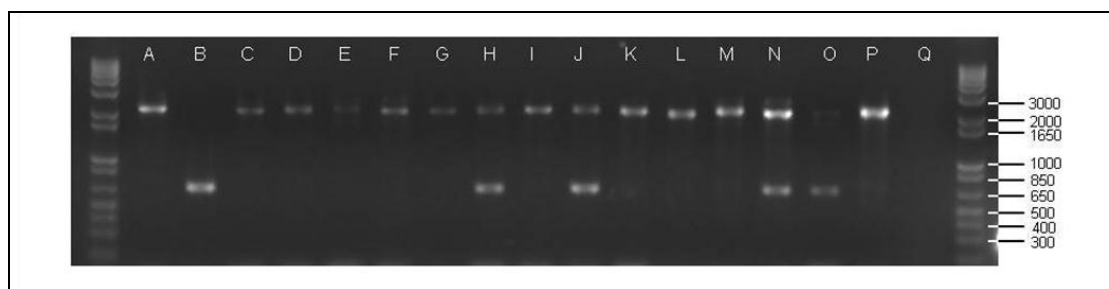
Figure 17. Alignment of the inferred translated sequence of *Tci-cat-4* generated from the clones of the individual worms, with introns removed manually from sequences 1a, 1b, 1c, 2a, 2b and 2c. The sequences are compared with an original reference sequence for the gene generated from cDNA previously (Nisbet *et al.*, 2008). The underlined asterisks highlight the residues thought to form the GTP binding pocket and the hatched box the residues thought to bind to a pteridine ring structure (Moore *et al.*, 2000). The grey shaded box corresponds to those residues believed to be the sites of phosphorylation (Du *et al.*, 2009). These residues are identical across all the sequences. Blue indicates acidic residues, pink indicates basic residues, green indicates hydroxyl, amine and basic residues and red indicates small and hydrophobic residues. "*" identifies the residues in that column that are identical in all sequences, ":" identifies the conserved substitutions and "." identifies the semi-conserved substitutions.

2.4 Discussion

In this chapter, differential transcriptions of *cat-4* mRNA levels amongst stages were observed in two species of parasitic nematode. Serotonin is known to play a role in the developmental arrest of a wide range of animals (Popova *et al*., 1984; Sze *et al*., 2000) and it had been hypothesised that serotonin synthesis, as a means of controlling larval development, may be a reason for the high levels of *Tci-cat-4* expression in the iL_3 stage of *T. circumcincta* (Nisbet *et al*., 2008). The evidence obtained here for *D. viviparus*, i.e. that *Dvi-cat-4* transcription levels were not significantly different between L_5 derived from hypobiotic and non-hypobiotic isolates, implied that it is unlikely that the GTP-Cyclohydrolase pathway has involvement in this form of hypobiosis, at least in this species. This was confirmed by results from suppression subtractive hybridisation experiments between hypobiotic and non-hypobiotic *D. viviparus*, where transcripts representing elements of the serotonin pathway were not identified as being different between the two datasets (Strube *et al*., 2007). From the *D. viviparus* transcription data obtained here, it would appear that GTP-Cyclohydrolase does not play a role in regulation of within-host hypobiosis. Furthermore, there was no difference in mean gene transcript levels in L_3 from two isolates that had been subjected to incubation at 4 °C, a process that induces hypobiosis in the field isolate HannoverDv2000.

In *C. elegans*, dauer larvae have been shown to have increased transcription of the *cat-4* gene than other stages (Jeong *et al*., 2009) so there are potential parallels in the biology of *cat-4* expression in L_3 of parasitic nematodes. The data presented here showed that *cat-4* was transcribed at high levels in both *D. viviparus* and *T. circumcincta* pre-parasitic stages (levels measured were 187 times higher in day 4 *D. viviparus* larvae than in eggs, and 3447 times higher in *T. circumcincta* L_3 than in eggs). The mean levels of *Dvi-cat-4* transcript increased 12-fold during the transition between the L_1 and L_3 stages of *D. viviparus*. This increase in mean transcript levels was observed in both isolates, providing further evidence that GTP-Cyclohydrolase is potentially relevant for development or survival of larvae while on pasture. This peak in transcript level could also be occurring in *T. circumcincta* between L_1 and L_3

stages, but this was not detected here, as intermediate L_2 stages were not examined due to constraints on parasites available. In *C. elegans*, G-coupled receptors have been linked with both the control of formation of, and exit from, dauer state (Jones *et al.*, 2001; Kim *et al.*, 2009). This requires accumulation of particular transcripts before transition. It has been hypothesised that these receptors are involved in the chemical sensing of the dauer pheromone that directs which pathway the animals follow. Accumulation of specific transcripts of genes prior to life-cycle stage transition occurs in a number of organisms including the sea snail, *Haliotis asinine*, in which a widespread change in gene expression occurs prior to the metamorphosis event that changes free-swimming larval stages into sea-bottom dwelling adults. Within the gene expression changes that occur, one cluster of genes, mainly of unknown function, but including the *kakapo* gene involved in microfilament activity and genes involved in cellular metabolic processes, steadily increase in expression with time and remain high until the metamorphosis occurs (Williams *et al.*, 2009).

At days 5 and 10 post hatching, *Dvi-cat-4* transcript levels were significantly lower in the vaccine isolate than in the field isolate ($p<0.001$ and $p=0.032$ respectively). While this could represent a biological difference between isolates, as the initial peaks in transcript were of similar magnitude and occurred at a similar time point; this is unlikely, particularly as at all the other time points in the transition between L_1 and L_3 there were no significant differences. The differences seen in L_5 males and mixed sex groups are unlikely to be biologically significant because the levels of transcript are so low, although differences in serotonin levels between the sexes has been observed previously in *C. elegans* (Loer and Rivard, 2007).

The preliminary investigations into the expression patterns of *Tci-pah-1* over the life-cycle and *Tci-cat-4* over a period of 10 weeks may provide information that could help inform future experiments. The changes in *Tci-cat-4* suggest that the L_3 are not metabolically inactive, but in a similar way to the dauer larvae of *C. elegans*, are altering gene expression levels as required, or in preparation for a later event (Jones *et al.*, 2001). If the increase in *Tci-pah-1* observed in the L_1 and L_3 were to be

confirmed in future, then it would appear that the melanisation pathway (Figure 5) may be the more likely requirement for the increased levels of GTP-Cyclohydrolase. Previous work in *C. elegans* identified expression of *pah-1* in the hypodermis (Loer *et al*., 1999), and if levels of transcript reflect *cat-4* transcript patterns, then the hypothesis for GTP-Cyclohydrolase being required for melanisation would have more credence. If this work were to be repeated, further sequencing of the *Tci-pah-1* gene may enable different primer and probe sets to be designed that would have less of an inhibitory effect and generate more accurate qPCR efficiencies.

Analysis of gene sequences of *Tci-cat-4* from individual *T. circumcincta* larvae showed that, despite the highly conserved nature of this gene, there were sequence differences at the DNA level within and between worms. The existence of a putative full-length gene sequence in genomic DNA without introns may suggest either genomic DNA derived from gut-commensal or extraneous bacteria [protein encoding genes in bacteria do not possess introns; (Edgell *et al*., 2000)] or the presence of a pseudogene. It is unlikely to be a bacterial transcript, as the phylogenetic tree (Figure 11) clearly shows the clustering of the bacterial amino acid sequences distant from the nematode GTP-Cyclohydrolase sequences. Given the high levels of *cat-4* mRNA, it is possible that some of the transcript has been retrotranscribed and incorporated into genomic DNA. This has been described for genes in other organisms (D'Errico *et al*., 2004), and describes a situation whereby mRNA is inserted back into the genome, leading to the presence of retrotransposed (retroposed / retrocopies) of genes. However, as none of these sequences contained a termination codon within the coding sequence and the level of variation within the sequences was no higher than that seen in the proposed functional gene sequences, it cannot be ruled out that these sequences could represent functional genes. It has been suggested that the genomic location of retrotransposed duplicates may be key to whether transcription occurs, with those near other coding regions being more likely to be expressed (Hahn, 2009), with the hypothesis that retrocopies can be under the regulatory framework of nearby genes. A study into the 1000 retroposed copies of genes within the human genome demonstrated that of these, 120 had evolved into functional genes (Vinckenbosch *et al*., 2006). Until a complete genome has been

sequenced and annotated for *T. circumcincta*, it would be difficult to identify the location of these duplicates without undertaking primer walking experiments (Kieleczawa et al., 1992). Indeed, the presence of high levels of variation within individual parasites is one of the main hurdles faced by scientists attempting to annotate the genomes of parasitic nematodes (Mitreva *et al*., 2011).

It may also be possible that gene duplication events within the genome have occurred to service demand for the production of the large quantities of *Tci-cat-4* transcript; if such a high volume is needed in a short period of time, then a higher number of genomic template copies could be useful. This has been observed, for example, in the duplication of the esterase genes responsible for organophosphate resistance in the aphid *Myzus persicae*, whereby higher numbers of copies of the gene were observed in resistant individuals which were proposed to have enabled the aphids to survive pesticide treatment (Srigiriraju *et al*., 2009). Ohno (1970) proposed previously "When the metabolic requirements of an organism dictates the presence of an enormous amount of a particular gene product, the incorporation of multiple copies of the gene locus by the genome often fulfils that requirement". With a thousand-fold increase in the level of *cat-4* transcript produced by *T. circumcincta* L_3 compared to eggs, this would suggest that metabolic requirements may be assisted by relevant gene duplication. These high levels of *cat-4* transcript appear to be unique to the Clade V nematodes, with 86 % of all ESTs for GTP-Cyclohydrolase in Nembase (www.nematodes.org; accessed May 2011) found within the Clade V species (11 % Clade IV; 1 % Clade III; 2 % Clade I). This may reflect that there are higher numbers of ESTs in public databases for Clade V species (310,751 *vs* 207,911 for Clade IV, 117,007 for Clade III and 43,811 for Clade I) but even when the number of *cat-4* ESTs are analysed as a percentage of total ESTs for that Clade, the levels are found to be five times higher in Clade V nematodes compared to other Clades.

If the requirement for high levels of *cat-4* transcript occurred after the divergence of the nematode Clades, this could provide further evidence for the role of gene

duplication. Analysis of the number of copies of the salivary amylase gene in humans identified a higher number of copies in populations that ate a higher level of starch in their diet than in populations such as those living in the Yakut whose diet was predominantly fish and therefore low in starch (Perry *et al*., 2007). This is also seen in other primates, with chimpanzees and bonobos having low levels of expression of the gene encoding salivary amylase and ingesting low levels of starch, while the cercopithecines (an old-world family of primates that include baboons and macaques) consume a very high level of starch and have much higher expression levels. For recent (in evolutionary terms) changes in transcript requirement, it would be predicted that the multiple copies of the gene would remain highly similar in sequence in order to perform the same function. The high level of sequence similarity seen among different copies of the *Tci-cat-4* gene would appear to fit this hypothesis, with gene duplication events enabling higher levels of enzyme to be produced. Why the Clade V nematodes in particular require such high levels of *cat-4* will be explored in later chapters.

In this chapter the role of GTP-Cyclohydrolase was explored in the context of hypobiosis using a model organism *D. viviparus*. Model organisms have been used widely since Gregor Mendel studied the inheritance characteristics of pea plants in the 1850s, and have provided insights into the study of disease in many different species. It must always be remembered, however, that the outcomes of an experiment in a model system may not transfer exactly to another species. In these experiments the model organism is a closely related species from the same Clade, with a similar life-cycle, albeit with a different host predilection site than *T. circumcincta*. As such, it is inferred that the findings here, that GTP-Cyclohydrolase is not involved in the hypobiosis of *D. viviparus*, will apply to other parasitic nematodes, including *T. circumcincta*, that are more technically challenging to study. Alongside the evidence for a lack of role in intra-host hypobiosis, this chapter has also demonstrated the possible role of GTP-Cyclohydrolase in development between the pre-parasitic larval stages of both *T. circumcincta* and *D. viviparus*. The potential increase in phenylalanine hydroxylase gene transcription in these same stages suggests that the requirement for tyrosine is possibly greater in free-living

stages. One possible requirement for this is the production of melanin, which will be explored in greater detail in later chapters.

3 Investigation of function by using a GTP-Cyclohydrolase inhibitor *in vitro*

3.1 Introduction

Studies into the function of nematode gene products can enable a clearer understanding of their role in the basic biology and survival of the parasite. Functional studies can take a number of different approaches (Hashmi *et al*., 2001), but often include studying the effect of inhibition, either at the DNA or protein level. In an attempt to understand the role of GTP-Cyclohydrolase in human disease, a biochemical inhibitor has been used to study the effect on the cells and pathways (Ikemoto *et al*., 2008); by understanding the effects of absence of the enzyme it is possible to gain a clearer view of its function under normal conditions. Mutations in the human GTP-Cyclohydrolase gene are implicated in a wide range of diseases including atypical phenylketonuria (Thöny and Blau, 1997), Parkinson's disease (Curtius *et al*., 1984) and Alzheimer's disease (Barford *et al*., 1984). Of 628 patients with tetrahydrobiopterin (BH_4) deficiencies worldwide listed on the BIODEF database (Blau *et al*., 1996; accessed January 2009), 26 of these were due to mutations in the GTP-Cyclohydrolase gene, resulting in severe neurological symptoms that could not be treated with a low phenylalanine diet, but instead with the administration of L-Dopa and 5-hydroxytryptophan. Without GTP-Cyclohydrolase, the conversion of phenylalanine to tyrosine cannot occur, leading to toxic accumulations of phenylalanine in the bloodstream, which causes damage to the nervous system (Blau *et al*., 2001). The chemical inhibitor of GTP-Cyclohydrolase, 2,4-diamino-6-hydroxypyrimidine (DAHP) was first described as an inhibitor of biopterin-dependent growth of the protozoan *Crithidia fasciculate* (see Rembold, 1964), and since then has been used to inform on the role of GTP-Cyclohydrolase in the BH_4 pathways (Ikemoto *et al*., 2008; Kobayashi *et al*., 1991). DAHP is similar in biochemical structure to the ring structure component of GTP (Figure 18), so competes directly for GTP-Cyclohydrolase binding (Xie *et al*., 1998). DAHP also acts as an indirect inhibitor of GTP-Cyclohydrolase in mammals by activating the GTP-Cyclohydrolase Feedback Regulatory Protein (GFRP); this occurs because DAHP is also similar in structure to the ring component of BH_4 (Xie

et al., 1998). It is hypothesised that two pentamers of GFRP bind on either side of the GTP-Cyclohydrolase decamer to block the activity of the enzyme (Bader *et al.*, 2001). The role of GFRP in nematodes has not yet been studied.
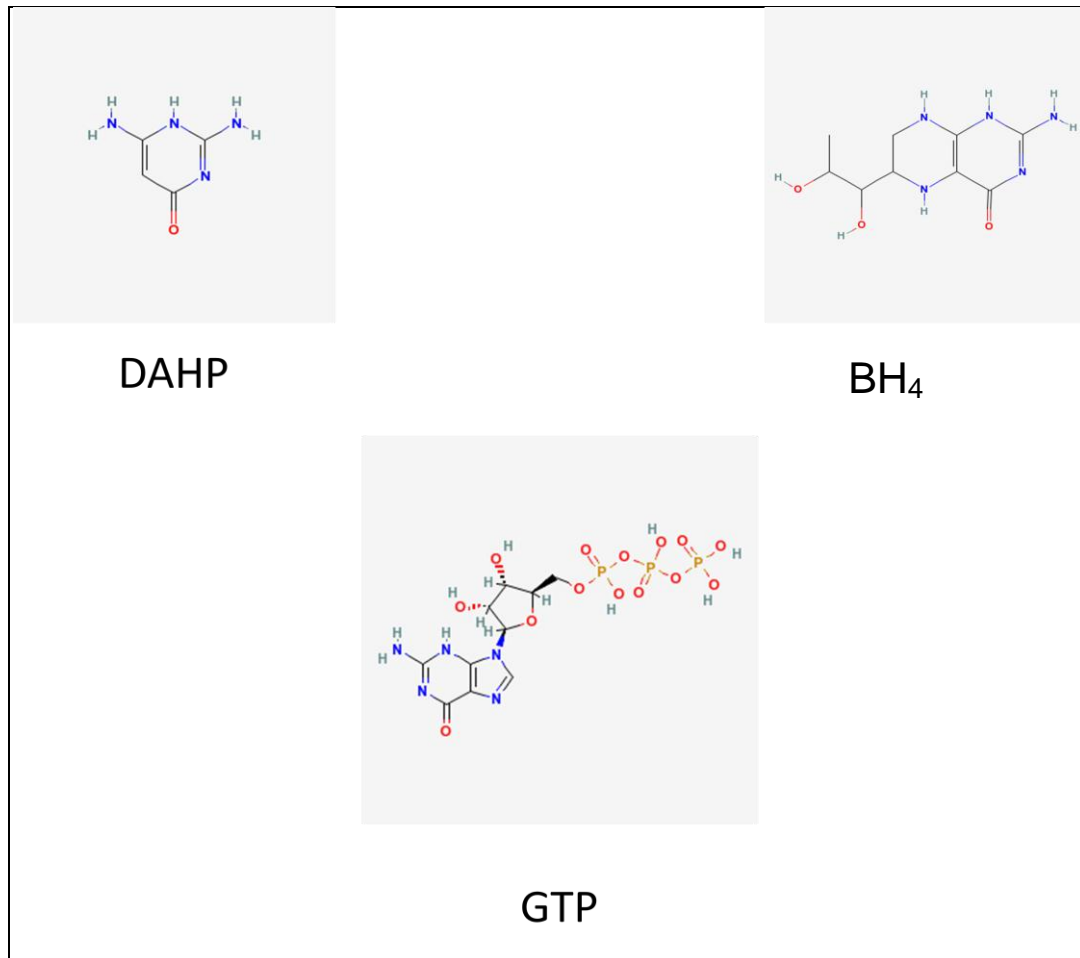
Figure 18. The biochemical structures of DAHP, BH$_4$ and GTP (NCBI PubChem).

It is also possible to silence specific genes using RNA interference (RNAi). Originally termed 'co-suppression' in 1990, the technique was discovered by researchers investigating colour in petunias (Napoli *et al.*, 1990); however, it was research into *C. elegans* by Fire *et al.* (1998) that led to the breakthrough that enabled the technique to be harnessed for use across the scientific community. It was identified that the introduction of double-stranded RNA (dsRNA) into an organism could selectively silence genes in a process that was both systemic and heritable (Fire *et al.* 1998). A simplified description of the mechanism of action is shown in Figure 19. The natural role of RNAi is proposed to be an ancient, evolutionarily-conserved

mechanism of defence against viruses which possess dsRNA in their genome (Wang *et al*., 2006), with recent research demonstrating that negative strand viruses could be effectively targeted by the RNAi pathway (Mueller *et al*., 2010). It was originally hypothesised that the RNAi pathway was exclusive to the cytoplasm, but recent investigations provide evidence for a role within the nucleus (Ketting, 2011).
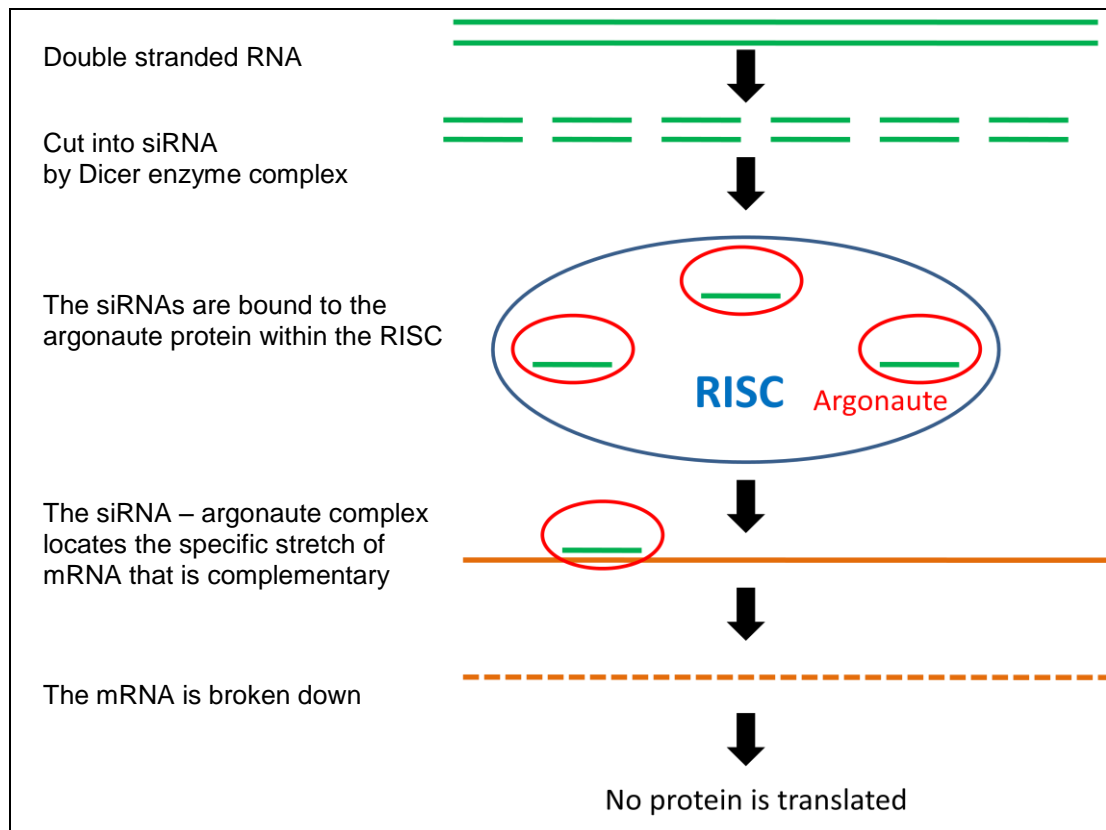
Figure 19. A summarised version of the mechanism of action of RNA interference. Double stranded RNA (dsRNA) that corresponds to the sequence for a specific gene is synthesised artificially and then exposed to the cells. The dsRNA is cut into short interfering RNA (siRNA) by an enzyme complex Dicer. The siRNAs are then bound to the argonaute protein within the RNA Induced Silencing Complex (RISC). The complementary sequence of mRNA is located by the siRNA-argonaute complex, at which point the mRNA is broken down resulting in a lack of translation to protein.

RNAi has been used across a wide range of disciplines (eg. Kimber *et al*., 2007; Pecot *et al*., 2011; Song *et al*., 2010), but unfortunately for the nematode research community, the transfer of the technique to parasitic species has not been straightforward (Geldhof *et al*., 2006; Knox *et al*., 2007). Successful knockdown of

transcript has been sporadic, despite numerous different methodologies and life-cycle stages being tested. Viney and Thompson (2008) hypothesised that the lack of success could either be due to inadequate exposure of the target mRNA to the dsRNA or that parasitic nematodes did not possess the full complement of genes required for the RNAi pathway. Recent bioinformatics analyses of the genomic and transcriptomic datasets of 13 parasitic nematode (both animal and plant parasitic species) revealed that, despite species differences, the genes required for effective RNAi were present (Dalzell *et al*., 2011). The proteins involved in siRNA synthesis and processing were well conserved with between 6 and 9 of the proteins involved in this process in *C. elegans* found in the parasitic species. All of the parasite species appeared to be capable of some dsRNA amplification, although none of the species studied possessed the full complement of proteins required for this role in *C. elegans*. For dsRNA spread, the apparent lack of *C. elegans* orthologues in the parasitic species suggests that sequence divergence may have occurred such that bioinformatics techniques are unable to identify the sequences as successful RNAi has been reported in species such as *Brugia malayi* which appear to lack the required genes (Dalzell *et al*., 2011). This suggests that the variability observed with RNAi in parasitic nematodes is derived from problems with delivery rather than a fundamental lack of RNAi processing proteins (Maule *et al*., 2011). RNAi has not been reported in *Teladorsagia circumcincta* so, in this chapter, silencing of the gene encoding GTP-Cyclohydrolase (*Tci-cat-4*), as well as the gene encoding β-tubulin isoform 1 (referred to from this point at *Tci-tub-1*) and a non-nematode derived gene, was examined.

The aims of the work described in this chapter were to inhibit GTP-Cyclohydrolase function *in vitro* and to explore the effects of inhibition on *T. circumcincta*. Inhibition of a gene that is highly upregulated in larval stages, using both RNAi and chemical inhibition techniques, could provide key insights into the role of the enzyme within in the parasite.

3.2 Materials and Methods

3.2.1 RNAi

3.2.1.1 Amplification of gene fragments

To examine the effect of gene silencing of *Tci-cat-4*, RNAi experiments were performed. Fragments of *Tci-cat-4*, *Tci-tub-1* (NCBI accession Z69258.1) and *lacZ* (200 – 260 bp) were amplified by PCR from *T. circumcincta* L_3 cDNA (Section 2.2.2) or from pGEM-T plasmid (Promega) using gene-specific primers (Table 5) and the Advantage 2 polymerase mix (Clontech). PCR conditions were 95 °C for 1 min, followed by 30 cycles of 95 °C, 53 °C (*Tci-tub-1* and *lacZ*) or 58 °C (*Tci-cat-4*) and 68 °C 1 min, followed by a final extension step of 1 min at 68 °C. The *lacZ* fragment was used as a negative control as a sequence that showed no significant similarity to any nematode gene when searched using the BLASTn tool on NCBI. Negative controls are important for RNAi experiments as lipofectin has been shown to be toxic if used without dsRNA (Omindi *et al*., 2003), and so all soaking experiments must contain a dsRNA negative control. β-tubulin isoform 1 had been shown previously to be successfully silenced in RNAi experiments in *Haemonchus contortus* (see Samarasinghe *et al*., 2011), so was included here as a gene that may have a higher likelihood of producing a knockdown effect. The sequence for *Tci-tub-1* needed to be confirmed, as this isotype had not been studied in the isolate of *T. circumcincta* under study. First, the PCR product was ligated into the pGEM-T vector (Promega), transformed into competent *E. coli* JM109 cells (Promega) and then sequenced (Eurofins MWG) to confirm that the sequence did not contain any of the restriction sites that would be used for sub-cloning (sequences shared 97 % identity to the reference sequence Z69258.1 at the nucleotide level and were 100 % identical at the amino acid level). The PCR fragment of interest was then excised from the pGEM-T vector using the restriction enzymes *NcoI* and *HindIII*, purified using the PCR purification kit (Qiagen) and ligated using T4 ligase (Promega) into the pL4440 vector (Addgene plasmid 1654) that had been linearised using the same enzymes. For the *Tci-cat-4* and *lacZ* amplicons, gene fragments were ligated directly into the pL4440 vector using either the restriction enzymes *NcoI* and *HindIII* with T4

ligase (*Tci-cat-4*) or the Infusion technique (Clontech; *lacZ*) according to manufacturers' instructions.

3.2.1.2   Production of double-stranded RNA

The pL4440 plasmids containing the amplicons were linearised with two different restriction enzymes (in separate reactions) to produce linear DNA with a T7 promoter upstream of the amplified gene fragment (*NcoI* and *HindIII* for *Tci-cat-4*; *SalI* and *XmaI* for *Tci-tub-1* and *lacZ*). Double-stranded RNA (dsRNA) was then produced by *in vitro* transcription using the T7 Ribomax express kit (Promega) following manufacturer's instructions, resuspended in 80 µl nuclease-free water, quantified (Nanodrop® ND-1000 UV-Vis Spectrophotometer) and stored at −80 °C until needed.

3.2.1.3   Incubation of *T. circumcincta* in dsRNA

L_3 of the MTci2 strain of *T. circumcincta* were used for the RNAi experiments. Two thousand L_3 were exsheathed using sodium hypochlorite (Glaser and Stoll, 1940) for each dsRNA incubation. The L_3 were washed three times in PBS, followed by a further three washes in Earle's balanced salt solution (Sigma) combined with penicillin (167 units / ml), streptomycin (33 µg / ml) and fungizone (1.25 µg / ml). After the final wash, the volume of L_3 was reduced to 30 µl by centrifugation and aspiration of the supernatant. Three replicate incubations were set up for each of the three genes being tested (*Tci-tub-1, Tci-cat-4* and *lacZ*). Separately, 40 µg of each dsRNA in solution were combined with 1 µl lipofectin (Invitrogen) and 0.2 µl RNAsin and incubated for 10 min at room temperature. The incubated dsRNA / lipofectin solutions (10 µl) were then added to the L_3 to give a final volume of 40 µl and incubated at 37 °C for 72 h in an atmosphere of 5 % CO_2. After incubation, the L_3 were washed three times in PBS to remove the dsRNA and snap frozen before storage at -80 °C.

3.2.1.4 Analysis of RNAi effect

RNA extractions from nematodes incubated as described in Section 3.2.1.3 were performed using Trizol (Invitrogen) according to manufacturer's instructions. Five µl of the resulting total RNA in aqueous solution were used for cDNA synthesis using the AffinityScript Multiple Temperature cDNA synthesis kit (Agilent) using oligo(dT) primers with synthesis of first strand cDNA occurring over 1 h at 42 °C. Semi-quantitative PCR was performed using Biomix reagents (Bioline) with gene-specific primers (Table 6) that were designed to span an intron to rule out genomic DNA amplification. The full genomic sequence of *Tci-tub1* was not known, but a partial sequence had been published previously (Silvestre and Humbert, 2002), with one identified intron contained within the sequence amplified by the primers used. Any genomic amplification would therefore be at least 874 bp, but could be larger if further introns were present. Three genes were investigated in each cDNA sample: *elongation factor 1α* (*Tci-ef-1a*) which had been used previously as a reference gene in *T. circumcincta* (see Chapter 2), *Tci-cat-4* and *Tci-tub-1*. Samples of 5 µl were removed from each reaction at 25, 30, 35 and 40 cycles to assess by agarose electrophoresis the amplification of the PCR products representing each transcript in a semi-quantitative fashion. Samples were loaded onto 1 % agarose / TAE gels containing GelRed (Cambridge Bioscience) and examined using UV (Appendix 8.1.1).

Table 5. Primer sequences used to generate gene fragments for cloning *Tci-tub*-1, *Tci-cat-4* and *lacZ* into the pL4440 plasmid. The underlined bases in the *Tci-cat-4* primers refer to the sequence for two restriction enzymes (*NcoI* and *HindIII*) that were introduced to enable insertion into the pL4440 vector. The underlined bases in the *lacZ* primers refer to the pL4440 vector sequence that was added to the end of the primers to enable cloning using the Infusion system (Clontech).

Gene	Forward primer sequence	Reverse primer sequence	Product length (bp)
Tci-tub-1	5'-TGCCACTCTTTCTGTACACC-3'	5'-CGAAACCTGGCATGAAGAAG-3'	260
Tci-cat-4	5'-ATCCCATGGGTTCTCTCTATGTGAGCATCATCTAGTGCC-3'	5'-TCAAAGCTTCTTGCCTCTATCACAACTGCAACACC-3'	216
lacZ	5'-ATTCGATATCAAGCTTCCTGTGTGAAATTGTTATCCGC-3'	5'-CGGTATCGATAAGCTTAATACGCAAACCGCCTCTC-3'	201

Table 6. The oligonucleotide primer sequences used in the semi-quantitative PCR to assess the effect of RNAi.

Gene	Forward Primer sequence	Reverse Primer sequence	Product length (bp)	
			cDNA	gDNA
Tci-cat-4	5'-CGTCAAGGGCTTCTGAAAAC-3'	5'-GGTGGTGGCATTGATCTTCT-3'	414	1725
Tci-ef-1α	5'-TGAGCGTAAGGAAGGAAATG-3'	5'-CGTATAACCAGCAGCGATTT-3'	428	486
Tci-tub-1	5'-TGCCACTCTTTCTGTACACC-3'	5'-TCATTACTCCTCGGGGTACG-3'	798	≥874

3.2.2 Chemical inhibition of GTP-Cyclohydrolase

T. circumcincta parasite material was collected from a sheep experimentally infected with MTci2 as described previously (Section 2.2.1.2). DAHP was purchased from Sigma Aldrich and dissolved in distilled water. Solutions were prepared fresh each day as a range of concentrations from 1 – 26 mM.

3.2.2.1 Egg Hatch Test

In order to minimise the degree of embryonic development, *T. circumcincta* eggs used in this test were collected from sheep faeces (Section 2.2.1.2) and used in the test within 4 h. Suspensions containing 100 eggs in 100 µl of tap water were dispensed into 24-well tissue culture plates containing DAHP test solutions (1 – 26 mM in distilled water) to give a final volume of 2 ml. The eggs were incubated for 48 h at 26 °C in the dark and then fixed with helminthological iodine (Jackson *et al*., 2004) to allow the number of eggs and larvae to be counted using a compound microscope.

3.2.2.2 Larval Feeding Inhibition Test

Recently-hatched *T. circumcincta* first stage larvae (L_1) were Baermannised (Baermann, 1917) at 25 °C for approximately 1 h allowing the larvae to migrate through a 20 µm mesh. Migrated L_1 were adjusted to produce a suspension containing 100 L_1/100 µl tap water and 100 µl of L_1 suspension were dispensed into separate 1.5 ml microcentrifuge tubes containing DAHP test solutions (as above). Following 2 h of incubation at 20 °C, 7.5 µl of Fluorescein isothiocyanate (FITC) labelled *E. coli* (see Álvarez-Sánchez *et al*., 2005) were added to each tube, and then vortexed gently prior to being placed horizontally in a 25 °C incubator in the dark and incubated for a further 18 h. Post-incubation, the tubes were centrifuged at 2000 ×*g* for approximately 20 s and 500 µl of the supernatant removed and discarded. Five µl of the pellet containing the L_1 were transferred onto a microscope slide and placed on a hot plate (at 50 °C) for approximately 30 s to immobilize the L_1. The slide was examined under a fluorescence microscope (Axiovert 25, Zeiss). L_1 that

had fed on the FITC labelled *E. coli* glowed green when placed under fluorescent light, while those that had not fed remained dark (Figure 20). The number of fed and unfed L_1 on each slide was counted.
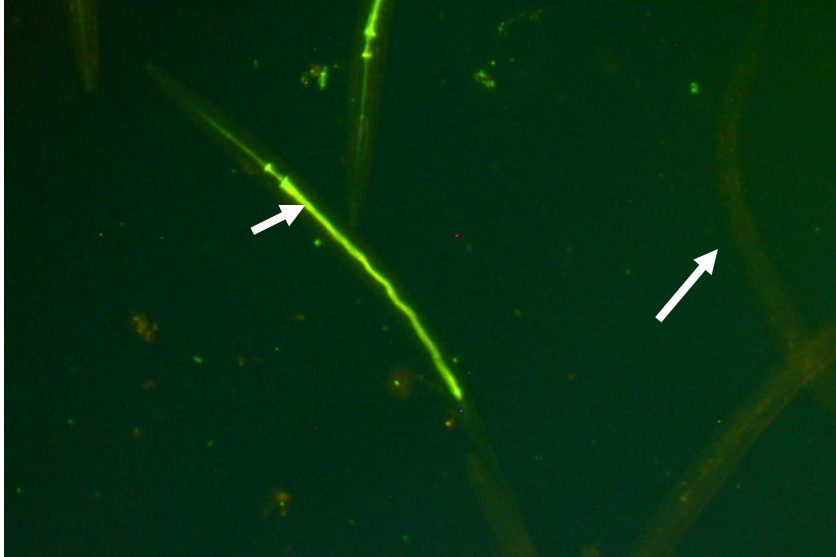

Figure 20. First stage *T. circumcincta* larvae (L_1)as viewed under a fluorescence microscope. Larvae that have fed on the fluorescent *E. coli* and those that have been unable to feed are highlighted.

3.2.2.3 Larval Development Test

One hundred and twenty µl of DAHP test solution, at concentrations from $0 - 26$ mM, were dispensed into the wells of a 96-well tissue culture plate. Twenty µl of Earle's balanced salt solution with 1 % yeast extract were added to provide a growth medium. One hundred eggs in 10 µl water and amphoteracin-B (Sigma) at 5 µg / ml were added to the plate giving final DAHP concentrations of 0 -20.8 mM in a final volume of 150 µl. The plate was incubated at 22 °C for 7 days in the dark and then fixed with helminthological iodine as above. The proportions of the different life-cycle stages were then counted for each concentration.

3.2.2.4 Larval Migration Inhibition Test

T. circumcincta L_3 that had been stored for less than six months at 4 °C were Baermannised to ensure that only L_3 capable of migration were used in the test. The

volume was adjusted to produce a suspension containing 100 L_3/100 µl water. One hundred L_3 and 1 ml of each of the DAHP test solutions were combined in 1.5 ml microcentrifuge tubes and centrifuged at 2000 $\times g$ for 1 min. The supernatant was discarded and the process repeated to ensure that the L_3 were incubated in the correct concentration of DAHP. After 2 h at 26 °C, a further centrifugation and removal of supernatant was performed to reduce the volume of L_3 / DAHP solution to 200 µl. The L_3 suspensions were transferred to migration chambers containing 1800 µl of the DAHP solutions at the relevant concentrations and a 25 µm migration filter (Demeler *et al.*, 2010). L_3 that were affected by the inhibitor did not migrate through the mesh filter to the bottom of the well. After a further 2 h, the L_3 were fixed with helminthological iodine and the numbers of migrated and non-migrated L_3 counted using a compound microscope.

All bioassays were conducted in duplicate (larval feeding inhibition test and migration inhibition test) or triplicate (larval development test and egg hatch test) and repeated on different days, with parasite material collected from sheep of the same age in two consecutive years. Probit analysis of the response to DAHP was performed using Genstat (12th Edition) and the effective dose at which 50 % of the worms were prevented from hatching, feeding, migrating or developing to L_3 (ED_{50}) estimated. For the larval development test a generalised linear model was also performed using Minitab (version 12) comparing the number of eggs that had developed through to L_3 stage in the different concentrations of DAHP (Lind *et al.*, 2005).

3.2.2.5 Electron Microscopy

In order to identify if cuticular changes were occurring in L_1 exposed to DAHP, eggs were extracted in the same way as above, but were then incubated in distilled water or 26 mM DAHP for 48 h. The emerging L_1 were Baermannised and fixed for 1 h in 4 % glutaraldehyde, 1 % osmium tetroxide, 3 % sucrose in 0.1 M sodium cacodylate buffer. The L_1 were washed for 1 h in 0.1M sodium cacodylate buffer with 3 % sucrose before dehydration in a series of ethanol steps (50 %, 70 %, 90 % then 100

%). Samples were 'critical point' dried and then examined using an Hitachi S-4700 Scanning Electron Microscope. These experiments were performed at the University of Edinburgh, with the kind assistance of Dr Stephen Mitchell.

3.2.2.6   *C. elegans* cultures for sensitivity to DAHP

To assess for generalised toxicity of DAHP against nematodes, *C. elegans* wildtype N2 strain and the GTP-Cyclohydrolase *cat-4* deletion mutant LC35 (provided by Prof. Curtis Loer, University of San Diego) were grown from eggs on plates containing DAHP: Nematode Growth Media (NGM; Appendix 8.1.5.1) plates were prepared as well as NGM plates containing 26 mM DAHP, added after autoclaving. Eggs from lysates of each strain of worm were then transferred onto the NGM and NGM+DAHP plates that had been seeded with *E. coli* strain OP50 as a food source and incubated at 20 °C for two weeks. The plates were examined daily to assess worm development.

3.2.3   GTP-Cyclohydrolase Feedback Regulatory Protein (GFRP)

To examine whether *T. circumcincta* possessed the gene encoding the GTP-Cyclohydrolase Feedback Regulatory Protein (GFRP), a stepwise process was undertaken. Firstly, the coding sequence for *gfrp* in *C. elegans* was BLASTn searched against the partial *T. circumcincta* genome sequence available at the Sanger Institute. A *T. circumcincta* sequence with 70 % nucleotide identity over 144 bases was identified (tc-13d23.p1k on www.sanger.ac.uk), from which oligonucleotide primers were designed to amplify the short section of coding sequence (shown in yellow in Figure 21). 'Semi-quantitative PCR' was performed using these primers on *T. circumcincta* cDNA generated previously in Section 2.2.1.2. Reactions contained 15 µl 2x Biomix (Bioline), 5 µl of each of the forward and reverse gene-specific primers at 2 µM, and 1 µl cDNA template (diluted with 4 µl water). Cycling conditions were 94 °C for 5 min, followed by 40 cycles of 30 s at 94 °C, 30 s at 52 °C and 30 s at 72 °C, followed by a final 10 min extension at 72 °C. Samples of 5 µl were removed after 25, 30, 35 and 40 cycles and were examined on a 1 % agarose gel containing TAE and GelRed. To generate the full coding sequence, the gene

specific reverse primer (Figure 21) was used with the TriplEx forward primer to amplify products from an L$_4$ SMART cDNA library [Clontech; (Nisbet *et al.*, 2011)] and the Advantage 2 polymerase (Clontech). PCR conditions were 94 °C for 1 min, followed by 35 cycles of 94 °C for 30 s, 54 °C for 30 s and 68 °C for 1 min, followed by a final extension step of 1 min at 68 °C. The PCR product was purified and ligated into pGEM-T (Promega) using T4 ligase. The plasmids were transformed into JM109 competent *E. coli* cells (Promega), incubated for 1 h at 37 °C in a shaking incubator in the presence of SOC medium, and then spread on LB medium plates containing 100 µg / ml ampicillin, 0.5 mM IPTG and 80 µg/ml X-Gal. Colony PCR was performed on white colonies using the gene-specific primers (Figure 21), with positive colonies selected for amplification. Plasmids were amplified in 10 ml LB medium with 100 µg / ml ampicillin at 37 ° C for 18 h, at 200 rpm. The plasmids were purified using the Wizard plus SV minipreps kit (Promega) and sequenced (Eurofins MWG).

```
Query:  107 TCACCA-AGCAGTTGGGAAATGAGTTTCAGGAATACATAACAAAATGGTCGCCACGAAAA 165
            |||||  | || | | ||| |   ||  |||||||| | |||   |   | || ||  ||
Sbjct:    2 TCACCGCATCAAT-GTGAATTTTTTTGCAGGAATATGTGACAGCGTTAGCACCTCGTCAA 60


Query:  166 GTGCTCAACCAATTGGAAAAAGAGGGATGGACAGTGGTCGCTATGGCTGGAATCGGACAA 225
            || ||  ||| | | ||||| ||||| |||| |||||| |  ||||| || ||||| |||
Sbjct:   61 GTACTAGACCTACTAGAAAAGGAGGGTTGGAAAGTGGTTGGAATGGCCGGTATCGGTCAA 120


Query:  226 ACTTGTGCTTGGACTTTATATAAA 249
            ||||||| |||||||| | |||
Sbjct:  121 ACTTGTGCATGGACTTTACACAAA 144
```
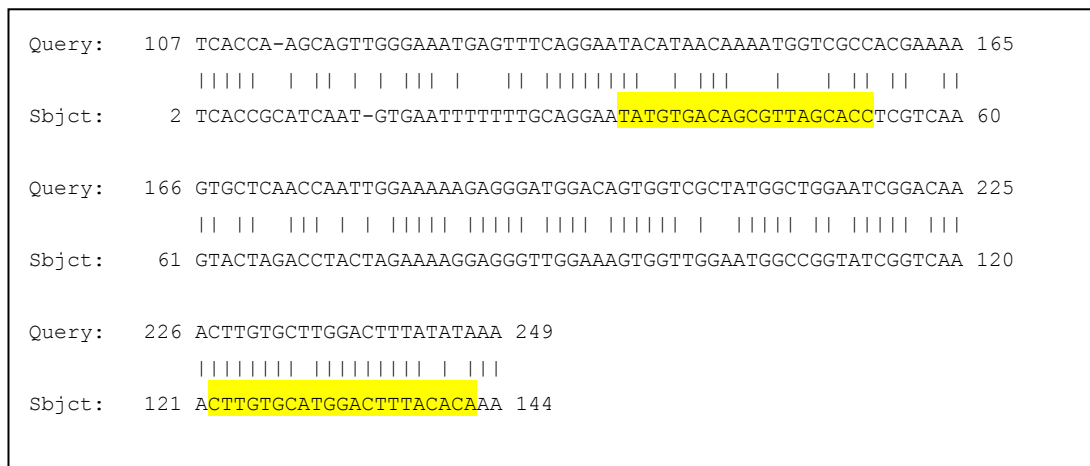
Figure 21. An alignment between the *C. elegans* GTP-Cyclohydrolase Feedback Regulatory Protein sequence (Query) and the *T. circumcincta* genome sequence read "tc-13d23.p1k" (Sbjct) generated at www.sanger.ac.uk. The bases highlighted in yellow were used to design oligonucleotide primers to amplify the appropriate sequence from *T. circumcincta*.

3.3 Results

3.3.1 RNAi

L_3 which had been exposed to *Tci-tub-1* and *Tci-cat-4* dsRNA appeared healthy and active after the 72 h incubation, but L_3 exposed to *lacZ* dsRNA appeared less active with more detritus present in the fluid at the end of the experiment. RNA was extracted successfully from all samples exposed to *Tci-cat-4* and *lacZ* dsRNA, but only from two of the three samples incubated with *Tci-tub-1* dsRNA. Semi-quantitative PCR indicated that partial silencing of the *Tci-tub-1* mRNA had occurred, as shown in Figure 22, with a reduction in *Tci-tub-1* transcript from L_3 that had been soaked in *Tci-tub-1* dsRNA compared to those L_3 that had been soaked in *Tci-cat-4* dsRNA. This result was consistent across both *Tci-tub-1* and all *Tci-cat-4* replicates.
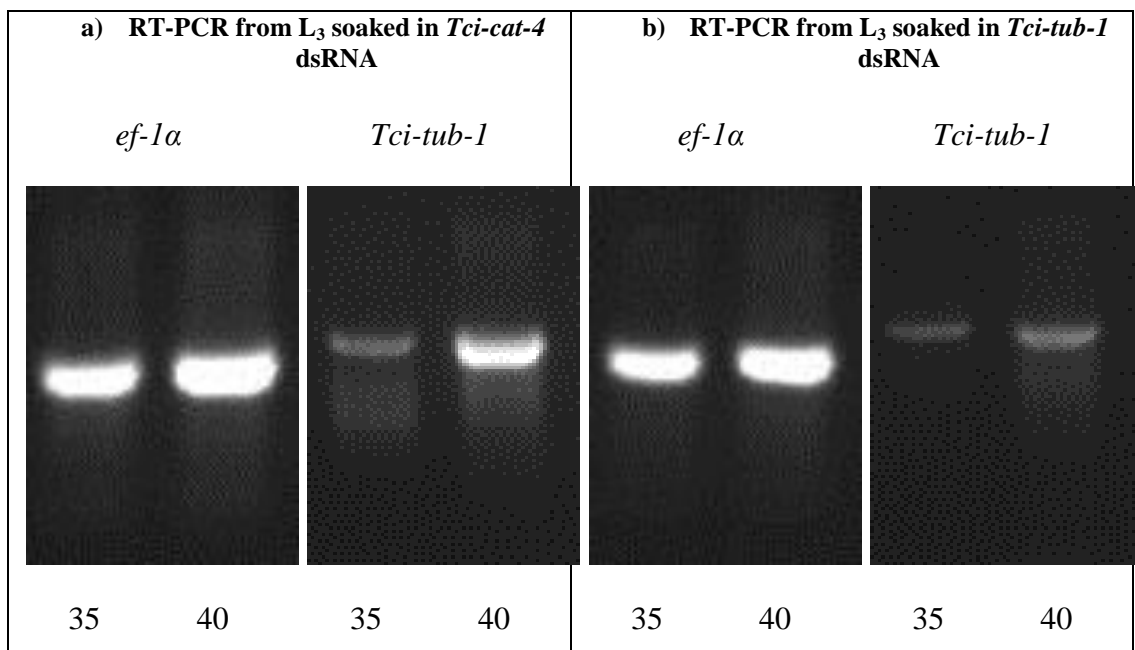

a) RT-PCR from L_3 soaked in *Tci-cat-4* dsRNA				b) RT-PCR from L_3 soaked in *Tci-tub-1* dsRNA			
ef-1α		*Tci-tub-1*		*ef-1α*		*Tci-tub-1*	
35	40	35	40	35	40	35	40

Figure 22. Semi-quantitative amplification of *Tci-ef-1α* and *Tci-tub-1* from different cDNA from the populations of L_3 that had been exposed to (a) *Tci-cat-4* dsRNA or (b) *Tci-tub-1* dsRNA. The amplification of the reference gene *Tci-ef-1α* over 35 and 40 cycles was similar for both populations. Amplification of the gene for *Tci-tub-1* was reduced in the PCR using cDNA produced from L_3 that had been exposed to *Tci-tub-1* dsRNA compared to those exposed to *Tci-cat-4* dsRNA, suggesting a partial silencing of the *Tci-tub-1* gene. These results are from the first replicate, but are representative of the second replicate.

No reduction was seen in the *Tci-cat-4* transcript levels of the L_3 that were exposed to *Tci-cat-4* dsRNA (Figure 23), with PCR products of equivalent intensity observed in both samples. This occurred consistently across all replicates.
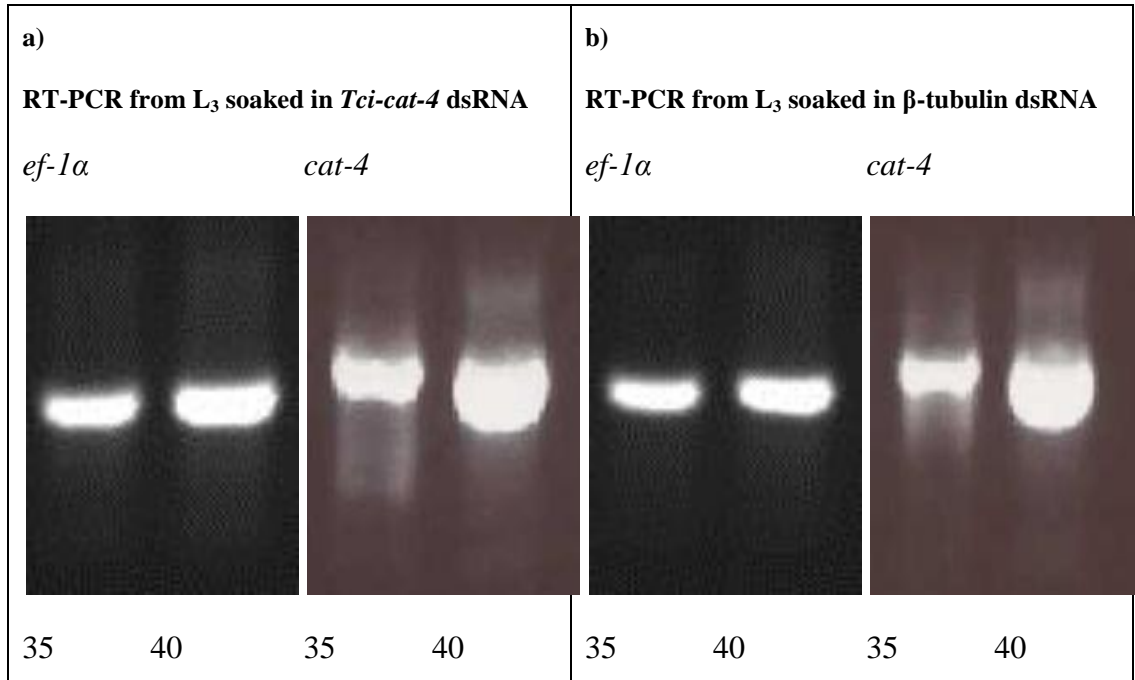

Figure 23. Semi-quantitative amplification of *Tci-ef-1α* and *Tci-cat-4* from different cDNA from samples of L_3 that had been exposed to (a) *Tci-cat-4* dsRNA or (b) *Tci-tub-1* dsRNA. Amplification of the *Tci-cat-4* gene was consistently high across larvae that had been exposed to both *Tci-cat-4* dsRNA and *Tci-tub-1* dsRNA, demonstrating that no silencing had occurred.

In L_3 that were exposed to non-nematode *lacZ* dsRNA, reductions in intensity of PCR products of *Tci-cat-4, Tci-tub-1* and *Tci-ef-1a* were observed (as shown in the example in Figure 24). In this example, the degree of amplification of *Tci-ef-1a* was comparable with that in PCRs containing cDNA from L_3 that had been exposed to both *Tci-tub-1* and *Tci-cat-4*. However, the amplification of the *Tci-ef-1a* sequences from L_3 that had been exposed to *lacZ* was reduced. This pattern was observed across all the genes (i.e. reduction in *Tci-tub-1* and *Tci-cat-4* amplification products observed in L_3 that had been exposed to *lacZ* dsRNA). This was not observed with the other dsRNA, so *Tci-tub-1* levels were not affected by *Tci-cat-4* dsRNA and vice versa.
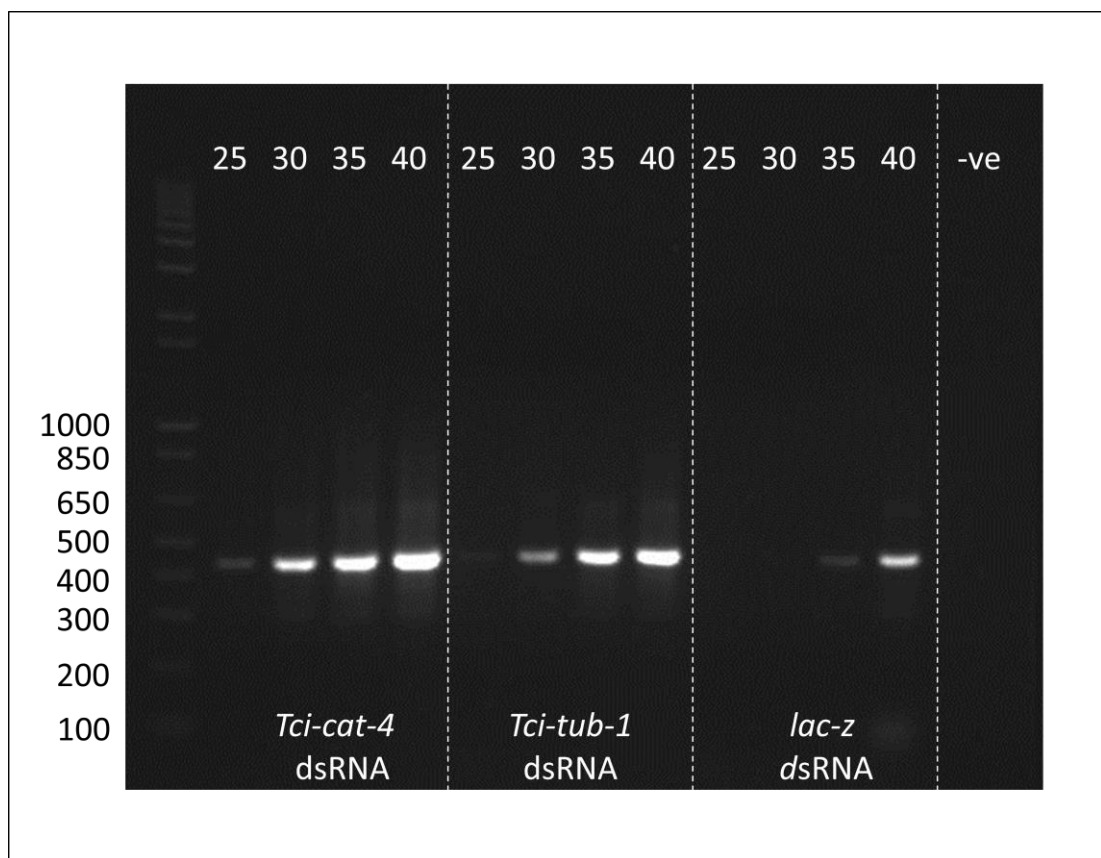

Figure 24. Semi-quantitative amplification of *ef-1a* from cDNA of L_3 that had been exposed to either *Tci-cat-4, Tci-tub-1* or *lacZ* dsRNA, with bp sizes shown for the 1 kb plus ladder (Invitrogen).

3.3.2 Bioassays

There was no measurable effect of DAHP on eggs, L_1 and L_3 in the egg hatch test, the feeding test, or the migration test, with similar patterns of behaviour observed in individuals exposed to the highest concentrations of DAHP compared to control samples which had been incubated in water alone (Figure 25). Probit analysis confirmed that none of the parasites exposed to DAHP behaved in a manner that was significantly different from control larvae or eggs. A small proportion (<5 %) of L_1 that hatched in the egg hatch test in the highest concentration of DAHP appeared to have abnormal cuticle development (Figure 26).
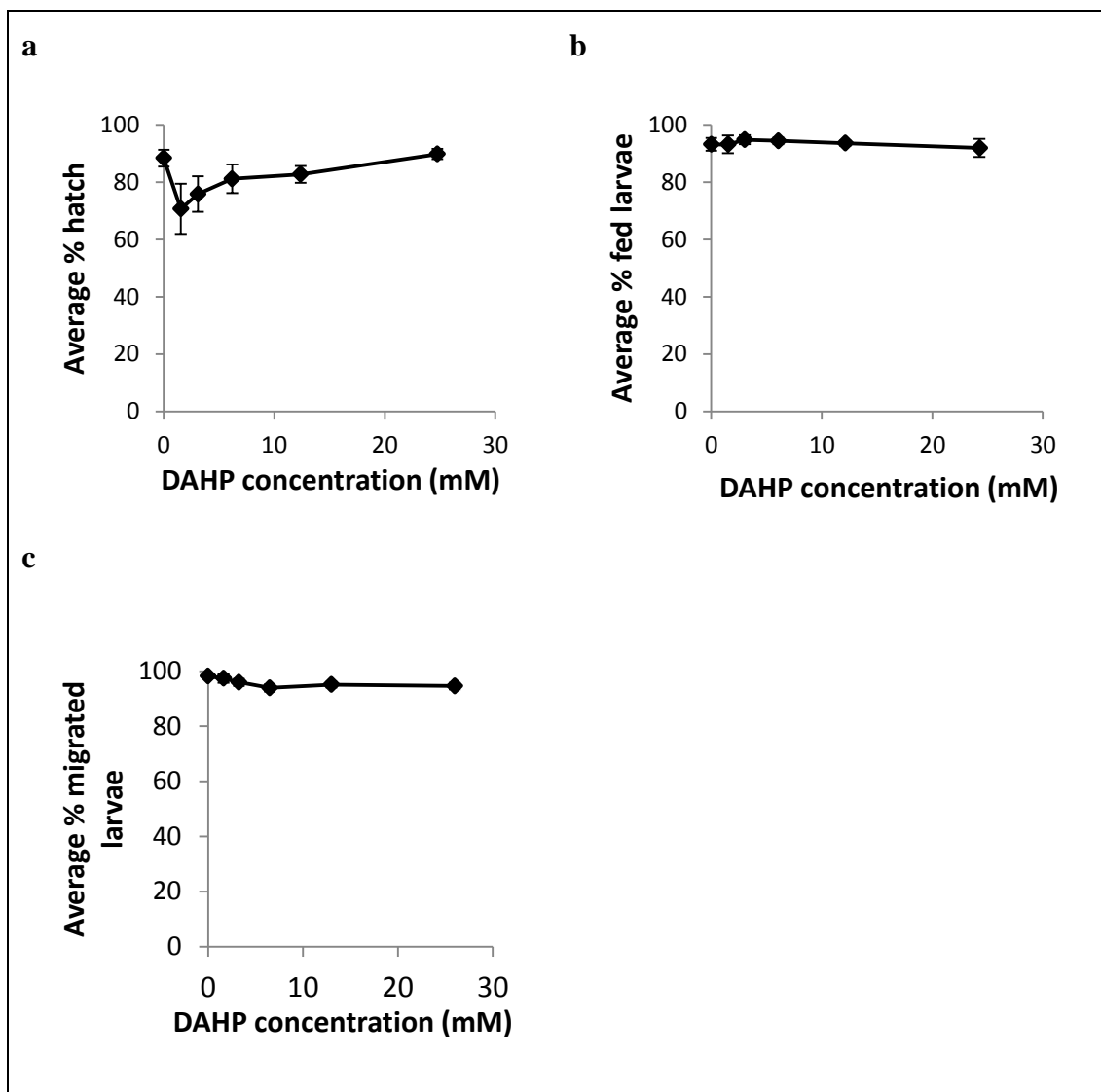

Figure 25. The effect of DAHP on a) egg hatch rates (n = 11), b) larval feeding (n = 4) and c) larval migration (n = 4). Error bars show the standard error of the mean.
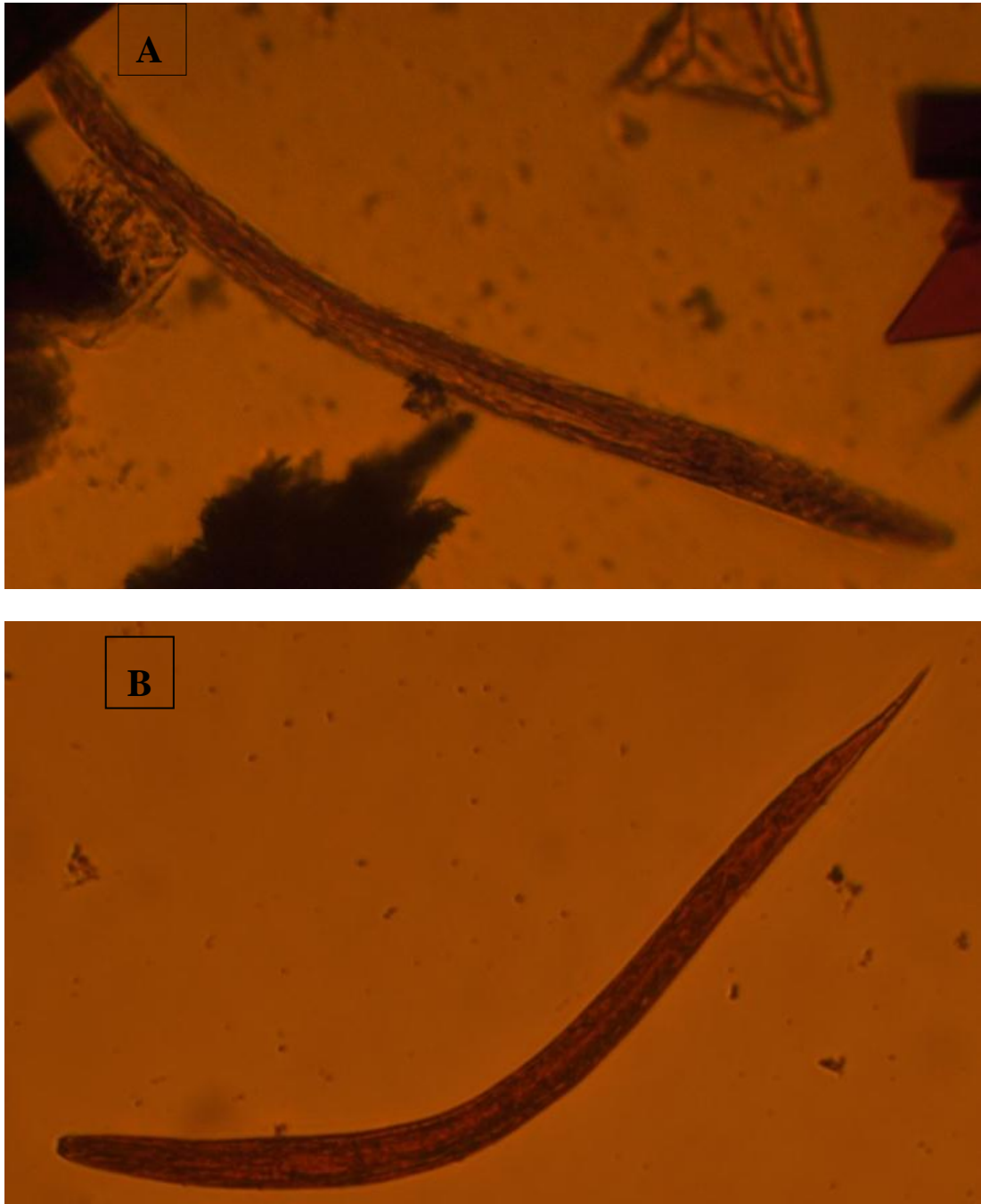
Figure 26. Changes to the cuticle were visible in a small proportion of L_1 that had hatched in 26 mM DAHP. The cuticle of the DAHP-treated L_1 (A) appears damaged, with a more uneven surface, when compared to an L_1 incubated in the aqueous solution only (B).

In the larval development test, higher concentrations of DAHP tested significantly reduced the proportion of eggs that developed to L_3 ($p < 0.001$), as shown in Figure 27 and Figure 28. The effective dose at which 50 % of eggs failed to reach L_3 (ED50) was 5.5 mM. At the highest concentration of DAHP (20.8 mM), 73 % ($\pm$ 1.1

% SEM, n=21) of the individuals were L_1, compared to just 11 % ($\pm$ 2.0 % SEM, n=21) in the controls. The proportion of L_3 enumerated in samples was 83 % ($\pm$ 2.7 % SEM, n=21) in the controls and 3 % ($\pm$ 2.3 % SEM, n=21) when incubated in the highest DAHP concentrations. There was also an increase in the proportion of L_2 observed after incubation in the highest DAHP concentrations; 5 % ($\pm$ 1.4 % SEM, n=21) in the controls *vs* 21 % ($\pm$ 6.3 % SEM, n=21) in 20.8 mM DAHP. The main effect of DAHP was on the larval stages, as 96 % ($\pm$ 1.1 % SEM, n=21) of eggs hatched, even when incubated in the highest concentrations of DAHP.
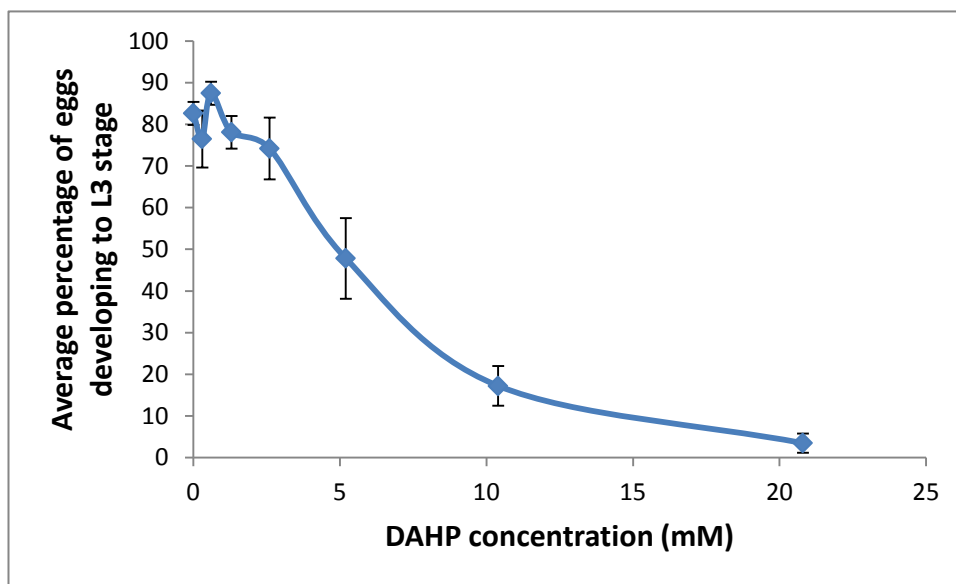

Figure 27. The average percentage of eggs reaching L_3 after seven days of incubation with DAHP ; error bars represent the standard error of the mean (n = 21). In the controls, 83 % of eggs developed through to L_3, whereas only 3 % developed to L_3 in the presence of 20.8 mM DAHP. The effective dose at which 50 % of the eggs failed to reach L_3 (ED50) was 5.5 mM.
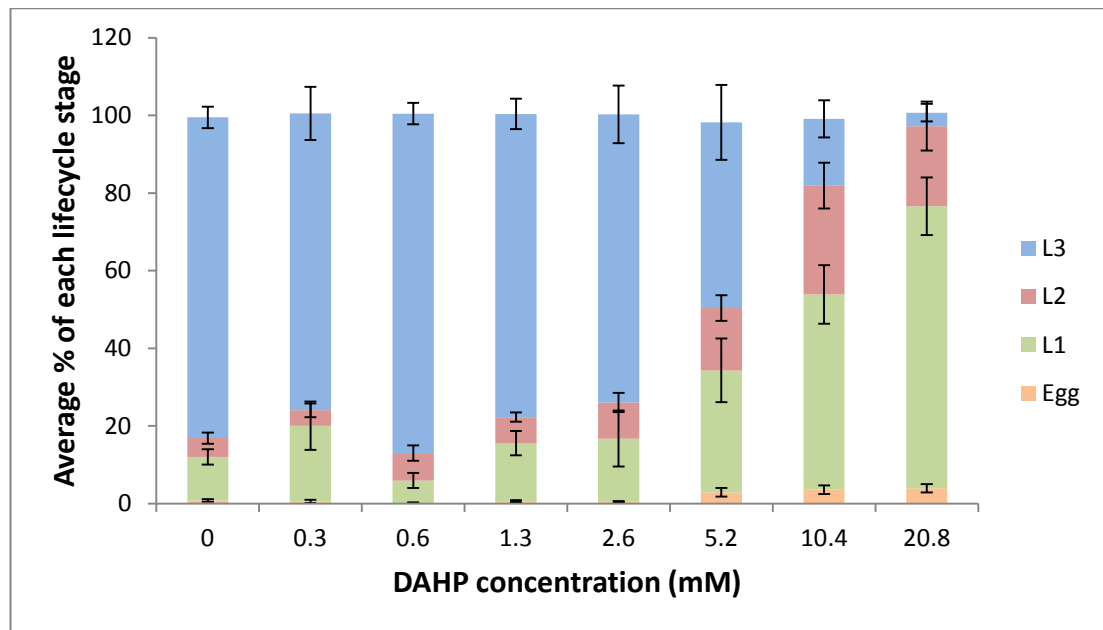
Figure 28. The average percentage of each of the *T. circumcincta* life-cycle stages present after seven days incubation in different concentrations of DAHP , with error bars showing the standard error of the mean (n = 21).

Both the wild-type N2 and mutant *cat-4* strains of *C. elegans* were able to develop normally to fully mature, egg-laying adults in the presence of 26 mM DAHP. At least five generations were observed on the plates before overcrowding induced the devlopment of dauer larvae.

3.3.3 Scanning Electron Microscopy

The scanning electron micrographs (Figure 29) revealed that *T. circumcincta* L_1 have a star-shaped triradiate mouth, similar to that described previously for *H. contortus* L_3 (Lichtenfels *et al.*, 1990). In contrast to later stages and other nematode species, *T. circumcincta* L_1 had what appeared to be an excretory pore approximately 2.5 μm from the anterior end (shown in Figure 29a with the white arrow). This was observed in a number of L_1, with no other pores seen elsewhere on the cuticle. The electron microscopy did not identify any major differences in the cuticles of the L_1 that had been treated with DAHP compared to the L_1 that had been incubated in water, although it was noted that during the fixing and drying process, control L_1 formed clumps, whereas the DAHP-treated larvae remained separate.
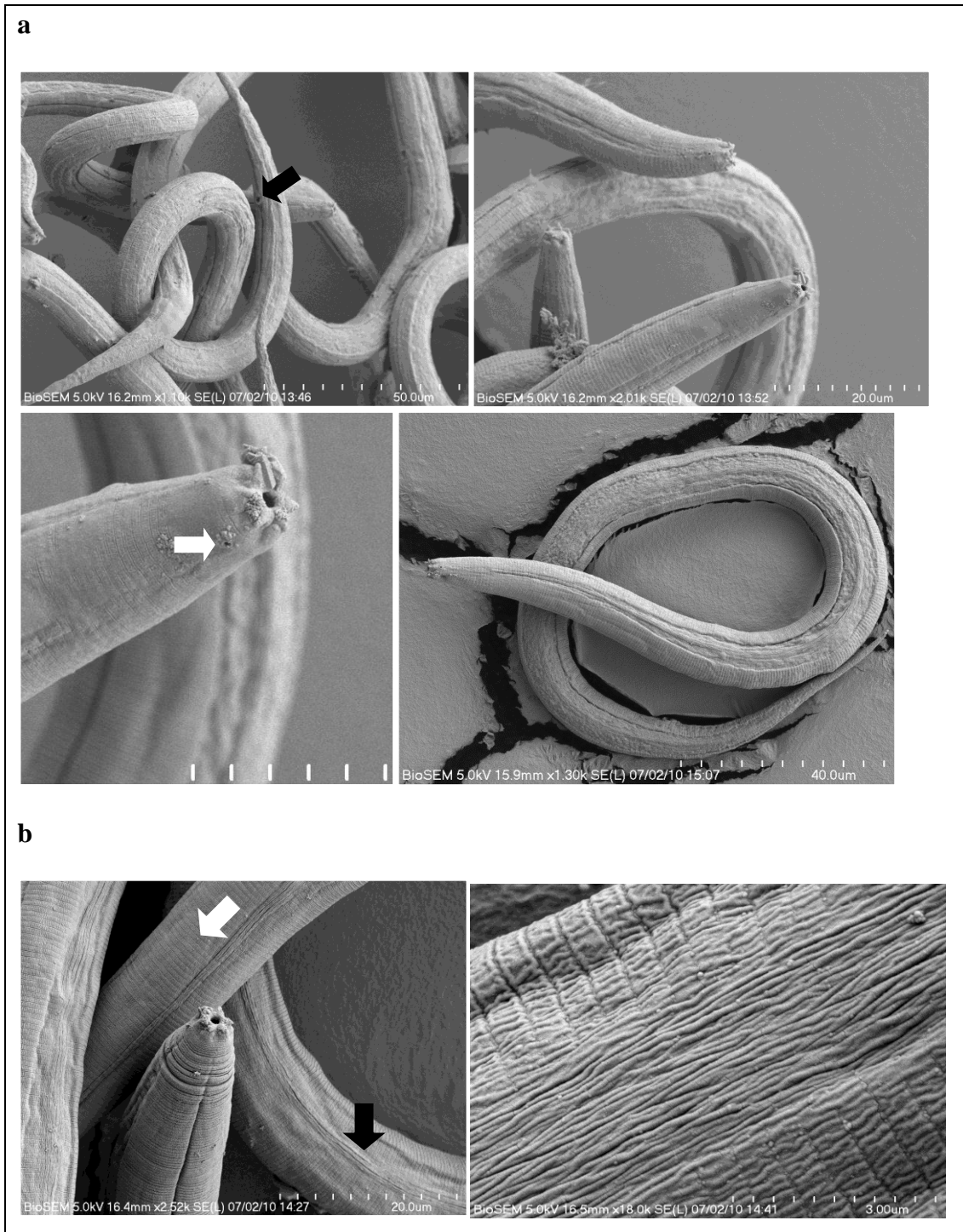
Figure 29. Electron micrographs of the a) control *T. circumcincta* L_1 and b) DAHP-treated *T. circumcincta* L_1. The anus is shown with a black arrow (a) and the possible excretory pore with a white arrow (a). The alae (black arrow in b), annuli (white arrow in b) and mouth all appear intact.

3.3.4 GTP-Cyclohydrolase Feedback Regulatory Protein

Full-length cDNA sequence for *Tci-gfrp* was amplified from the L$_4$ cDNA library. The associated sequence has been deposited on the European Bioinformatics Institute database with accession number HE577178. The deduced amino acid sequence aligned closely with that of other species as shown in Figure 30, with a 71 % identity to the *C. elegans* sequence. *Tci-gfrp* was PCR amplified from cDNA of all life-cycle stages tested. Bands were of equivalent intensity for all stages (although amplification of the reference gene *ef-1α* was not as stable in the egg sample, so this stage was discounted from analysis) as shown in Figure 31.

```
Rat             MPYLLISTQIRMEVGPTMVGDEHSDPELMQQLGASKRRVLGNNFYEYYVNDPPRIVLDKL 60
Mouse           MPYLLISTQIRMEVGPTMVGDEHSDPELMQHLGASKRSVLGNNFYEYYVNDPPRIVLDKL 60
B taurus        MPYLLISTQIRMEVGPTVVGDEHSDPELMQHLGASKRSVLGNNFSEYYVNDPPRIVLDKL 60
Human           MPYLLISTQIRMEVGPTMVGDEQSDPELMQHLGASKRRALGNNFYEYYVDDPPRIVLDKL 60
Zebrafish       MPYILISTQIRLETGPTMVGDEYSDPSIMNYLGARKITVLGNNFSEYHVDEPPRLVLDKL 60
Xenopus         MPYVLISTQIRMETGPTIVGDEFSDTQLMAQLEADKRTVLGNNFSEYCVNEPPRVTLNKL 60
C elegans       MPYILISTQIRMECGPTMVGDGESDPKLMGTLEAKLTKQLGNEFQEYITKWSPRKVLNQL 60
T circumcincta  MPYMLISTQIRLEVGPTFVGDGESDKELMARLRANPSRQLGNEFVEYVTALAPRQVLDLL 60
                ***:*********:* ***.***  ** .:*   * *    ***:* **  .  .** .*: *


Rat             ECRGFRVLSMTGVGQTLVWCLHKE----------- 84
Mouse           ECKGFRVLSMTGVGQTLVWCLHKE----------- 84
B taurus        ERRGFRVLSMTGVGQTLVWCLHKE----------- 84
Human           ERRGFRVLSMTGVGQTLVWCLHKE----------- 84
Zebrafish       DKIGYRVVSMTGVGQTLVWCLHKESSNTL------ 89
Xenopus         EKLGYRVVSMTGVGQTLVWCLHKE----------- 84
C elegans       EKEGWTVVAMAGIGQTCAWTLYKCDDEKEENGMNF 95
T circumcincta  EKEGWKVVGMAGIGQTCAWTLHKED--------- 85
                .   *: *:  * *.***   * *.*
```

Figure 30. ClustalW alignments of the GFRP amino acid sequences from different vertebrate and invertebrate species. There is a high level of homology between all the sequences. Amino acid sequences were downloaded from NCBI with the following accession numbers: Rat (AAH86944), Mouse (NP_796131), *Bos Taurus* (NP_001107998), Human (BAG34813), *Danio rerio* (NP_957056), *Xenopus (Silurana) tropicalis* (NP_998880). The *C. elegans* sequence was downloaded from wormbase Y38C1AA.13.
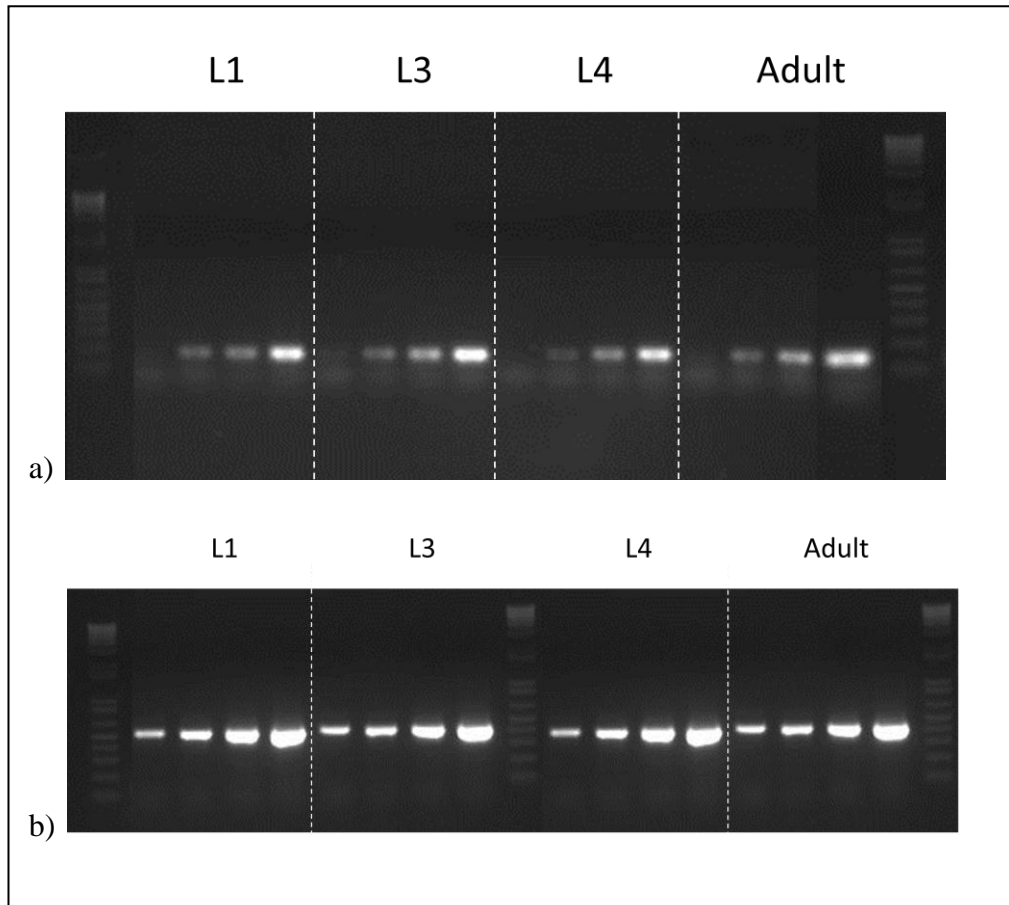
Figure 31. Semi-quantitative PCR of a) *Tci-gfrp* and b) *Tci-ef-1α* cDNA from different life-cycle stages of *T. circumcincta* over 25, 30, 35 and 40 amplification cycles. Amplification of *Tci-ef-1α* remained relatively consistent over the life-cycle.

3.4 Discussion

In this chapter, the effect of a GTP-Cyclohydrolase inhibitor *in vitro* and the use of RNAi were reported. Successful RNAi in *T. circumcincta* has never been reported previously and this technology has had mixed success when used in other parasitic nematode species (Hussein *et al.*, 2002; Islam *et al.*, 2005; Issa *et al.*, 2005). In plant parasitic nematodes, it appears to be a more reliable technique than in animal parasitic nematodes (Bakhetia *et al.*, 2008; Kimber *et al.*, 2007; Shingles *et al.*, 2007). Here, the partial knockdown of the *Tci-tub-1* transcript in *T. circumcincta* L$_3$ with gene-specific dsRNA was demonstrated. The result was comparable to that seen in *H. contortus*, in which this gene was silenced in a number of experiments using the same methods (Samarasinghe *et al.*, 2011). The experiments using *T. circumcincta* were performed once, with only two replicates analysed for effect, so it would be beneficial to repeat the tests, both with *Tci-tub-1* and with other *T. circumcincta* genes to have complete confidence in the RNAi effect in this species. However, as an initial result, it looks promising.

The lack of effect of the *Tci-cat-4* dsRNA on *T. circumcincta* appears to correlate with the current hypotheses surrounding the mechanism of action of RNAi within parasitic nematodes (Rosso *et al.*, 2009); the larvae used in these experiments had been artificially exsheathed in sodium hypochlorite and then stored in an incubator rather than developing inside the abomasum of the sheep. Under such artificial conditions it appears that the genes most susceptible to silencing are those that come into direct contact with the dsRNA, located either in the digestive system or involved in sensory apparatus (Samarasinghe *et al.*, 2011). The localisation of *cat-4* within *T. circumcincta* has yet to be confirmed but it could be hypothesised that it has a similar pattern to that seen in *C. elegans,* i.e. in the serotonergic and dopaminergic neurons, as well as in the head and body wall muscles (Sze *et al.*, 2000). With a similar localisation pattern in *T. circumcincta*, the *Tci-cat-4* would not have been directly exposed to the dsRNA. The substantial reduction, but not complete silencing, of the *Tci-tub-1* transcript suggests that it may have been successfully silenced within exposed surfaces, for example the digestive system, but not in the rest of the body.

The generalised knockdown of transcript after incubation with dsRNA produced from the non-nematode *lacZ* gene has been documented previously; Dalzell *et al*. (2009) identified that nine, non-nematode dsRNAs designed for use as controls produced inhibitory effects on nematode physiology when used in RNAi screens of the plant parasitic nematodes, *Meloidogyne incognita* and *Globodera pallida*. In that study, the dsRNA was generated from *Lycopersicon esculentum* chloroplast-specific ribosomal protein, G-protein β-subunit, C14 cysteine protease, and a sucrose synthase; from *Solanum tuberosum* ethylene receptor and ADP-ribolysation factor; from *Malus domestica* oxygen evolving protein and intergenic spacer receptor region; and neomycin phosphoterase derived from the vector pEGFP-N1. The dsRNA from these genes was capable of inducing an inhibitory effect on motility of the nematodes, which increased in effect with increasing concentration of dsRNA and with decreasing length of dsRNA fragment (Dalzell *et al*., 2009). Despite the visible effect on phenotype (reduced motility), no transcriptional changes were identified using RT-PCR. As recovery from the deleterious effects induced by the non-nematode dsRNA was observed with time, it was speculated that the time period in which knock-down was occurring was not the time period in which RNA samples were taken. In the study by Dalzell *et al.*, the length of dsRNA that induced the strongest effect on motility was 229 bp; dsRNA was generated from the same gene in a larger 486 bp format that was inhibitory, but to a lesser extent. The *lacZ* dsRNA used in the experiments in this chapter was 201 bp, so if the size of the fragment is important, then a more deleterious effect may have been observed in these experiments. Ma *et al.,* (2006) hypothesised that within dsRNAs used in RNAi experiments in *Drosophila melanogaster*, short regions of CAG, CAU, CAA or CAC repeats could produce significant off-target effects, and should, if possible, be avoided. These repeats were not observed to be present in either the *lacZ* sequence used in the current research or in any of the non-nematode sequences used in the experiments of Dalzell *et al*., (2009) This could mean that further 'deleterious sequences' may be present in these dsRNAs that are yet to be identified. The role and relative importance of the different aspects of dsRNA are still being explored (Yoo *et al*., 2008).

The "RISC saturation hypothesis" is one that has arisen since the transition of RNAi from experimental technique to clinical trial procedure (Grimm *et al*., 2010), whereby widespread toxic effects are seen if the concentrations of gene-specific dsRNA are too high. If saturation of the Argonaute protein family can cause toxicity in a concentration-dependent mechanism, then it is also possible that incorrect processing of the non-nematode dsRNA leads to a similar saturation effect, resulting in toxicity to the organism. To examine this process more fully, it would be interesting to explore the point at which the toxicity is occurring. With further experiments using siRNAs it may be possible to identify whether the non-nematode dsRNAs are inhibitory at the point of interaction with Dicer, or if the inhibition occurs within RISC. If the mechanism is linked to Argonaute then siRNAs would produce the same toxic effect as dsRNAs, however, if the failure of the pathway is occurring upstream of Argonaute then non-target siRNAs should produce little effect on the nematodes. With more widespread use of non-nematode genes, it may be possible to identify the features of the sequences that are causing the off-target effects in a similar way to the discovery of the CAN repeats in *Drosophila* (Ma *et al*., 2006).

As a result of the inability to selectively silence *Tci-cat-4* using RNAi, the function of the enzyme was investigated using the chemical inhibitor, DAHP. These experiments demonstrated that DAHP could disrupt larval development of *T. circumcincta*. In *C. elegans*, however, the *cat-4* deletion mutant is able to develop normally, albeit with an increased sensitivity to external chemicals (Weinshenker *et al*., 1995). This implies that although the transcript and enzyme may perform the same biological role in both species (see chapter 5), parasitic nematodes require GTP-Cyclohydrolase to develop and survive in the experimental conditions tested, whereas the free-living species does not. The high levels of *Tci-cat-4* present in larval stages (Chapter 2) would support this argument; in a resource-limited environment it seems unlikely that large amounts of energy would be utilised in the production of a transcript unless it was critical for survival and development. The

fact that the *C. elegans cat-4* and N2 strains were both able to survive and develop normally on NGM containing high levels of DAHP demontrates that the effects on larval development seen in *T. circumcincta* were unlikely to have been caused by a generalised toxicity of the chemical. Since *C. elegans* is able to develop normally without GTP-Cyclohydrolase (as demonstrated by the continuing survival of the *cat-4* strain), if any effect of the DAHP had been seen on either strain then this would have been evidence for toxicity outwith the specific inhibition of GTP-Cyclohydrolase.

In contrast to the effects of DAHP on *T. circumcincta* larval development, there were no effects on hatching rates, feeding rates or migration rates. This indicates that specific developmental events and processes are affected by the inhibition of GTP-Cyclohydrolase. The structural similarities between DAHP and the ring structure component of GTP have previously led to the assumption that enzymic inhibition could be due to competitive binding to GTP (Harada *et al*., 1993), however, subsequent research has shown that a second mechanism of inhibition also occurs via GFRP (Xie *et al*., 1998). Structural studies on rat GFRP have shown that it is a 9.5 kDa protein that forms pentamers, two of which form a sandwich with the decamer of GTP-Cyclohydrolase to block its activity (Bader *et al*., 2001). GFRP provides a mechanism to prevent toxic accumulation of phenylalanine and increased levels of BH_4 lead to an increase in the biosynthesis of the feedback protein (Harada *et al*., 1993). As DAHP is also similar in structure to the ring structure of BH_4, it can activate GFRP, particularly in tissues that normally express the feedback protein (Chavan *et al*., 2006; Kolinsky and Gross, 2004).

To date, little research has been done into the role of GFRP in nematodes. In demonstrating that transcript levels of GFRP do not seem to change dramatically across the life-cycle, despite widely different levels of GTP-Cyclohydrolase transcript, it appears that the processes involved in GTP-Cyclohydrolase regulation may be similar to that observed in other systems. In murine livers, GFRP levels remain constant under different GTP-Cyclohydrolase levels and the control of GTP-

Cyclohydrolase enzyme production is at the mRNA transcript level (Tatham *et al*., 2009). Within the brains of rats, GFRP was found to be located in the serotonergic but not the dopaminergic neurons (Kapatos *et al*., 1999), suggesting that the inhibition of GTP-Cyclohydrolase is not a common mechanism throughout all tissues expressing GTP-Cyclohydrolase. It is possible therefore that in *T. circumcincta* the high levels of GTP-Cyclohydrolase required in $L_1 - L_3$ are under transcriptional control, and operate outwith the regulation of GFRP. In mammalian tissues that do not express GFRP, the levels of DAHP required to inhibit GTP-Cyclohydrolase are higher (Kolinsky and Gross, 2004). If nematodes do not co-express GFRP then the levels of DAHP required to inhibit GTP-Cyclohydrolase would need to be higher as inhibition is due only to competitive binding for GTP (Figure 18).

Although no cuticular changes were observed in L_1 that developed in the presence of DAHP using scanning electron microscopy, it is possible that the preparation process could have eliminated those L_1 that were damaged. In order to use the cleanest possible sample, the L_1 were Baermannised before fixing. This could have selected out L_1 with gross deformity to the cuticle. Alternatively, the effects of the DAHP on the L_1 could be beneath the surface. If this experiment was to be repeated it would be preferable not to Baermannise the L_1, but instead to work with the cleanest possible sample of eggs, and accept that a level of detritus would be unavoidable in the electron microscopy sample.

In this chapter the effects of a chemical inhibitor of GTP-Cyclohydrolase have been demonstrated and, for the first time the use of RNAi in *T. circumcincta* was described. These techniques have enabled further understanding of the role of GTP-Cyclohydrolase in the development of larvae, which will be further investigated in later chapters.

4 Expression of recombinant GTP-Cyclohydrolase for localisation and the elucidation of function in strongylids

4.1 Introduction

The location of a protein within the body of an organism can provide clues as to the function of that protein within a particular species, for example, the production of a recombinant serpin in *Haemonchus contortus* enabled the localisation of the protein to the gut cells of the parasite, linking functions of the protein to the digestion activities of the worm (Yi *et al*., 2010). Information on function can then be utilised for the design of therapeutics; for example, in the case of the *Haemonchus* galactose-containing glycoprotein complex (H-gal-GP) that has been used as a vaccine candidate to prevent *H. contortus* infection, the knowledge that it was expressed within the gut of the adult worms meant that it was a potential "hidden antigen" suitable for therapeutic targeting by ingested antigen specific antibodies (Ekoja and Smith, 2010). The role of GTP-Cyclohydrolase within the serotonin and dopamine pathways has been explored in mammals using antibodies raised against recombinant GTP-Cyclohydrolase. In rats, antibodies raised in mice against a recombinant GTP-Cyclohydrolase maltose binding protein fusion identified that the expression of the protein within the brain was concentrated in the nucleus of neurons, in contrast to the predominantly cytoplasmic localisation pattern of tryptophan hydroxylase (Elzaouk *et al*., 2004). Investigations using antibodies raised against the recombinant version of mice GTP-Cyclohydrolase showed the localisation of the protein to the caveolae (microdomains within the plasma membrane) of the aorta, with the caveolae appearing to provide an inhibitory mechanism of control of GTP-Cyclohydrolase expression (Peterson *et al*., 2009). These studies highlight the variety of functions that GTP-Cyclohydrolase performs in different tissues and organisms, and emphasises the complexity of trying to understand the function in *Teladorsagia circumcincta*.

Large numbers of experiments have been undertaken to produce recombinant versions of nematode proteins for use as vaccine candidates, such as the aspartic and cysteine proteases in human hookworm infections (Hotez *et al*., 2003). Much of the

focus of veterinary parasitic nematode recombinant protein production has been on proteins localised to the gut (Knox and Smith, 2001). This focus has been driven by the protective effects of vaccines generated from native material, but invariably, the recombinant proteins have failed to provide the same level of protection as native gut extracts (Smith and Zarlenga, 2006). In the filarial nematodes *Onchocerca volvulus* and *Brugia malayi* responsible for widespread human morbidity in the Tropics, a recombinant excretory / secretory protein (ES62) was generated using the yeast expression system *Pichia pastoris* (see Egan *et al*., 2006). Despite appearing to form stable tetramers, dimers and monomers, antibodies raised against this recombinant protein in mice were unable to recognise native protein. Investigations revealed that significant glycosylation events were occurring in the production of the recombinant, which affected the secondary structure of the protein rendering it unsuitable for its purpose as a vaccination candidate (Egan *et al*., 2006). The large numbers of recombinant vaccine candidates for control of veterinary helminths have been reviewed in detail (Geldhof *et al*., 2007); a common thread running through many of the studies has been a need to overcome obstacles in producing soluble recombinant proteins that exist in a conformation that is bound by the host antibodies and / or recognised by other arms of the immune response. Seemingly promising proteins from *in vitro* studies have failed to produce the desired results of protecting the host from infection and reducing the morbidity associated with parasitic nematodes (Geldhof *et al*., 2007). Recombinant proteins are often able to provide information about the native protein that would be otherwise unknown from *in silica* analysis of the amino acid sequence, but antibodies raised against them may not recognise the native protein and this has implications for both localisation and vaccination studies.

In order to identify the cellular and sub-cellular location of GTP-Cyclohydrolase expression in *T. circumcincta*, antibodies that bind to the native protein need to be generated, and to produce such antibodies, a suitable recombinant protein must be generated because the technical challenges of purifying native protein are currently too great. The issue of producing a recombinant protein that is soluble in aqueous buffers and that can be used to generate antibodies capable of binding native protein is one that has affected the study of GTP-Cyclohydrolase in other nematode species.

In *Ostertagia ostertagi*, for example, a recombinant GTP-Cyclohydrolase (95 identical to *T. circumcincta* GTP-Cyclohydrolase) was produced using the prokaryotic *Escherichia coli* pET-15b expression system (Moore *et al*., 2000). This system adds an N-terminus polyhistidine region to the recombinant protein, but does not contain a signal peptide for transfer of the protein to the periplasmic fraction. The recombinant protein produced by this method was not soluble in aqueous buffers and required 6 M urea to solubilise it prior to purification. Antibodies raised in rabbits against the recombinant GTP-Cyclohydrolase were unable to bind native GTP-Cyclohydrolase on Western blots of soluble extracts of *O. ostertagi* L_3 and adult worms, although localisation using immunogold labelling with the antibody did suggest the presence of native GTP-Cyclohydrolase in the body wall muscles and cells of the intestine in sections of the L_3 stage. The expression of soluble recombinant GTP-Cyclohydrolase in *E. coli* has not been straightforward in a wide range of species, for example, *Triticum aestivum* (see McIntosh *et al*., 2008) and *Plasmodium falciparum* (see Stephens *et al*., 2011).

The 3D structure of GTP-Cyclohydrolase (Figure 3) provides a possible explanation as to why these solubility issues have been encountered. As a protein that forms a decamer, the visible epitopes of the native protein are likely to be a complex mixture of residues derived from multiple monomers (Nar *et al*., 1995a). This complex structure is likely to be the reason that re-folding recombinant proteins that have been solubilised in urea has not been successful. With this in mind, a number of different strategies were utilised to produce an aqueous soluble recombinant *T. circumcincta* GTP-Cyclohydrolase that could be used to generate antibodies that could then be used for immunolocalisation studies and ELISA experimentsto detect and quantify the native protein in different life-cycle stages.

The most commonly used systems for the production of recombinant proteins are prokaryotic systems that use *E. coli* as an expression system (Baneyx, 1999). Production of recombinant proteins in prokaryotes is relatively quick and inexpensive, with expression occurring within hours of induction (Roman *et al*., 1995). Eukaryotic expression systems yield protein more slowly [days or weeks

rather than hours (Higgins, 2001)], but can provide some of the post-translational modifications that can be crucial for the production of recombinants that are bound by antibodies that recognise native protein (Mann and Jensen, 2003).

Each of the expression systems used previously in this project has been used to produce soluble recombinant proteins and as such, the reasoning behind using them is explained below.

4.1.1 Prokaryotic systems

4.1.1.1   pET-SUMO

The pET SUMO system was developed after the discovery of the <u>S</u>mall <u>U</u>biquitin-related <u>Mo</u>difier that was found to increase solubility of proteins it was covalently linked to (Saitoh *et al*., 1997). This system has since been used to produce soluble recombinant proteins that have previously proven difficult to produce in soluble form using other prokaryotic systems. One example of this is the human protein fibroblast growth factor 21 (FGF21) that is a potential drug target for diabetes due to its role in glucose homeostasis. Previous attempts to produce recombinant FGF21 had resulted in the production of insoluble recombinant proteins in inclusion bodies, but the use of a SUMO fusion protein enabled a soluble recombinant protein to be produced that was capable of lowering the blood glucose levels when administered to diabetic rats (Wang *et al*., 2010).   If the difficulties in producing recombinant GTP-Cyclohydrolase are due to production of insoluble recombinant protein in inclusion bodies, then this system has potential to provide the solution to producing a soluble recombinant GTP-Cyclohydrolase.

4.1.1.2   pET-22b(+)

The pET-22b(+) expression vector contains a pelB leader sequence that enhances localisation to the periplasm of bacterial cells, but previous studies have identified that removing this leader sequence can increase the solubility of recombinant proteins (Zhuge *et al*., 2007): in the production of recombinant *T. circumcincta* macrophage migratory inhibitory factor-like tautomerase (Tci-MIF-1), dopachrome

tautomerase activity was only achieved with the soluble recombinant enzyme after the protein was expressed without the pelB leader sequence (Nisbet *et al*., 2010). Other studies using pET-22b(+) have also produced soluble recombinant protein, such as the *H. contortus* cystatin that was functionally active and could inhibit cysteine proteases, and antibodies raised against it in a rabbit were capable of binding native protein in immunolocalisation studies (Newlands *et al*., 2001). Given these results for *T. circumcincta* and *H. contortus* proteins, it is possible that a soluble recombinant GTP-Cyclohydrolase could be produced using this system.

4.1.1.3   Maltose Binding Protein

A third prokaryotic system that has been used for the production of recombinant GTP-Cyclohydrolase from other species is the maltose binding protein system (Witter *et al*., 1996a; Witter *et al*., 1996b). This protein has been shown to act as a molecular chaperone for the protein it is fused to (Fox *et al*., 2001; Kapust and Waugh, 1999), thus increasing the solubility of previously insoluble proteins by assisting in protein folding. Within this project, this system will be the only one used that does not use a poly-histidine region ("His-tag") for purification. It has been shown previously that differences can occur between proteins as to the accessibility of the His-tag for purification (Debeljak *et al*., 2006), so if the conformation of GTP-Cyclohydrolase does not appear to allow nickel affinity purification then this system will provide an alternative, as the binding is to an amylose resin rather than nickel.

4.1.2 Eukaryotic systems

4.1.2.1   *Pichia pastoris*

Pichia pastoris has been used widely for the production of recombinant proteins as it grows to a high cell density and secretes induced proteins into the cell culture medium (De Schutter *et al*., 2009; Romanos, 1995). It is also capable of performing post-translational modifications such as folding and glycosylation that cannot be performed in prokaryotic systems (Macauley-Patrick *et al*., 2005). Analysis of a recombinant esophageal gland-specific pectate lyase from *Meloidogyne javanica* expressed in *P. pastoris* showed that glycosylation events occurred during the culture

process, confirming the location of predicted glycosylation sites within the nematode protein sequence (Doyle and Lambert, 2002). Expression is not always straightforward, however, with proteins with a high degree of homology derived from the same species expressing to different degrees. This was the case with the cathepsin B cysteine proteases from the human hookwork *Necator americanus*, where four distinct cathepsin B sequences were identified and cloned, but only one was expressed successfully in a soluble form using *Pichia* (see Ranjit *et al*., 2008). Although the structures of prokaryotic and eukaryotic GTP-Cyclohydrolase are very similar (see Section 1.5), and therefore it would seem logical to hypothesise that the 3D structural assembly of *T. circumcincta* should be provided by a bacterial expression system, if they are not, then using *P. pastoris* could provide the means for the production of an appropriately folded protein.

4.1.2.2   *Caenorhabditis elegans*

A more recent development is that of manipulating *C. elegans* to express genes of interest from other nematode species. It had been shown previously that parasitic nematode genes could be expressed in *C. elegans* (Kwa *et al*., 1995) and, more recently, this has been harnessed with the addition of a polyhistidine tag to the protein so that the expressed protein can be purified. For example, Murray *et al*. (2007) expressed an *H. contortus* cathepsin L cysteine protease in *C. elegans* under the control of a *C. elegans* cathepsin L promoter sequence. After purification using nickel affinity chromatography, the recombinant protein was shown to be enzymically active and glycosylated. Because of the close genetic relationship between *C. elegans* and other Clade V parasitic nematode species (Blaxter *et al*., 1998), it is hypothesised that many of the post-translational modifications and protein processing mechanisms could be conserved (Britton and Murray, 2006). If this were the case, then a recombinant protein produced in *C. elegans* could potentially be more similar in structure to the native protein than that which can be achieved using bacterial or yeast expression systems. As this system is still under development, and has not yet been tested with proteins from any other species than

H. contortus, there is the possibility that it could be used for a variety of nematode species and proteins.

All of the above systems were utilised in this project in an attempt to produce a recombinant *T. circumcincta* GTP-Cyclohydrolase and each will be discussed in this chapter. If a recombinant GTP-Cyclohydrolase could be produced then the aim would be to use this protein to raise specific antibodies for use in immunohistochemistry on sections of *T. circumcincta* to show where the native protein is expressed. Alongside this, antibodies raised against the recombinant protein could be used in ELISA experiments to detect levels of native GTP-Cyclohydrolase using soluble extracts of tissues of different life-cycle stages of *T. circumcincta*. Although the transcript levels for *Tci-cat-4* are known and have been discussed in Chapter 2, no studies into the levels of the protein have been performed. As transcript levels do not always reflect protein concentrations (Griffin *et al.*, 2002), detecting levels of GTP-Cyclohydrolase protein within the worm using antibodies raised against a recombinant protein could provide further insight into the role of GTP-Cyclohydrolase in the life-cycle of *T. circumcincta*.

4.2 Materials and Methods

4.2.1 Prokaryotic expression

T. circumcincta L$_3$ complementary DNA (cDNA; generated in Section 2.2.2) was used as a template in polymerase chain reaction (PCR) to amplify the *Tci-cat-4* gene for cloning into the different bacterial expression systems. Primers were designed to include additional nucleotides for restriction enzyme sites, and to keep the initiation methionine codon in frame, if necessary. The termination codon was omitted from the reverse primer for the pET-22b(+) sequence as the termination codon was present in the plasmid sequence. The primers for PCR amplification (shown in Table 7) were used with the Advantage 2 PCR polymerase (Clontech) as this contains a proof-reading enzyme to minimise PCR-induced errors. PCR conditions were 95 °C for 1 min, followed by 30 cycles of 95 °C for 30 s, 60 °C for 30 s and 68 °C for 1 min, followed by a final extension step of 1 min at 68 °C. Amplicons of the correct size were purified using a PCR purification kit (Qiagen). The purified PCR products and relevant plasmid vectors were digested with restriction enzymes if required (*XhoI* and *NdeI* for pET-22b(+); *HindIII* and *XmnI* for pMAL-p2x). The inserts were ligated into the plasmids using T4 ligase (Promega) as per manufacturer's instructions. The plasmids were transformed using heat-shock into competent *E. coli* cells [JM109 (Promega) for pET-22b(+) and pMAL-p2x and Mach 1-T1^R (Invitrogen) for pET-SUMO].

Table 7. Primer sequences used for amplification of *Tci-cat-4* for cloning into the prokaryotic expression vectors. Bases in green represent the initiation methionine codon, and those in red the termination codon. Underlined bases highlight the location of the restriction sites used and lower case bases represent additional bases added to keep the sequence in the correct frame or to add bases before the restriction site to improve digestion by the restriction enzyme.

Expression System	Forward Primer	Reverse Primer
pET-SUMO	5'-ATGGCTAGCGAAAGCGGT-3'	5'-TCACTTTTTGTTGATGAGATT CAAG-3'
pET-22b(+)	5'-tccagatataCATATGGCTA GCGAAAGCGGTTTC-3	5'- ggtggtgCTCGAGCTTTTTGTTGA TGAGATTCAAGAATTCTTC-3'
pMAL-p2x	5'-gaggGAACGATTTCaATGGC TAGCGAAAGCGGTTTCCTTTC-3'	5'- agtgccAAGCTTTCACTTTTTGTTG ATGAGATTCAAGAATTCTTCTCTGG-3'

Transformed *E. coli* cells were incubated for 1 h at 37 ° C in a shaking incubator in the presence of super optimal culture (SOC) medium and then spread on Luria-Bertani (LB) medium plates with the appropriate selective antibiotic, i.e. 100 µg / ml ampicillin for pET-22b(+) and pMAL-p2x, 50 µg / ml kanamycin for pET-SUMO. Colony PCR was performed on stabs of single colonies using gene specific primers (forward: 5'-CGTCAAGGGCTTCTGAAAAC-3', reverse: 5'-GGTGGTGGCATTGATCTTCT-3'). Transformed cells were cultured overnight in 10 ml LB medium with the appropriate selective antibiotic at 37 ° C for 18 h in a shaking incubator. Plasmids were purified using the Wizard plus SV minipreps kit (Promega) and sequenced (Eurofins MWG). Plasmids containing *Tci-cat-4* in the correct frame and orientation were transformed into *E. coli* competent cells; BL21 codon plus (DE3)-RIPL (Stratagene) for pET-22b(+) and pMAL-p2x or BL21 Star One Shot *E. coli* (Invitrogen) for pET-SUMO. Once colonies containing the correct size inserts had been identified using Colony PCR (using the primers described above), transformed cells were cultured in LB-kanamycin (pET-SUMO), LB-ampicillin-chloramphenicol [pET-22b(+)] or rich medium (1 % tryptone, 0.5 % NaCl, 0.2 % glucose) with ampicillin and chloramphenicol (pMAL-p2x). Samples (500 µl) were taken once the absorbance at 600 nm had reached 0.4-0.6 before protein expression was induced by the addition of 1 mM IPTG. After induction of protein expression, processing for each technique was performed as appropriate for the system (as described in the following Sections).

4.2.1.1 pET-SUMO

Samples (500 µl) were taken from the cell cultures at the point of induction with IPTG and once every hour for 6 h. These samples were centrifuged at 18,000 *x g* for 30 s and the supernatant discarded. The cell pellets were re-suspended in 500 µl lysis buffer [50 mM potassium phosphate (pH 7.8), 400 mM NaCl, 100 mM KCl, 10 % glycerol, 0.5 % Triton X-100, 10 mM imidazole]. The samples were then frozen at $-$ 80 °C and thawed at 42 °C three times before being centrifuged at 18,000 *x g* for 1 min to separate the soluble and insoluble fractions. The supernatant was removed for further analysis and the 'insoluble' pellet re-suspended in 500 µl lysis buffer.

Thirteen µl of each soluble and insoluble fraction were loaded onto a NuPAGE 4 – 12 % gel as described in Appendix 8.1.2, separated by electrophoresis and visualised using SimplyBlue safestain (Invitrogen). These pilot studies identified that there was a strong band representative of the recombinant GTP-Cyclohydrolase visible 4 h after induction with IPTG. This protein was present in the insoluble fraction, and insoluble in aqueous buffers.

For larger scale production, a 50 ml culture of LB-kanamycin was inoculated with BL21 Star One Shot *E. coli* (Invitrogen) cells containing the GTP-Cyclohydrolase-SUMO fusion plasmid. This culture was incubated for 2 h at 37 °C with shaking, before IPTG to a final concentration of 1 mM was added. After 4 h, the culture was centrifuged at 3000 *x g* for 10 min, the supernatant discarded and the pellet stored at – 80 °C. The cell pellet was defrosted on ice, re-suspended in 5 ml binding buffer 1 (Appendix 8.1.4.3; at this point the buffers did not contain urea), sonicated for 20 s twice and then re-frozen at -80 °C. The cell suspension was thawed and incubated in 1 % Triton-X in binding buffer 1 for 30 min on ice before centrifugation at 18,000 *x g* for 10 min. The supernatants were removed and discarded. The cell pellets were re-suspended in 100 µl binding buffer 1 (Appendix 8.1.4.3) and then 1 ml binding buffer 2 (Appendix 0) added. The suspension was then centrifuged at 18,000 *x g* for 10 min to ensure that the sample loaded onto the column did not contain any debris. A 1 ml HisTrap™ HP column (GE Healthcare) was attached to a peristaltic pump and flushed with distilled water at a flow rate of 1 ml / min. It was then equilibrated with binding buffer 1 before the sample was loaded onto the column using a syringe (a small amount of the sample was retained for analysis by gel electrophoresis). The column and sample were incubated for 30 min at room temperature. The column was washed with 5 ml binding buffer 2 at 1 ml / min, with the flow-through collected. The proteins were then eluted with 1 ml elution buffer 1b (Appendix 8.1.4.11), 1 ml elution buffer 2b (Appendix 8.1.4.12), 1 ml elution buffer 3b (Appendix 8.1.4.13) and 3 ml elution buffer 4b (Appendix 8.1.4.14); these buffers represent a stepwise increasing gradient of imidazole from 50 mM to 500 mM. Protein gel electrophoresis (NuPAGE system, Invitrogen) was used to determine which eluates contained the highest concentration of purified recombinant protein

(Appendix 8.1.2). A band of the predicted size was observed in two of the eluates, and the identity of this protein was confirmed using Peptide Mass Fingerprinting using Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) analysis. The 1 ml fraction containing the purified protein was diluted with 200 ml SUMO protease buffer [50 mM Tris-HCl, pH 8, 0.2 % Igepal (NP-40), 150 mM NaCl, 1 mM DTT] and mixed gently overnight to attempt re-folding / solubilisation.

A 50 ml sample of this protein suspension in protease buffer was removed to attempt to cleave the SUMO fusion protein. Imidazole was added to a sample of SUMO protease buffer to a final concentration of 20 mM so that a nickel affinity column could be equilibrated with the same concentration as the sample solution. A 1 ml HisTrap™ HP column (GE Healthcare) was attached to a peristaltic pump and flushed with distilled water at a flow rate of 1 ml / min. It was then equilibrated with the SUMO protease buffer + imidazole for 5 min. The 50 ml sample was then loaded onto the column at a rate of 1 ml / min. Ten microlitres SUMO protease (Invitrogen) were mixed with 1 ml SUMO protease buffer and loaded onto the column and incubated at 30 °C for 2 h. The cleaved GTP-Cyclohydrolase should then have no longer been bound to the SUMO fusion or the nickel column so it was attempted to elute the cleaved protein using 9 ml protease buffer into 1 ml fractions. The fractions were subjected to polyacrylamide gel electrophoresis (Appendix 8.1.2).

A second method of cleavage was also performed. In this method, the GTP-Cyclohydrolase-SUMO-fusion proteins were concentrated using Amicon ultracentrifuge concentrators (10 kDa cutoff; Milipore). The 50 ml sample was concentrated to 200 µl, after which a sample was loaded onto a NuPAGE gel to examine if the protein was still intact. After confirmation that the protein had not been affected, or lost, during concentration, 20 µl of the concentrated fusion protein were added to 20 µl 10 x SUMO protease buffer + salt (Invitrogen), 150 µl water and 10 µl SUMO protease. This was incubated at 30 °C for 6 h with samples taken at 1, 2, 4 and 6 h. These samples were loaded onto a NuPAGE gel to assess the success of

the cleavage process. This process was also repeated with 170 µl of the fusion protein (with no water).

4.2.1.2 pET-22b(+)

Samples (500 µl) were taken from the cell cultures at the point of induction with IPTG and once every hour for 6 h. These samples were centrifuged at 18,000 $x g$ for 30 s and the supernatant discarded. The cell pellets were re-suspended in 500 µl binding buffer 1 (Appendix 8.1.4.3). The samples were then frozen at $-80\,°C$ and thawed at 42 °C three times before 500 µl 20 % Triton X-100 were added and the sample incubated on ice for 30 min. The samples were then centrifuged at 18,000 x g for 1 min to separate the soluble and insoluble fractions. The soluble proteins, contained within the supernatant, were removed and the insoluble proteins re-suspended in 500 µl binding buffer 1. Thirteen microlitres of each soluble and insoluble fraction were loaded onto a NuPAGE gel as described in Appendix 8.1.2 and subjected to visualisation as described in the preceding Section. The process was also repeated using cultures that were incubated at room temperature overnight, rather than at 37 °C in an attempt to increase solubility of the recombinant protein.

4.2.1.3 Maltose Binding Protein

A pilot experiment was performed to assess the solubility of recombinant GTP-Cyclohydrolase produced in this system and whether it would bind to amylose resin. Eighty millilitres of Rich Broth (1 % tryptone, 0.5 % yeast extract, 0.5 % NaCl, 0.2 % glucose, 100 µg / ml ampicillin) were inoculated with an overnight culture of cells containing the fusion plasmid. The culture was grown to reach an OD_{600} of 0.5 as determined by spectrometry, a 1 ml sample taken ('sample 1' – uninduced cells) and IPTG added to the culture to a final concentration of 0.3 mM. After 2 h, a 1 ml sample was removed ('sample 2' – induced cells). The remaining culture was split into two fractions and centrifuged at 4000 $x g$ for 20 min at 4 °C. The supernatants were discarded. One cell pellet was re-suspended in 5 ml column buffer (20 mM Tris HCl pH 7.4, 200 mM NaCl, 1 mM EDTA) and frozen overnight at $-20\,°C$. The

suspension was defrosted and sonicated eight times in 15 s bursts before being centrifuged at 9000 $x\,g$ for 20 min at 4 °C. The supernatant was retained as the crude extract ('sample 3') and the pellet re-suspended in column buffer ('sample 4' – insoluble fraction). Two hundred microlitres of amylose resin (New England Biolabs) were centrifuged to remove the storage buffer and the amylose resin re-suspended in column buffer. A sample of 50 µl of the resin in column buffer was then mixed with 50 µl of the crude extract and incubated for 15 min on ice. The mixture was centrifuged for 1 min, the supernatant discarded and the resin retained ('sample 5' – protein bound to amylose). The other cell pellet from the original culture was re-suspended in 10 ml 30 mM Tris-HCl pH 8, 20 % sucrose. EDTA, to a final concentration of 1 mM, was added and the sample incubated at room temperature with shaking for 10 min. The sample was then centrifuged at 8000 $x\,g$ for 10 min at 4 °C. The supernatant was discarded and the pellet re-suspended in 10 ml ice-cold 5 mM $MgSO_4$. This was mixed for 10 min on ice and then centrifuged at 8000 $x\,g$ for 10 min at 4 °C and the supernatant retained ('sample 6' – cold osmotic shock fluid). All six samples were loaded onto a NuPAGE gel and examined as above. A band of the predicted size was observed in the soluble phase of a number of samples. The identity of the fusion protein was confirmed using Peptide Mass Fingerprinting using Matrix-assisted laser desorption / ionization time of flight (MALDI-TOF).

Amylose resin was prepared into a 3 ml column and washed with 8 column volumes of column buffer (20 mM Tris-HCl pH 7.4, 200 mM NaCl, 1 mM EDTA) at a rate of 1 ml / min. The remaining 39 ml of the crude extract generated above (sample 3) were then loaded onto the column at 1 ml / min (200 µl were retained as a pre-loading comparison). The column was washed through with 12 column volumes of column buffer, and the flow-through retained. The proteins were then eluted using five column volumes of elution buffer (column buffer + 10 mM maltose) and the fractions collected in 1 ml samples which were subjected to analysis on a NuPAGE gel.

Cleavage of the fusion protein was attempted using Factor Xa (New England Biolabs). To assess the time taken to cleave the fusion protein, 20 μl fusion protein solution were mixed with 1 μl Factor Xa (diluted to 200 μg /ml). Samples were removed at 0, 2, 4, 8 and 24 h and analysed by polyacrylamide gel electrophoresis. Cleavage appeared to start immediately, as determined by the changing banding pattern on the gel, but it took 8 -24 h for the fusion protein to have been cleaved completely. The remainder of the purified fusion protein was cleaved with Factor Xa at room temperature for 24 h. In order to remove the protease and maltose binding protein from the purified recombinant, it was necessary to dialyse the proteins into a buffer (20 mM Tris-HCl pH 8, 25 mM NaCl) that could be loaded onto a MonoQ column (GE Healthcare) to perform anion exchange chromatography. The column was equilibrated with the above buffer before the sample was loaded. Protein concentrations in the eluates were measured using the absorbance at 280 nm. Once the protein concentration had plateaued after washing, the proteins were eluted using an elution buffer that increased the salt concentration in a gradient over 30 min (20 mM Tris-HCl pH 8, 1 M NaCl). Fractions of 0.5 ml were collected during this time. This process was repeated using the same elution buffer at pH 7, rather than pH 8, as the proteins were not separated using the first technique. As little protein was recovered, further investigations into the cleavage process were required.

In order to assess the presence of the GTP-Cyclohydrolase protein and to establish if it was being damaged during cleavage, western blots were performed (see Appendix 8.1.3 for methodology). The primary antibody used in these blots was derived from rabbits that had been immunised with an insoluble, *E. coli*-expressed recombinant GTP-Cyclohydrolase from *O. ostertagi* generated using the pET-15b expression system (Moore *et al*., 2000). This antibody was kindly donated by Professor Eileen Devaney (University of Glasgow). The secondary antibody was a goat anti-rabbit immunoglobulin HRP conjugated antibody (Dako). Western blots were performed on pre- and post- cleavage samples (from above), as well as samples after dialysis to assess at what point the GTP-Cyclohydrolase was being lost. A band of the predicted size for the cleaved GTP-Cyclohydrolase was also sent for identification by Peptide Mass Fingerprinting using MALDI-TOF.

4.2.2   Eukaryotic expression 1: *Pichia pastoris*

A PCR product was generated from *T. circumcincta* L3 cDNA (generated as in Section 2.2.2) using primers shown in Table 8 and the Advantage 2 PCR kit (Clontech). PCR conditions were 95 °C for 1 min, followed by 30 cycles of 95 °C for 30 s, 60 °C for 30s and 68 °C for 1 min, followed by a final extension step of 1 min at 68 °C. The PCR product was purified using a PCR purification kit (Qiagen) and ligated using T4 DNA ligase (Promega) into the dephosphorylated (shrimp alkaline phosphatase; Promega) pPICZαC vector (Invitrogen) after digestion with the restriction enzymes, *Cla1* and *Xba* (Roche).

Table 8. Primer sequences used to amplify GTP-Cyclohydrolase from *T. circumcincta* for expression in yeast (*Pichia pastoris*). The methionine initiation codon is shown in green, the introduced restriction enzyme recognition sites are underlined and additional bases added to keep the sequence in frame or to extend past the restriction site are shown in lower case. The termination codon was removed from the reverse primer so that the termination codon utilised was the one after the 3' His tag, encoded by the plasmid.

	Primer sequence	Restriction site introduced
Forward	5'-gaagc<u>ATCGAT</u>aATGGCT AGCGAAAGCGGTTTC-3'	*ClaI*
Reverse	5'-tttttgt<u>TCTAGA</u>TTTTTGTTG ATGAGATTCAAGAATTCTTC-3'	*XbaI*

The ligated plasmids were transformed using heat-shock into JM109 competent *E. coli* cells (Promega) and incubated overnight at 37 °C on low-salt LB (1 % tryptone, 5 % NaCl, 5 % yeast extract) plates containing 25 μg / ml zeocin. Positive colonies were identified by colony PCR using the primers shown in Table 8 and then grown in 10 ml low-salt LB-zeocin overnight at 37 °C in a shaking incubator. The plasmids were purified using Wizard plus SV minipreps kit (Promega), digested with the restriction enzyme *Pme1* (New England Biolabs) and purified using a PCR purification kit (Qiagen). Separately, 200 ml of YPD (<u>Y</u>east extract <u>P</u>eptone <u>D</u>extrose medium; 1 % yeast extract, 2 % peptone, 2 % dextrose) were inoculated with X-33 *P. pastoris* cells (Invitrogen) and grown overnight at 29 °C with shaking. The cells were harvested by centrifugation at 1500 *x g* for 5 min at 4 °C. The

supernatant was discarded and the cells re-suspended in 1 ml ice-cold water, the volume was increased to 200 ml once all the cells had been re-suspended. Centrifugation was repeated three more times. After the first centrifugation step, the cells were re-suspended in 100 ml ice cold water, after the second centrifugation step, in 4 ml ice cold 1 M Sorbitol and after the final centrifugation step in 1 ml ice cold 1 M Sorbitol. A mixture of 70 µl of these competent X-33 cells and 30 µl digested plasmid was placed into a pre-cooled electroporation cuvette and placed on ice for 5 min. Electroporation was conducted at 1500 V and 200 Ω. Immediately afterwards, 1 ml ice-cold 1 M Sorbitol was added and the mixture transferred to a tissue culture tube for incubation at 30 °C for 2 h (without movement). The cells were spread onto YPDS-zeocin plates (1 % yeast extract, 2 % peptone, 2 % dextrose, 1 M Sorbitol, 2 % agar, 100 µg/ml zeocin) and incubated at 30 °C for 3 days. Positive colonies identified by colony PCR using the 5' AOX1 (5´-GACTGGTTCCAATTGACAAGC-3´) and

3' AOX1 (5´-GCAAATGGCATTCTGACATCC-3´) primers, were selected and grown in BMGY (1 % yeast extract 2 % peptone, 100 mM potassium phosphate at pH 6, 1.34 % YNB, 4×10^{-5} % biotin, 1 % glycerol) overnight at 29 °C in a shaking incubator. The cells were harvested by centrifugation and resuspended in BMMY (1 % methanol instead of 1 % glycerol) and incubated overnight at 29 °C in a shaking incubator for 72 h. Every 24 h, 1 ml of cells were harvested and 100 % methanol to a final concentration of 0.5 % was added. After three days, the cells were harvested by centrifugation at 2000 x g for 5 min at 4 °C. The supernatant was mixed with an equal volume of 2 x binding buffer 1 (40 mM sodium phosphate, 1 M NaCl, 40 mM imidazole pH 7.4). The sample was purified on a 1 ml HisTrap™ HP column (GE Healthcare), as described in Section 4.2.1.1 A band, as identified on polyacrylamide gel, of the predicted size for recombinant GTP-Cyclohydrolase appeared only in the flow-through, so it appeared that the protein had not bound to the nickel column. This technique was repeated twice more with the same results, so the identity of the protein in the flow-through was investigated using Peptide Mass Fingerprinting using MALDI-TOF.

4.2.3 Eukaryotic expression 2: *C. elegans*

Primers were designed (Table 9) and used in PCR with *T. circumcincta* L_3 cDNA (prepared as described in Section 2.2.2) with the *Pfu* enzyme mix (Agilent) in the proportions 5 µl 10x Buffer, 1 µl 25 mM dNTPs, 1 µl 'GTP-CH His For' primer 10 µM, 1 µl 10 µM 'GTP-CH His R1' primer (1st round) or 'GTP-CH His R2' primer (2nd round), 0.4 µl *Pfu* polymerase and 40.6 µl water. Cycling conditions were, 1 min at 95 °C, 35 cycles of 95 °C 1 min, 53 °C (R1) or 59 °C (R2) for 30 s, 72 °C for 4 min, followed by a final extension of 72 °C for 5 min. The PCR product from the first amplification was purified using the PCR purification kit (Qiagen) and diluted 1 / 100 in water before being used as template for the second round. After purification of the second round product, it was A-tailed (Promega) and ligated into pGEM-T easy (Promega) using T4 ligase. The ligated plasmids were transformed into JM109 competent *E. coli* cells (Promega) by heat shock, incubated for 1 h at 37 °C in a shaking incubator in the presence of SOC medium and then spread on LB medium plates containing 100 µg / ml ampicillin, 0.5 mM IPTG and 80 µg/ml X-Gal. Colony PCR was performed on selected white colonies using the 'GTP-CH His For' and 'GTP-CH His R2' primer combination, with positive colonies selected for amplification by culture. Cells were grown in 10 ml LB medium with 100 µg / ml ampicillin at 37 ° C for 18 h in a shaking incubator. The plasmids were purified using the Wizard plus SV minipreps kit (Promega) and sequenced using T7 and SP6 primers (Eurofins MWG). Once confirmation of the insertion of the correct in-frame sequence had been received (Figure 32), the plasmids were digested using the restriction enzymes, *NotI* and *XhoI*, and ligated into a second vector (pABR09) that could be used for microinjection into *C. elegans*.

Table 9. Oligonucleotide primer sequences used to generate a GTP-Cyclohydrolase construct with a his-tag at the C-terminus of the protein for expression in *C. elegans*.

Primer name	Primer sequence	Primer features
GTP-CH His For	5'-gcggccgcATGGCTAGCGAA AGCGGTTT-3'	Introduced *NotI* site at start of amplicon
GTP-CH His R1	5'-gtggtggtggtggtggtgCTTTTTGTT GATGAGATTCAAGAATTC-3'	End of the *Tci-cat-4* gene, with 6 histidine residues added
GTP-CH His R2	5'-ctcgagTCAgtggtggtggtggtggtg gtggtggtggtgCTTTTTG-3'	End of the *Tci-cat-4* gene, an additional 4 histitidine residues (10 in total), a termination codon and a *XhoI* site added

Figure 32. Diagram of the construct that was inserted into a plasmid and injected into *C. elegans* for recombinant protein production.

The plasmids were microinjected into the gonads of *C. elegans* hermaphrodite N2 individuals, along with a green fluorescent protein reporter fusion construct with a cuticular collagen gene that is ubiquitously expressed throughout the organism (*dpy7:gfp*). This was performed at the University of Glasgow by Dr Brett Roberts. Once individual worms had developed with the green fluorescent protein phenotype, these were picked individually onto NGM plates to form stable lines of worms that expressed the *cat-4* and *dpy-7:gfp* genes. One hundred worms were homogenised in SDS loading buffer and loaded onto a 4 − 12 % SDS PAGE gel. A Western blot was performed using an anti-His (C-terminus) primary antibody raised in mice (Invitrogen) and an anti-mouse IgG HRP secondary antibody (Promega), visualised using an ECL detection kit (GE Healthcare). As a band of the predicted size was visible, large scale production of the protein was carried out, as follows.

To produce large numbers of transformed *C. elegans* to express the Tci-CAT-4 protein, 35 peptone-rich plates (Section 8.1.5.2) were seeded with 100 µl freshly starved individual worms and incubated with BL22 *E. coli* as a food source at 20 °C for five days. Worms were washed off the plates using ice cold 0.1 M NaCl, and centrifuged at 6000 x g for 5 min. The supernatant was removed and the pellet of worms resuspended in ice cold 0.1 M NaCl to a volume of 20 ml. An equal volume of ice-cold 60 % sucrose was added, and mixed gently, before centrifugation of 2500 x g for 5 min was performed. The 'clean' worms that collected in a layer above the sucrose were aspirated and transferred quickly into ice cold 0.1 M NaCl for centrifugation at 6000 x g for 5 min. The supernatant was removed and a further two washes in ice cold 0.1 M NaCl performed. After the final wash, the worms were resuspended in 5 ml 0.1 M NaCl, with 5 ml 2x lysis buffer added (100 mM NaH_2PO_4, 600 mM NaCl, 40 mM imidazole; pH 8). The worms were then frozen overnight at -80 °C. The frozen pellet was thawed on ice and sonicated on ice ten times for 10 s with a 30 s pause between each cycle. The worms were then homogenised in 1 ml aliquots using a handheld glass homogeniser and centrifuged at 15,000 x g for 3 min. The cleared lysate was removed and the centrifugation step repeated to ensure the removal of all the insoluble proteins. The pellets from both centrifugation steps were then resuspended in 1x lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole; pH 8), and the process of sonication, homogenisation and centrifugation repeated to ensure the maximal recovery of soluble protein. The cleared lysates from all the centrifugation steps were pooled and mixed with 200 µl nickel-nitrilotriacetic acid (Ni-NTA) agarose resin (Qiagen) and incubated on a roller for 2 h at 4 °C. The resins and lysate mixture were poured into a 1 ml polypropylene column (Qiagen) and the flow-through collected. The column was washed with 2 ml wash buffer 1 (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole; pH 8), followed by three, 2 ml washes with wash buffer 2 (50 mM NaH_2PO_4, 300 mM NaCl, 40 mM imidazole; pH 8). The protein was then eluted in six fractions, each of 150 µl using elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole; pH 8). Samples of all fractions were separated on a NuPAGE gel (Invitrogen) stained with SimplyBlue safestain (Invitrogen) to identify those containing protein of the estimated size of GTP-Cyclohydrolase.

As the eluted fractions contained multiple bands, the samples were diluted in binding buffer 1 without imidazole so that the final imidazole concentration was 20 mM. A 1 ml HisTrap™ HP column (GE Healthcare) was attached to a peristaltic pump and flushed with distilled water at a flow rate of 1 ml / min. It was then equilibrated with binding buffer 1 (Appendix 8.1.4.3) before the sample was loaded onto the column (a small amount of the sample was retained for comparison on a gel). As the volume containing the protein was large, the sample was re-circulated through the column for 1 h. The column was washed with 5 ml binding buffer 1 at 1 ml / min, with the flow-through collected. The proteins were then eluted with 1 ml elution buffer 1a (Appendix 8.1.4.6), 1 ml elution buffer 2a (Appendix 8.1.4.7), 1ml elution buffer 3a (Appendix 8.1.4.8) and 3 ml elution buffer 4a (Appendix 8.1.4.9). Polyacrylamide gel electrophoresis (NuPAGE system, Invitrogen) was used to determine which eluates contained the purified protein (Appendix 8.1.2). The eluates containing 'purified protein' were also examined using Western blotting. The fractions were probed with both an anti-his antibody directly conjugated to HRP (Sigma) and the anti-recombinant *O. ostertagi* GTP-Cyclohydrolase serum described in Section 4.3.4. A purified band of the predicted size that was visible on the protein gel and was detected by both sets of antibodies was sent for Peptide Mass Fingerprint Analysis using MALDI-TOF analysis (Bruker Ultraflex II).

A second batch of worms was harvested as described above, but only purified using the nickel column method described in the above paragraph, without using the Ni-NTA agarose. Samples of the proteins purified in this way were analysed by LC-MS/MS (Bruker AmaZon 3-D Ion trap)

4.2.4 Human monoclonal GTP-Cyclohydrolase antibody

The *T. circumcincta* and human sequences for GTP-Cyclohydrolase share 75 % identity at the amino acid level. As such, a monoclonal antibody (MO1) clone 4A12, raised in mice against human GTP-Cyclohydrolase (Abnova) was used in an attempt to detect native *T. circumcincta* GTP-Cyclohydrolase. A PBS soluble extract was

made from ~ 50, 000 L_3 that were snap frozen in liquid nitrogen, and then ground to a powder in a mortar and pestle with liquid nitrogen. Ten millilitres of PBS were added and the mixture continued to be ground until a liquid suspension was formed. The mixture was then centrifuged at 12,000 $x\ g$ for 15 min at 4 °C. The supernatant was retained as the S1 extract, and stored at − 80 °C until required. A sample of S1 extract was loaded onto a NuPAGE gel. This was then transferred to nitrocellulose membrane and probed with the monoclonal antibody, diluted 1 / 1000 with TNTT. A secondary rabbit α mouse immunoglobulin / HRP antibody (DakoCytomation) was used, and the blot develop using DAB.

4.3 Results

4.3.1 pET-SUMO

Recombinant GTP-Cyclohydrolase was produced using the pET-SUMO system (Figure 33, Lane 1); however the protein was insoluble in aqueous buffers. The protein was solubilised using urea which enabled it to be purified by nickel affinity chromatography (Figure 33, Lanes 5 and 6). The excised band was confirmed by MALDI analysis to be the GTP-Cyclohydrolase-SUMO fusion, with 59 % sequence coverage and 42 % intensity (a measure of protein concentration as a proportion of the total protein in the sample) as shown in Figure 34. When samples of the purified SUMO fusion protein were cleaved using the SUMO protease, no bands were visible on a polyacrylamide gel after cleavage.   This occurred after cleavage using both techniques, and as such it was likely that the protein was unable to remain in solution post-cleavage.
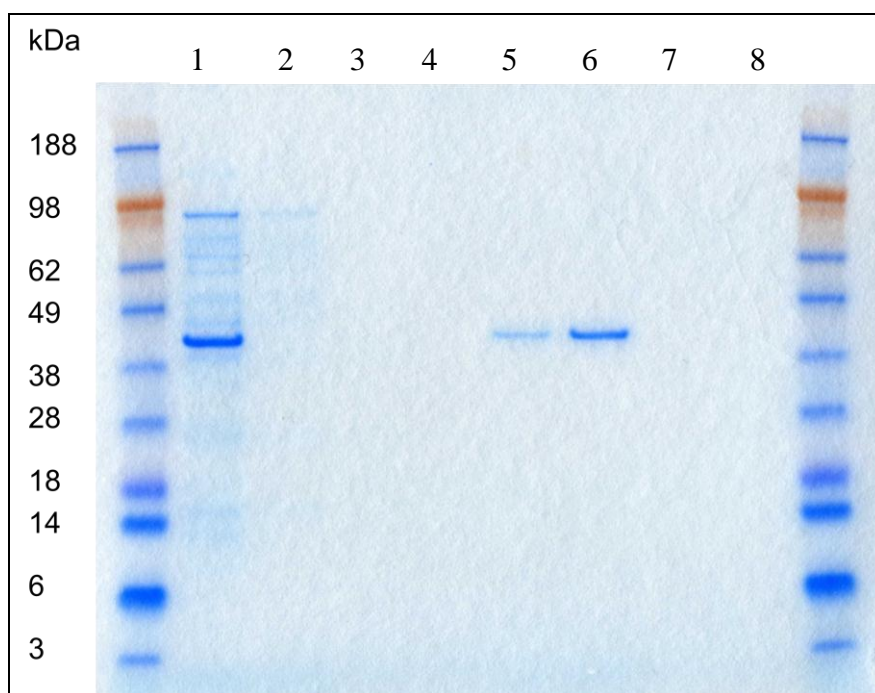

Figure 33. Polyacrylamide gel image of protein products of expression of GTP-Cyclohydrolase in pET-SUMO vector that were insoluble in aqueous buffers (lane 1). The flowthrough of the nickel column is shown in lane 2 and the eluted fractions shown in lanes 3 – 8. The purified recombinant GTP-Cyclohydrolase was identified in lanes 5 and 6. The proteins are shown alongside SeeBlue® Plus2 Pre-Stained standard (Invitrogen).

Figure 34. Sequence coverage results of the GTP-Cyclohydrolase SUMO fusion from the MALDI analysis. Residues shown in red represent peptides derived from GTP-Cyclohydrolase fusion which were matched to the theoretical sequence of amino acids generated from the DNA sequence. The greyscale reflects intensity, with the darker the grey the more intense the peak was in the spectra. The yellow highlighted residues represent potential N-glycosylation sites.

4.3.2 pET-22b(+)

Recombinant GTP-Cyclohydrolase was produced in the pET-22b(+) system, but it was insoluble in aqueous buffers (Figure 35). Bands of the expected size were visible in the 'insoluble induced lanes' of the polyacrylamide gel. As a recombinant *O. ostertagi* GTP-Cyclohydrolase had been produced previously using pET-15b (Moore *et al*., 2000), and this had been shown to be insoluble in aqueous buffers it was not deemed useful to attempt to solubilise this protein using urea as any antibodies raised against it may be less likely to bind to the native protein.
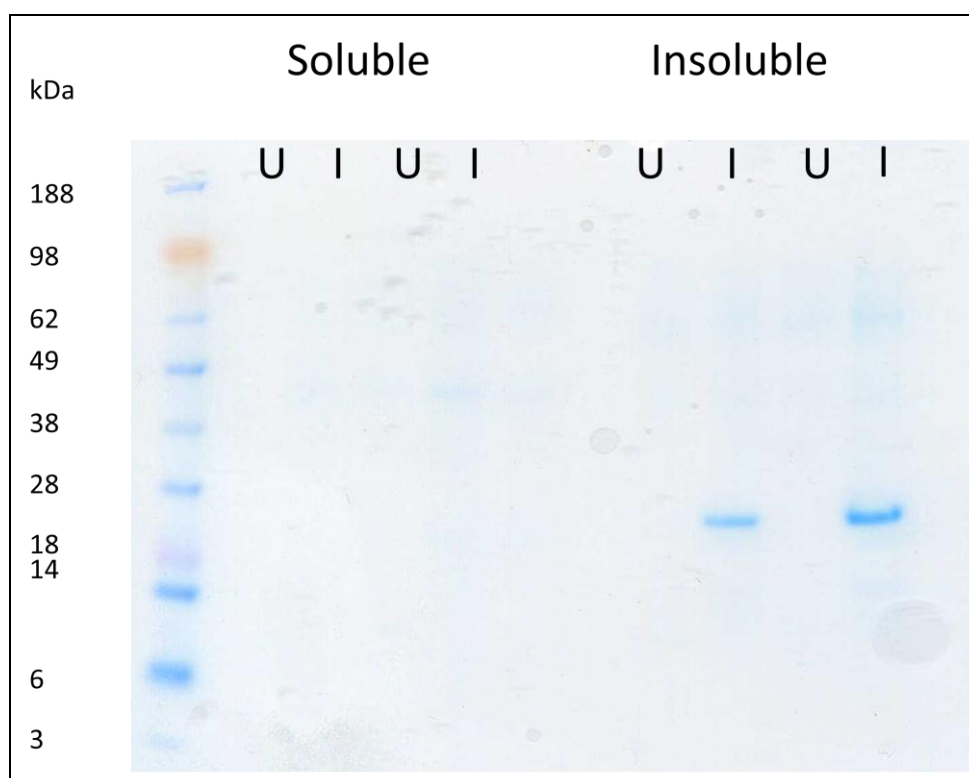

Figure 35. Polyacrylamide gel image of the protein products of expression of GTP-Cyclohydrolase in pET-22b(+). Two different cultures were split into the insoluble and soluble fractions both pre- (Uninduced – U) and post-induction (Induced – I) with IPTG. In both cultures, the band of the predicted size for GTP-Cyclohydrolase appeared in the insoluble fraction. The proteins are shown alongside SeeBlue® Plus2 Pre-Stained standard (Invitrogen).

4.3.3 *Pichia pastoris*

When material derived from the supernatant of the *P. pastoris* culture was subjected to polyacrylamide electrophoresis, a band of the predicted size was observed (lane 8, Figure 36) indicating that a recombinant GTP-Cyclohydrolase soluble in aqueous

buffers had been produced. However, this protein did not bind to the nickel column but was identified in the 'flow-through' sample after nickel affinity chromatography (lane 2, Figure 36). MALDI analysis of this band indicated that the protein was not GTP-Cyclohydrolase, but a bacterial outer membrane protein (sequence coverage 29 %, intensity 38 %) suggesting contamination of the culture.
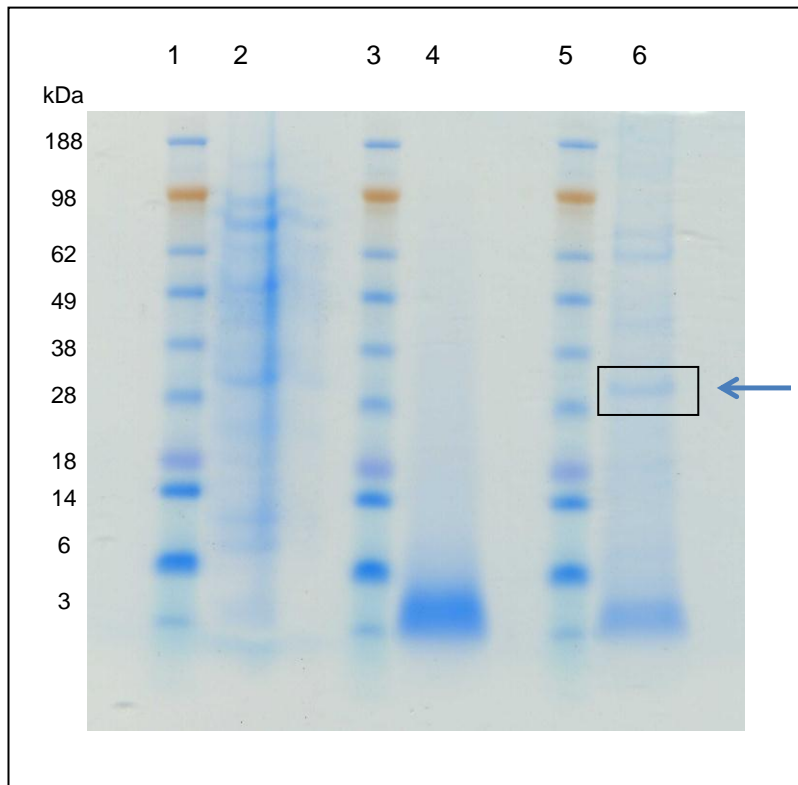

Figure 36. Polyacrylamide gel image of the protein products of expression of GTP-Cyclohydrolase in *P. pastoris*. The marker SeeBlue® Plus2 Pre-Stained standard (Invitrogen) is shown in lanes 1, 3 and 5. Lane 2 shows the proteins that did not bind to the nickel column during nickel affinity chromatography. Lane 4 shows the proteins that did bind to the nickel column and were eluted using imidazole. Lane 6 shows the pre-purified total soluble fraction for comparison. The band highlighted with the arrow was the predicted size for GTP-Cyclohydrolase but was observed in the unbound lane 2 rather than the bound lane 4.

4.3.4 Maltose Binding Protein

The pilot study using the maltose binding protein expression system showed that soluble recombinant GTP-Cyclohydrolase was produced as a fusion protein. The study also showed that the fusion protein bound to the amylose resin, and thus could

be purified using chromatography. MALDI analysis of the MBP-fusion confirmed its identity with 41 % intensity and 46 % sequence coverage as shown in Figure 37.

Figure 37. Sequence coverage of the MALDI analysis of the GTP-Cyclohydrolase MBP fusion protein. Residues shown in red represent peptides which were matched to the theoretical sequence. The greyscale reflects intensity, with the darker the grey the more intense the peak in the spectra. The yellow highlighted residues represent potential N-glycosylation sites.
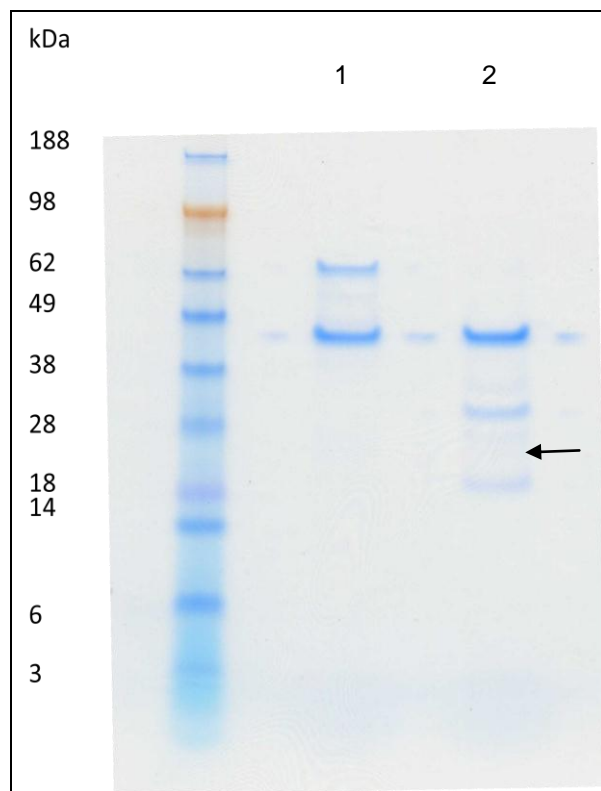
Figure 38. Polyacrylamide gel image of the recombinant GTP-Cyclohydrolase produced using the maltose binding protein expression system . Lane 1 shows the GTP-CH-MBP fusion protein (67 kDa) and the MBP (43 kDa). After digestion with Factor Xa (lane 2), the MBP remained and the GTP-Cyclohydrolase fusion protein had been cleaved into MBP (43 kDa) and GTP-Cyclohydrolase (23.8 kDa), highlighted with the arrow. The two bands above and below the GTP-Cyclohydrolase band in lane B are Factor Xa, which consists of two disulphide linked chains of 30 and 15 kDa. The proteins are shown alongside SeeBlue® Plus2 Pre-Stained standard (Invitrogen).

Large quantities of the GTP-Cyclohydrolase-MBP fusion protein were purified using an amylose column (lane 1, Figure 38). However, after cleavage with Factor Xa, small quantities of pure GTP-Cyclohydrolase were obtained (Lane 2, Figure 38). Only a very faint band of the predicted size for GTP-Cyclohydrolase was visible after electrophoresis. Factor Xa cleaves proteins after the recognition site Ile-Glu-Gly-Arg, which is not present within the theoretical GTP-Cyclohydrolase sequence. After Western blot analysis using rabbit α *O. ostertagi* recombinant GTP-Cyclohydrolase serum (Moore *et al*., 2000), it appeared that the GTP-Cyclohydrolase produced using the MBP system was degraded (Lane 2, Figure 39). There was some non-specific binding by the antibody to the proteins present in material that had not been incubated with Factor Xa (Lane 1, Figure 39), but there was a band that identified the MBP-GTP-Cyclohydrolase fusion at 67 kDa. After cleavage with Factor Xa, (lane 2) there was a smear of proteins suggesting that breakdown was

occurring, which would also explain why only a faint band was visible on the protein gel. Lane 3 of Figure 39 represents the proteins present after the dialysis into the buffers required for anion exchange chromatography (to remove the Factor Xa and MBP). This also revealed that most of the proteins were lost during dialysis, so the small quantities of GTP-Cyclohydrolase present after cleavage were further diminished. This added further evidence to the real-time graphical representations of protein concentration that were observed during the anion exchange chromatography in which only very low levels of protein were detected (not shown).
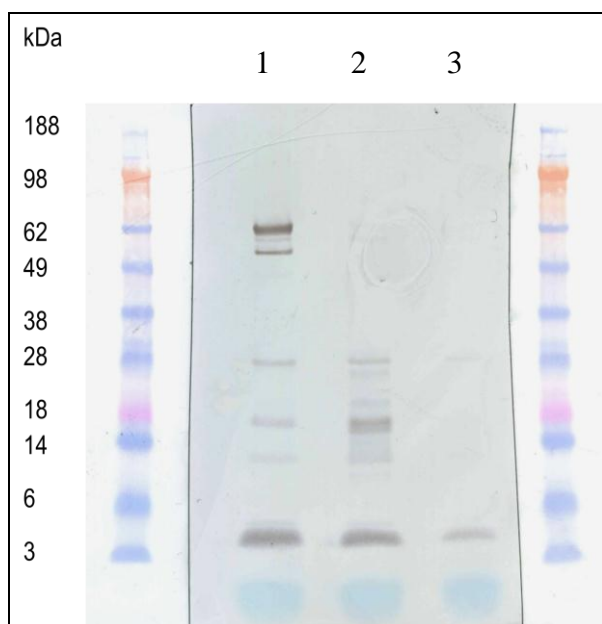
Figure 39. Western blot showing the presence of GTP-Cyclohydrolase breakdown products after cleavage with factor Xa. Lane 1 shows the pre-cleavage sample, which shows the presence of a band of ~ 4 kDa, suggesting breakdown of the GTP-Cyclohydrolase MBP fusion may have occurred at this point. In lane 2, further degradation products from 12 – 28 kDa are visible after factor Xa cleavage. Lane 3 showed that dialysis of the proteins caused most of the remainder of the proteins to be lost. The proteins are shown alongside SeeBlue® Plus2 Pre-Stained standard (Invitrogen).

4.3.5 *C. elegans*

The transgenic *C. elegans* expressed the gfp construct (Figure 40). A high proportion of the worms had incorporated the plasmid as an extrachromasomal array (approximately 50-70 % of the worms displayed the gfp phenotype in any one generation) which meant that recombinant protein recovery should also have been high. A protein of the predicted size of GTP-Cyclohydrolase was purified from the

recombinant *C. elegans* worms (Figure 41). This protein was also detected by an anti-His antibody in pilot experiments performed by Brett Roberts at the University of Glasgow and the anti-*O.ostertagi*-GTP-Cyclohydrolase antibodies (Moore *et al*., 2000) on a Western blot (data not shown). The protein was not detected in wild type N2 worms by either of these antibodies, suggesting that it was GTP-Cyclohydrolase that had been produced in the recombinant worms. Peptide mass fingerprint analysis indicated that the protein at ~26 kDa (Lane G, Figure 41) was a *C. elegans* stress induced protein 1 (*sip-1*), NCBI accession number CAA84703 (sequence coverage score of 79.9 %).
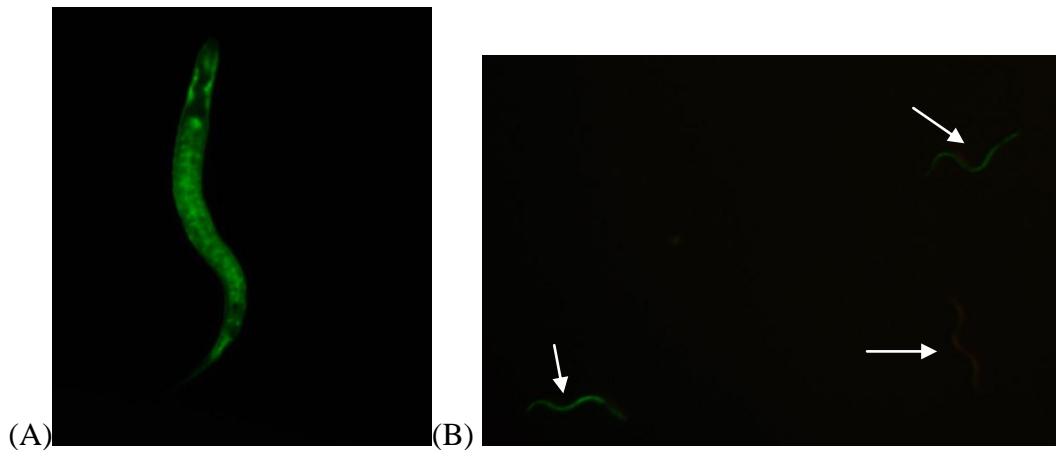

(A)   (B)

Figure 40. Transgenic *C. elegans* expressing the gfp construct that was used as a marker of plasmid uptake (A). On the right panel (B), three worms are highlighted, one which has not taken up the construct (bottom right) and two that had (bottom left and top right). The worms expressing gfp should also have taken up the GTP-Cyclohydrolase plasmid construct.
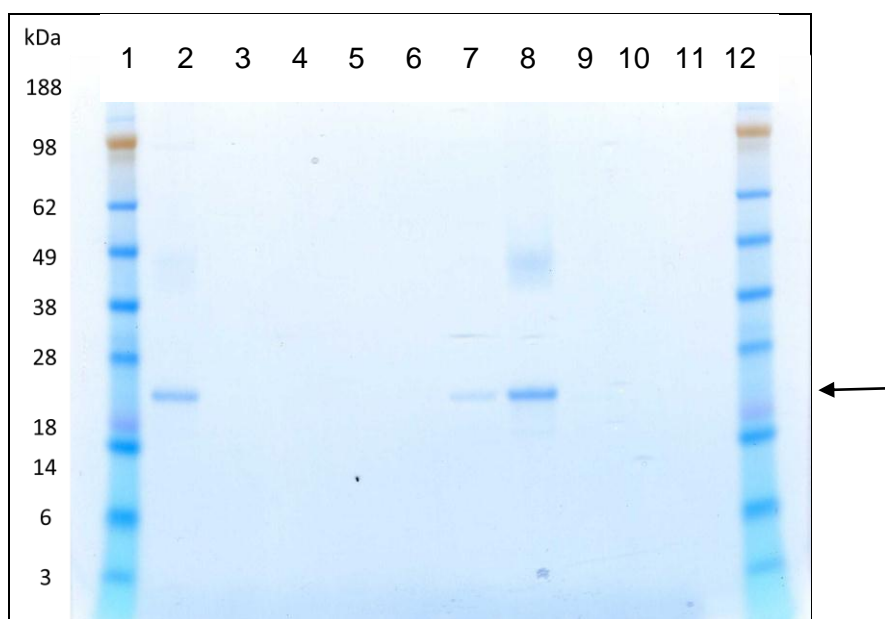
Figure 41. Polyacrylamide gel image of the proteins recovered from the second round of purification of the aqueous soluble proteins by nickel affinity purification from transgenic *C. elegans*. Lane 2 shows the diluted and buffer exchanged fraction that was produced from the first round of purification (NiNTA bead purification) and used as the starting point for the second round (His-trap column). Lane 3 represents the flowthrough, and lanes 4-11 the fractions obtained using increasing concentrations of imidazole. Bands of the predicted size are visible in lanes 7 and 8. The proteins are shown alongside SeeBlue® Plus2 Pre-Stained standard (Invitrogen) in lanes 1 and 12.

Because of the result of the MALDI analysis, a second batch of transgenic *C. elegans* was produced and harvested to ensure that the recombinant protein had not been lost during the purification steps. The expression products were analysed by SDS-PAGE (Figure 42). When sections of this gel (indicated on Figure 42) were analysed by LC-ESI-MS/MS analysis, many proteins were identified (1707 protein hits), but *T. circumcincta* GTP-Cyclohydrolase was not one of them. *E. coli* GTP-Cyclohydrolase was identified, but *C. elegans* GTP-Cyclohydrolase was not. A wide range of *C. elegans* and *E. coli* proteins were identified as would be expected from the worms and their food source. Section A (Figure 42) contained the proteins present prior to nickel affinity purification, and if GTP-Cyclohydrolase was present it should have been contained within this slice; if the protein was being trapped on the nickel column it should have been identified here. Section B contained the proteins of the predicted size to contain GTP-Cyclohydrolase in the eluted fraction from the nickel affinity column. Section C contained the region of antibody recognition with the anti-GTP-Cyclohydrolase antibody (Figure 39). As *T. circumcincta* GTP-

Cyclohydrolase was not identified, it was decided not to proceed any further with attempts to produce a soluble recombinant GTP-Cyclohydrolase.
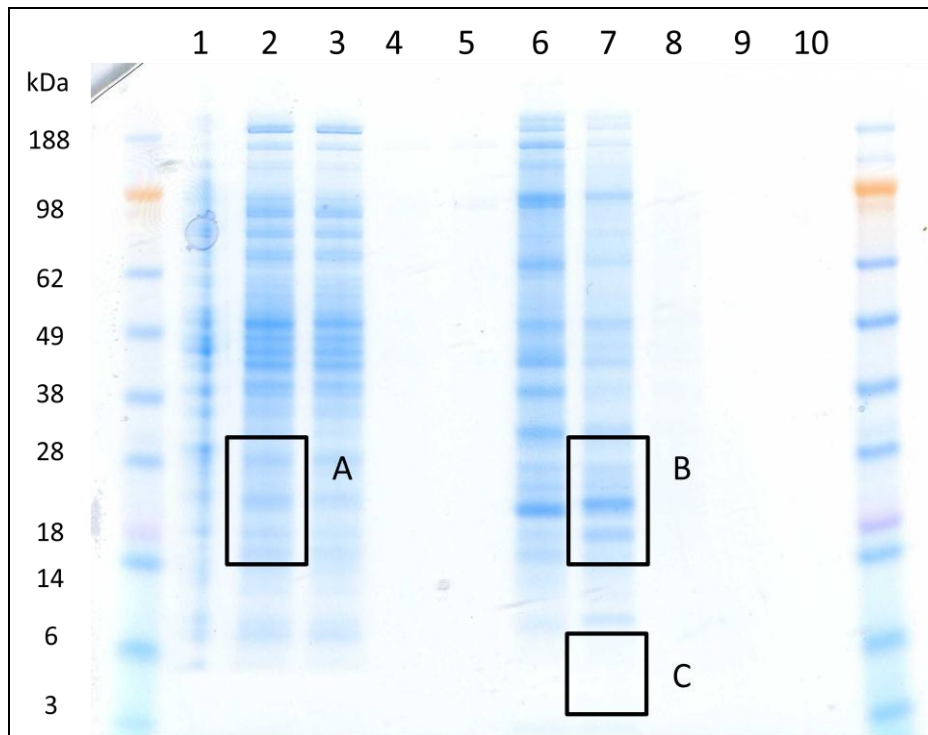
Figure 42. Polyacrylamide gel image of the proteins recovered from the soluble fraction of *C. elegans* after the second batch of protein production and purification using nickel affinity chromatography. Lane 1 contained the insoluble fraction, lane 2 the pre-column sample, lane 3 the flowthrough from the His-trap column and lanes 4 – 10 the samples eluted with increasing imidazole concentrations. Sections A, B and C were excised and analysed using LC-ESI-MS/MS analysis. The proteins are shown alongside SeeBlue® Plus2 Pre-Stained standard (Invitrogen).

4.3.6 Monoclonal antibody against human gch1

The monoclonal antibody that detected human gch1 did not detect the *T. circumcincta* GTP-Cyclohydrolase on a Western blot, despite the relative conservation of amino acid sequence, and hypothesised structural similarities between the proteins of the two species.

4.4 Discussion

In this chapter, the *T. circumcincta* recombinant GTP-Cyclohydrolase was shown to be insoluble following expression in the pET-SUMO and pET-22b(+) bacterial systems and no intact recombinant protein was produced via the yeast expression system. Soluble recombinant protein was produced using the maltose binding system, but the protein degraded during the enzyme cleavage step. Initial studies suggested a promising result for the production of a soluble GTP-Cyclohydrolase using *C. elegans*, but proteomic analysis demonstrated that the protein thought to be *T. circumcincta* GTP-Cyclohydrolase on SDS-PAGE was instead a stress induced protein, *sip-1*. Many *C. elegans* proteins, including *sip-1*, are rich in histidine residues, with 10 out of a total 159 amino acids representing histidine, including the final residue of the protein. Although *sip-1* does not contain the traditional polyhistidine tag that is utilised in nickel affinity chromatography, it is possible that in the absence of any other protein binding to the nickel, *sip-1* bound via the histidine residues described. As the tertiary structure of this protein is not known, it is also possible that, when folded, a number of the histidine residues could be located close to each other, forming an effective binding site for the nickel affinity column. This leaves the question as to whether the *C. elegans* produced the recombinant protein which could not then be purified or whether it was never produced in the first place. The plasmid containing the *Tci-cat-4* His tagged construct was co-injected with a *dpy-7:gfp* construct so that worms that had taken up the plasmids into extrachromosomal arrays could be identified (Chalfie et al., 1994). In these experiments, while the frequency changes each generation, the proportion of worms expressing gfp was approximately 50 - 70 % (as identified by counting fluorescent worms). With such a high proportion of worms expressing the gfp, the recovery of GTP-Cyclohydrolase should have been successful. As the transgenic worms produced a stress-induced protein (Ding and Candido, 2000), and the wild-type worms did not, this suggests that recombinant GTP-Cyclohydrolase production may have been occurring, but that it was deleterious to the worms. The wild-type worms were harvested in exactly the same way as the recombinant worms, so the production of the stress protein was not likely to be due to harvesting or growing conditions.

Alongside this, there is the data from the LC-ESI-MS/MS analysis, that showed that of the 1700 proteins identified in the fractions examined, bacterial GTP-Cyclohydrolase was present, but no nematode GTP-Cyclohydrolase. Given the large numbers of *C. elegans* proteins identified using this technique, if any GTP-Cyclohydrolase was present as protein within the worms it should have been detected. This may suggest that *C. elegans* do not store GTP-Cyclohydrolase as a protein, but use it immediately for the production of BH_4. As it is known to be the rate-limiting factor in the production of BH_4 (Tegeder *et al*., 2006), perhaps the addition of extra GTP-Cyclohydrolase, albeit that of another species, was used by the free living species to produce extra BH_4 for use in the production of the biogenic amines, particularly because with this expression system there is no over-expression, just constitutive expression of the protein. This process of increased BH_4 synthesis may have then resulted in unforeseen physiological stress for the worms, thus causing the production of the stress protein identified. SIP-1 is known to be expressed in the embryo of developing *C. elegans* (Ding and Candido , 2000), and as such, it would appear that the physiological consequences of increased GTP-Cyclohydrolase occurred early on in development (if that was the cause of the stress). GTP-Cyclohydrolase Feedback Regulatory Protein [GFRP (Milstien *et al*., 1996)] has been confirmed by the amplification of cDNA encoding the gene in *C. elegans* (see www.wormbase.org), and it is possible that with the addition of superfluous GTP-Cyclohydrolase, then the feedback mechanism reduced the levels of enzyme. In order to pursue the expression of recombinant GTP-Cyclohydrolase in *C. elegans*, it would be advantageous to inject the construct plasmid into *Ce-cat-4* deletion mutants to ascertain whether the production of recombinant GTP-Cyclohydrolase is possible against the background of a worm that is unable to produce its own GTP-Cyclohydrolase. Another approach would be to inject the construct into a GFRP deletion mutant (as yet unavailable) to assess whether GTP-Cyclohydrolase production would be possible if the feedback loop had been disabled. Before this was attempted however, experiments should be undertaken to assess the viability of *C. elegans* individuals that have had the feedback gene removed, as increased levels of native GTP-Cyclohydrolase may prove problematic for the worms.

The use of *C. elegans* for the production of recombinant parasitic nematode proteins is a novel approach and still under development. To date, soluble recombinant proteins, for example *H. contortus* cathepsin L (*cpl-l*) and H11 (a potential vaccine candidate) have been produced (Murray *et al*., 2007), but no *T. circumcincta* recombinant proteins have been expressed in *C. elegans* thus far. Both *H. contortus* proteins are known to express within the gut cells of nematodes (Andrews *et al*., 2005; Neveu *et al*., 2003), which may provide further clues as to the mechanisms required for successful recombinant protein production. The *Ce-cpl-1* promoter was used for all of the proteins attempted so far including GTP-Cyclohydrolase in the present work (Murray *et al*., 2007), it may be possible that this promoter is only appropriate for proteins that are naturally expressed within the gut. As discussed in Chapter 3, if GTP-Cyclohydrolase is not expressed within the gut cells, but within the hypodermis, then it may be that *Ce-cpl-1* is not an appropriate promoter as it could lead to the production of GTP-Cyclohydrolase in a part of the body where the protein is not normally expressed. With more time, it would be interesting to examine whether using the *Ce-cat-4* promoter region would result in recombinant GTP-Cyclohydrolase expression in tissue that it is normally expressed in, thus reducing the stress to the individual nematodes. Although, if the issue was that the GTP-Cyclohydrolase was being used by the worms, then this approach may not alleviate that problem, but accentuate it.

It would be advantageous to repeat the process of attempting to produce a recombinant GTP-Cyclohydrolase in *C. elegans* and varying the protocol. Alternatives could include using GFRP mutants (as discussed earlier), or inserting artificial introns into the gene construct, as there is evidence that the production of proteins in nematodes is enhanced by the presence of introns: this was initially demonstrated with myosin proteins, and was shown to be independent of intron sequence, suggesting that it is the splicing that is necessary rather than sequence information within the intron (Okkema *et al*., 1993). Alternatively, the *Tci-cat-4* gene could be amplified from genomic DNA, thereby incorporating the gene specific introns into the plasmid. In bacterial and yeast expression systems, recombinant

protein production is under the control of strong inductors; for example T7 in the bacterial systems (Tabor and Richardson, 1985), AOX1 in *P. pastoris* (see Cereghino and Cregg, 2000) and these facilitate large quantities of recombinant protein production. Using the methods described in this project, there is no mechanism for the overexpression of a recombinant protein in *C. elegans*, expression can only be constitutive. However, recent research may have provided a solution to this, with the use of codon adaptation. Redemann *et al.* (2011) identified that *C. elegans* genes that were expressed at high levels preferentially used certain codons for which there were a larger quantity of tRNAs available. This observation was then used to develop software (*C. elegans* codon adapter, available online at http://worm-srv3.mpi-cbg.de/codons/cgi-bin/optimize.py) that could be used to design artificial sequences that were able to increase the expression of G-protein regulators using synthetic genes. Using this program to synthetically alter the sequence of *Tci-cat-4*, or indeed any other protein of interest could potentially increase yield recovered from transgenic worms. Using *Ce-cat-4* as an example, according to the software, changing the first 21 bases of coding sequence from

ATGTCCAGAATTGAGAACGAA to ATGTCCCGTATCGAGAACGAG would retain the amino acid sequence of Met Ser Arg Ile Glu Asn Glu, but would increase the 'codon adaptation index' from 0.6 to 1, and theoretically increase the expression of recombinant GTP-Cyclohydrolase. It is not yet known if the function of the synthetic genes will perform in the same manner as artificially-altered, native genes, but this system merits further investigation.

Of the other systems examined in this chapter to produce recombinant protein, the second most promising was the maltose binding protein fusion system; using this, a soluble recombinant GTP-Cyclohydrolase was produced, but it was unable to be cleaved without degradation. This system has been used to produce recombinant forms of mammalian GTP-Cyclohdrolase (Witter *et al*., 1996b) and, given the conserved nature of the protein, this does provide hope that a soluble recombinant *T. circumcincta* could be produced. Again, with more time, it would be advantageous to explore the other expression vectors within the maltose binding protein system to see whether it would be possible to produce a protein that could be cleaved from the

fusion protein without degradation, or to attempt other cleavage techniques. When the re-solubilised pET-SUMO fusion protein was cleaved, the GTP-Cyclohydrolase was unable to remain in solution, suggesting that there is an issue with the stability of the bacterially expressed recombinant protein. Previous attempts to produce a recombinant GTP-Cyclohydrolase from the closely related nematode *O. ostertagi* produced a protein that had to be solubilised in urea, and antibodies raised against it were unable to bund to native protein on a Western blot (Moore *et al*., 2000).

The production of a recombinant *T. circumcincta* GTP-Cyclohydrolase has not been straightforward, a feature that has been observed for other species, such as the malarial parasite *Plasmodium falciparum*. The folate pathway is an established anti-malarial drug target (Nzila *et al*., 2005) and as such there has been focus on the role of GTP-Cyclohydrolase as a potential novel target (Lee *et al*., 2001). Despite this interest, it was only after the addition of a molecular chaperone to increase the accuracy of protein folding, that a soluble recombinant *P. falciparum* GTP-Cyclohydrolase was produced (Stephens *et al*., 2011). The molecular chaperone used in this case was heat shock protein 70, known to be expressed in a ubiquitous manner in eukaryotes and believed to prevent the misfolding of proteins (Thulasiraman *et al*., 1999). The sequence for *Ce-hsp-70* has been deposited on Wormbase (www.wormbase.org), and a BLAST search of the *T. circumcincta* genome (accessed September 2011; Sanger Institute) identified a region with 60 % identity to the *Ce-hsp-70* gene. This sequence could be used to design primers to generate full-length sequence for *Tci-hsp-70*, which could be co-expressed in a bacterial expression system with GTP-Cyclohydrolase in an attempt to increase the solubility, and potential activity of the recombinant protein.

Without the production of a soluble recombinant GTP-Cyclohydrolase, it has not been possible in this project to produce antibodies against the enzyme that would have enabled immunolocalisation experiments to take place. As such, the tissues within *T. circumcincta* that express GTP-Cyclohydrolase are still unknown, and until any further research is carried out, comparisons will have to be drawn from the

model free-living nematode *C. elegans* and from the previous work performed with *O. ostertagi* (Moore *et al*., 2000). The localisation of GTP-Cyclohydrolase to all serotonergic and dopaminergic neurons as well as the body wall in *C. elegans* (see Sze *et al*., 2002) may be a conserved expression pattern. It has been shown that the parasitic nematode *H. contortus* possess serotonergic neurons that have a similar location to those of *C. elegans* (Rao *et al*., 2011); so it is plausible that a key enzyme in the production of serotonin should also be present in those neurons. As for the role within the body wall, it could be that GTP-Cyclohydrolase is important for the production of melanin (to be explored in the next chapter), or it could be required for the crosslinking of tyrosine and collagen within the cuticle (Yang and Kramer, 1999). If the production of a soluble recombinant GTP-Cyclohydrolase is not possible using any of the above suggested alternative methods, then an alternative strategy for localisation may have to be investigated. This could include in-situ hybridisation, which has been used with success to localise expression patterns of heat shock protein 20 in *H. contortus* to the intestine and reproductive organs of adult worms (Hartman *et al*., 2007). The use of green fluorescent protein as a gene marker was used in this chapter to identify the *C. elegans* individuals that had taken up the plasmids as an extrachromasomal array (Mello *et al*., 1991). If the *Tci-cat-4* gene were to be labelled with a fluorescent marker, it may be possible to assess the expression pattern within the nematodes directly. The major obstacle to be overcome before this could be achieved, however, is the ability to culture offspring from parasitic nematodes *in vitro*. In *C. elegans*, the plasmids are microinjected into the gonads of the hermaphrodites, which enables the embryos to incorporate the plasmids as an extra-chromosomal array. With parasitic nematodes, the obstacle of sexual rather than asexual mating exists, and the fact that this occurs inside the mammalian host. While the aims of this chapter have not been realised, it should be possible in the future to produce a soluble recombinant GTP-Cyclohydrolase from *T. circumcincta* using some of the methods described in this discussion.

5   Use of *Caenorhabditis elegans* to investigate the role of GTP-Cyclohydrolase in nematode melanisation

5.1   Introduction

Caenorhabditis elegans was first isolated from soil in Algeria in May 1897 (Maupas, 1900), and the descriptions given at that time suggested that the species would be well suited to further experimental investigation. It was 50 years later, however, before the scientific community started to realise the potential of free-living nematode species as alternatives to studies on the model species *Drosophila melanogaster* for testing of the toxicity of newly developed antibiotics and for newly emerging genetic studies (Gochnauer and McCoy, 1954; Nigon and Dougherty, 1950). Since then, the characterisation of *C. elegans* has been so detailed that the identity of every embryonic cell has been named and it was the first eukaryotic organism for which the complete genome sequence was published (The *C. elegans* Sequencing Consortium, 1998). As was acknowledged by Maupas in his original description, more studies into parasitic nematodes had been undertaken at that time, due to their "large size and practical importance" (Maupas, 1900). The difficulties in culturing parasitic nematodes existed then as now (Smith and Zarlenga, 2006), with, in most cases, the need for donor animals to be infected to maintain the lifecycle. The descriptions by Brenner (1974) of the ease with which *C. elegans* could be maintained in culture, with a rapid life-cycle, gave a further impetus to research in this species and it is now used ubiquitously for research in a wide variety of disciplines.

Recent interest in the use of *C. elegans* as a tool for the elucidation of information regarding gene function in parasitic nematodes has taken a number of forms. One method utilised has been that of functional complementation or gene rescue. Described by the 1995 Nobel Laureate Edward Lewis (1945), functional complementation has been used to ascertain whether a wild-type gene can restore the function of a defective mutant, and thus demonstrate that the mutation is occurring in

the same gene as the wild-type. This was traditionally performed using genes from the same species (for example, Rusconi *et al*., 2005) but has now been used to identify the functions of orthologous genes between different species (for example, Malonek *et al*., 2005). If a gene of unknown function from another species can restore the function of a *C. elegans* mutant with a clear and known phenotype, then it can be hypothesised that the genes are performing the same function. Generally, as more is known about gene function in *C. elegans* compared to parasitic nematodes, then the established information on gene function can be used to infer roles in the parasitic species. This has been performed to examine the role of glutamate gated chloride (GluCl) channels in ivermectin resistance in *H. contortus*, by restoring normal motor function of the *C. elegans avr-14* mutant with the orthologue from the parasitic nematode (Yates *et al*., 2003). The role of β-tubulin in benzimidazole resistance was also explored using this technique, with the expression of different isotypes of the *H. contortus* β-tubulin isotype I gene conferring resistance or sensitivity on the *C. elegans ben-1* (benzimidazole sensitive) mutants (Kwa *et al*., 1995).

There is a large body of research surrounding the role of GTP-Cyclohydrolase in *C. elegans* (see Horvitz *et al*., 1982; Loer and Kenyon, 1993; Sze *et al*., 2000). If this knowledge could be applied to parasitic nematode research, then the increased level of understanding regarding the function of GTP-Cyclohydrolase could be exploited to further understand the role of this enzyme in *Teladorsagia circumcincta*. It has been shown that *C. elegans cat-4* mutant worms contain lower levels of melanin in their cuticles than wild-type worms (Calvo *et al*., 2008). Of the two main pathways proposed for study in this project (Figure 5, Chapter 1), the serotonin pathway was explored in Chapter 2. This chapter will explore the role of GTP-Cyclohydrolase in the melanisation pathway, using functional complementation to assess whether the *Tci-cat-4* gene is capable of restoring cuticular integrity in the *Ce-cat-4* mutants.

The 'leaky cuticle' of the *Ce-cat-4* mutants allows for an easy identification of the *cat-4* phenotype as mutant worms are killed by exposure to lower concentrations of

bleach or anthelmintics than wild-type worms (Loer *et al*., 1999). Melanin in the cuticles of nematodes has been hypothesised to protect them from oxidative stress (Calvo *et al*., 2008), which may be particularly important as the main respiratory surface is the cuticle. Alongside protection from oxidative stress, there is also the possibility that cuticular melanin could provide protection from external factors such as ultraviolet (UV) light. UV radiation causes damage to tissues through DNA damage, such as the insertion of cyclobutane pyrimidine dimers (CPDs), whereby two pyrimidine bases join adjacently (most commonly thymine-thymine) which then disrupts normal DNA coding sequence (Ikehata and Ono, 2011). In mammals, DNA damage can lead to skin cancer or immune suppression (Nishigori *et al*., 1996; Venema *et al*., 1990), and the deleterious effects of UV exposure can continue beyond the initial insult with increased levels of oxidative stress in cells (Peak *et al*., 1984). As *C. elegans* live in the soil, these worms are less likely to be exposed to UV light than parasitic nematodes, the free-living stages of which live on pasture. Indeed, trichostrongyle L_3 have been shown to be adversely affected by high levels of UV: studies on *T. circumcincta*, *H. contortus* and *Nematodirus battus* were performed in Petri dishes placed on turf inside Perspex boxes that either allowed the passage through, or blocked, UV light. The L_3 of the three species were then exposed to natural sunlight on a range of days with different levels of cloud cover and artificial UV conditions and the proportion of larvae alive after exposure were counted. This showed that larvae were susceptible to UV to different degrees, with *H. contortus* surviving at higher doses than the other species (van Dijk *et al*., 2009).

Much of the focus of research into GTP-Cyclohydrolase in nematodes thus far has focused on gene transcript levels (Abubucker *et al*., 2009; Moore *et al*., 2000; Nisbet *et al*., 2008). It is not practical to measure GTP-Cyclohydrolase activity directly, however it is possible to utilise the biochemical pathway shown in Figure 43 (Bezin *et al*., 1998). Under normal conditions the conversion of GTP to BH_4 occurs as described earlier (Figure 4) and is summarised in Figure 43. However, if alkaline phosphatase and potassium iodide are added to the enzyme reaction, then neopterin is produced instead (Bezin *et al*., 1998). As neopterin can be measured using a commercially available test, levels of this compound measured after the addition of

alkaline phosphatase and potassium iodide can be used to provide an indirect measure of the levels of activity of GTP-Cyclohydrolase (He and Rosazza, 2003). Here, this technique will be used to assess GTP-Cyclohydrolase activity levels in soluble extracts obtained from various developmental stages of *C. elegans* and *T. circumcincta*.
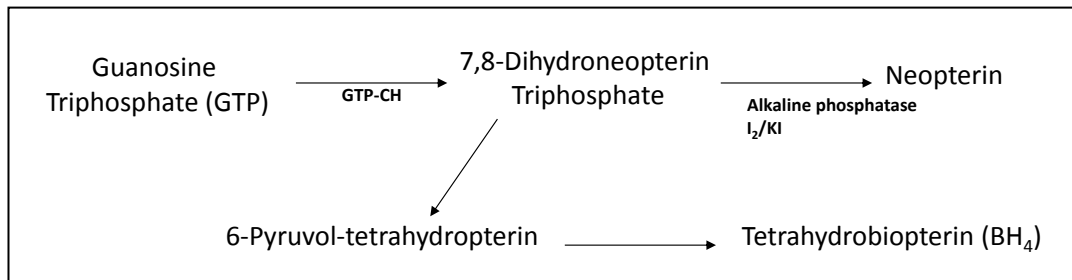
Figure 43. The biochemical pathway that can be utilised to measure neopterin levels to give an indirect assessment of GTP-Cyclohydrolase levels of activity.

In this chapter, functional complementation experiments will be described examining whether the *Tci-cat-4* gene can restore the cuticular integrity of the *Ce-cat-4* mutants. Levels of melanin within cuticles of *T. circumcincta* larvae were measured after exposure to sunlight, to assess the impact of UV on levels of this molecule. And finally, samples from both of these experiments, as well as material derived from various stages of *T. circumcincta* were examined to determine GTP-Cyclohydrolase activity levels in PBS soluble extracts.

5.2   Materials and Methods

5.2.1   Functional complementation with *Tci-cat-4*

5.2.1.1   Production of transgenic lines of *C. elegans*

Genomic DNA was extracted from the N2 strain of *C. elegans* using the DNeasy blood and tissue kit (Qiagen).   This DNA was used as template for PCR amplification of a 1500 bp region of the *C. elegans* 5' untranslated region (UTR) of *Ce-cat-4* using the primers *Ce-1500-F* and *Ce-1500-R* (sequences shown in Table 10; primers designed from sequence available on www.wormbase.org).   PCR was performed using the Advantage 2 polymerase mix (Clontech) with the cycling conditions 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s and 68 °C for 3 min 30 s, with a final extension step of 10 min at 68 °C.   The PCR product was purified and digested along with the pGEM-T plasmid (Promega) using the restriction enzymes *NcoI* and *SacII*.   Digested products were purified using the PCR purification kit (Qiagen) and ligated using T4 ligase (Promega) to form the 'pCe5'UTR plasmid' (Figure 44).   The 'pCe5'UTR plasmids' were transformed using heat-shock into JM109 competent *E. coli* cells (Promega), incubated for 1 h at 37 °C with 200 rpm movement in the presence of SOC medium and then spread on LB agar plates containing 100 µg / ml ampicillin, 0.5 mM IPTG and 80 µg/ml X-Gal. Colony PCR was performed on selected colonies using the *Ce-1500-F* and *Ce-1500-R* primers, with colonies positive for PCR products selected for amplification. Plasmids were amplified in 10 ml LB medium with 100 µg / ml ampicillin at 37 ° C for 18 h with 200 rpm movement. The 'pCe5'UTR plasmids' were purified using the Wizard plus SV minipreps kit (Promega) and sequenced (Eurofins MWG).

Table 10. Oligonucleotide primer sequences used to generate PCR products for the construction of the rescue plasmids

. Underlined bases represent those that correspond to the restriction sites that were introduced into each sequence. *Ce-1500-F* and *R* were used to amplify the 1500 bp 5' UTR from *C. elegans*. *Tc-cat-4-F* and *R* were used to amplify the *cat-4* gene from *T. circumcincta*. *Ce-cat-4-F* and *R* were used to amplify the *cat-4* gene from *C. elegans*. *Ce-500-F* and *R* were used to amplify the 500 bp 3' UTR from *C. elegans*.

| Primer Name	Primer Sequence 5' ... 3'	Restriction Sites introduced	Tm
Ce-1500-F	atc<u>ccatgg</u>TCATTCGTCATCACCTCCTCCC	*NcoI*	62 °C
Ce-1500-R	ttt<u>ccgcggaagctt</u>TTTGATATTATGATGTTGATAGAGTGGGTAAAG	*HindIII, SacII*	62 °C
Tc-cat-4-F	gag<u>aagctt</u>ATGGCTAGCGAAAGCGGTTTCC	*HindIII*	62 °C
Tc-cat-4-R	ggc<u>actagt</u>TCACTTTTTGTTGATGAGATTCAAGAATTCTTCT	*SpeI*	62 °C
Ce-cat-4-F	gag<u>aagctt</u>ATGTCCAGAATTGAGAACGAAAGCG	*HindIII*	62 °C
Ce-cat-4-R	ggc<u>actagt</u>TTAGCGTTTGTTGATAAGATTAAGGAATTCTTC	*SpeI*	62 °C
Ce-500-F	ggc<u>actagt</u>AAATTCTAGTCATTTCCTTTTTTACTTGTTTTTTTGG	*SpeI*	62 °C
Ce-500-R	gag<u>catatg</u>ATATGTCACAAAAGATTACATGTTTTATCAACAAAATG	*NdeI*	62 °C
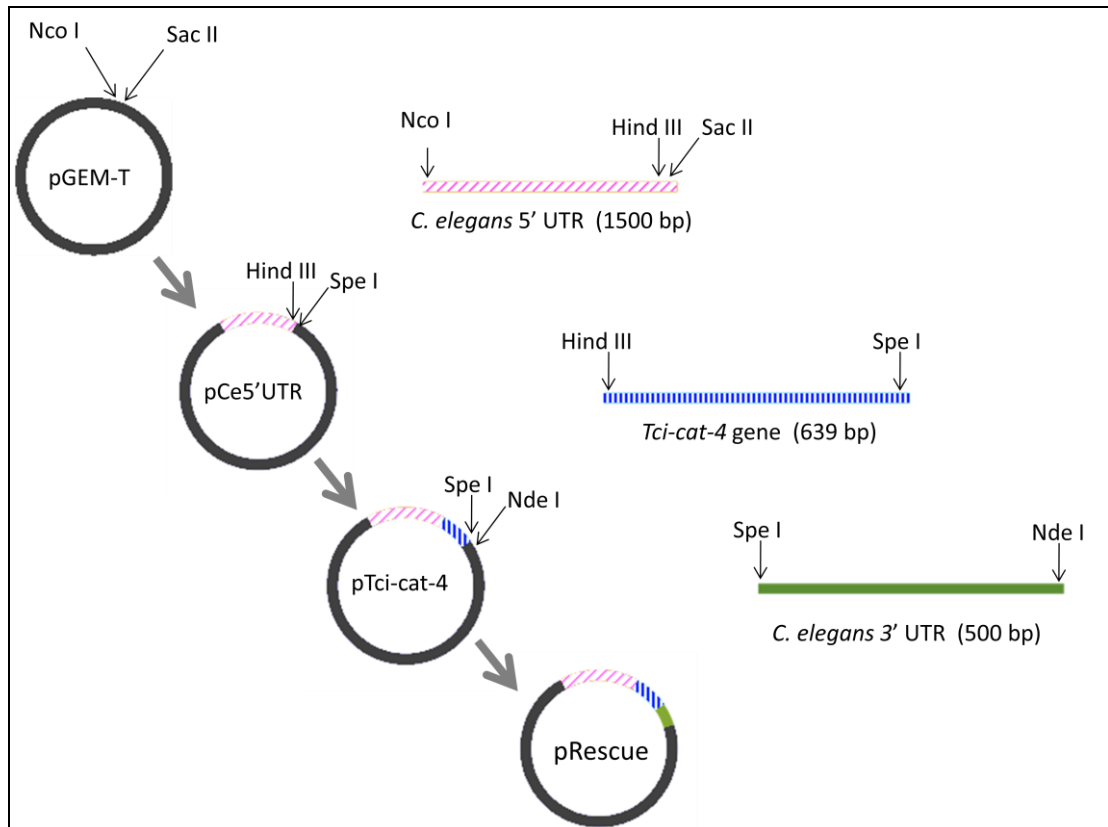
Figure 44. The location of enzyme restriction sites involved in the assembly of the rescue plasmids for functional complementation. *NcoI* and *SacII* were used to insert the *C. elegans* 5' UTR into pGEM-T to make the 'pCe5'UTR plasmid'. *HindIII* and *SpeI* were then used to add the *Tci-cat-4* gene in to construct the 'pTci-cat-4 plasmid'. Finally, the rescue plasmid was completed by the addition of the *C. elegans* 3' UTR using the *SpeI* and *NdeI* enzymes. This process was repeated with the *Ce-cat-4* gene to produce control rescue plasmids.

Full-length genomic DNA for the *cat-4* gene from *C. elegans* and *T. circumcincta* was amplified by PCR from the genomic DNA of both species using the primers *Ce-cat-4-F* and *Ce-cat-4-R,* and *Tc-cat-4-F* and *Tc-cat-4-R,* respectively (Table 10). The PCR products and the pCe5'UTR plasmid were digested using the restriction enzymes *HindIII* and *SpeI*, purified using the PCR purification kit (Qiagen) and ligated using T4 ligase (Promega) to form the pCe-cat-4 (control) and pTci-cat-4 plasmids (Figure 44). Transformation, amplification and sequencing were performed as described for the pCe5'UTR plasmid.

Finally, the 3' UTR for the *cat-4* gene from *C. elegans* was amplified by PCR using the *Ce-500-F* and *Ce-500-R* primers (Table 10). The PCR product and the pCe-cat-4 and pTci-cat-4 plasmids were digested using the restriction enzymes *SpeI* and *NdeI*, purified using the PCR purification kit (Qiagen) and ligated as described above to form the pRescue plasmids (Figure 44). Transformation, amplification and sequencing was performed as described for the pCe5'UTR plasmid. After confirmation of the correct assembly of the construct by sequence analysis (Eurofins MWG), the rescue plasmids were purified using a plasmid mini kit (Qiagen) to ensure the highest possible DNA quality, and eluted into nuclease-free water. Rescue plasmids were injected into the distal arm of the gonad of hermaphrodite *C. elegans* individuals of the *Ce-cat-4* deletion mutant LC81 (kindly provided Prof. Loer, University of San Diego; injections performed by Dr Collette Britton, University of Glasgow). Plasmids containing the rolling gene *Ce-rol-6* were injected at the same time so that individual worms that had taken up both the plasmids could be identified visually (Kramer *et al*., 1990). Stable lines for both the *C. elegans* control rescue plasmids and the *T. circumcincta* rescue plasmids (referred to from now on as Ce rescue and Tc rescue for clarity) were maintained on Nematode Growth Media (NGM) plates (Section 8.1.5.1), with individual worms showing the rolling phenotype selected by successive transfer to fresh plates (Brenner, 1974).

5.2.1.2   Assessment of functional complementation

a) Individual transformed worms were picked and lysed as described in Section 2.2.6 and then used as templates for PCR reactions to confirm the presence or absence of the *cat-4* gene. Five microlitres of each lysate were used as template for a series of PCR reactions containing 15 µl 2x Biomix (Bioline), 5 µl of each of the *Ce-cat-4F* and *Ce-cat-4R* (or *Tci-cat-4F* and *Tci-cat-4R*) gene-specific primers at 2 µM. Cycling conditions were 94 °C for 5 min, followed by 40 cycles of 30 s at 94 °C, 30 s at 57 °C and 90 s at 72 °C, followed by a final 5 min extension at 72 °C.

b) Total melanin concentrations for a sample of each strain (N2, LC81, Ce rescue and Tc rescue) of *C. elegans* were measured using methodology adapted from Haywood *et al.* (2006), Scoville and Pfrender (2010) and Herbert and Emery (1990). Synchronised cultures of each of the strains were grown on peptone-rich medium (Section 8.1.5.2) seeded with the BL22 strain of *E. coli*. When individual worms had reached adulthood, they were washed off in M9 buffer (Section 8.1.5.7), counted so that each sample contained 6,000 adult worms, and the volume reduced by aspiration to 30 µl once the worms had settled. The worms were then placed into 1 ml 5 M NaOH for 24 h at 40 °C. Fifty milligrams of *Sepia* melanin (Sigma) were also mixed with 5 M NaOH and incubated for 24 h at 40 °C. After the incubation, the samples were centrifuged at 3,000 *x g* for 3 min to pellet any non-solubilised material. The *Sepia* melanin sample was diluted 10-fold and then in a series of doubling dilutions to produce a standard series. The optical density of the samples and melanin standards was then measured using a spectrophotometer at 350 nm. A sigmoidal dose-response (variable slope) curve was generated using the absorbance values from the standards and the melanin concentrations of the samples were calculated from this using Graphpad Prism 4 software. This was repeated three times for each strain of *C. elegans*, derived from three different plates of worms. A one-way ANOVA was performed using Minitab (version 15).

c) Alongside the measurement of melanin in the worms, the sensitivity of the worms to external agents was measured using bleach. Individual adult worms were picked out using a platinum wire and placed into 10 % sodium hypochlorite solution (10 – 15 % available chlorine; Sigma) at room temperature. The time taken for each worm to stop moving in the test solution was measured using a stopwatch. This was performed with 24 individual worms from each strain, with means and standard errors calculated. A one-way ANOVA was performed using Minitab (version 15).

5.2.2 Exposure of *T. circumcincta* L₃ to natural sunlight

The effect of natural sunlight on the production of melanin in *T. circumcincta* was assessed by placing 20,000 L_3 in 100 ml water in a 150 mm Petri dish in direct sunshine in Edinburgh, UK, on each of three days in March and May 2011. A second Petri dish of L_3 was placed alongside the first, but was protected from direct sunshine by a loose covering of aluminium foil. After 5 h, the L_3 from each Petri dish were concentrated to 30 µl by centrifugation and the total melanin produced assessed using the test described previously (Section 5.2.1.2). As the variation within the groups was not equal, the results were log transformed before analysis with a 2-sample t-test using Minitab (Version 15). Measurements of the UV intensity during the exposure were taken every 5 min by the Health Protection Agency at their recording station in Glasgow, which is on a similar latitude to where the experiments took place (55.86 °N).

5.2.3 GTP-Cyclohydrolase activity assay

5.2.3.1 Nematode material

To perform the enzyme assay, PBS soluble extracts were made from a number of different samples of worms. For each sample, the worms were snap frozen in liquid nitrogen, and then ground to a powder in a mortar and pestle with further liquid nitrogen added. PBS (2 - 10 ml, depending on the sample volume) was added and the mixture continued to be ground until a liquid suspension was formed. The mixture was then centrifuged at 12,000 $x \ g$ for 15 min at 4 °C. The supernatant was retained as the S1 extract, and stored at – 80 °C until required.

T. circumcincta material was collected as described in Section 2.2.1.2, with the addition of the collection of L_5 worms, harvested at 14 days post infection. In addition to each life-cycle stage (egg, L_1, L_3, L_4, L_5, adult), 1.2 million L_3 were divided amongst six 150 mm Petri dishes in 100 ml water. Three of the Petri dishes were placed outside in Edinburgh on 17th August 2011 between 9.30 am and 12 noon (sunny conditions). The remaining three dishes were also placed outside during this time, but were protected from sunlight by a covering of aluminium foil. After this

period, the L$_3$ were concentrated by settling under gravity and the water removed by aspiration. The L$_3$ were then frozen at $-$ 80 °C and S1 extracts prepared as above.

For *C. elegans*, cultures of each of the strains under investigation were grown on peptone-rich medium (Section 8.1.5.2) seeded with the BL22 strain of *E. coli*. When the worms had exhausted the food source, the mixed life-cycle stages were washed off in M9 buffer (Section 8.1.5.7), allowed to settle on ice and the supernatant removed. The worms were then frozen and S1 extracts prepared as described above.

The protein concentrations of the samples to be tested in the enzyme assay were calculated using a bicinchoninic acid (BCA) assay (Pierce) using bovine serum albumin standards diluted with PBS.

In total, there were two biological replicates of each of the *T. circumcincta* life-cycle stages, with each biological replicate being made up of hundreds of thousands of individual eggs, L$_1$ or L$_3$, or thousands of L$_4$, L$_5$ and adults. For the sunlight- or dark-exposed *T. circumcincta* L$_3$, there were three biological replicates. For the *C. elegans* strains there was only one biological replicate as the populations are clonal. Extracts of each biological replicate were employed in the enzyme assay in triplicate to provide technical replicates.

5.2.3.2   Enzyme assay

GTP was dissolved in Buffer A [0.05 M Tris, 0.05 M KCl, 2.5 mM EDTA, 10 % glycerine (v/v), pH 7.8] to a concentration of 500 μM. Ninety microlitres of GTP / Buffer A were combined with 10 μl of the protein extracts and incubated for 1 h in darkness at 37°C. The reactions were terminated by addition of 590 μl Buffer A and 100 μl acidic iodine solution (1 % I$_2$ and 2 % KI in 1 M HCl) and kept at room temperature for 15 min. After this, 100 μl of 1 M NaOH were added before a final incubation with 5 units of alkaline phosphatase at 37 °C for 45 min. At this point the

commercial neopterin test was performed as per manufacturer's protocol (ELItest; Brahms). Briefly, this involved the samples and standards (human serum of known neopterin concentrations) being mixed with a neopterin / alkaline phosphatase conjugate and incubated on a neopterin-antibody coated microtitre plate for 2 h at room temperature in the dark. The wells were washed four times before the substrate (4-nitrophenyl phosphate) was added and incubated for 30 min at room temperature. The reactions were stopped using 2 M NaOH and the absorbance read at 405 nm. Neopterin concentrations were calculated for the samples from the standard curve generated by the human serum samples provided with the kit. The neopterin concentrations were then divided using the original protein concentration of the sample to give concentrations of neopterin of nmol per mg protein. Means and standard errors were calculated for each sample and an ANOVA performed using Microsoft Excel.

5.3 Results

5.3.1 Functional complementation

PCR analysis of the individual worm lysates confirmed that the *Ce-cat-4* gene was present only in the N2 and Ce rescue strains as shown in Figure 45. The *Tci-cat-4* gene was only present in the Tc rescue strain (Figure 46).
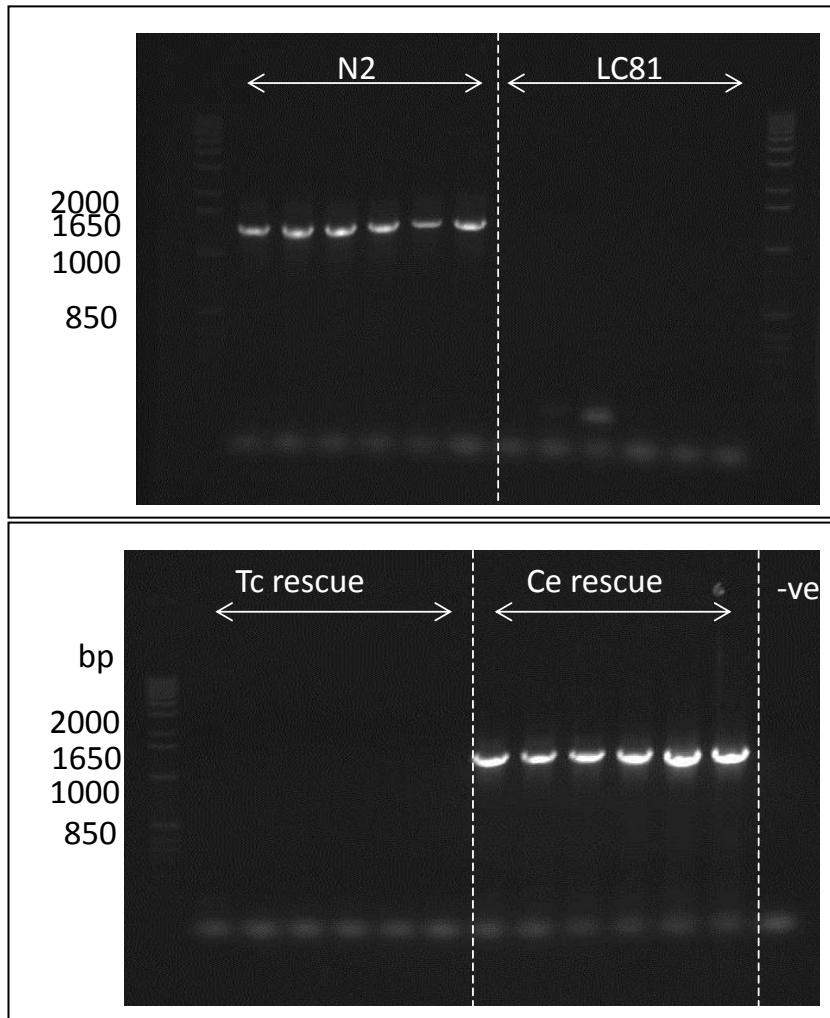

Figure 45. Image of the agarose gel of the PCR products generated from individual worm lysates from the different *C. elegans* strains using the *Ce-cat-4* primers. The *Ce-cat-4* gene was only amplified from the N2 and Ce rescue strains. The DNA ladder shown is 1kb plus (Invitrogen) and the negative (no template) control is shown in the last lane (-ve).
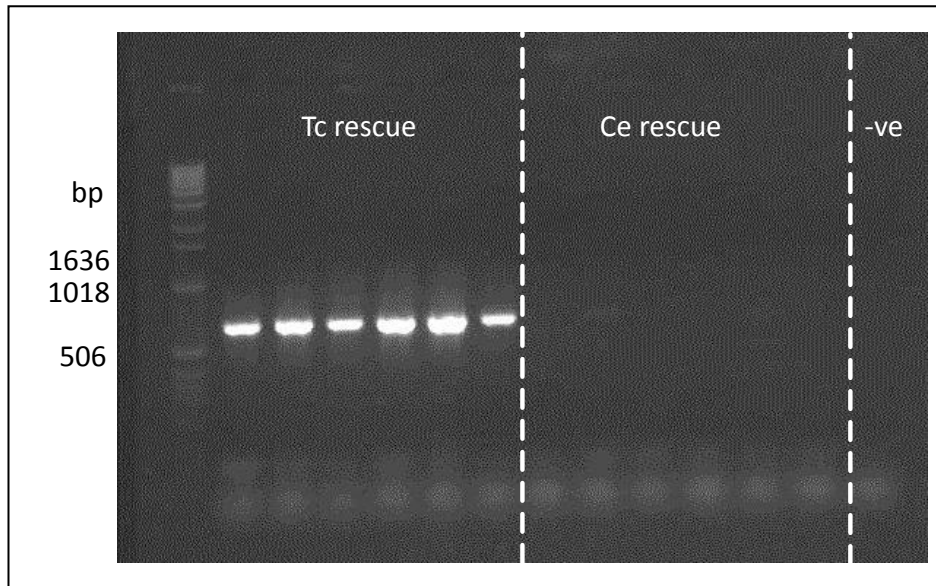
Figure 46. Image of the agarose gel of the PCR products generated from individual worm lysates from the Tc rescue and Ce rescue strains using the *Tci-cat-4* primers. PCR products are shown alongside the 1 kb DNA ladder (Invitrogen) and the negative control is shown in the last lane (-ve).

The results of the melanin assay on the different strains of *C. elegans* are shown in Figure 47. Levels of melanin were 25 % higher in the N2 strain than in the LC81 *cat-4* mutants (p = 0.037). Although melanin levels appear higher in the two rescue strains than in the *cat-4* mutants, the levels were variable and the difference was not significant (Figure 47). As these experiments were performed on mixed populations of rescued and non-rescued individuals this is not an unexpected result.
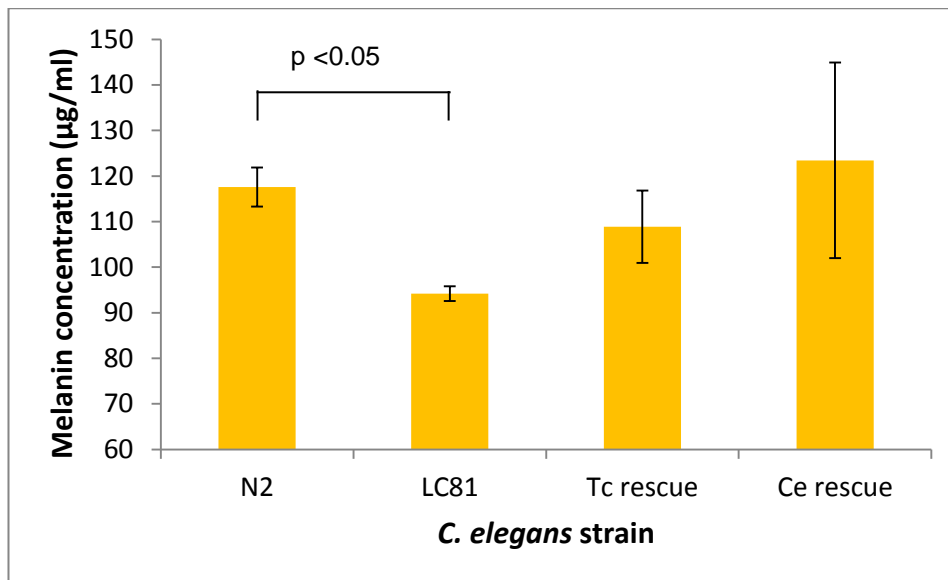
Figure 47. The levels of melanin, as measured using a spectrophotometer, within the different strains of *C. elegans*. Error bars represent the standard error of the mean of three replicates of 6000 individuals.

The average time taken for the different strains to stop moving in 10 % sodium hypochlorite solution is shown in Figure 48. The *Ce-cat-4* mutant strain LC81 became stationary quicker (14 s ± 0.69 s) than the wild-type N2 strain (58.75 s ± 2.13 s) and the two rescue strains, Ce rescue (63.04 s ± 2.2 s) and Tc rescue (58.96 s ± 1.59 s) ($p < 0.001$). There was no significant difference between the wild-type strain and either the *C. elegans* control rescue strain or the strain that had been injected with the *T. circumcincta cat-4* gene ($p = 0.249$), thus demonstrating that function had been restored in both strains.
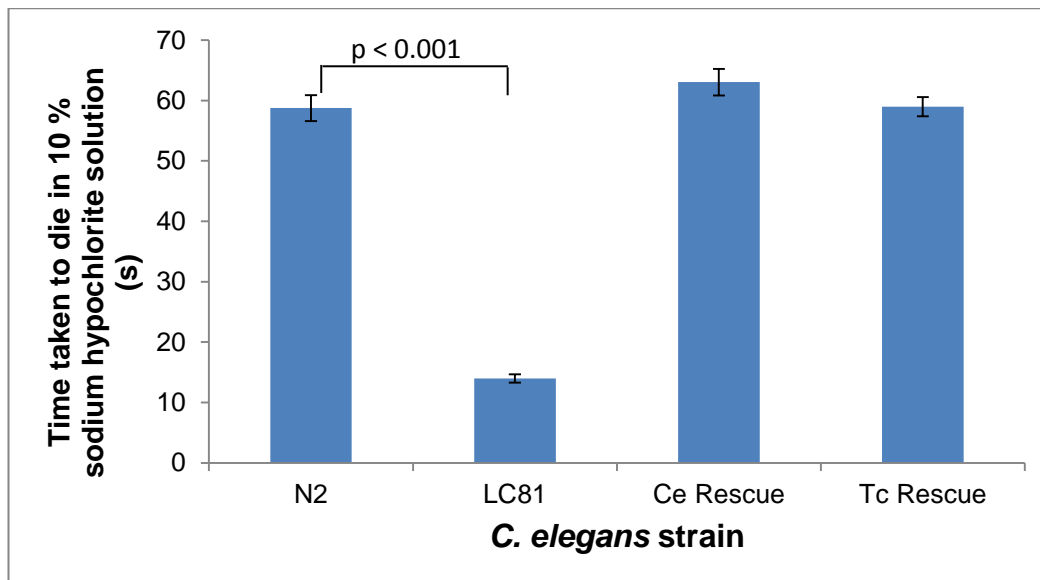
Figure 48. The time taken for the different strains of *C. elegans* to die in 10 % sodium hypochlorite solution. Error bars represent the standard error of the mean, n = 24.

5.3.2 Exposure of *T. circumcincta* L$_3$ to natural sunlight

On the three days in Spring 2011 that were chosen to perform these experiments, weather conditions were similar in Edinburgh and Glasgow (no rain at either location, with only occasional cloud cover in Glasgow), so the UV data for Glasgow should be comparable to the conditions experienced by the L$_3$ in Edinburgh. As the UV measurements show in Figure 49, on the first day of testing in March, the UV levels remained below that which is considered by the World Health Organisation to be damaging to human health (2.5, www.who.int), but for the two dates in May, UV was above this level for much of the period of testing. Reductions in UV levels were due to intermittent clouds passing over the testing site, but as there are very few days in which a cloud-free sky is experienced in both Edinburgh and Glasgow at the same time, this could not be avoided.
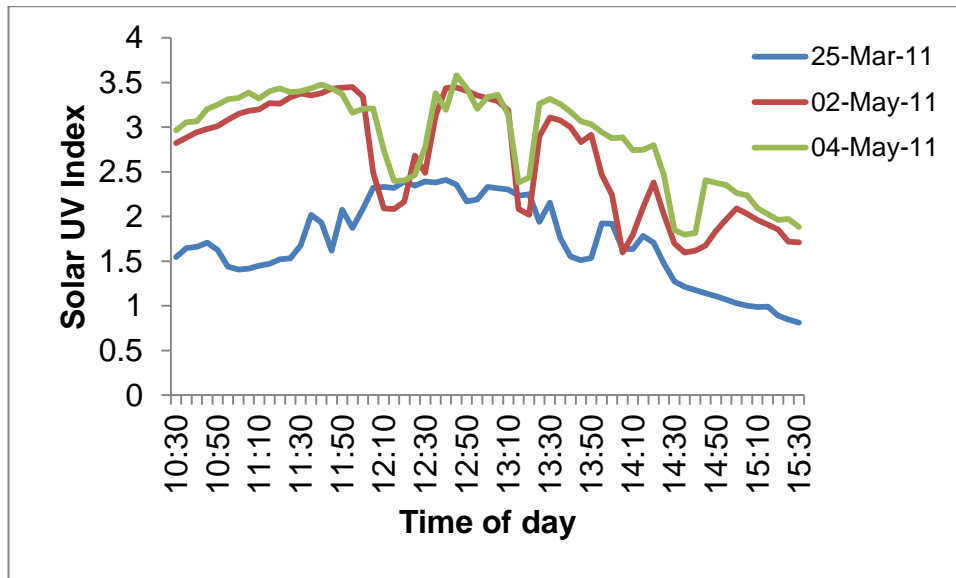
Figure 49. Solar UV index measurements taken at the Southern General Hospital, Glasgow, UK, on the three days that the melanin assay was performed. Data points represent mean scores over 5 min periods taken continuously throughout the day (data courtesy of the Health Protection Agency).

The results of the melanin assay using the *T. circumcincta* L_3 exposed to sunlight or dark conditions on the three days are shown in Figure 50. The levels of melanin detected in the larvae were significantly higher (P=0.013) in those that had been exposed to sunlight were higher (202.2 μg / ml ± 46.3) than in those larvae that had been kept in darkness (64.3 μg / ml ± 4.7). Death was observed in a proportion (5 – 10 %) of the larvae exposed to sunlight in both days in May (not observed in March or in any of the larvae kept in the dark). The higher levels of melanin in light-exposed L_3 were obvious to the naked eye, with the colour of the supernatant a brown colour in the tube containing those larvae that had been incubated with NaOH after exposure to sunlight (Figure 51). The low levels of variation in melanin levels observed within the group not exposed to sunlight suggests that the experiment is repeatable and the higher level of variation in the sunlight-exposed group is likely to be due to the effects of varying levels of UV experienced over the three days.
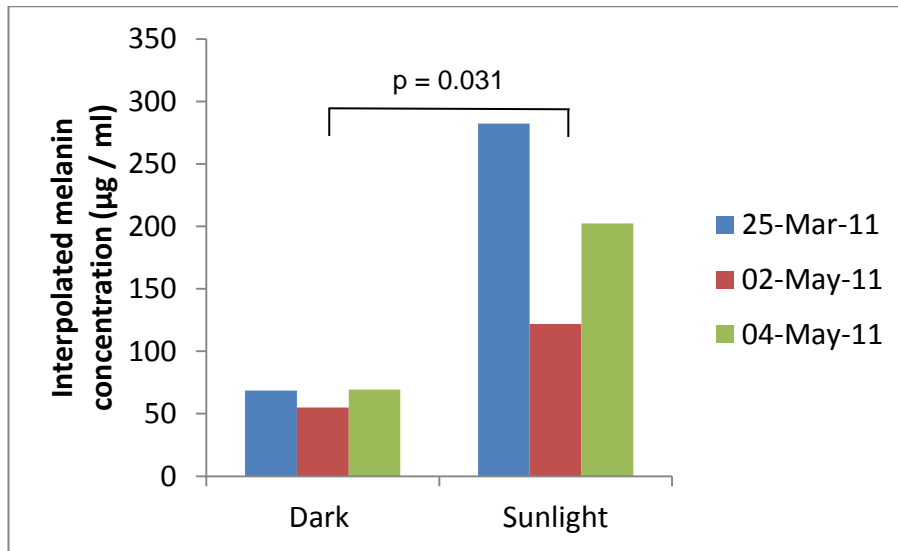
Figure 50. Melanin concentrations measured in *T. circumcincta* L_3 exposed to either sunlight or darkness on three days in 2011.
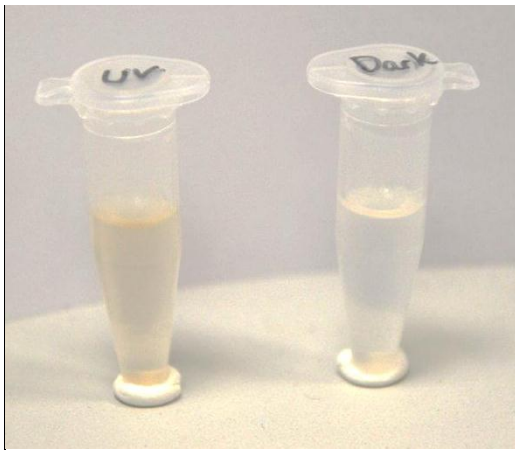
Figure 51. The soluble fraction of suspension of the L_3 that had been exposed to either UV or darkness after 24 h incubation in NaOH.

5.3.3 GTP-Cyclohydrolase activity assay

Differences in GTP-Cyclohydrolase activity (as measured by neopterin synthesis) were obtained in different life-cycle stages of *T. circumcincta*, with highest levels observed in L_1 and L_3 stages (Figure 52 and Figure 53). Levels in the L_5 and adult worms were significantly lower than the other stages in both biological replicates ($p < 0.05$), but this is not annotated on the graphs for clarity. In the first biological

replicate (Figure 52), there was a significant difference between the L3 and L4 stages (p=0.009). In the second biological replicate (Figure 53), there was a significant difference between the L_1 and L_4 stages (p = 0.03).
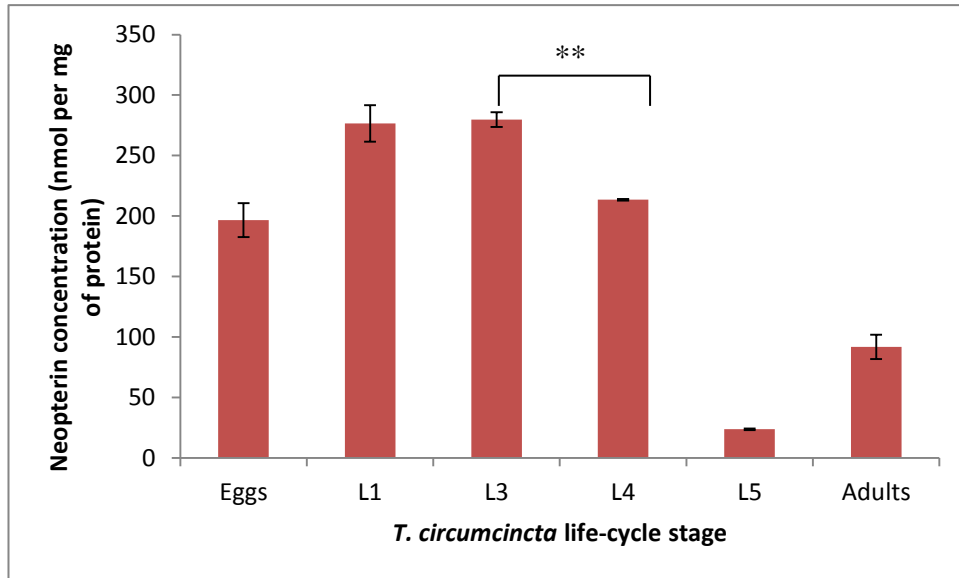
Figure 52. Neopterin concentrations per mg protein measured in the first biological replicate of the different life-cycle stages of *T. circumcincta*. Error bars represent the standard error of the means calculated from three technical replicates for each sample.
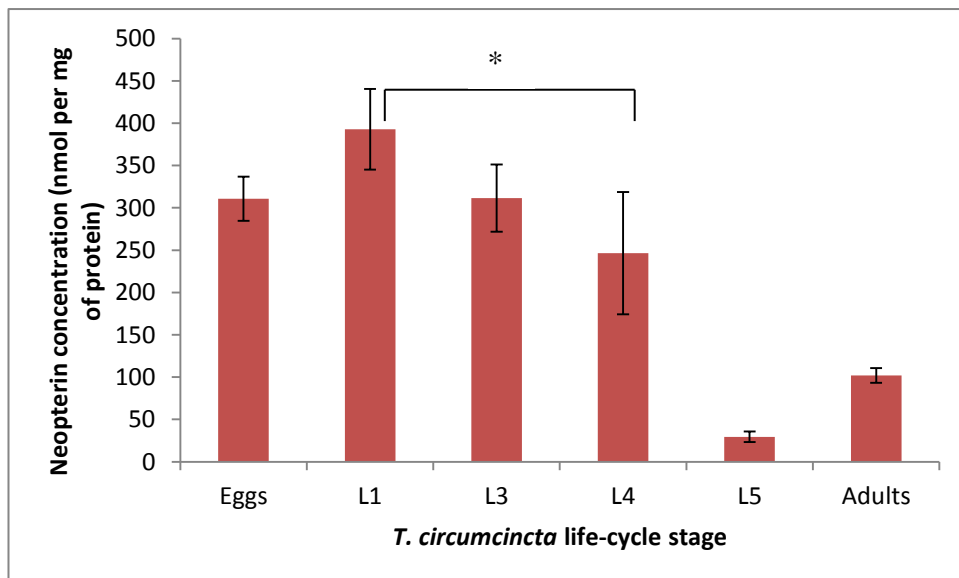
Figure 53. Neopterin concentrations per mg protein measured in the second biological replicate of the different life-cycle stages of *T. circumcincta*. Error bars represent the standard error of the means calculated from three technical replicates for each sample.

No significant differences were observed in the neopterin concentrations of any of the *C. elegans* strains (p = 0.839, Figure 54), however it is notable that the average neopterin concentrations were approximately 20 fold lower than in *T. circumcincta* L$_3$, and the sensitivity of the assay may be very low at these concentrations.
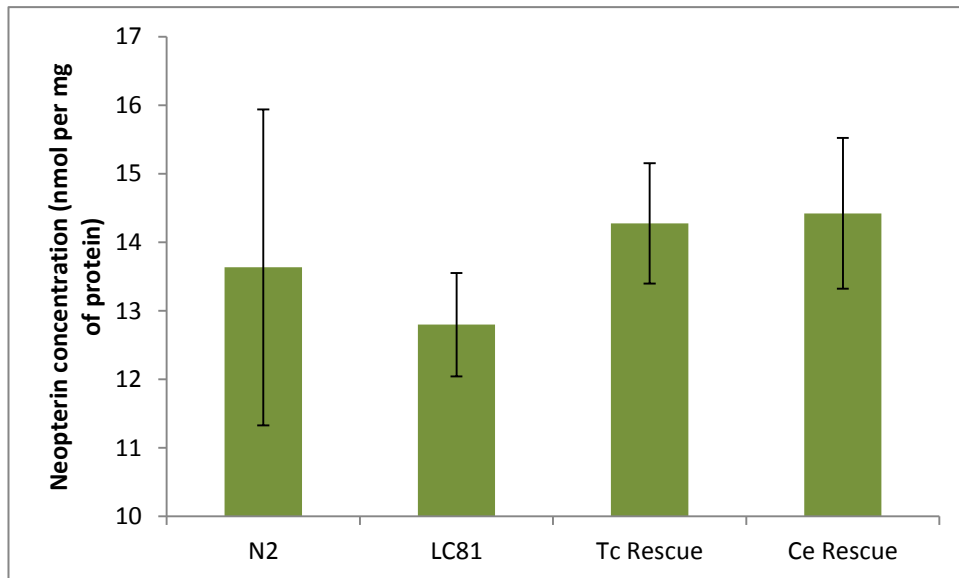
Figure 54. Neopterin concentrations concentrations per mg protein measured from the different *C. elegans* strains. The error bars represent the standard errors of the means calculated from three technical replicates of each sample.

T. circumcincta L$_3$ that were exposed to sunlight had 32 % higher GTP-Cyclohydrolase activity levels than those kept in darkness as shown in Figure 55 (p = 0.019).
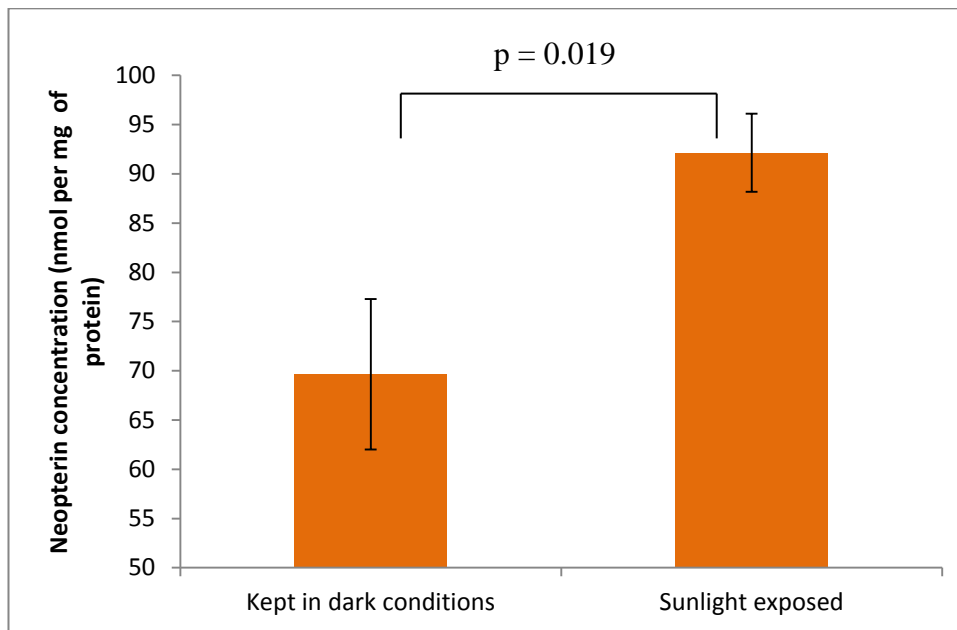
Figure 55. Mean neopterin concentrations per mg protein measured in *T. circumcincta* L$_3$ that were exposed to sunlight or kept in the dark. Error bars represent the standard errors of the means of three technical replicates of three biological replicates for each sample.

5.4 Discussion

This chapter has described the first reported functional complementation experiment using a gene from *T. circumcincta* to restore function in mutant *C. elegans*. That *Tci-cat-4* could restore the integrity of the cuticle of the mutant individuals to that comparable with the wild-type suggests that the function of the protein is conserved between the two species. Most of the previous functional complementation experiments performed using *C. elegans* and parasitic nematode genes have been to investigate the role of either anthelmintic resistance genes (Cook *et al*., 2006), or potential vaccine candidates (Britton and Murray, 2002). It is known that GTP-Cyclohydrolase is required for a number of biochemical pathways within *C. elegans* (see Chase and Koelle, 2007) and other organisms (Thöny *et al*., 2000), such that it plays a role in a number of different physiological processes (see Figure 5). In this chapter, the role of GTP-Cyclohydrolase was explored within the context of the melanisation pathway. It was observed that *T. circumcincta* L_3 that are exposed to natural sunlight had increased levels of GTP-Cyclohydrolase activity. It was also shown that L_3 exposed to sunlight produce a dark pigment that was soluble in sodium hydroxide, and is hypothesised to be melanin. It is known that *C. elegans cat-4* mutants have lower levels of melanin in their cuticles than wild-type worms (Calvo *et al*., 2008 and Figure 47 herein), and through the functional complementation experiments here it was demonstrated that the *Tci-cat-4* gene was capable of performing the same role as *Ce-cat-4* in restoring cuticular integrity. Together, these data suggest a role for GTP-Cyclohydrolase in synthesising melanin, presumably in the cuticles, of the pre-parasitic larval stages of *T. circumcincta*.


According to earlier studies, the total solar UV score required to induce significant larval death in *T. circumcincta* is 200 (van Dijk *et al*., 2009). For the three days studied in this project, the total UV scores were (in chronological order) 140.8, 246.8 and 269.74. The observation that larval death occurred in a proportion of the individuals from the second two dates appears to agree with the data from van Dijk *et al.* (2009), as these were the days on which the total UV score was above 200. One difference between the two studies was that in the studies performed in Bristol the L_3

were left outside for 24 h (van Dijk *et al.*, 2009), whereas in the current experiments, the L_3 were only exposed to sunlight for 5 h, as the aim was not to kill the parasites but to investigate melanisation. If the total UV scores for the 5 h period that the L_3 were exposed are taken into account, then the values are 104.8, 161.7 and 175.6 (chronological order). This suggests that shorter periods of high intensity UV are sufficient to induce some death in *T. circumcincta* L_3, and the value of 200 should be used as a 24 h value, rather than a total score for the period of exposure. The change in colour of the supernatant derived from the L_3 after exposure to sunlight was visible by eye, and suggests a rapid (hours rather than days) production of melanin within the L_3. As this change occurred over a short period of time (the larvae were only exposed to the sunlight for 5 h), the mechanisms for the production of melanin may already be in place. As has been shown previously in this thesis, the production of *cat-4* transcript occurs in high levels in the free-living L_1-L_3 stages of *T. circumcincta*; i.e. the stages that are exposed to sunlight. As UV can be lethal to the nematodes, prioritising resources to produce the rate-limiting factor in an enzyme pathway that could prove critical for survival may have been an adaptive survival strategy.

The effect of UV on nematodes is one that could help to explain other patterns of nematode biology. It has been shown that survival of *Ostertagia ostertagi* L_3 is significantly reduced if pasture is cut down to $5 - 7$ cm on a regular basis (Fernandez *et al.*, 2001), thus allowing deeper penetration of UV into the sward. The grazing patterns of mammalian hosts may also impact nematode survival; it has been observed that the parasitic nematodes that infect rabbits (*Graphidium strigosum, Passalurus ambiguus* and *Trichostrongylus retortaeformis*) survive for shorter periods on grass than those that infect other species (Dunsmore and Dudzinski, 1968; Evans, 1940). Rabbits graze grass very close to the ground (Petrovan *et al.*, 2011), leaving little protection for the nematodes from UV; with continued exposure, survival times may be reduced. It would be interesting to investigate whether the infective L_3 of *G. strigosum, P. ambiguus* and *T. retortaeformis* would survive for longer periods if artificially placed onto longer swards. Research into the migratory patterns of *H. contortus* on herbage in Britain over the course of $1946 - 1947$ (Rees,

1950), showed that the peak numbers of L$_3$ found on herbage occurred at different points in the day depending on the time of year. In June, when UV levels were at their strongest, the peak numbers of L$_3$ in grass were observed 8 h before and after midday (i.e. 4 am and 8 pm); when UV levels would have been much lower in intensity. In December, however, peak L$_3$ numbers were observed 2 – 4 h before and after midday when the effects of UV would have been much lower than in the summer. To avoid the high levels of UV, the L$_3$ would have to migrate down toward or into the soil.

The main process discussed in this chapter has been that of the role that melanisation plays in nematodes exposed to UV. Melanin is known to play a number of different roles within eukaryotes (Riley, 1992). One function, particularly described in insects, is that of providing structural stability of the exo-skeleton by providing cross-linking of proteins. For insect cuticles this provides the main outer structural support (Riley, 1997). In *C. elegans* (and other nematodes), the structure of the outer cuticle plays a key role in survival, providing an impervious barrier to prevent desiccation and protect the worm from harmful elements of the environment (Page and Johnstone, 2007). In nematodes, the main respiratory surface is the cuticle, and as such it is exposed to the highest levels of oxygen (Fujii *et al.*, 2011); melanin has been previously hypothesised to protect *C. elegans* against the resulting oxidative stress (Calvo *et al.*, 2008). As it has now been shown that *Tci-cat-4* is capable of restoring the integrity of the cuticle of *Ce-cat-4* mutants, it can be speculated that the cuticular role of melanin in *C. elegans* is also required in *T. circumcincta*. The pre-parasitic stages of *T. circumcincta* are exposed, as *C. elegans* individuals are, to oxygen in the air so protection against oxidative stress would be beneficial. The cuticle of L$_3$ *T. circumcincta* may be required to protect individuals for many months; as such, the cross-linking of proteins performed by melanin would provide a solid structural basis for the cuticle of the pre-parasitic stages.

Alongside investigations into melanisation, this chapter has also focussed on GTP-Cyclohydrolase enzyme activity. Attempts to correlate large-scale transcript and

proteomic data studies have found low levels of correlation between the two (Greenbaum *et al.*, 2003), despite the requirement for RNA to produce the proteins. It had been suggested that the discrepancies found in large-scale studies of the yeast *Saccharomyces cerevisiae* were due to unidentified post-transcriptional control mechanisms of the proteins that meant that mRNA levels failed to predict protein concentrations (Griffin *et al.*, 2002). However, more recent research in mice suggests that this could have been due to limitations in technology, rather than a genuine biological effect (Kislinger *et al.*, 2006). With advances in proteomic and transcriptomic methodologies, it appears that a more direct correlation between mRNA and protein levels can be observed in some cases; of 1758 mice gene / protein pairs, only 503 were observed to have no correlation, with many of these encoding genes that are expressed in the liver before protein secretion to the bloodstream and would therefore fall through the analysis techniques (Kislinger *et al.*, 2006). As levels of GTP-Cyclohydrolase had not been reported in nematodes previously, it was unclear as to whether the high levels of transcript observed in the pre-parasitic stages of *T. circumcincta* would also mean high levels of the protein. The data here suggests that this is the case, with higher GTP-Cyclohydrolase activity levels in the pre-parasitic stages compared to L_5 and adult *T. circumcincta*. The data from *C. elegans*, in which no statistical difference was observed between the wild-type N2 strain and the *Ce-cat-4* deletion mutant, LC81, suggests that there is a sensitivity issue with this assay, which given the indirect nature of the assay is not unexpected. The lack of difference between the GTP-Cyclohydrolase activity levels between the strains, despite one strain being mutant and therefore unable to produce any GTP-Cyclohydrolase, could also be reflected in the biology of *C. elegans*. The neopterin levels synthesised by all four strains were much lower than any of the measurements for *T. circumcincta*. In the proteomic analysis of the soluble fraction of *C. elegans* (Section 4.3.5), no *C. elegans* GTP-Cyclohydrolase was detected, which gives rise to the possibility that GTP-Cyclohydrolase activity is under close regulation and active enzyme is only transiently present in the adult worms. Given the potential lack of sensitivity in the enzyme assay, the trend for higher neopterin levels, and presumably GTP-Cyclohydrolase in the pre-parasitic stages, is probably an underestimate of the genuine biochemistry. To accurately quantify the levels of GTP-Cyclohydrolase in

the different life-cycle stages of *T. circumcincta*, some of the recent advances in proteomics could be harnessed to provide quantitative measurements of a large number of proteins within the different stages. Techniques such as differential in gel electrophoresis (DIGE) can be harnessed to examine the differences in protein concentration amongst multiple samples (Marouga *et al*., 2005), or the peak intensity on liquid chromatography mass spectrometry can be analysed *in silico* to provide a measure of protein concentration (Ahn *et al*., 2007). If the production of a recombinant *T. circumcincta* GTP-Cyclohydrolase had been successful then an ELISA could have been performed using antibodies raised against the recombinant; this could have facilitated quantification of GTP-Cyclohydrolase in the soluble extracts of different life-cycle stages of the parasite as well as immunolocalisation experiments.

Taken together, the results from this chapter suggest that one requirement for the high levels of *Tci-cat-4* transcript observed previously in the L_1 - L_3 stages of *T. circumcincta* is for the production of melanin to aid survival of the nematodes whilst on pasture, through protection from UV and perhaps oxidative stress.

6   Discussion

Parasitic gastroenteritis is an increasingly important problem facing small ruminant farmers in the UK, with the rise in resistance to all but the newest class of anthelmintics now widespread (Sargison *et al*., 2007). With this in mind, it is important to develop a further understanding of the biology of parasites that cause disease, so that future drugs can be designed. By increasing knowledge about parasitic nematodes it may be possible to identify points in the life-cycle at which they are at their most vulnerable, and use therapeutics. The transition from the free-living L_3 stage to the parasitic L_4 stage had been identified as a time-point in which dramatic physiological changes rapidly occur in *Teladorsagia circumcincta*, and other closely related species (Nisbet *et al*., 2008). As L_3, individuals must survive on pasture for prolonged periods of time, without the ability to take on additional resources. The environmental conditions on pasture can vary, with fluctuations in temperature, moisture and UV. When L_3 are ingested, individuals must adapt to an environment with a temperature rise to 39 °C and a rapid drop in pH to the acidic environment of the abomasum. If the key mechanisms involved in this transition could be identified then the possibility of producing an anthelmintic that targets the L_3 on ingestion exists. With this in mind, when a number of independent studies into gene expression patterns in parasitic nematodes identified that one enzyme was highly up-regulated in the L_3 stage, it was hypothesised that GTP-Cyclohydrolase could be a key target in the transition to parasitism (Abubucker *et al*., 2009; Moore *et al*., 2000; Moser *et al*., 2005; Nisbet *et al*., 2008). As such, the role of GTP-Cyclohydrolase in the life-cycle of *T. circumcincta* was explored in this thesis using a number of different techniques.

GTP-Cyclohydrolase catalyses the rate-limiting steps in the synthesis of BH_4, which is required for a number of different biochemical pathways. The two main pathways explored in this thesis were the conversion of tryptophan to serotonin and phenylalanine to melanin. It had been hypothesised that the production of serotonin was required to regulate the process of larval arrest, as *C. elegans* deletion mutants for tryptophan hydroxylase (*tph-1*) are more likely to enter the dauer pathway, even

when environmental conditions are good (Sze *et al*., 2002). The dauer pathway in *C. elegans* and the hypobiotic state in parasitic nematodes had been compared before (Hotez *et al*., 1993), but investigations into the role of serotonin and GTP-Cyclohydrolase in hypobiosis had not been undertaken. Using qPCR, analysis of transcript levels of GTP-Cyclohydrolase in two isolates of the bovine lungworm *Dictyocaulus viviparus* showed that there were no differences in *Dvi-cat-4* levels between the isolate that underwent hypobiosis and the isolate that did not. Comparable levels of *Dvi-cat-4* were observed both at the point of hypobiosis and earlier on the life-cycle. This suggested that GTP-Cyclohydrolase regulated levels of serotonin did not play a role in the hypobiosis of *D. viviparus*, which was further validated by a large-scale analysis of genes from hypobiotic and non-hypobiotic L_3s that did not identify any of the serotonin pathway genes in the data (Strube *et al*., 2007).

The peak in GTP-Cyclohydrolase gene transcript levels occurred during the transition between the L_1 and L_3 stage for both isolates of *D. viviparus*. The highest levels of *Tci-cat-4* transcript were also observed in the L_1 and L_3 stages of *T. circumcincta*, suggesting that the requirement for GTP-Cyclohydrolase is conserved between the parasitic nematodes and occurs in the pre-parasitic larval stages. The results of the enzyme assay of GTP-Cyclohydrolase activity levels appeared to confirm that the transcript levels were also representative of protein levels in the different life-cycle stages. Despite a lack of sensitivity, arising from the indirect nature of the assay, there was a trend for higher levels of GTP-Cyclohydrolase activity in the L_1 and L_3 stages of *T. circumcincta*. The effect of the chemical inhibitor DAHP *in vitro*, showed that the greatest effect was on larval development, with 83 % of individual eggs developing to L_3 in control samples with DAHP absent, but only 3 % developing in the presence of the highest DAHP concentrations. No effect was observed on rates on larval feeding, migration or egg hatching, suggesting that the role of GTP-Cyclohydrolase is in the transition between the larval stages.

If the requirement for the high levels of GTP-Cyclohydrolase in parasitic nematodes is focussed on the $L_1 - L_3$ stages of the life-cycle, then the role for the melanisation pathway is more prominent. Previous research has shown that the cuticles of *Ce-cat-4* mutants had lower levels of melanin in their cuticles than wild-type individuals (Calvo *et al.*, 2008). This was confirmed in the *cat-4* mutants used herein (Figure 47). The functional complementation experiments described in this thesis showed that the GTP-Cyclohydrolase gene from *T. circumcincta* was capable of restoring the integrity of the cuticle of the *C. elegans cat-4* mutants as measured by exposure to sodium hypochlorite. This implies that the gene is capable of performing the same role in both species and that GTP-Cyclohydrolase has a role in maintaining the structure of the nematode cuticle. The most likely basis for this role is through melanisation of the cuticle, which would provide crosslinking of proteins and protection from oxidative stress and UV (Riley, 1997). Confirmation of the presence of melanin in *T. circumcincta* came from the visual change in colour observed between the larvae that were exposed to natural sunlight and those that were kept in the dark. The levels of melanin increased, and the GTP-Cyclohydrolase activity levels were significantly higher in the sunlight exposed larvae.

Taken together, the data from this project suggests that GTP-Cyclohydrolase is required by the pre-parasitic larval stages to produce melanin within the cuticle. This may protect individual larvae from UV whilst on pasture. As the cuticle from the L_2 stage is retained as an outer sheath around the L_3 stage, it seems logical that the high levels of *cat-4* transcript observed in the L_2 stage, helps to provide a double layer of protection for the larvae. By producing high levels of melanin in the L_2 cuticle, the larvae may be able to maximise protection, and utilise the influx of nutrients during this phase. When the larvae moult to L_3, retaining the L_2 cuticle, they no longer feed. The high level of *cat-4* transcript produced during this non-feeding stage suggests that the production is critical for survival. If this theory holds true, then despite the melanin in the outer sheath, it appears further production is required to enable the larvae to survive on pasture. The harmful effects of UV on nematodes has been shown before (van Dijk *et al.*, 2009), and was shown in this project with a proportion of *T. circumcincta* L_3 being killed after exposure to 5 h of sunlight exposure. Under

field conditions, the larvae can migrate down herbage to avoid UV, and this has been shown to be the case (Rees, 1950). However, there must be a balance; if L_3 exist only in the soil and remain protected from UV, then opportunities of being ingested are reduced. The free-living nematode *C. elegans* has traditionally been described as a soil-dwelling organism, although one study failed to locate it in soil but found it resident on snails instead (Caswell-Chen *et al.*, 2005). Whether resident in snails, soil, or a laboratory, the levels of UV exposure experienced by an individual *C. elegans* are likely to be lower than that experienced by an individual *T. circumcincta* L_3 on pasture. This environmental difference is probably the reason that *C. elegans* individuals can survive as *cat-4* mutants, whereas *T. circumcincta* individuals require GTP-Cyclohydrolase to complete normal development to L_3.

The functional complementation experiments also highlighted an interesting feature with regards to the production of a recombinant GTP-Cyclohydrolase. As the functional complementation experiments showed that *C. elegans* individuals were capable of producing *T. circumcincta* GTP-Cyclohydrolase, this adds further weight to the hypothesis that the *C. elegans* worms used for recombinant Tci-CAT-4 production were producing the poly-histidine tagged GTP-Cyclohydrolase, but that it was either being used by the individuals or proving to have a toxic effect and, as such, was regulated by a feedback mechanism. Now that it is known that *C. elegans* is capable of producing *T. circumcincta* GTP-Cyclohydrolase, further adjustments to the protocol used for the production of a recombinant protein could be undertaken to attempt to produce a soluble recombinant protein. As the functional complementation experiments were performed using the *Ce-cat-4* promoter sequences, these could be used instead of the *Ce-cpl-1* promoter to assess whether production of the protein in the tissues that it is normally expressed in would improve the success rate. The recombinant protein could also be produced in a *Ce-cat-4* mutant strain, so as to reduce the overall levels of GTP-Cyclohydrolase within the tissues. If having too much GTP-Cyclohydrolase causes a physiological imbalance then, by removing the *C. elegans* protein, only the recombinant *T. circumcincta* protein will be present, which may reduce the negative impact of the protein.

A wide variety in the pigmentation colours are seen in different vertebrate groups, with some teleost fish possessing five different pigment cell types, as opposed to the one type of melanocyte cells found in mammals and birds (Bagnara, 1998). Alongside the variety of pigment cells, there is also evidence that genome and gene-specific duplication events have produced an increased number of genes for the production of the conserved melanocyte cells, including those encoding GTP-Cyclohydrolase. Some species of fish (pufferfish, stickleback and seabream) have now been identified as possessing three distinct groups of GTP-Cyclohydrolase genes (Braasch *et al.*, 2007). Some of this duplication is likely to have occurred during the Fish Specific Genome Duplication event, but as one of the 'extra' group of genes is only found in a small number of species, it appears likely that individual gene duplication events involving GTP-Cyclohydrolase have occurred, adding to the variety of pigments produced by those species. In *C. elegans*, there is only one copy of the *cat-4* gene encoding GTP-Cyclohydrolase. If, as the data suggests in chapter 2, *T. circumcincta* may possess more than one copy of the *cat-4* gene within the genome then this would enable a greater quantity of melanin to be produced. The comparisons with the fish demonstrate that with an evolutionary advantage, gene duplication of the *cat-4* could become fixed in the genome of certain species.

It has been shown previously in *C. elegans* that *cat-4* gene expression is affected by different *cis*-regulatory modules in the serotonergic and dopaminergic neurons (Flames and Hobert, 2009). Small regulatory sequences were found to exist for five genes in the dopamine pathway (*cat-4*, *cat-2*, *bas-1*, *cat-1* and *dat-1*) with mutations in regulatory sequences for all of the genes affecting either serotoninergic neurons or dopaminergic neurons. For *cat-4*, eight different promoters were studied, with mutations causing different expression patterns in the different dopaminergic and serotonergic neurons. The regulatory sequences were found to be highly conserved in other *Caenorhabditis* species, and in mammalian species such as human and mouse (Flames and Hobert, 2009). The genome sequences are not available to study whether *T. circumcincta* also possess similar regulatory sequences for *Tci-cat-4* (they

appear $1 - 5$ kb from the start codon for the gene) but given the apparently highly conserved nature of the sequences in different species of animals, it would seem likely that they do. If this is the case, then the control mechanisms for the expression of *Tci-cat-4* may be different for the serotonin and the dopamine pathways. If as this research has suggested, the high levels of *Tci-cat-4* transcript observed in the stages living on pasture are required to produce melanin, through the dopamine pathway, then the ability of the worms to regulate *Tci-cat-4* expression to only those tissues that require melanin would be beneficial and would prevent an over-production of serotonin unnecessarily. As GTP-Cyclohydrolase is required for more than one biochemical pathway, it is likely to be performing multiple different functions within the nematodes at different stages in the life-cycle as the requirement for serotonin and melanin changes. This thesis does not argue that there is only one function for GTP-Cyclohydrolase, but instead that the large requirement for the enzyme in the pre-parasitic larval stages is primarily for the production of melanin.

There has been a large focus of research recently on climate change, and its impact on all aspects of life including agriculture. The changes occurring are likely to impact on parasitic nematodes, with resulting changes in disease patterns. Kenyon *et al.* (2009) described how recent outbreaks of parasitic gastroenteritis could be linked to changes in local climatic conditions. These included outbreaks of teladorsagiosis in $7 - 9$ week old lambs which were believed to have been caused by the over-winter survival of L_3 on pasture (Kenyon *et al.* 2009). According to the data collected from Scotland over the period $1961 - 2004$, there was a 3.3 % increase in the number of annual sunshine hours over that period, with the highest rise seen in the autumn [13.8 % (Barnett *et al.*, 2006)]. As the data from this thesis and previous research (van Dijk *et al.*, 2009) suggests that the levels of UV and sunshine are critical for the survival of *T. circumcincta*, then there is the possibility that an increase in sunshine hours could affect the annual distribution of the parasite. Between 1961 and 2004, there was a reduction in the total number of nights with ground frosts by 27.8 and the growing season was extended by more than 4 weeks (Barnett *et al.*, 2006). Taken together, these data suggest that the chances of survival of parasitic nematodes in winter on pasture are greater, which could result in patterns of disease earlier in the

season, before UV levels rise to that which proves detrimental to the larvae (van Dijk *et al*., 2009). It is important to minimise the effects of parasitic gastroenteritis on very young lambs, as it has been shown that those lambs that have a check in growth due to parasitic nematodes early on in life, never recover from that and perform less well than their peers throughout (Coop *et al*., 1977). One of the original aims of this thesis was to examine the role of GTP-Cyclohydrolase and assess its potential as a drug target. The enzyme has shown to be an unlikely candidate for further investigation for potential therapeutics, as its function appears not to lie in the transition to parasitism, but in the survival of the larvae on pasture.

There are many aspects of this research that it would be beneficial to continue investigating in the future. Now that it has been demonstrated that *T. circumcincta* are susceptible to RNAi, albeit only to dsRNA of specific genes, it would be advantageous to investigate whether the genes that are susceptible to silencing are those expressed within the gut, and if so, whether other mechanisms of exposing the individuals to dsRNA enables other genes to be silenced. The observation that non-nematode derived dsRNA caused a deleterious effect on the larvae in a similar fashion to that observed previously in plant parasitic nematodes (Dalzell *et al*., 2009) provides a particularly intriguing avenue of research. Using short interfering RNAs derived from non-nematode sources could be a mechanism of identifying whether the problem is caused pre- or post-dicer. RNAi experiments have historically been particularly successful in *C. elegans*, so it would be interesting to perform the same non-nematode derived dsRNA experiments in *C. elegans* to assess whether the observation is specific to the parasitic nematodes, or phylum-wide. If it is a parasitic-nematode phenomenon then the possibility exists for examining further the differences between the free-living and parasitic species.

The use of *C. elegans* for recombinant protein production is one that should have an increased focus in the future. In this thesis, although the production of a purified soluble recombinant GTP-Cyclohydrolase was not achieved, the functional complementation results showed that *C. elegans* had the capacity to produce *T.*

circumcincta GTP-Cyclohydrolase in a form that was correctly folded so as to be enzymically active. It should be possible to achieve the same result with a poly-histidine tag so that the protein could be purified using nickel-affinity chromatography. This would enable the immunohistochemistry studies and ELISA experiments to be performed that would enable further understanding about the function of this enzyme to be determined.

Finally, the area of research that may prove to be the most technically challenging would be to study the effect of sunshine and UV levels on *T. circumcincta* larvae on pasture. This would require pasture that is free of infective L_3 prior to study, and then split into a number of different study areas. Using variables of pasture height, shade and time, it would be interesting to observe the survival times of infective L_3 on pasture. If the hypotheses derived from this thesis are accurate, then the larvae should survive for the shortest period of time on grass with the shortest sward length exposed to full sunlight, while those larvae in the shade on longer grass should survive the longest.

In summary, this thesis has demonstrated that the pre-parasitic stages of *T. circumcincta* produce higher levels of GTP-Cyclohydrolase transcript and enzyme than the parasitic stages and that the L_3 have higher levels of melanin after exposure to sunlight. This suggests that GTP-Cyclohydrolase is required for the survival of the parasitic larvae whilst on pasture.

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8   Appendix

8.1   General laboratory procedures and solutions

8.1.1   PCR and cloning reagents

8.1.1.1   Tris-acetate-EDTA (TAE) buffer

A concentrated 50 x solution of TAE was made by combining the following:

242 g Tris base

57.1 ml Glacial acetic acid

100 ml 0.5M EDTA (pH 8)

Water to 1 litre.

8.1.1.2   DNA Loading buffer

A 6 x gel loading buffer was made by combining the following:

0.25 % Bromophenol blue

0.25 % Xylene cyanol FF

40 % (w/v) Sucrose in water

This was stored at 4°C.

8.1.1.3   1 % agarose gel

4 g agar, 8 ml 50 x TAE and 40 μL 10,000 x Gel Red were combined and the volume made up to 400 ml with dH_2O. This was heated on full power in a microwave for approximately 5 min and then cooled to 45 °C before use. The gel was then poured into a gel electrophoresis tank with a comb pre-positioned to give the correct number and size of wells. Once the gel had set, the comb was removed and 1 x TAE poured over to submerge the gel. The samples were loaded alongside a Ready-Load™ 1 kb DNA Ladder (Invitrogen) and the tank attached to a power supply. 100 V were applied for approximately 45 mins and the gel visualised using UV.

8.1.1.4   SOC media

In 100 ml of distilled water:

2 g Bacto®-tryptone

0.5 g Bacto®-yeast extract

0.05 g NaCl

1 ml 250 mM KCl

This was autoclaved for 15 mins. Once the liquid had cooled to below 60 °C, the final two ingredients were added:

0.5 ml filter-sterilised $MgCl_2$ (1.9 g $MgCl_2$ in 10 ml water)

2 ml filter-sterilised glucose (1.8 g glucose in 10 ml water)

Aliquots were taken and stored at -20 °C.

8.1.1.5   LB (Luria-Bertani Medium)

10 g Bacto®-tryptone

5 g Bacto®-yeast extract

10 g NaCl (5g for low-salt LB)

The volume was made up to 1 litre with distilled water and the pH was adjusted to 7.5 with NaOH. This was then autoclaved to sterilise. For LB agar plates, 15 g agar were added prior to autoclaving.

8.1.2 Protein Gel Electrophoresis

Thirteen microlitre aliquots of each sample to be tested were taken and added to 5 µl 4 x sample buffer (Invitrogen) and 2 µl sample reducing agent (Invitrogen) before being heated to 70 °C for 10 mins. NuPAGE® Novex 4-12 % Bis-Tris gels were rinsed with distilled water before use and inserted into a NuPAGE tank (Invitrogen). Running buffer was prepared by mixing 50 ml 20 x NuPAGE® running buffer (Invitrogen) with 950 ml distilled water. Of this diluted running buffer, 200 ml was removed and added to 500 µl NuPAGE® Antioxidant (Invitrogen). The buffer containing the antioxidant was poured into the central chamber containing the gel, the buffer without antioxidant was poured into the outer chamber. The samples were loaded alongside 8 µl SeeBlue® Plus2 Pre-Stained standard (Invitrogen) and 200 V applied for the appropriate time for the proteins to resolve (35 mins). On

completion, the gel was removed from the plastic casing and placed in a container with distilled water on an orbital shaker at room temperature. The water was changed twice after 5 mins, then 20 ml SimplyBlue™ SafeStain (Invitrogen) were added and left to rock for 2 h. The stain was poured off and the gel left to de-stain in water overnight.

8.1.3 Western Blot

A nitrocellulose membrane filter paper sandwich (Invitrogen) and 6 pad sponges (Invitrogen) were saturated with western transfer buffer (see below for recipe). The sponges were compressed to ensure no air bubbles were trapped that would impede the blotting of the proteins. A protein gel was run as described above but not stained; instead once the proteins had resolved, the gel was removed and placed onto the wet filter paper. The nitrocellulose membrane was lined up with the gel to ensure accurate transfer and another filter paper placed on top. This was placed on top of two sponges in the transfer pod with another four sponges placed on top. The pod was secured tightly in the tank with transfer buffer filling the centre and water providing coolant for the rest of the tank. Current at 25 V was applied for 1 h to ensure accurate transfer of the proteins from the gel to the membrane. TNTT was used to block the membrane overnight at room temperature (once the ladder had been separated off into distilled water). The primary antibody, diluted to 1/1000 in TNTT, was poured over the membrane and left to rock gently for 1 h. The antibody was washed off and three 10 min washes with TNTT were performed. The process was repeated with the secondary antibody and three TNTT washes. The bands were visualised using the SigmaFast 3,3′-diaminobenzidine tablet set (Sigma) and the reaction stopped with distilled water.

8.1.3.1 Western Transfer Buffer   Stored Room Temperature

850 ml Distilled water

100 ml Methanol

50 ml NuPAGE transfer buffer (20 x; Invitrogen)

8.1.3.2   TNTT

50 ml 1 M Tris

146.1 g NaCl

2.5 ml Tween 20

0.5 g Thimerasol

Made up to 5 litres with distilled water and the pH adjusted to 7.4 using HCl.

8.1.4   Solutions for protein purification

8.1.4.1   Lysis Buffer

0.3 ml 1 M KH_2PO_4

4.7 ml 1 M K_2HPO_4

2.3 g NaCl

0.75 g KCl

10 ml glycerol

0.5 ml Triton X-100

68 mg imidazole

The above were mixed in 90 ml of distilled water and the pH adjusted to 7.8 with HCl. The volume was adjusted to 100 ml with distilled water and then stored at 4°C.

8.1.4.2   0.1M Sodium Phosphate Buffer (pH 7.4)

3.1 g $NaH_2PO_4 \cdot H_2O$

10.9 g NaH_2PO_4

Made up to 1 l with distilled water

8.1.4.3   Binding Buffer 1

20 ml 0.1 M Sodium Phosphate Buffer

80 ml Distilled water

2.92 g NaCl

0.136 g Imidazole

8.1.4.4   Binding Buffer 2

100 ml Binding Buffer 1

48 g Urea

8.1.4.5   Elution Buffer Stock (without urea)

80 ml 0.1M Sodium Phosphate Buffer

320 ml Distilled water

11.68 g NaCl

8.1.4.6   Elution Buffer 1a

100 ml Elution Buffer Stock

0.34 g Imidazole

8.1.4.7   Elution Buffer 2a

100 ml Elution Buffer Stock

1.36 g Imidazole

8.1.4.8   Elution Buffer 3a

100 ml Elution Buffer Stock

2.38 g Imidazole

8.1.4.9   Elution Buffer 4a

100 ml Elution Buffer Stock

3.4 g Imidazole

8.1.4.10 Elution Buffer Stock (with 8 M urea)

80 ml 0.1M Sodium Phosphate Buffer

320 ml Distilled water

11.68 g NaCl

192 g Urea

8.1.4.11 Elution Buffer 1b

100 ml Elution Buffer Stock

0.34 g Imidazole

8.1.4.12 Elution Buffer 2b

100 ml Elution Buffer Stock

1.36 g Imidazole

8.1.4.13 Elution Buffer 3b

100 ml Elution Buffer Stock

2.38 g Imidazole

8.1.4.14 Elution Buffer 4b

100 ml Elution Buffer Stock

3.4 g Imidazole

8.1.5 Solutions for use with *C. elegans*

8.1.5.1   Nematode Growth Medium (NGM)

3 g NaCl

17 g Agarose

2.5 g Peptone

975 ml Distilled water

This was autoclaved to sterilise and then the remainder of the ingredients added:

1 ml 5 mg / ml cholesterol in 100 % ethanol

1 ml 1 M $CaCl_2$

1 ml 1 M $MgSO_4$

25 ml 1 M KPO_4 buffer (108.3 g KH_2PO_4, 35.6 g K_2HPO_4, Water to 1 litre)

NGM plates were stored at room temperature for 3 days to dry before *E. coli* OP50 cells were spread on the surface.

8.1.5.2   Peptone rich medium

1.2 g NaCl

20 g Peptone

25 g Agarose

Water to 1 litre and autoclaved before the final ingredients added:

1 ml 5 mg / ml cholesterol in 100 % ethanol

1 ml 1 M $CaCl_2$

1 ml 1 M $MgSO_4$

25 ml 1 M KPO_4 buffer

8.1.5.3   S Basal

5.85 g NaCl

1 g K_2HPO_4

6 g KH_2PO_4

1 ml Cholesterol (5 mg/ ml in ethanol)

Water to 1 litre

Sterilised by autoclaving.

8.1.5.4   Trace metals solution

1.86 g EDTA

0.69 g $FeSO_4 \cdot 7H_2O$

0.2 g $MnCl_2 \cdot 4H_2O$

0.29 g $ZnSO_4 \cdot 7H_2O$

0.025 g $CuSO_4 \cdot 5H_2O$

Water to 1 litre

Sterilised by autoclaving and stored in the dark.

8.1.5.5   1 M Potassium Citrate pH 6

20 g citric acid monohydrate

293.5 g tri-potassium citrate monohydrate

Water to 1 litre

Sterilised by autoclaving

8.1.5.6 S Medium

1 l S Basal

10 ml 1 M Potassium citrate pH 6

10 ml trace metals solution

3 ml 1 M $CaCl_2$

3 ml 1 M $MgSO_4$

8.1.5.7 M9 Buffer

3 g KH_2PO_4

6 g Na_2HPO_4

5 g NaCl

1 ml 1 M $MgSO_4$

Water to 1 litre

Sterilised by autoclaving