BIOLOGY OF DEVELOPMENTAL ACTIVATION OF INFECTIVE *TRICHINELLA SPIRALIS*

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ABSTRACT

The initiation of further development is fundamental to the infectious processes of parasitic nematodes. I have examined early developmental activation of *Trichinella spiralis* larvae during host invasion, with particular emphasis on gene regulation and the timing of events. Using a novel approach, changes in tissue specific transcriptional activity were observed in live larvae during the infectious process with the fluorescent nucleic acid dyes SYTO12 and acridine orange. Simultaneously, the metabolic switch from anaerobic metabolism, characteristic of the infective stage, to aerobic metabolism, as found in the enteral stages, was tracked by measuring activities of the key regulatory enzymes phosphoenolpyruvate carboxykinase and pyruvate kinase, as well as isocitrate dehydrogenase (NADP) activity, and used as a co-indicator for developmental activation. Both metabolic enzyme activities and transcription patterns were found to change in response to host death, liberation from the nurse cell, and exposure to components of the host stomach environment. The role of amphidial neurones in developmental regulation was examined using FITC based labelling, and at least part of the initial developmental processes were discovered to be under amphidial control. Changes to the surface properties of larvae during the process of infection were monitored using the fluorescent lipid probe PKH26, and gave evidence for temporal regulation of activation-related alterations of larval structure and/ or physiology. Further, RNA-fingerprinting was carried out to identify specific genes associated with, or regulating, developmental activation. Some putatively differentially expressed transcripts were identified, but could not be completely characterised to date.

The results give a clear indication that the activation processes of *T. spiralis* infective larvae occur at a much earlier time than previously thought, and are stimulated upon liberation of the larvae from the nurse cell inside the host stomach. Further, regulation of development appears to be under transcriptional control, and tissue specific transcription is initiated early during the infectious process, perhaps immediately after release from the nurse cell.

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There are more things in heaven and earth,
Horatio,
than are dreamt
in your philosophy.

William Shakespeare
Hamlet, Act I, Scene V

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ABBREVIATIONS

AF18	5-*N*-(octadecanoyl) aminofluorescein
bp	nucleotide base pairs
CA	catecholamine
cAMP	cyclic AMP
DEPC	diethylpyrocarbonate
DiI	1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanin perchlorate
DiO	3,3'-dioctadecyloxacarbocyanine perchlorate
DTNB	5,5'-dithio-bis(2-nitrobenzoic acid)
E64	trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane
ESA	excretory/ secretory antigens
FDA	fluorescein diacetate
FITC	fluorescein isothiocyanate
g	centrifugal force expressed as multiplication of gravity
HBSS	Hank's balanced salt solution
ICDH	isocitrate dehydrogenase
IP_3	inositol trisphosphate
L1 – L4	the four larval stages of nematodes
NA	noradrenaline
nt	nucleotide
PCR	polymerase chain reaction
PBS	sterile phosphate buffered saline
PEPCK	phosphoenolpyruvate carboxykinase
p.i.	post infection
PITC	phenyl isothiocyanate
PK	pyruvate kinase
PMSF	phenylmethylsulfonyl fluoride
RAcD	randomly amplified cDNA
SDS-PAGE	denaturing polyacrylamide gel electrophoresis
SEM	scanning electron microscopy
UTR	untranslated region

1. INTRODUCTION

Since its discovery in 1835 by James Paget, *Trichinella spiralis* has changed in status from a pathologists' curio to a major public health challenge in central Europe at the turn of the last century, and to a substantial economic burden for the European Union (EU) at the present. Before the crucial observation of disseminating *T. spiralis* larvae in the flesh of a deceased patient diagnosed with typhoid fever, by Friedrich von Zenker in 1860, it was probably common to misdiagnose trichinellosis as typhus abdominalis. During the decades following the disease's clinical recognition, the great outbreaks of trichinellosis in Germany, some of which resulted in mortality rates of up to 30%, afforded urgency to the call for measures to control this parasitic infection (Campbell, 1983c). The ensuing trichinoscopy programme introduced in Europe greatly reduced the incidence of human trichinellosis in this geographical area, and is still successfully controlling the disease today. This control measure of the domestic cycle of *T. spiralis*, however, costs the high burden of an estimated US$ 570 million per year in the EU (Pozio, 1998).

Occurrence of *Trichinella* infection in both domestic and sylvatic cycles has been shown to be worldwide in the past three decades, spanning every continent (Kim, 1983). From 1995 to June 1997 alone, 10038 cases of human infections from 22 countries were reported to the International Commission on Trichinellosis (*ICT Country Status Report*, 1998, http://www.krenet.it/ict/). The majority of cases were reported from Eastern Europe (7220), followed by Latin America with 1725 reported cases.

Classified in the order Trichurata, (phylum Nematoda, class Adenophorea), *Trichinella spiralis* (Owen, 1835) *sensu stricto*, actually is one of 5 sibling species which cannot be differentiated by morphological criteria, but have been shown to comprise 8 gene pools. All members of the proposed species complex share the same life cycle and ecological niche within their hosts, and differ mainly in geographical distribution. Some biochemical differences and variation in pathogenicity, however, have been identified among the proposed species (Pozio *et al*, 1992).

A cosmopolitan parasite of vertebrates, *T. spiralis* seems to have a strikingly low host specificity, infecting a wide spectrum of mammalian species (Campbell, 1983b). The single most important factor contributing to the "ecological success" and geographical range of *Trichinella* is meat consumption (Despommier, 1993).

A peculiarity of the life cycle of *T. spiralis* is that all developmental stages of the parasite can be found within one individual host; the same animal functions as definitive and transmission host. Infective juveniles enter the host via the oral route with contaminated meat. Upon reaching the small intestine, the larvae enter the mucosal epithelium intracellularly and rapidly moult four times to adulthood (males attain a length of ≈ 1.5 mm, females reach twice this size); a process which requires ≈ 28 h (Kozek, 1971a & 1971b). Copulation takes place about 30 h post infection (p.i.). Born juveniles are carried away by the hepatoportal system through the liver, the heart, the lungs, and then the somatic circulation. Upon reaching skeletal muscle, the juveniles penetrate individual muscle fibres, grow, and become quiescent. Although infected muscle cells eventually become encapsulated by the host immune reaction, the parasites seem to actively maintain their host cells, transforming them into 'nurse cells', through as yet uncharacterised interactions. When thus infected muscle tissue is ingested by the next host, the infective larvae are liberated from the surrounding tissue in the stomach, and initiate the next cycle upon reaching the small intestine (Despommier, 1993).

BIOLOGY OF INFECTION

Following the release from the surrounding muscle tissue by digestion with pepsin-HCl in the stomach, the infective larvae pass into the duodenal lumen where they receive the environmental cues directing them into the cytoplasm of the enterocytes, leading to the establishment of infection. *T. spiralis* locates at the level of the platform of the crypts and above, and actively enters new cells as the enterocytes migrate up to and around the parasites (Despommier, 1993). The juvenile worms live within the naked cytoplasm of a row of columnar epithelial cells (Stewart *et al,* 1987;

Despommier, 1993), and develop to adulthood in this niche (Kozek, 1971a).

In order to successfully establish an infection, the hitherto quiescent infective larvae must rapidly change their behaviour pattern, adapt to the new environment, and resume development. How the infective larvae regulate their development, what signalling methods they use, and to what environmental stimuli they respond, are not fully understood. The present study, therefore, set out to explore the signalling pathways and levels of gene regulation involved in controlling the resumption of development observed in *T. spiralis* larvae during the infectious process.

Initiation of development: Activation signals

Transmission stages of intestinal parasites entering via the oral route depend on adequate and timely activation to prevent passage past the suitable microhabitat, and out of the host. These infective stages must therefore be receptive to specific environmental cues which relay information guiding the parasites to their correct microhabitats, and which stimulate resumption of development. The variety of stimuli to which an intestinal nematode can respond are still being elucidated, although several investigations with intestinal nematodes and platyhelminths have been carried out (Smart, 1989; Proudfoot *et al*, 1993a & 1993b). Studies concerning the critical activational cues encountered by infective intestinal nematode stages have shown that temperature, undissociated carbonic acid or the dissolved gaseous CO_2 content, pH, reducing agents, and bicarbonate ions are generally important for activation *in vitro* (Rogers, 1960; Proudfoot *et al* 1993b). The very complex environment within the gut, where the levels of metabolites, gases, pH and intestinal secretions alter both radially and longitudinally within the lumen, and also fluctuate with the host's daily feeding cycle, will require a dynamic response in site finding behaviour from the parasites (Smart, 1989). There are, therefore, a number of potential cues to which an intestinal parasite could respond to modulate its behaviour to its best advantage.

An investigation of stimuli that induce physiological and biochemical changes in *T. spiralis* larvae during the establishment of infection in the intestine has revealed that trypsin and bile seem to be important cues (Stewart *et al,* 1987). One of the more obvious changes effected by exposure to trypsin and bile *in vitro*, shown by that study, is the temperature dependent behavioural transition from coiling to migrating movements. Infective larvae which have not responded to 'activational' cues remain in a characteristic coiled position, uncoiling periodically to a limited extent, but invariably returning to a fully coiled state; whereas activated larvae are fully uncoiled, motile and probing their environment. These behavioural changes are also observed during the developmental transition which occurs *in vivo,* when larvae invade their new intestinal niche. According to the reported observations of Stewart *et al*, (1987), allowing pepsin-HCl isolated worms to remain in pepsin-HCl solution will not result in activation-related changes. Placing them in nutrient solution, however, will result in a 50% increase in activation-related behaviour (at 37° C only). *T. spiralis* larvae which have been incubated in a nutrient solution containing 5% bile and 0.25% trypsin will invariably start to respond with activation-related behaviour. Juvenile worms isolated from the intestines of rats at 1 h post infection behaved as unactivated infective larvae described above, while juveniles isolated from rat intestines 2 h post infection exhibited the same behaviour as worms treated with trypsin and bile in nutrient solution *in vitro*.

Treatment of pepsin-HCl isolated larvae with either trypsin or bile results in a significant alteration in the outer surface of the worm, most notably the loss of the surface accessory layer (Stewart *et al*, 1987). Modha *et al* (1994) confirmed that activation of *T. spiralis* larvae with medium containing trypsin and bile coincides with the loss of the accessory layer as seen by electron microscopy. Further, the authors report diffused organisation within the cuticle, which in unactivated larvae was highly ordered in distinct strata, and an accumulation of rough endoplasmic reticulum and enlarged mitochondria in the hypodermis. These changes have been interpreted as potential preparations for a moult. Correlating with these observations, *in vitro* sugar uptake of

juveniles 2 h after enteral infection has been demonstrated to be similar to levels observed in trypsin and bile treated muscle larvae, which take up sugar at a much higher level than untreated infective larvae (Stewart *et al*, 1987).

A rapid change in the surface lipid of a variety of nematodes, including *T.spiralis,* has been described during the transition to the next (definitive) host (Proudfoot *et al,* 1993a & 1993b). This involves an alteration in the lipophilicity of the cuticle, as seen by the insertion of the fluorescent lipid analogue 5-*N*-(octadecanoyl)aminofluoresceine (AF18) only after exposure to mammalian tissue culture conditions. In *T. spiralis*, the increase in surface lipophilicity coincides with loss of the surface accessory layer, and perhaps resorption of the cuticle (Modha *et al*, 1994). Proudfoot *et al* (1993a and 1993b) have also reported that infective larvae have no affinity for AF18 immediately after being recovered by acid-pepsin digestion, but that significant insertion could be achieved after exposing the larvae to trypsin and bile in medium at 37° C.

These observations on *T. spiralis* transition to the enteral environment, taken together, have led to the conclusion that trypsin and bile (in mammalian culture medium) are part of the critical cues for developmental activation. Stewart *et al*, (1987) have suggested that *in vivo*, the infective L_1 larvae are primed by exposure to host enteric elements (trypsin and/or bile), through alterations in surface structure, to receive and respond to environmental cues that induce behavioural and biochemical changes. Presumably, larvae altered by stripping of the surface accessory layer are better able to receive environmental cues than those with intact surfaces. The accessory layer may therefore act as a barrier that prevents the reception of environmental cues which could elicit an activation response at an inappropriate time and place. Scanning electron microscopy studies of infective larvae reveal that no amphidial pores or other sensory structures are visible with the surface accessory layer intact, though two amphidial pores and several other sensory structures are clearly visible in adult worms (which do not have an accessory layer) (Kim & Ledbetter, 1980).

In vitro exposure to trypsin and bile in mammalian culture medium, therefore, seems to stimulate the transition from quiescent infective muscle larva to active enteral juvenile stage in *T. spiralis*. This *in vitro* activation protocol has opened the doors for further characterisation of the biochemical signalling which controls the life-cycle stage transition.

Initiation of development: Intracellular signalling

The surface property changes, observed during the transition to the enteral environment, have been used as a marker to monitor activation of infective *T. spiralis* larvae *in vitro*. It has thus been possible to examine some of the intracellular signalling pathways which lead to activation. Introducing the calcium chelator diazo-2AM into infective larvae, prior to activation with trypsin and bile, results in a significant reduction in AF18 insertion (Modha *et al*, 1994). However, addition of exogenous cAMP, to raise intracellular cAMP levels, results in restoration of AF18 insertion to values comparable to those of activated larvae. This suggests that a type I (calcium stimulated) adenylate cyclase may regulate at least a part of the activation process. Interestingly, Proudfoot *et al* (1993b) report exactly the opposite, finding that the depletion of cAMP elicited transformation. Nonetheless, their observations indicate that Ca^{+} channel blockers inhibit transformation, also implicating Ca^{+} involvement in activation.

With the use of the fluorescent lipid probe PKH26, the larval accessory layer can be labelled directly, and the dynamics of the shedding process observed by fluorescence microscopy (Modha, Kusel & Kennedy, 1995). At sub-activation conditions, a continual shedding and replacing of the accessory layer can be observed. The loss of the accessory layer upon activation with trypsin and bile containing medium does not result in replacement, but rather in a permanent loss. Introduction of exogenous inositol trisphosphate (IP_3), Ca^{2+}, or cAMP into infective larvae revealed an increase in the rate of accessory layer shedding. This phenomenon is distinct from the activation observed upon exposing the larvae to components of the enteric environment, but may indicate that second

messengers are involved in a continual regulation of the cuticle surface. Introduction of exogenous IP_3, Ca^{2+}, or cAMP into infective larvae also resulted in an increase in AF18 insertion, indicating that these molecules may also mediate some morphological changes which are observed at activation. Effects of depletion of intracellular Ca^{2+} could be reversed by cAMP, but not IP_3, leading to the conclusion that IP_3 comes first in the signalling pathway, followed by Ca^{2+}, and then cAMP. Where or how the intracellular signalling cascade may fit into the pathway inducing activation remains unknown, and its biological significance remains to be investigated.

ROLE OF CHEMOSENSATION IN DEVELOPMENT

A major method organisms have for monitoring their environment is chemosensation. Among the metazoa, specialised cells of the nervous system are utilised for sensing specific chemicals. Morphological descriptions of a variety of free-living and parasitic nematodes have shown that, in general, the anterior sensory organs of nematodes consist of circles of structures arranged in a hexaradiate pattern. This pattern comprises a head with six lips containing 12 labial sensilla, four cephalic sensilla, and two amphids (McLaren, 1976). Although the functions of these structures are not fully understood, there is strong evidence that the sensilla perform a mechanosensory role, while the amphids function in chemosensation (Bird & Bird, 1991; Ashton & Schad, 1996). There are, however, quite a few exceptions to this general plan, and especially in parasitic nematodes, a number of other, species specific, putative sensory cells and structures have been identified (McLaren, 1976). Regrettably, very little work has been carried out on the sensory systems of *T.spiralis,* and it is therefore still uncertain how this parasite perceives its environment. On the whole, the neurobiology of *T. spiralis* is still largely unexplored, but must be taken into account if niche selection behaviour and developmental regulation are to be understood (Despommier, 1993). Alterations in nematode behaviour or developmental pathways, in

response to environmental cues, depend upon the processing of information in the nervous system (Bird & Bird, 1991).

Aid in focusing research concerning chemosensation and neuroregulation in *T.spiralis,* and understanding of the parasite's neurobiology, may be found by using a well described model, the free living secernentean nematode *Caenorhabditis elegans*. Although there are some pronounced differences between the Secernentea and Adenophorea in the detailed structure of sensory receptors (Bird & Bird, 1991), perhaps the basic principles of neuroregulation are evolutionarily conserved sufficiently to allow parallels to be drawn between *T. spiralis* and *C. elegans.*

C. elegans can undergo alternative development at the moult between the second larval (L2) and third larval (L3) stages, entering a state of dormancy, termed dauer larva (Wood, 1988). The nematodes monitor food availability and their own population density by specific chemosensory cues, and direct development to the dauer state if food is scarce and/ or the population exceeds a certain threshold. If conditions become favourable again, the nematodes can recover from the dormant dauer larva state, and resume normal development. Virtually every tissue responds to the dauer-inducing signal by assuming a specific, reversible morphological state (Albert & Riddle, 1983). This feature makes *C. elegans* a good model for elucidating chemosensory control of development, induced by environmental cues, in nematodes. It has been argued that the dauer state is directly comparable, and homologous, to the developmental arrest observed in infective stages of parasitic nematodes (Bird & Bird, 1991).

Eleven classes of chemosensory cells with endings exposed to the environment have been proposed in *C. elegans*. The roles of many of these cell types in developmental decisions have been examined by killing individual cells or groups of cells using a laser microbeam, and evidence is strong that environmental stimuli which initiate developmental decisions in *C. elegans* are interpreted by its nervous system. Experiments have shown that a subset of the chemosensory cells is

involved in decisions to form dauer larvae, and whether to recover from the dauer stage based on environmental conditions (Bargmann, Thomas & Horvitz, 1990; Bargmann & Horvitz 1991). Developmental processes normally regulated by chemosensory inputs are not regulated after the deaths of some chemosensory neurones. Positive and negative environmental signals are evaluated in the decision to form dauer larva: food and pheromone levels together determine the developmental outcome.

Neurones as regulators of development

Many of the mutations which have been identified causing a dauer-defective phenotype, in which developing *C. elegans* fail to form dauer larvae, also cause structural defects in the ciliated sensory neurones (Bargmann, Thomas & Horvitz, 1990). The chemosensory cells prevent dauer formation even in mutants in which the sensory endings of these cells are defective. These results suggest that these chemosensory cells are active in the absence of environmental stimulation, since killing of these cells results in inappropriate dauer formation. When three of the chemosensory cells (ADF, ASG, ASI) are killed together in young animals, dauer larvae are formed regardless of environmental conditions. Thus these cells function to prevent dauer formation in presence of an adequate environment. Other neurones, (the ASJ cells) are crucial for the recovery from the dauer stage. In contrast, the ADF; ASG; and ASI neurones can be killed without inhibiting recovery from the dauer stage (Bargmann, Thomas & Horvitz, 1990). It has therefore been concluded that the activity of some chemosensory cells is required for *C. elegans* to develop to adulthood.

These findings indicate that the ground state of development in the absence of chemosensory cell function is dauer formation. More detailed studies have shown that either the ADF or ASI cells are necessary and sufficient to prevent entry into the dauer stage (Bargmann & Horvitz, 1991). ADF, ASI, and possibly ASG regulate the decision between dauer

formation and development to adulthood by providing a signal that stimulates normal development (or inhibits dauer formation) (Bargmann & Horvitz, 1991). The integration of food and pheromone inputs could take place within these sensory cells. By contrast, for dauer recovery, it appears that the ASJ neurones must be activated by external stimuli: these neurones do not trigger normal development in the absence of environmental stimuli.

Changes occurring in the dauer larva affect many cell types, giving an example of neuronal regulation of the development of both neuronal and non-neuronal tissues. Perhaps mechanisms like those used by *C. elegans* for neuronal control of dauer formation and recovery are used by parasitic nematodes to evaluate environmental signals and regulate their development accordingly. *C. elegans* recovery from the dauer larva state may rely on similar mechanisms as does initiation of development of infective *T. spiralis* larvae upon receiving activation cues in the host enteric environment.

GENETICS OF DIFFERENTIATION

Maintenance of particular states of gene activity seems prerequisite to continuance of a developmental stage. The arrested development (dormancy) of the *T. spiralis* infective stage larvae in the host muscle tissue, therefore, must rely on the maintenance of a differentiated state. Consequently, changes in the maintenance of gene activity must take place upon activation of the larvae in the host intestine. This regulation of development and differentiation involves both initial signalling and maintenance circuits.

Absolutely nothing is known about genetic or molecular control of development throughout the *T. spiralis* life cycle. *C. elegans,* however, may yet again provide a good model for genetic analysis of the maintenance of gene activity in nematodes.

To date, studies have failed to provide evidence for epigenetic modifications of DNA or chromatin in *C. elegans* for any obvious part of its

development. This includes absence of chromatin diminution, lack of gene control by methylation, absence of sequence rearrangements, and no evidence of anomalous resistance to restriction enzyme digestion of genomic DNA. Presumably regulatory circuits are set up to maintain differential gene expression, positive feedback loops being most likely (Hodgkin, 1994).

Temporal co-ordination of developmental events

The genes involved in developmental regulation can be classified into structural genes, which are responsible for structural components contributing to morphology (morphogenesis genes), and temporal genes, which define the timing of developmental change. In *C. elegans,* some genes have been identified which are involved in temporal control of developmental events; for example, the hypodermal larva-to-adult switch is controlled by a number of genes, mutations in which will prevent formation of the adult cuticle. Dauer larva formation presents a simple developmental switch integrating environmental and temporal information. Parasite transmission stages reaching infectivity, and infective stages recovering from dormancy in the process of infecting a new host, display a similar integration of temporal developmental regulation and environmental cues. Temporal control of dauer larva development therefore presents a good model system for examining processes which control timing of development in *T.spiralis*. The problem to be addressed is how genes interact to co-ordinate the temporal control of multiple developmental events in diverse cell types and at different developmental stages. Liu and Ambros (1989) investigated whether the general temporal control genes involved in the larval adult (L/A) switch of *C. elegans* also affect timing of other distinct developmental pathways, specifically dauer formation.

Heterochronic mutations cause stage-specific transformations in larval development patterns such that cells adopt fates characteristic of cells that normally occur earlier or later in the same lineage. The nature of

these cell fate transformations suggests that cells of equivalent developmental potential are generated at successive stages and that heterochronic genes determine the temporal component of their fates. Liu and Ambros (1989) found that the same genes (*lin-4* and *lin-14*) regulate the stage of dauer initiation (at the L2 moult) and are at least partly responsible for the L/A switch (at the L4 moult). It was further demonstrated that the L/A switch and dauer initiation are executed independently and are not causally related. However, as the authors point out, the pathway of environmental control is separate from the pathway of temporal control defined by *lin-4* and *lin-14* genes. Since differentiation in response to environmental signals seems to be mediated by specific neurones, the *lin-4* and *lin-14* genes may regulate the stage of dauer larva formation by controlling a signal (or response to a signal) originating from particular neurones. Temporal decrease in *lin-14* activity is required for the normal succession of stage-specific developmental patterns. Unchanging abundance of the *lin-14* transcript during development indicates that downregulation of the *lin-14* product must occur at the post-transcriptional level (Ambros & Moss, 1994). Downregulation of *lin-14* has been shown to be linked to the long 3' untranslated region (3' UTR). Experiments indicate that *lin-4* acts upstream of *lin-14* in a regulatory pathway, and that *lin-4* is a negative regulator of *lin-14*, playing a critical role in *lin-14* downregulation (Lee, Feinbaum & Ambros, 1993; Wightman, Ha & Ruvkun, 1993). Recent characterisation of the *lin-4* gene has revealed that it encodes two very small RNAs of 22 and 61 nucleotides (Lee, Feinbaum & Ambros, 1993). Further, it appears that the active gene product of *lin-4* is not protein but RNA. It has therefore been suggested that *lin-4* RNA interacts directly with the 3' UTR of the *lin-14* mRNA; and thus negatively affects translation of the *lin-14* mRNA (Lee, Feinbaum & Ambros, 1993; Wightman, Ha & Ruvkun, 1993). *lin-4* transcripts accumulate in the L1 stage several hours after hatching, and around the time that the level of *lin-14* protein begins to decline. Thus, the downregulation of *lin-14* is generated by a temporally controlled increase in the activity of *lin-4*, which may in turn be triggered by a 'food signal' that

initiates postembryonic development (Ambros & Moss, 1994). *lin-14* therefore seems to function as a negative regulator of L3 specific programs. Further, the level of *lin-14* activity affects at which stage dauer larvae may be formed. Animals that have reduced *lin-14* activity may form precocious dauer larvae, while animals with increased *lin-14* activity may form retarded dauer larvae after the L3 stage (Ambros & Moss, 1994).

Information on developmental regulation at the molecular and genetic levels is still lacking for parasitic nematodes. To date, investigations into genetic control of development of parasitic nematodes have not been possible, due to the limitations imposed by parasitic life cycles. Thus, the genetic studies possible with *C. elegans* have given first insights into regulatory genes and molecular pathways in nematode development. Molecular analysis of developmental regulation in parasitic nematodes can benefit from these insights, by giving an awareness of the various potential interactions of regulatory molecules. Regulatory genes may be multifunctional, such as *lin-4* and *lin-14* (which regulate the stage of dauer initiation and are at least partly responsible for the L/A switch), and integrate with control mechanisms which underlie environmental influences. Temporal genes may control the signal (or response to a signal) from a particular neurone. At least some regulators underlie post-transcriptional control, but they in turn are regulated by differentially transcribed RNA. These examples point the way for an improved understanding of the questions which we ask about regulation of development in parasitic nematodes.

Regulatory genes in development

Work on *C. elegans* has given an indication of the types of genes which potentially might play a role in developmental decision making and integration of environmental information with temporal regulation in nematodes. Some genes critical for the initiation of dauer larva formation have been identified, though regrettably no genes have been implicated in

dauer recovery (which would be more directly relevant to activation of *T. spiralis* larvae). Nonetheless, the principal mechanisms in question should give insights which will aid in recognising various aspects of regulatory mechanisms during activation of infective stages of parasitic nematodes.

Epistasis analysis has allowed the ordering of genes into a formal pathway controlling dauer formation in *C. elegans* (Vowels & Thomas, 1992). The particular anatomical site of function of the genes in the dauer pathway is not known with the exception of *daf-6* and *daf-10*, which are required for the production of normal chemosensory amphid and phasmid sensilla (main groups of chemosensory neurones). Only one of the dauer-constitutive genes, *daf-11*, depends on normal chemosensory endings, making it a good candidate for encoding a component of the chemosensory mechanism that controls dauer formation (Vowels & Thomas, 1992). The gene pathway leading to dauer formation has been divided into those genes acting upstream or in the chemosensory endings, and those acting downstream of the chemosensory endings. Observations have satisfied the prediction that the dauer-inducing stimuli act on specific chemosensory cells. Downstream genes, such as *daf-16* and *daf-18*, are components of the tissue specific differentiation events that occur in response to activation of the dauer pathway. Mutations in these genes block the formation of only some dauer tissues, rather than the induction of dauer formation *per se*. Vowels & Thomas (1992) propose that the nine genes required for normal cilium structure prevent dauer-inducing conditions from de-repressing dauer formation. Five *daf*-constitutive genes (mutations in which will cause dauer formation despite favourable environmental conditions) have been shown to act in the same place of the pathway as the laser kill of the ADF, ASI, and ASG neurones. These five genes are good candidates to encode functions required for the repression of dauer formation by ADF, ASI, and ASG, either presynaptically or postsynaptically. Two of the genes are *daf-1* and *daf-4*. Georgi, Albert & Riddle (1990) have characterised the *daf-1* gene, which specifies an intermediate step in the genetic pathway controlling dauer larva development. The *daf-1* gene potentially affects cells receiving signals from amphidial neurones, and precedes genes (*daf-9* and *daf-15*)

in which mutations cause abnormalities in morphogenesis of the dauer larva. It is therefore likely that the *daf-1* gene plays a central role in signal transduction. Molecular analysis of the *daf-1* gene revealed that it specifies a protein kinase significantly similar to the *raf* proto- oncogene family of serine/ threonine protein kinases, with a trans-membrane domain characteristic of signal peptides (Georgie, Albert & Riddle, 1990). The authors speculate that the protein could be a cell surface receptor directly involved in processing environmental cues into developmental decisions, or, alternatively, that the kinase might affect the differentiation of a neurone that is needed for signal transduction. Further work has linked the *daf-1* serine/ threonine kinase receptor to a family of TGF-β, bone morphogenetic protein (BMP), and activin binding type II receptors (Massague *et al*, 1992). The *daf-4* gene has been shown to encode a receptor protein kinase similar to the *daf-1*, activin, and TGF-β receptor serine/ threonine kinases (Estevez *et al*, 1993). *In vitro* studies have shown that the *daf-4* receptor will bind BMPs.

Control of gene expression: Developmental transition

The molecular aspects of transition from the dauer state to continuation of normal development in *C. elegans* have been studied mainly by comparisons of transcriptional activity *pre-* and *post-* recovery. Recovery of worms from the dauer state is accompanied by an ordered temporal sequence of gene expression. Run-on transcription assays have shown that within 75 min after placement in food, or shortly after commitment to recovery, bulk mRNA transcription increases two-fold. RNA polymerase II (rpo II) mediated transcription is decreased in dauer larvae (to a level of 11-17%) when compared to developmentally active stages. Within one hour of transfer to fresh medium containing food, an increase in Rpo II activity to a rate of 23% of that in growing worms can be detected (Dalley & Golomb, 1992).

In contrast, Reape & Burnell (1991a & 1991b) observed that neither actinomycin D nor α-amanitin seem to affect recovery from the dauer

state, and hence concluded that transcription is not necessary for initiation of the recovery sequence. Continuation of normal development, involving the next molt, however, was shown to depend on transcription. Reape and Burnell hypothesised that the worms synthesise and store a sufficient amount of mRNA (or pre-messenger RNA) before entry into the dauer state, which they will require if they receive the signal to initiate a recovery sequence. It has been demonstrated that inhibition of protein synthesis (with cycloheximide, anisomycin, oxytetracycline, and puromycin) during the recovery period can retard or prevent dauer larva recovery (Reape & Burnell, 1991b). None of the protein synthesis inhibitors affected general motility of the worms, and the authors postulated that the effect of protein synthesis inhibitors on pharyngeal pumping in recovered larvae may be mediated through the pharyngeal sensory or motor nervous system. Considering the legendary impermeability of dauer larvae, (Wood, 1988), it is probably safe to assume that the inhibitors used in the above studies failed to penetrate into the larvae before or during the early recovery stage. The observed effects all took place some time after initiation of feeding. The putative requirement for transcriptional activity for the initiation of dauer recovery could, therefore, not be tested.

Control of gene expression: Basis for some regulatory mechanisms

RNA processing is potentially an important factor in control of gene expression in nematodes. The discovery of mRNA maturation by *trans*-splicing of 5' leader sequences in nematodes (Nilsen, 1993) revealed new aspects of nematode gene expression with implications for the understanding of regulatory mechanisms.

The occurrence of polycistrons and linked *trans*-splicing have been reported in *C. elegans* (Speith *et al*, 1993). A leader sequence (SL) is *trans*-spliced on to the 5' end of primary transcripts during the maturation of the pre-mRNA. Splicing to SL1 does not appear to be linked to maturation of polycistronic transcripts, but to the generation of capped 5'

ends from primary transcripts with a 5' intron-like region. Surprisingly, *trans*-splicing of SL2 is linked to maturation of polycistrons. It is speculated that the SL must play some part in determining initiation of translation. SL2-like leaders might be differentially regulated, either spatially or temporally, offering additional controls over gene expression (Blaxter & Liu, 1996). SLs therefore generate and mark the 5' end of mature mRNAs, whether derived from monocistronic (SL1) of polycistronic (SL2) precursors, potentially effecting both mRNA stability and turnover as well as translational capability, or availability, of the mRNA.

The occurrence of *trans*-splicing, both with SL1 and SL2, has also been observed in *T. spiralis* (Blaxter & Liu, 1996). It remains to be investigated if *trans*-splicing plays a significant part in regulation of gene expression with respect to resumption of development in infective larvae of parasitic nematodes, such as *T.spiralis*.

THE OBJECTIVE

As a direct consequence of the complex life cycles of many parasitic nematodes, often involving multiple hosts, very little is known about the molecular regulation of development in these parasites. The transition of the quiescent infective stage to the next host, with ensuing resumption of development, is of interest not only due to the complexity of the underlying interactions, but also because of its importance to the parasite/ host relationship.

At present, the understanding of the regulatory processes which control the resumption of development of infective *T. spiralis* larvae is still in its infancy. The main aim of the present study was, therefore, to determine at which levels gene expression is regulated with respect to the initiation of development of infective *T. spiralis* larvae during the infectious process. Further aims included the characterisation of genes important in resumption of development in the infective larvae, and elucidation of signalling pathways which lead to initiation of development.

2. EXPOSURE OF INFECTIVE LARVAE TO THE ENTERAL ENVIRONMENT: DIFFERENTIAL RNA EXPRESSION

INTRODUCTION

Biochemical aspects of the transition from the developmentally quiescent infective stage to the developmentally active intestinal form have been investigated in *T.spiralis*. These studies have attempted to ascertain the environmental cues for, and timing of, developmental activation (Stewart *et al*, 1987), morphological changes in response to activation (Modha *et al*, 1994; Stewart *et al*, 1987), and metabolic responses to the translocation to the enteral microhabitat (Stewart, 1983). The observations resultant from these investigations have given strong evidence that infective larvae resume development in the host small intestine after exposure to trypsin and bile. Based on this model of development, investigations into the intracellular signalling pathways responsible for putative developmental activation have been conducted. The involvement of second messengers in the control of activation-induced changes to the surface of *T. spiralis* infective larvae was investigated using membrane-permeant photo-activatable caged' compounds to alter intracellular levels of inositol trisphosphate (IP_3), calcium ions (Ca^{2+}) and cyclic AMP (cAMP). Consequently, the existence of a linear second messenger pathway involving the sequential release of IP_3, Ca^{2+} and then cAMP was proposed (Modha, Kusel & Kennedy, 1995). Nonetheless, where the signalling mechanisms act and what effects they have on gene expression of the developing larvae remain to be described. There are no molecular markers for resumption of development, and changes in gene expression during *T. spiralis* development remain wholly uncharacterised. Nothing is known about how *Trichinella* infective stages control their development at the molecular level.

The present study was conducted in order to describe changes in gene expression at the transcriptional level upon resumption of development of infective larvae. Any differentially transcribed genes would

serve as molecular markers of development, as well as giving a starting point for dissection of the activational pathway. Based on the trypsin and bile stimulated activation paradigm, mRNA of acid-pepsin isolated (putatively unactivated) larvae was compared with mRNA from *in vitro* activated larvae. To this end, PCR based RNA fingerprinting techniques were chosen as the most efficient and effective means of screening for differentially expressed transcripts. cDNA from polyadenylated mRNA was amplified with PCR using either arbitrary primers or oligo (dT) with 5' spliced leader primers. Reaction products were compared using polyacrylamide gel electrophoresis and silver staining. The results indicate that infective larvae may initiate developmentally regulated transcription at a time point prior to the exposure to trypsin and bile.

MATERIALS AND METHODS

Parasites obtained from experimental infections in mice were exposed to components of the mammalian intestinal environment *in vitro*. In order to determine whether the larvae respond to these stimuli by initiating the transcription of previously inactive genes, RNA from larvae that had not been exposed to enteral components was compared with RNA from exposed larvae using randomly amplified cDNA (RAcD) produced by arbitrary priming of cDNA in the polymerase chain reaction (PCR).

Parasite maintenance and recovery

Laboratory infections of *T. spiralis* were maintained in BALB/c mice, and larvae were recovered between 6 weeks and 3 months post infection. Infected mice were killed by fracturing the spine, skinned, and eviscerated with exception of the heart and lungs. The carcasses were subsequently homogenised, and then digested in aerated 0.5% pepsin (1 Anson unit/g, purchased from BDH), 0.5% HCl, in 0.9% saline at 37° C for two hours. After filtering the digest mix through a fine mesh (250 μm) steel sieve, the filtrate containing the larvae was subjected to four sedimentation steps in 1 L 0.9% saline, separating the larvae from residual murine material, which remains in suspension. Obtained larvae were washed three times in 0.9% saline, and then resuspended in 5 ml saline. 30 μl of larval suspension were examined under the light microscope in order to estimate the number of larvae present, and to ascertain the condition of the larvae.

Recovered larvae were separated from any remaining debris and nurse cells by sucrose floatation as follows: Larvae were centrifuged in 10 ml 50% w/v sucrose/ sterile distilled water at 1700 ***g*** for 7 min. Under these conditions cellular debris will pellet, and nurse cells form diffuse a band at the centre of the centrifuge tube while larvae float on the surface of the

sucrose solution. The top larval layer was aspirated and washed three times in ice-cold sterile phosphate buffered saline (PBS).

In order to separate live larvae from dead larvae, Percoll centrifugation was carried out as follows: Larvae were layered on top of 5 ml 45% Percoll / PBS in 10 ml conical tubes and centrifuged at 800 ***g*** for 20 min. The top layers were aspirated and discarded, and the pellet of live larvae resuspended in PBS. The larvae were subsequently washed three times in PBS.

Larvae to be activated by exposure to components of the host enteric environment were stored at 4° C for a maximum of 16 h before activation and subsequent sucrose and Percoll purification steps. All larvae were frozen in liquid nitrogen immediately after purification and stored at -70° C until RNA extraction was carried out.

In vitro activation by exposure to components of the host enteric environment

Activation with trypsin and bile in medium, as described by Modha, Kusel & Kennedy (1995), was carried out as follows.
Larvae (about 25000) were suspended in 170 µl RPMI 1640 medium (GibcoBRL). 20 µl 50% bile were added to give a final concentration of 5%, and 10 µl 5% trypsin were added to give a final concentration of 0.25%. The larvae were subsequently incubated at 37° C for 45 min. After the incubation, 10 µl of larval suspension were examined by light microscopy to monitor the status of the larvae, and to check for the behavioural change, from coiling to migrating, which accompanies activation as described by Stewart *et al* (1987). Following activation, the larvae were washed three times in de-ionised distilled H_2O, and subjected to the sucrose and Percoll purification steps described above. After freezing in liquid nitrogen, the larvae were stored at -70° C.

Total RNA extraction

200 µl lysis buffer (0.1 M Tris (pH 8.0), 0.2 M NaCl, 2% SDS, 0.2 M EDTA (pH 8.0)), equilibrated at 65° C, were added to 30 µl packed volume of

frozen larvae. 12.5 µl proteinase K (10 mg/ml) were added to the lysis solution, and the larvae were incubated at 65° C for 90 min. After the incubation, 1 ml TRIzol reagent (GibcoBRL) was added, and the preparation remained a further 10 min at 65° C. The lysate was then rapidly cooled on ice and centrifuged at 12000 ***g*** for 10 min at 4° C. The supernatant was transferred to a fresh tube, and incubated at 65° C 10 min. One chloroform extraction step (0.2 ml) followed, centrifugation taking place at 12000 ***g*** for 15 min at 4° C. The aqueous phase was transferred to a fresh microcentrifuge tube, and 0.5 ml isopropanol added. After 10 min incubation at room temperature, the sample was centrifuged at 12000 ***g*** for 10 min at 4° C. The supernatant was removed, and the RNA pellet washed three times in 75% ethanol. After air drying, the RNA pellet was dissolved in 50 µl DEPC treated H_2O.

The RNA yield was estimated by measuring the absorbence at 260 nm of 2 µl RNA suspension in 500 µl sterile H_2O. 3 µl of RNA suspension were run on a 1% agarose gel at 100 V for 60 min, stained with 0.5 µg/ml ethidium bromide, and viewed under UV illumination to check the sample for degradation. All RNA samples were stored at -70° C.

DNA digestion

Elimination of contaminant DNA from RNA samples.
Reactions were carried out in 250 µl total volume, consisting of 160 µl DEPC H_2O, 25 µl DNAasel reaction buffer (x10), 25 µl DNAasel (25 U), 0.3 µl Human Placental RNAase Inhibitor (30 U), and 40 µl RNA suspension (approximately 40 µg RNA). The samples were incubated at room temperature for 15 min. Following the incubation, 30 µl 25 mM EDTA (pH 8.0) were added, and the samples heated to 65° C for 10 min. One phenol/chloroform (50:50) extractions and one chloroform extraction (300 µl) were carried out to remove all enzymes. The RNA was precipitated in 345 µl EtOH and 15 µl NaAct (2 M) at -20°C for 2 h. Collection of the RNA was carried out by centrifugation at 4° C for 30 min, and the RNA pellet was washed twice in ice cold 75% EtOH. After air drying, the RNA pellet was suspended in 50 µl DEPC H_2O.

RNA yield was estimated by measuring absorbance at 260 nm as described above. 3 µl of RNA suspension were electrophoresed on a 1% agarose gel as described above. All RNA samples were stored at -70° C.

Reverse Transcription

Polyadenylated mRNA was reverse transcribed using oligo (dT) primers. For each 20 µl reaction, the following were added into a nuclease free 0.5 ml microcentrifuge tube: 5.0 µl RNA sample (1 µg RNA/µl), 1.0 µl oligo $(dT)_{12-18}$ primer (500 µg/ml), and 5.0 µl DEPC H_2O.
The reaction was heated to 70° C for 10 min, then quickly cooled on ice and collected by brief centrifugation. The following were then added to each reaction: 4.0 µl 5x First Strand Buffer (GibcoBRL), 1.0 µl dNTP (10 mM), 2.0 µl DTT (0.1 M), and 1.0 µl RNasin Ribonuclease Inhibitor (38 U/µl) (Promega).
The reaction was warmed to 42° C for 2 min, after which 1.0 µl (200 U) SuperScript II reverse transcriptase (GibcoBRL) was added to each reaction. The reactions were carried out at 42° C for one hour, and stopped by heating to 70° C for 15 min.
First strand products were stored at -20° C.

Second strand synthesis

Initially, second strand synthesis was performed on all cDNA samples. However, since no differences in PCR products from only first strand or double stranded cDNA template were observed, only first strand cDNA was used in later experiments.

RNase inhibitor and enzymes were removed from the first strand sample by standard phenol/ chloroform extraction, followed by ethanol precipitation (Sambrook, Fritsch & Maniatis, 1989). The precipitated cDNA was dissolved in 20 µl H_2O.

On ice, the following were added, in order, to the 20 µl first strand mixture: 91.8 µl H_2O, 32 µl 5x Second Strand Buffer (94 mM Tris-HCl (pH

6.9), 453 mM KCl, 23 mM $MgCl_2$, 750 µM β-NAD, 50 mM $(NH_4)_2SO_4$), 3 µl 10 mM dNTPs, 6 µl 0.1 M DTT, 2 µl *E. coli* DNA ligase (7.5 U/µl), 4 µl *E. coli* DNA Polymerase I (10 U/µl), 1.0 µl *E. coli* RNase H (2 U/µl). The complete reaction mixture was incubated for 2 h at 16° C. 10 U of T4 DNA polymerase were added per µg first strand cDNA, and the incubation was continued at 16° C for 5 min. The enzymes were inactivated by heating to 65° C for 10 min.

Removal of primers and buffers from cDNA sample

Primers, enzymes, and dNTPs were initially removed from the cDNA samples using Microcon 50 microconcentrators (Amersham) as follows. The volume of the first- or second- strand reaction was increased to 200 µl with sterile H_2O. One chloroform extraction step (200 µl chloroform) was carried out (incubation for 2-3min at room temperature, centrifugation at 12000 ***g*** for 10 min). The aqueous phase, containing the cDNA, was heated to 95°C for 5 min, then immediately cooled on ice. The cDNA solution was added to a Microcon 50, and centrifuged at 12000 ***g*** for 6 min. The retentate (about 20 µl) was spun out at 1000 ***g*** for 3 min, and increased to 200 µl volume with sterile H_2O. The Microcon 50 centrifugation was repeated twice, and the final retentate (about 20 µl) stored at -20° C.

As an alternative to the above procedure, a simpler method using GlassMax minicolumns (GibcoBRL) was employed for later experiments. 100 µl of sterile H_2O were equilibrated at 65° C. Binding solution (5 M NaI) was added to the DNA sample at a 4.5:1 (v/v) ratio. The sample was added to a spin column and centrifuged at 13000 ***g*** for 20 s. The column was washed three times with 400 µl of cold (4°C) wash buffer (GibcoBRL), with a final centrifugation at 13000 ***g*** for 1 min. 40 µl of the preheated H_2O buffer were added to the column, and the cDNA was eluted by centrifuge at 13000 ***g*** for 20 s. The eluted cDNA was stored at -20° C.

Spliced leader and oligo (dT) primed PCR

PCR was carried out on the cDNA using primers to the nematode mRNA 5' *trans*-spliced leaders SL1 (GGTTTAATTACCCAAGTTTGAG) and SL2 (GGTTTTAACCCAGTTACTCAAG), and to the poly-A tail.
Hot start PCR was carried out as follows: In a 0.5 ml microcentrifuge tube 2.0 µl cDNA (≈ 50 ng/µl), 1.0 µl spliced leader primer (20 pmol/µl), 1.0 µl primer oligo $(dT)_{18}$ (20 pmol/µl), and 21.0 µl sterile H_2O were overlaid with mineral oil and heated at 95° C for 5 min. The temperature was lowered to 80° C while the following reaction mix is added to each tube: 5.0 µl x10 PCR buffer (final concentration: 50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100), 3.0 µl $MgCl_2$ (25 mM), 1.0 µl dNTP (10 mM), 0.20 µl Taq (5 U/µl), and 15.80 µl sterile H_2O.
Controls contained reaction mix and primers but no cDNA, or cDNA and reaction mix without primers.
Cycling conditions were 94° C for 1 min, 55° C for 1 min and 72° C for 2 min for 35 cycles followed by a 10 min extension at 72° C. To establish the optimal annealing temperature, a range from 50° C to 58° C was tested.

Positive control

As a positive control, amplifications with a primer (TTATTCCTTGCCCATACAGC) to the 43 kDa glycoprotein described by Vassilatis *et al.* (1992) and oligo $(dT)_{18}$ were carried out as described for the spliced leader PCR.

Arbitrarily primed PCR

Oligodeoxyribonucleotide (oligo) primers (10-mers) of arbitrary sequence (see *Arbitrary primer sequences* below) were used to amplify the cDNA to generate stage specific RAcD patterns. For each reaction, one primer was used either singly, in combination with a oligo (dT) primer, or in combination with one other arbitrary primer. 10 different primers, part of a primer set obtained from Genosys, were used in total for the RAcD

analysis. In the initial screen 3 separate reactions were set up for each condition tested: 3 reactions per primer per reaction per activational state of larvae.

Reaction conditions were as follows: each 20 µl reaction contained buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100), 1.5 mM $MgCl_2$, 200 µM dNTPs, 20 pmol arbitrary primer, optionally 20 pmol oligo (dT) primer, 60 ng cDNA, and 1.0 U *Taq* polymerase. No hot start was carried out, but reactions were assembled and kept on ice until the thermal cycler heating block had pre-heated to 94° C.
Controls contained reaction mix and primers, but no cDNA, or reaction mix and cDNA, but no primers.

Cycling conditions were 94° C for 1 min, 35° C for 1 min and 72° C for 2 min for 2 cycles, followed by 94° C for 1 min, 40° C for 1 min and 72° C for 2 min for 33 cycles followed by a 10 min extension at 72° C.

Arbitrary primer sequences

(P1) ACGCTACATC	(P6) AAGATAGCGG
(P2) CGAAACAGTC	(P7) CCTATCCGTT
(P3) CTTACACTTG	(P8) GATTGCGTTC
(P4) GTTAGTGGCA	(P9) CGTTCGTGTA
(P5) ATCTGAGGAG	(P10) TGCTGTGAAC

Electrophoresis and visualisation of PCR products

PCR products were electrophoresed through 6% polyacrylamide (30:1 *bis*-acrylamide) mini gels (BioRad), or 16 cm gels (Pharmacia), at 8 V/ cm. Visualisation of the DNA was by silver staining as follows.
Gels were fixed in 10% ethanol and 0.007% acetic acid in a total volume of 100 ml for 5 min. A further 50 ml fixing solution containing 0.2 g silver nitrate were added, and the gel stained for 10 min. The gel was washed in de-ionised H_2O for 20 s, and 2 min after a water change. The washed gel was developed immediately in 150 ml 0.75 M NaOH and 0.007% formaldehyde until bands appeared. After development was finished, the

gel was washed twice in fixing solution (same as above), photographed, and dried using a BioRad gel dryer for future reference.

RESULTS AND DISCUSSION

The aim of the experiments was to find genes which are differentially transcribed in response to, or as part of, putative developmental activation. Through the identification of such genes, the nature of the observed activation might be established, as well as any potential sequence in changes of gene expression observed as a result of the activation process. Differentially transcribed genes were to be used as markers in further studies on the signalling pathways involved in activation.

To achieve this aim, mRNA from infective larvae, isolated by acid-pepsin digest, was compared to mRNA from recovered larvae which had been exposed to components of the host enteral environment *in vitro*. This comparison was made by means of arbitrary and spliced leader specific amplification of the reverse transcribed mRNA using PCR.

Recovery and activation of infective larvae

Recovery of infective larvae by acid-pepsin digest of parasitised muscle tissue is a well-established method which is routinely carried out in many laboratories (Campbell, 1983). Although this method consistently yields relatively pure preparations of infective larvae, the sensitive nature of RAcD analysis demanded a safeguard against any possible contamination with host material. Sucrose centrifugation proved to be an efficient and reliable method to remove any nurse cells or other murine cells from the samples. Examination of purified infective larvae revealed consistently clean samples, devoid of nurse cells or the debris sometimes visible in parasite preparations which have not been subjected to sucrose centrifugation.

The basis of the transcriptional activity comparison, the *in vitro* activation, is based on work previously carried out by other investigators. Work on the structure and biochemistry of infective larvae after invasion of the host intestine *in vivo*, and after exposure of infective larvae to various

components of the host's enteral environment *in vitro,* by Stewart *et al* (1987), has led to the utilisation of the activation protocol as described in this report. The correlation between the observed *in vitro* and *in vivo* changes have led to the term 'activation' being used to describe the transformation catalysed by exposure to trypsin and bile in medium (Modha *et al*, 1994). The success of the activation procedure described in this work was determined by observation of the behavioural change from coiling to migrating behaviour. All parasite samples treated with trypsin and bile in medium were activated, as judged by this standard.

mRNA analyses

RNA extraction and reverse transcription

Total RNA was extracted by an adaptation of standard methods which have generally been shown to yield good quality RNA (Jackson, Hayden & Quirke, 1991; Sambrook, Fritsch & Maniatis, 1989). Gel analysis of DNaseI digested total RNA revealed minimal degradation, as far as detectable by ethidium bromide staining (see Figure 1, p. 44, for example). As a critical starting point of the experimental work, the quality of the RNA was consistently deemed sufficient to give reliable results in RAcD analysis.

Reverse transcription was carried out with total RNA in order to avoid the loss of some rare mRNA species, inherent in most poly(A) RNA enrichment methodologies. Further, rare mRNA species enjoy greater protection from contaminant RNases in solutions of total RNA, than in enriched samples. This protection is afforded by the overabundance of non-messenger RNA (e.g. rRNA, tRNA), which acts as substrate for RNases.

Second strand synthesis was performed on all first strand templates during the pilot experiments in order to maximise potential binding sites for arbitrary primers during subsequent RAcD-PCR. Control arbitrarily primed PCR experiments with only first strand template, however, did yield the same results as those experiments conducted with double stranded

cDNA template. On the basis of these results, it was decided to discontinue second strand synthesis to keep the pre-PCR manipulation of the cDNA template to a minimum, in order to preserve template integrity and avoid loss of rare species as much as possible.

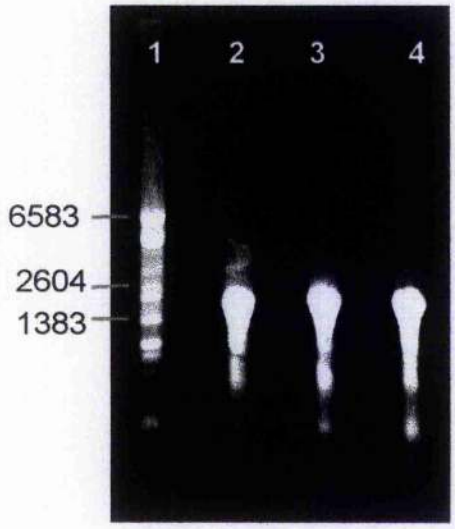
Removal of primers from the template cDNA was considered advisable in order to limit priming events to those primers chosen for PCR. This was necessary to limit reaction products to a manageable amount, and prevent unreproducible and spurious priming events.

Spliced leader and oligo (dT) PCR

As an initial screen for changes in transcription of any highly abundant *trans*-spliced mRNA during activation, PCR of cDNA from activated and unactivated larvae was carried out using oligo (dT) and spliced leader primers. The nematode 22 nt 5' *trans*-spliced leader sequences SL1 and SL2 (Nilsen, 1993) are thought to play an important function in the maturation of premessenger RNA, and are estimated to be present on 80% (SL1) and 3% (SL2) of *C. elegans* mRNAs (Blaxter & Liu, 1996). The phenomenon of mRNA *trans*-splicing seems to be ubiquitous among nematodes, and the presence of SL1 in *T. spiralis* has already been reported (Blaxter & Liu, 1996). Further, there is evidence that *trans*-splicing of SL2 is linked to the maturation of polycistrons (Speith *et al*, 1993). Hence, it is theoretically possible to detect either changes in transcription or post-transcriptional processing of *trans*-spliced genes using spliced leader PCR. Should *T. spiralis* infective larvae store premessenger polycistrons that are processed and translated only upon activation, SL2 PCR can potentially detect the mature mRNAs in a stage specific manner. Thus, expression analysis can be conducted in a manner independent of coding sequences. This could eliminate certain false negative errors, which can be expected in the case of stored premessenger RNA species, when primers to internal sequences are used. In this regard, both SL1 and SL2 primers are equally useful.

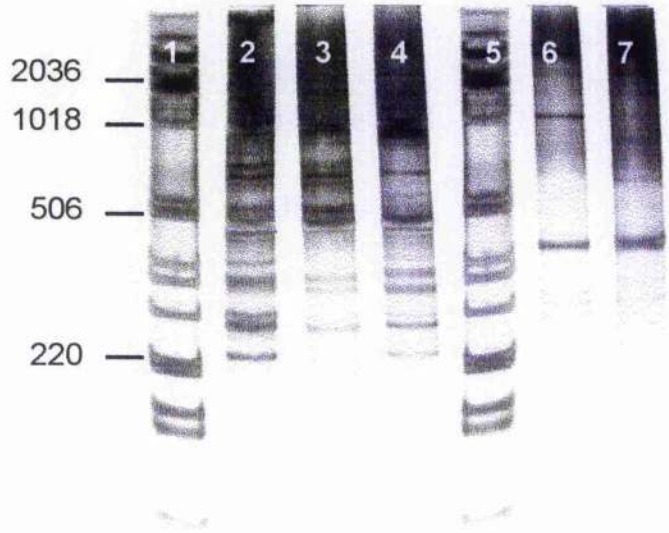
The results of the spliced leader PCR showed an abundance of products from both SL1 and SL2 primed reactions (see Figure 2, p. 44 and Figure 3, p. 46). The reaction results were, however, not reproducible

Figure 1. Total RNA from infective larvae electrophoresed through 1% agar and stained with 0.5 μg/ml ethidium bromide.



Lanes: (1) RNA size marker (Promega), sizes are given as number of nucleotides. (2 to 4) Total RNA from infective larvae.

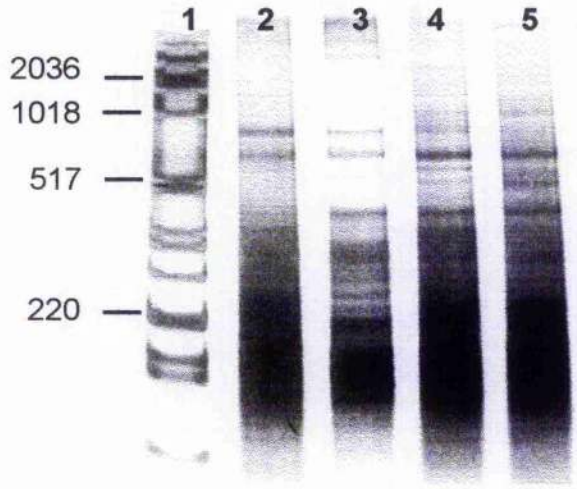
Figure 2. Products of spliced leader PCR electrophoresed through 6% polyacrylamide and silver stained.



Lanes: (1 & 5) 1 Kb DNA ladder molecular size marker, sizes given in number of base pairs. (2 to 4) SL1 and oligo (dT) primed amplified cDNA from unactivated (2 & 3) and activated (4) larvae. (6 & 7) SL2 and oligo (dT) primed amplified cDNA from unactivated larvae.

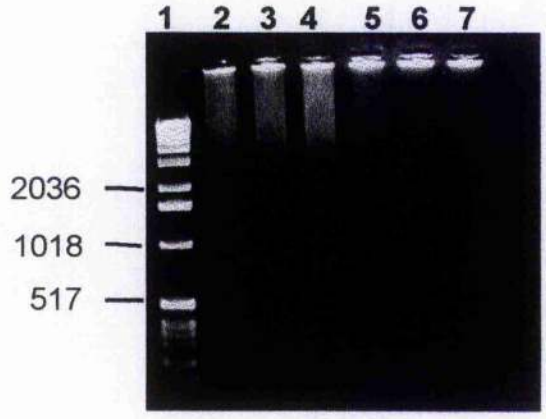
among experiments, using either set of primers. Further, the abundance of products caused a high background, which allowed the identification of, on average, only 20 prominent products. None of these products could be identified as being differentially amplified for either activated or unactivated samples. Notably, the variation between reaction products within experiments was minimal; i.e. PCR products obtained using the same reaction mix and thermal cycling run differed only minimally among replicates, and between activated or unactivated samples, even if the templates were from different sources. PCR products from different thermal cycling runs varied considerably, even if the templates were from the same source. Despite varying the concentrations of template, (from 20 ng to 100 ng), and primer, (20 pmol to 60 pmol), used in combination, as well as $MgCl_2$ concentration and annealing temperatures for a total of 126 reactions, the problems could not be alleviated. This lack of consistency in PCR products from different experiments is probably a function of the abundance of total *trans*-spliced mRNA amplification targets, which presumably makes the reactions very sensitive to even slightest variations in reaction conditions. These variations, caused by slight inherent deviations among ramp speeds and target temperatures of the thermal cycler between runs, as well as deviations among different preparations of reaction mixes caused by pipetting inaccuracies, are unavoidable. To test the sensitivity of the reactions, replicates of the same reactions were amplified at the same time using two different thermal cyclers. The results did show differences in reaction products obtained from each thermal cycler. This also implies that the products obtained from any one spliced leader PCR are not representative of the reaction templates as a whole. Possibly, the only solution to this problem would involve a reduction in the amount of template species. This may be accomplished by using oligo (dT) primers with an anchor of several nucleotides at the 5' end. In this study, however, it was chosen to proceed to arbitrary priming as a means of reducing reaction products to a manageable amount.

Figure 3. Products of spliced leader PCR with SL2 primer, electrophoresed through 6% polyacrylamide and silver stained.



Lanes: (1) 1 Kb DNA ladder molecular size marker, sizes given in number of base pairs. (2 & 4) SL2 and oligo (dT) primed amplified cDNA from unactivated larvae. (3 & 5) SL2 and oligo (dT) primed amplified cDNA from activated larvae. Products in lanes 2 & 3 were amplified in the same thermal cycling run, products in lanes 4 & 5 were amplified simultaneously in a thermal cycling run separate from 2 & 3.

Figure 4. Products of oligo (dT) and arbitrarily primed PCR, electrophoresed through 1.2% agarose and ethidium bromide stained.



Lanes: (1) 1 Kb DNA ladder molecular size marker, sizes given in number of base pairs. (2 to 4) arbitrary and oligo (dT) primed amplified cDNA from unactivated larvae; (5 to 7) the same reactions as 2 to 4, performed on template from activated larvae.

Arbitrarily primed PCR

RNA fingerprinting with PCR, using various forms of arbitrary priming (Liang & Pardee, 1992; Welsh *et al*, 1992), has been applied to a variety of experimental systems to detect differentially expressed genes. Such PCR based methods have the advantage of greater sensitivity, low template material requirements, efficiency, and cost effectiveness over older methodologies such as differential screening of cDNA libraries or subtractive enrichment methods (McClelland, Mathieu-Daude & Welsh, 1995).

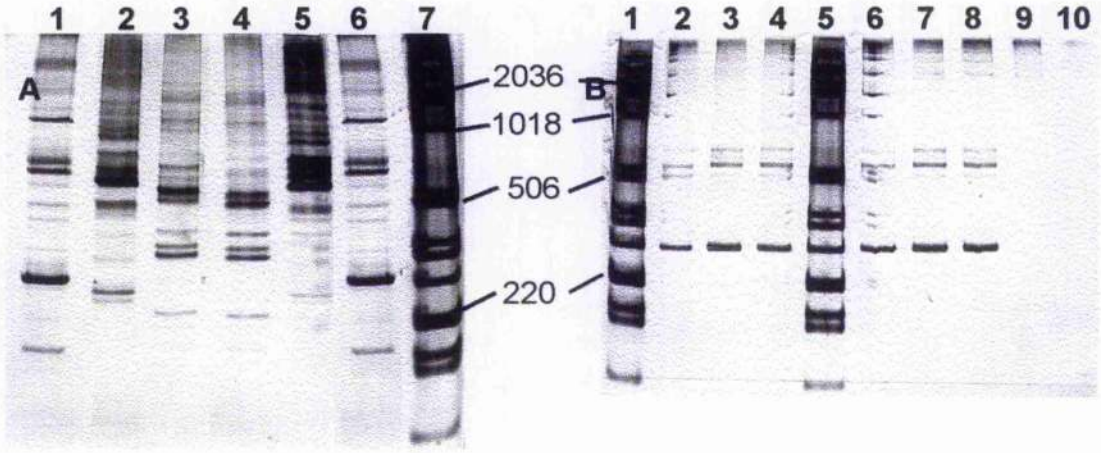
Arbitrary priming of templates in PCR amplification may serve a variety of purposes, and through variations of the reaction conditions the amount of products may be maximised or minimised. For the purposes of this study, reactions were designed to keep the number of products in a medium, and manageable, range, while still allowing representative sampling of the mRNA population with a realistic number of primers and reactions. To this end, the RADES method described by Murphy & Pelle (1994) was chosen as a basis for the design of the experiments used in this study. In this method, decamer primers of arbitrary sequence are used in the PCR amplification step, facilitating fairly stringent reaction conditions, which limit the number of products, reduce occurrence of random events, and improve the reproducibility of the experiments. The starting template for PCR was always oligo (dT) primed cDNA, differentiating this method from the well known RAP-PCR method described elsewhere (McClelland, Mathieu-Daude & Welsh, 1995). To further optimise the reactions, arbitrary primers were chosen to have 50% GC content, based on the AT richness, (only 35% G+C composition) of the *Trichinella* genome (Hammond & Bianco, 1992).

The results of PCRs primed by both one arbitrary primer and oligo (dT) were concatamers and an overabundance of products. Concatamers were visible just below the wells when the products were electrophoresed through agarose gels, with a high molecular weight smear of products trailing downstream (see Figure 4, p. 46). This product profile was consistent for ten different arbitrary primers tested at a range of annealing

temperatures (33° C to 40° C). The striking difference between this result, and the relatively low number of products obtained when only an arbitrary primer is used for amplification, (see below), is a function of the probability of any single primer functioning both forward and reverse on any one given template. The overabundance of products observed when oligo (dT) was used as the forward primer indicates that the chosen arbitrary primers had annealing sites on a large number of the opposite template strands. Although this made the combination of oligo (dT) and arbitrary primers useless for detecting single cDNA species, it was good evidence that the chosen arbitrary primers were adequate for representative sampling of the mRNA population. As subsequently shown by the results of the strictly arbitrary primed PCR described below, the number of templates for which any one arbitrary primer can function both forward and reverse is much reduced. In this case, representative sampling is achieved by carrying out multiple reactions with different primers.

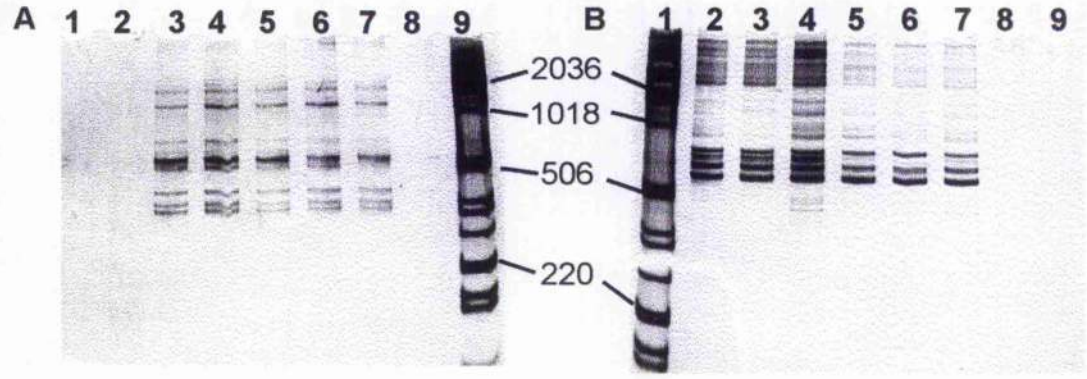
The purely arbitrarily primed PCR was carried out either with a single primer or with a combination of two primers. The number of products obtained from the singly primed reactions was primer dependent; a range from 100 to as few as 5 amplicons was observed, most reactions yielding about 40 products (see Figure 5, p. 49). The product profile obtained with any one primer was consistent among replicate reactions within an experiment (see Figure 5B and Figure 6, p. 49), as well as among different experiments using templates obtained from different RNA extraction and reverse transcription procedures, (see Figure 6, p. 49, comparing lane B4 with Figure 5, lane A5). This consistency enabled reliable comparisons between amplification patterns of unactivated and activated larval derived cDNA. However, no differentially amplified products were observed in any of the reactions. The reactions primed with two arbitrary primers did yield an overabundance of products in most cases, and proved less useful in comparisons of differential amplification patterns. As before, no differential amplification could be detected.

Figure 5. PCR products arbitrarily primed with a single primer, electrophoresed through 6% polyacrylamide and silver stained.



Lanes: (A7, B1 & B5) 1 Kb DNA ladder molecular size marker, sizes given in number of base pairs. (A1 to A3) amplified cDNA from unactivated larvae arbitrarily primed with a different primer per lane [P1, P2, P3]. (A4 to A6) the same reactions as A3 to A1, performed on template from activated larvae. (B2 to B4) amplified cDNA from unactivated larvae arbitrarily primed with primer P6. (B6 to B8) the same reactions as B2 to B4, performed on template from activated larvae. (9 & 10) Controls.

Figure 6. PCR products arbitrarily primed with a single primer, electrophoresed through 6% polyacrylamide and silver stained.



Lanes: (A9 & B1) 1 Kb DNA ladder. (A3 to A5) cDNA from activated larvae amplified with P5. (A6 to A8) unactivated cDNA amplified with P5. (B2 to B4) amplified cDNA from activated larvae primed with P2. (B5 to B7) unactivated cDNA amplified with P2. (A1, A2, B8, B9) Controls.

Conclusions

Although molecular aspects of the developmental biology of *T. spiralis* have remained undescribed until now, research has been conducted into biochemical and structural changes of the infective larvae as they establish an infection in the next host. The main aims of these studies have been to identify changes in the host / parasite interface during invasion, the timing of larval activation during the infectious process, and the signalling processes that lead to infective stage activation. The re-activation of *T. spiralis* has been noted to be accompanied by a change in the lipophilicity of the surface (Proudfoot *et al*, 1993a & 1993b), loss of the surface accessory layer; (Stewart *et al*, 1987; Modha *et al*, 1994), and metabolic changes (Stewart, 1983; Stewart, Raines & Kilgore, 1986), all occurring before the next molt. This evidence has given substantial support to the theory that infective larvae remain dormant until entry into the small intestine, and subsequent exposure to trypsin and bile (Despommier, 1983; Despommier, 1993).

The behavioural change observed after exposure of acid-pepsin isolated larvae to the enteric environment, on its own, may not be indicative of, or related to, developmental changes. In hookworms, (*Ancylostoma duodenale*), pharyngeal pumping and initiation of feeding are used as markers for resumption of development, but are preceded by secretion of the putative invasion factor ASP-1 by 6 h (Hawdon & Hotez, 1996). In *C. elegans* dauer larvae, however, pharyngeal pumping and resumption of feeding seem to precede any other changes during developmental re-activation (Wood, 1988; Reape & Burnell, 1991a). Thus, in nematodes, behavioural changes may or may not be linked to the same pathways as the biochemical and molecular markers of development. In infective *T. spiralis* larvae, nevertheless, the behavioural change is linked to changes in surface properties (Modha, Kusel & Kennedy, 1995), as well as structural changes. Migrating larvae have lost their surface coat and, at the electron microscopical level, display a diffused organisation within the cuticle, which in unactivated larvae is highly ordered in distinct strata. Further, an accumulation of rough endoplasmic reticulum and enlarged

mitochondria are evident in the hypodermis (Modha *et al*, 1994). No changes in larval physiology or structure have been detected in the absence of migratory behaviour. These collected observations are a strong indication that infective *T. spiralis* larvae do not restart development until exposure to the enteric cues of bile and trypsin, and, further, that the behavioural change is strongly linked to developmental activation. Thus, the *in vitro* activations in the here described experiments, monitored by tracking the behavioural change in the larvae, should represent the first step in resumption of development. This concept leads to the essential question of whether transcriptional activation is to be expected upon initial developmental activation. To date no information is available on the level of gene regulation involved in the type of structural changes observed in *Trichinella* larvae from comparable (analogous) systems. The extensive and structural nature of the changes makes control at the transcriptional level highly likely. More general observations from infective stages of other species of parasitic nematode, as well as from dauer larva emergence in *C. elegans,* give evidence that transcription initiation is an important part of resumption of development. Within 1 h of transfer to fresh medium containing food, *C. elegans* dauer larvae become committed to resume development (Golden and Riddle, 1984). Commitment to recovery is accompanied by a slight but reproducible increase in nuclear RNA polymerase II (pol II) activity to a rate approximately 23% that of actively growing worms. Exit from the dauer stage is accompanied by a temporally regulated pattern of transcription, with at least four distinct patterns of gene expression discernible (Dalley & Golomb, 1992). Although the contradictory results of pol II inhibition studies on dauer emergence with α-amanitin and actinomycin D only show effect during the post-dauer growth phase (Reape & Burnell, 1991a), it is quite probable that the inhibitors fail to penetrate the cuticle and are only taken up when the larvae are actively feeding. The compelling evidence, mainly from the work of Dalley & Golomb (1992) using nuclear run-on assays and northern blot analyses, clearly shows that transcription initiation is an important and early event in *C. elegans* dauer larva emergence. Petronijevic & Rogers (1983)

examined the switching on of 'parasitic gene sets' during the initiation of development in parasitic nematodes. They found actinomycin D to inhibit development of infective *Haemonchus contortus* to the fourth larval stage, but not to inhibit ex-sheathment of the infective larvae. Similar results were obtained with *Nippostrongylus braziliensis* (Bonner & Buratt, 1976). It has been argued that failure of the drug to penetrate might explain the lack of inhibition of ex-sheathment. In summary, all data collected in nematodes to date indicates that the resumption of development of arrested larval stages involves changes in gene expression at the transcriptional level. Why did the present study not pick up differences in any mRNA transcript abundance after putative activation of *T. spiralis* larvae?

PCR based RNA fingerprinting techniques have become well established and proven methods for examining stage or tissue specific transcription patterns (McClelland, Mathieu-Daude & Welsh, 1995). Although there are no previous reports on the frequency of *trans*-splicing in *T. spiralis*, the results of the here-described experiments indicate that *trans*-splicing of mRNA occurs extensively in *T. spiralis*. The issue with detecting differential expression of *trans*-spliced mRNA using spliced leader PCR remains one of relative abundance. The shear abundance of mRNAs with spliced leader sequence prevents the reliable and reproducible amplification of any one species among different experiments. The very nature of PCR ensures that, at non-optimal template concentrations, reactions will be driven in any one of several possible directions. Regrettably, it is the very universality of mRNA priming, which is what makes the method so attractive, that makes template optimisation an impossibility in the case of spliced leader and poly (dT) PCR. Another difficulty in detecting specific mRNA species, using spliced leader PCR, is that the reaction products are differentiated only by size, and not by any uniquely targeted sequences. Thus, it can be expected that a general background of reaction products will mask the presence of low abundance amplicons, as was indeed shown to be the case. Nonetheless, since replicates within experiments produced the same results, some limited

conclusions may be drawn from the data. None of the identifiable amplicons were differentially amplified, thus showing that none of the abundant *trans*-spliced mRNA seems to be differentially transcribed. None of the major changes, which one would associate with an initiation of extensive differentiation and growth, could be thus detected. Likewise, the RAcD failed to show the expected differences in transcription after activation. Although any negative results obtained by RAcD, or any other type of differential display analysis, must be considered as inconclusive, comparisons with the results obtained using similar methods in other biological systems, e.g. Murphy & Pelle (1994), may be indicative of the likely basis for the observed results. In the present case, it is highly probable that the lack of detectable differences in mRNA populations between *pre*- and *post*-activation larvae reflects a general lack of differences in transcription patterns. It is nonetheless notable that the RAcD analyses carried out in the here described study was by no means exhaustive, nor can it be considered to be completely representative of the biological facts.

Overall, it was considered a distinct possibility, at this stage of the investigation, that the *T. spiralis* larvae had initiated the sequence of events leading to resumption of development, and transcription of the required genes, before the *in vitro* activation. The most likely explanation, barring experimental artefacts, was that the larvae were activated upon liberation from the nurse cells during the recovery process. This would mean that, *in vivo,* larvae were actually activated in the stomach, and not, as thought until now, in the intestine. The changes observed after the *in vitro* activation with trypsin and bile would therefore be a continuation of an already started developmental process. There were, however, some distinct difficulties with this early activation theory. One major obstacle was the fact that the accessory layer of the cuticle was thought to exclude environmental stimuli from larval sense organs until stripped off by bile and trypsin in the intestine (Stewart *et al*, 1987). The investigation of the possibility of larval activation by environmental stimuli before loss of the accessory layer is subject of the following chapter.

3. ENVIRONMENTAL CUES AND TIMING OF ACTIVATION: ROLE OF THE ACCESSORY LAYER

INTRODUCTION

A model of *T. spiralis* developmental activation has been postulated, based on ultrastructural, biochemical and behavioural observations (Stewart *et al*, 1987; Modha *et al*, 1994). This model delineates activation of the quiescent infective stage via putative chemosensory cues within the host intestine. It has been suggested that trypsin and bile mediated loss of the surface accessory layer is important in facilitating chemosensation, by exposing the sensory neurone endings to the environment (Stewart *et al*, 1987). The accessory layer may, therefore, act as a barrier that prevents the reception of environmental cues, which could elicit an activation response at an inappropriate time and place.

Scanning electron microscopy (SEM) has revealed some structural differences in surface morphology between infective larvae and mature worms (Kim & Ledbetter, 1980). Adult worms have 14 clearly visible sensory structures, seen as pores, at the anterior end. Infective larvae, in contrast, have a completely smooth cephalic dome, the only distinguishable feature being the 1.5 μm oral slit. No other structures are visible in this region; notably no sensory structures can be detected. Lee, Wright & Shivers (1984) also recorded that the accessory layer extends over the entire surface, into the mouth and anal openings, and covers openings of the sense organs.

Analysis of mRNA of infective larvae, *pre-* and *post- in vitro* activation with trypsin and bile in mammalian culture medium, however, failed to show any differences in transcript profiles. Since initiation of development is expected to be accompanied by transcription of hitherto inactive genes (see previous chapter), the possibility of developmental activation at an earlier time was considered.

In *C. elegans,* amphidial neurones are critical in the regulation of development, especially in the process of initiating or terminating developmental arrest (Bargmann, Thomas & Horvitz, 1990; Bargmann & Horvitz, 1991). This regulatory process involves integration of environmental information with temporal gene regulation. Thus, amphid mediated chemosensation may well be critical in *T. spiralis* developmental activation.

The fact that the accessory layer covers the chemosensory receptors of the amphidial neurones of infective *T. spiralis* larvae is well documented. The question to be addressed here was whether the accessory layer prevents environmental cues from reaching the amphid receptors.

The larval surface

In many species of nematode, the cuticle surface may be covered with a carbohydrate-containing surface coat. In adenophorean nematodes, the surface coat may be secreted from epidermal gland cells, pores in the cuticle, or from the caudal glands (Bird & Bird, 1991). A variation of this kind of surface structure, the accessory layer, differs from the surface coat in that it is attached to, and moves with, the nematode. It is not a universal feature of nematodes, and appears only in some developmental stages of some species as a result of special functional/ anatomical requirements. The accessory layer of the first stage larvae of *T. spiralis* is an example of this kind of specialised structure.

The accessory layer of the cuticle appears early in the development of first-stage larvae of *T. spiralis* in nurse cells, and remains on the larvae through pepsin-HCl digestion *in vitro* (Despommier, 1983). It has been suggested that this accessory layer, which has been estimated to be about 15 nm thick, appears to remain in place until the entire cuticle is lost at the following moult, which takes place in the intestinal epithelium of the subsequent host (Wright & Hong, 1989). The majority of evidence, however points to the accessory layer being lost, or changing dramatically, early during the establishment of infection in the host

intestine (Modha, Kusel, & Kennedy, 1995; Modha *et al*, 1994; Stewart *et al*, 1987). All of the developmental stages subsequent to the infective L1 have been demonstrated to lack the accessory layer (Lee, Wright & Shivers, 1986).

Freeze-fracture studies of the surface accessory layer of infective larvae have been performed (Lee, Wright & Shivers, 1984; Wright & Hong, 1988; Gounaris, Smith & Selkirk, 1996), and a highly unusual ultrastructure of the larval surface was discovered. Two similar models have subsequently been proposed to account for the morphology of the accessory layer. Wright & Hong (1988) have proposed a model of the composition and structure of the accessory layer of infective (muscle stage) larvae involving a globular outer layer and a filamentous inner layer. According to this model, the outer layer consists of globular proteins, perhaps anchored by polysaccharide groups to the filamentous lipid layer. The proteins, as organised on the surface of the muscle larvae, are resistant to pepsin and trypsin. Notably, larvae which had the globular layer removed with detergents did not survive the stomach digest. Pepsin or trypsin did not degrade the predominantly lipid filamentous component of the accessory layer, even if the surface proteins had been removed with detergent. It is most likely that the filaments of the accessory layer are with a hydrophilic core, forming tubular micelles. Gounaris, Smith & Selkirk (1996) have also characterised the accessory layer and proposed a model of its structure. This model suggests that the accessory layer is composed of two extensive sheets of lipid in a non-bilayer configuration which overlay a conventional lipid bilayer. The non-bilayer configuration appears to be of the hexagonal type II (HII) arrangement with an estimated average diameter of the cylinders of 6.8 nm. This structure seems stable, remaining unchanged in response to variations in temperature between 20° C and 45° C, and shows no pH dependency of the adopted structures. This unusual configuration is probably stabilised by the associated (glyco)proteins, which are proposed to traverse both the distal HII cylinders and the outer monolayer. The authors further propose that the intestinal worms seem to have a differently arranged accessory layer, with different properties. According to both models, the accessory

layer is a highly hydrophobic structure, and potentially a barrier to any hydrophilic molecules.

Sensory neurones in *T. spiralis*

The nervous system of *T. spiralis* L1 larvae basically consists of a cephalic nerve ring that gives rise to four main nerve cords: two lateral, one dorsal, and one ventral. Nothing is known about the number of ganglia in the L_1 larvae, but the adult worm possesses six in its anterior end. There are no obvious sensory papillae at either end of the larvae (Despommier, 1983).

No detailed description of the sensory structures of *T. spiralis* has been recorded, but extensive studies have been carried out on other members of the phylum and class (McLaren, 1976). In general, the anterior sensory organs of nematodes consist of circles of structures arranged in a hexaradiate pattern. This pattern comprises a head with six lips containing 12 labial sensilla, four cephalic sensilla, and two amphids (Bird & Bird, 1991). There are some pronounced differences between the Secernentea and Adenophorea in the detailed structure of the sensory organs. Notably, the neuronal elements are more numerous in the Adenophorea than in the Secernentea, and the amphids are more prominent in the Adenophorea, containing more receptors (10-46) than do the Secernentea (3-15). The anterior sensory structures send sensory input to the anterior nerve ring through six nerve bundles. The processes from the non-amphidial sensilla have their cell bodies in front of the nerve ring in the anterior ganglion, whereas fibres from the amphids run past the nerve ring to their bipolar cell bodies in the lateral ganglia. Backwardly directed processes from sensillar bipolar neurones rejoin the process bundles and pass to the outer surface of the nerve ring, where they turn and enter the posterior part, move to the inner surface, and turn once again to the anterior region where they have their synaptic interactions. The process bundles from an amphid also run in the process bundles from each lateral labium. They run posteriorly past the nerve ring and connect with their bipolar neurones in the lateral ganglia as mentioned above.

Most of these nerve cells send processes into the ventral cord by way of the amphid commissures that project into the nerve ring. (Bird & Bird, 1991).

Amphids are the largest and most complex of the cephalic sense organs. The distal part of the amphid (fovea) opens to the exterior via a prominent pore through which material, apparently secreted by the amphidial gland, may flow. The distal part is connected by means of a pore with the amphidial duct, which merges posteriorly into the sensillar pouch (fusus) into which the amphidial dendrites protrude. The distal part, amphidial duct, and anterior part of the sensillar pouch are lined with cuticle. The posterior and major part of the sensillar pouch is formed by the amphidial gland. The amphid has many more receptors than do other sensilla (Bird & Bird, 1991; McLaren, 1976).

It has been demonstrated that the amphids are the site of integration of environmental information and temporal regulation, controlling developmental decisions in *C. elegans*. This fact, in conjunction with the high degree of development and structural complexity of the amphids in Adenophorean nematodes, makes it probable that amphid mediated chemosensation is crucial to developmental activation of *T. spiralis* infective larvae. The amphidial pores, however, are covered by the accessory layer of infective *T. spiralis* larvae until invasion of the intestine of the next host. The present study was conducted, therefore, to test whether infective larvae could receive environmental cues via the amphids before loss of the accessory layer, and thus resume development before entering the intestine of the next host.

MATERIALS AND METHODS

The initial criterion for activation of the infective larvae was the behavioural change from coiling to migrating, which had been described by Stewart *et al* (1987). Hence, initial experiments concentrated on establishing the environmental conditions necessary for inducing the migratory behaviour. To link this behavioural change to developmental activation, as has been documented (Modha, Kusel & Kennedy, 1995; Modha *et al*, 1994; Stewart *et al*, 1987), the correlation between changes in surface properties and behavioural changes were examined. To test the effect of the surface accessory layer on larval reception of environmental stimuli, amphid chemosensory receptor labelling was undertaken. Finally, conditions leading to the accessibility of amphids to environmental cues, changes in behaviour, and alterations of surface properties were all examined together.

Parasite maintenance and recovery

Parasites were maintained in BALB/c mice as before. Recovery of infective larvae was carried out as described in *Parasite maintenance and recovery*, p.33 (please note that the digest time for all experiments was 2 h, unless stated otherwise). It is important to note that the entire sedimentation process took about 1.5 h. The sucrose and Percoll centrifugations were not carried out, but sedimented larvae were used directly for experiments, or stored at 4° C.

After recovery, 30 µl of larval suspension were examined under the light microscope in order to estimate the number of larvae present and to ascertain the condition of the larvae.

Behaviour of infective larvae *pre-* and *post-* stimulation with environmental cues

Behaviour of larvae after acid-pepsin recovery

Observations were carried out with 300 larvae per experimental condition, and the experiments were repeated for three separate larval recovery processes. Acid-pepsin isolated larvae were suspended in PBS (6.8 mM $Na_2HPO_412H_2O$, 3.7 mM $NaH_2PO_42H_2O$, 154 mM NaCl, pH 7.2), or 0.9% saline pH 7.2, at room temperature (20° C), and viewed under the light microscope at 37° C in a hot room. Freshly isolated larvae, and larvae that had been stored at 4° C for 24 h, were incubated in RPMI 1640 medium at 37° C for 45 min, and subsequently observed under the light microscope at 37° C in a hot room. Larvae were also incubated in PBS at 37° C for 45 min and subsequently observed under the light microscope at 37° C.

Behaviour of larvae after exposure to trypsin and bile

Observations were carried out with larvae obtained from three separate recovery processes. Acid-pepsin isolated larvae (600) were suspended in RPMI 1640 medium (GibcoBRL) containing 5% bile (Sigma) and 0.25% trypsin type I (Sigma) and incubated at 37° C for 45 min. Subsequent to the incubation the larvae were washed three times in PBS equilibrated at 37° C, and viewed under the light microscope at 37° C in a hot room.

Staining of sensory neurones with fluorescein isothiocyanate

To examine whether the cuticle accessory layer of the infective larvae interferes with signal reception of the amphidial sensory endings, attempts at differential staining of the neurones before and after treatment with trypsin and bile were carried out. The fluorescent dye fluorescein isothiocyanate (FITC) was chosen since previous research has shown it to be effective in staining amphidial neurones of *C. elegans* (Hedgecock *et al,* 1985). It was postulated that the isothiocyanate group of the molecule would bind with protein receptors of the sensory neurone, the recycling of which would then stain the cell body and its processes.

All experiments were repeated at least three times for each batch of recovered larvae. Four larval recoveries were used for each experimental condition.

Autofluorescence control

Both unactivated larvae and larvae activated in medium containing trypsin and bile were examined under the fluorescence microscope with fluorescein and rhodamine filters for autofluorescence. Reference images were taken with a digital image capture device.

Staining of neurones in activated larvae

Larvae (≈1000) were activated *in vitro* with trypsin and bile in medium (see above), washed with medium three times, and maintained in medium at 37° C for the duration of the experiments. Aliquots of ≈50 larvae were subsequently suspended in 1 mM FITC in PBS at 37° C for 20 min. Following the incubation, the larvae were washed four to five times with PBS at 37° C. Samples of the FITC exposed larvae were then examined with a fluorescence microscope.

Staining of neurones in unactivated larvae

Separate aliquots of ≈50 unactivated larvae were incubated in 1 mM FITC in PBS at 20° C for 20 min. The larvae were washed four to five times with PBS and viewed with a fluorescence microscope. Staining of unactivated larvae with 1 mM FITC was also carried out at 37° C (same procedure as above).

Control incubations were carried out with the same PBS in which the FITC was dissolved. Incubation times were extended up to 2 h at 37° C.

Structural requirements for activational activity of selected compounds

After the discovery that FITC has an effect on larval behaviour in a manner analogous to activation with trypsin and bile, investigations into structural features critical to the activity of the compound were undertaken.

Derivatives of fluorescein and compounds with chemical structure analogous to parts of the FITC molecule were used in ascertaining which parts of the FITC interacts with the *T. spiralis* larvae, effecting the observed change in behaviour. The compounds were tested for activational activity under the same conditions as FITC. Comparison of results achieved with various chemicals and substructures of the molecules revealed which functional groups are important in the interaction leading to activation-type behaviour. All experiments were repeated at least three times for each batch of recovered larvae. Three larval recoveries were used for each experimental condition. Each experiment included a control incubation in the same PBS in which the test compound had been dissolved.

Fluorescein diacetate

Fluorescein diacetate (FDA) is a non -fluorescent compound derived from fluorescein by acetylation of the oxygens bound to the two outermost aromatic rings. This compound was chosen to test whether the triple ring structure found in FITC played a part in the observed activity. FDA taken up by cells is usually modified by endogenous esterases which cleave the acetate groups from the fluorescein group, making the molecule fluorescent.

A 10% solution of FDA in acetone was further diluted with PBS to yield a solution of 0.05% (1 mM) FDA in acetone/PBS. Unactivated larvae (300) were incubated in the 1 mM FDA solution at 37° C for 20 min. The larvae were subsequently washed 5 times in PBS and examined with a fluorescence microscope.

Unactivated larvae were incubated in 0.05% v/v acetone/PBS at 37° C for 20 min, then washed 5 times with PBS as a control.
Trypsin and bile activated larvae were also incubated in 1 mM FDA solution (see above) at 37° C for 20 min (subsequently washed 5 times with PBS).

All of the above experiments were replicated at 20° C.

Fluorescein

As the major molecular component of FITC, fluorescein was tested for activating activity. Unactivated larvae were incubated in 1 mM fluorescein in PBS at 37° C for 20 min, washed 5 times with PBS, and examined with a fluorescence microscope.

As a control, larvae were also exposed to fluorescein at sub-activation temperatures. The unactivated larvae were incubated in 1 mM fluorescein in PBS at 20° C for 20 min, then washed 5 times in PBS. Three groups of unactivated larvae which had been incubated in 1 mM fluorescein in PBS at 20° C for 20 min and subsequently washed 5 times with PBS, were incubated in PBS at 37° C for 20 min.

Fluorescein activation was also tested at physiologically more relevant concentrations. Larvae were recovered with a 35 min acid-pepsin digest in order to minimise any possible priming effect during the recovery procedure. Recovered larvae were incubated in 10 μM fluorescein at 37° C for 20 to 25 min. Incubation was also carried out in 0.05% agar containing 5 μM fluorescein at 37° C for 20 to 30 min. Controls were in 0.05% agar only, at 37° C, for the same time.

Phenyl Isothiocyanate

Although it is a potent poison, phenyl isothiocyanate (PITC) was tested for activity, being a molecular component of FITC. Unactivated larvae were incubated in 5 mM PITC in PBS at 37° C for 20 min and subsequently observed with a light microscope. Controls were incubated in 5 mM PITC in PBS at 20° C for 5 min to 20 min at 5 min intervals.

Dithioerythritol

Binding of the isothiocyanate group of the FITC molecule to a putative protein receptor of the larva by a simple chemical reaction involving disulfide interactions might be causing changes to the receptor responsible for mediating the observed change in behaviour. As an indicatory test whether simple thiol-reactions were involved, unactivated larvae were incubated in 5 mM dithioerythritol in PBS at 37° C for 20 min, then washed 4 times in PBS. Larvae were subsequently observed with a

light microscope at 37° C in a hot room. Controls were incubated in 5 mM dithioerythritol in PBS at 20° C for 20 min.

Activity of naturally occurring compounds

5-hydroxy tryptamine

After the discovery of the biological activity of fluorescein, natural substances with similar structure were sought. Serotonin was a good candidate, due its documented role in the activation of other helminths, and its synthesis in the proximal intestine (Mettrick, 1989). Larvae were recovered by a 35 min acid-pepsin digest to reduce any possible priming effect as much as possible. Recovered larvae were then allowed to cool down and checked for coiling behaviour. Larvae were incubated in 10 µM, 1 mM, or 10 mM 5-hydroxy tryptamine (serotonin) at 37° C for 20 min. Controls were incubated in saline under same conditions.

Activation-related changes in surface properties

Loss of the accessory layer, as associated with developmental activation by trypsin and bile containing medium, has been shown to correlate with changes in lipophilicity of the larval cuticle, as well as the cuticle's affinity for membrane linkers. The capability to detect these changes using fluorescent lipid probes made it possible to examine the nature of the observed activation following exposure of larvae to fluorescein. The question whether loss of the accessory layer observed upon activation is host- or parasite-induced could thus also be examined.

All experiments were repeated at least three times for each batch of recovered larvae. Three larval recoveries were used for each experimental condition.

Insertion of the lipid probe AF18 pre- and post-activation

All experiments were carried out using a solution of 0.0005% v/v 5-*N*-(octadecanoyl) aminofluorescein (AF18) obtained by a 1 in 100 dilution of 0.05% w/v AF18/ethanol solution with PBS.

Samples of larvae activated with trypsin and bile, as well as samples of unactivated larvae, were incubated with AF18 at 37° C for 5 min, and subsequently washed 4 times with PBS. The larvae were examined with a fluorescent microscope, and fluorescence was measured by digital gray level analysis (see *Image capture and analysis*, p. 66).

Larvae activated with 1 mM fluorescein in PBS at 37° C for 20 min were incubated with AF18 at 37° C for 5 min, and subsequently washed 4 times with PBS. Controls were incubated in PBS. Fluorescence was measured as above.

Labelling of the accessory layer with the lipid probe PKH26

Larvae were labelled with the fluorescent lipid probe PKH26 (Sigma) according to the manufacturer's instructions (3.0 µl lipid probe per 100 µl diluent 'C').

Unactivated, or trypsin and bile activated, larvae were incubated with PKH26 at 20° C for 10 min. The larvae were washed 3 times with chilled PBS (4° C) and viewed with a fluorescent microscope using a rhodamine filter. Reference images were taken with a digital image capture device.

Larvae activated with 1 mM fluorescein in PBS at 37° C for 20 min were incubated with PKH26 at 20° C for 10 min, and subsequently washed 3 times with chilled PBS. Controls were incubated in PBS as well as RPMI 1640 medium at 37° C prior to labelling as above. Larvae were also incubated in 1 mM fluorescein in PBS at 20° C for 20 min prior to labelling.

To clarify the timing of surface property changes, larvae were incubated with PKH26 immediately after 35 min digest recovery, involving only one 15 min sedimentation. Larvae from the same recovery were used to infect mice (2000 larvae per mouse), or were incubated in saline at 37° C for 60 min. The larvae were recovered from the infected animals 2 h post infection. The small intestines were removed from the mice, slit open, and rinsed three times with saline. The rinsed intestines were suspended in fine mesh netting and incubated in 50 ml saline at 37°C for 10 min. To ensure that only worms which had successfully invaded the epithelium would be used for experimentation, all sedimented worms were discarded.

A subsequent incubation was carried out in fresh saline at 37°C for 25 min, and recovered juveniles washed twice with saline and used immediately for surface labelling with PKH26.

Image capture and analysis

All experiments were documented by capturing live fluorescent or bright light images using a digital image capture device (custom made with a Bosch low light camera).

Image analysis was performed on the captured images using the free UTHSCSA ImageTool program (developed at the University of Texas Health Science Center at San Antonio, Texas and available from the Internet by anonymous FTP from ftp://maxrad6.uthscsa.edu).

RESULTS AND DISCUSSION

The surface accessory layer of *T. spiralis* seems to cover the entire surface of infective muscle larvae, as observed by scanning electron microscopy and freeze-fracture studies (Kim & Ledbetter, 1980; Lee, Wright & Shivers, 1984). The apparent loss of this surface structure during invasion of the host intestine has been attributed to the activity of trypsin and bile (Stewart *et al*, 1987). Further, a role in the regulation of amphid mediated chemosensation, via blockage of environmental stimuli, has been postulated for the accessory layer. Since amphidial neurones clearly play a crucial role in the regulation of development in *C. elegans*, specifically in aspects of control of developmental quiescence or activation, amphid-mediated chemosensation is probably pertinent to resumption of development in *T. spiralis*. The possibility of early activation of developmental processes in the stomach environment having become evident (as described in the previous chapter), it was important to test whether amphidial nerve endings were accessible to environmental stimuli despite the presence of the accessory layer. To achieve this, amphid labelling studies before and after *in vitro* activation with trypsin and bile were carried out.

Confirmation of *in vitro* behavioural activation conditions

The striking difference in behaviour, observed between larvae within nurse cells and enteral juveniles, is the most obvious marker of biological changes within the worm during the process of infecting the next host. This behavioural marker has been correlated with structural and physiological changes (Stewart *et al*, 1987; Modha *et al*, 1994), and has become accepted as an indicator for stage of development of *T. spiralis* larvae. The standard method for isolating muscle larvae, involving a lengthy (1 – 2 h) sedimentation procedure in saline, invariably yields larvae which display the same behaviour as larvae *in situ* within nurse cells (see Figure 7, p. 69). These larvae remain coiled, moving only by uncoiling and recoiling movements, which increase with respect to speed

and extent of movement as temperature increases. This observation has contributed to the theory that larvae do not respond to acid-pepsin digest (Despommier, 1983), and that the stomach phase of the infectious process is irrelevant to the life cycle (Despommier, 1993).

As a prelude to investigating sensory neurone interactions with the environment, it was considered important to test the effects of buffers and solutions, used for washing, suspending or incubating the larvae in this laboratory, on larval behaviour. This allowed standardisation of behaviour assays, and permitted the definite exclusion of the tested buffers and media from consideration for activating properties.

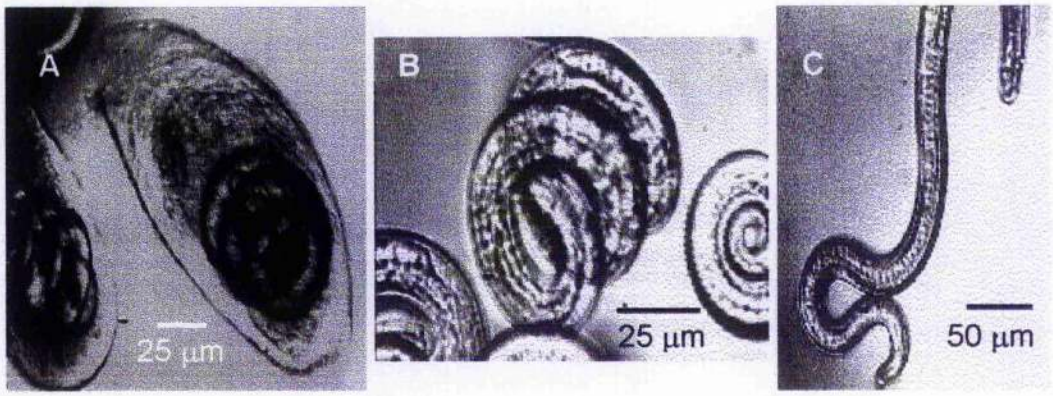
None of the tested solutions, PBS, RPMI 1640 medium, and 0.9% saline, had any effect on larval behaviour under the conditions tested. Neither the temperature, nor the length of incubation changed the activity of the tested solution. The temperature dependent variation in coiling rate, increasing to a maximum at 37° C, was equally evident in all experiments, regardless of the specific chemical environment. Rapid coiling and uncoiling of the posterior part of the larvae was observed, the anterior end always remaining tightly coiled. The fact that the experiments and microscopic observations were carried out in a constant environment of 37° C allowed for a continuous assessment, and eliminated artefacts due to environmental changes in transit from incubation to observation.

In accordance with previous reports, the incubation in trypsin and bile containing medium resulted in the change to migratory behaviour. This behaviour continued to be manifest even if the activating stimuli were removed and replaced with PBS. Active migration of the larvae was observed, as well as rapid probing with the anterior end in conjunction with pharyngeal pumping.

Staining of neurones with fluorescein isothiocyanate

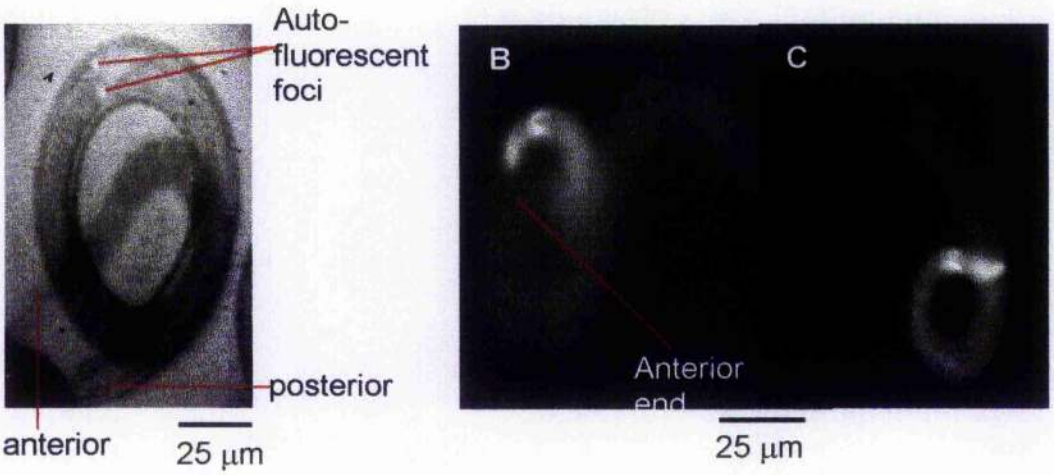
The labelling of sensory neurone endings and cell processes by non-invasive techniques was faced by several difficulties. Neurones are usually traced by microinjection of fluorescent dyes, like lucifer yellow, DiI

Figure 7. Larval behaviour in different microhabitats and developmental states.



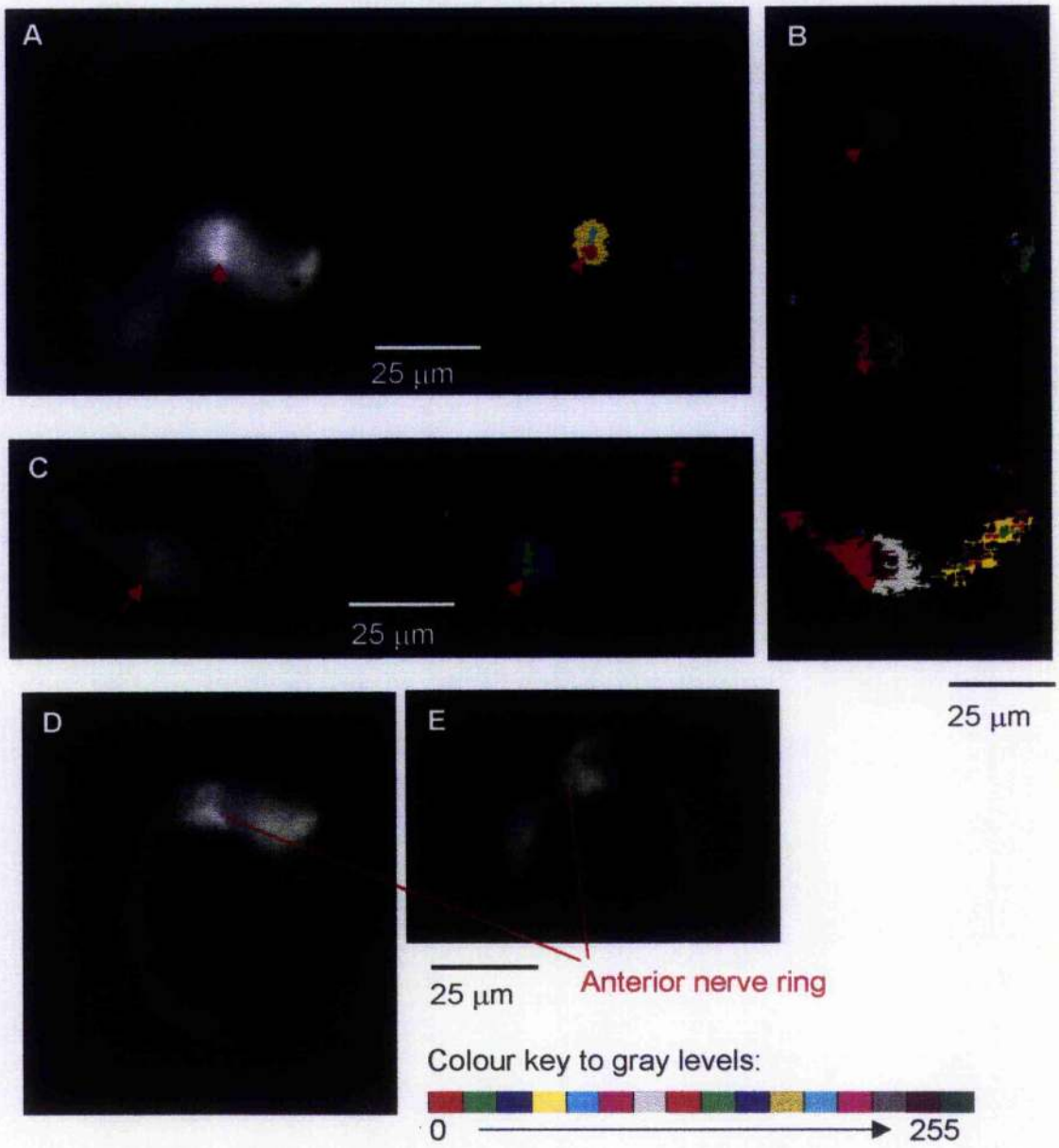
Nomarski interference photomicrographs of *T. spiralis* larvae: (A) *in situ* within isolated nurse cells, (B) isolated by 2 h acid-pepsin digest, in saline, and (C) developmentally active intestinal stage.

Figure 8. Fluorescence images of infective larvae.



(A) Larva activated with trypsin and bile, viewed under combination of phase contrast and fluorescence (fluorescein filter, ≈500 nm emission) microscopy. (B & C) Trypsin and bile activated larvae labelled with FITC and viewed with a fluorescence microscope. Cell bodies posterior to the nerve ring, with processes running anteriorly to the nerve ring, are visible. Labelling at the extreme anterior tip may involve filling of the buccal cavity.

Figure 9. Details of FITC labelled cell bodies.



Fluorescence photomicrographs and false colour gray level maps of FITC labelled neurones. (A) Arrow points to amphidial cell body. The gray level map clearly shows that two distinct bodies are labelled. (B & C) Two distinct cell bodies are labelled, as well as a posterior commissure. (D & E) Part of the anterior nerve ring, as well as the posterior amphidial cell bodies are labelled.

or DiO (Haugland, 1996). Even then, the tracing of cell processes may take many hours, which is rather impractical when examining rapid developmental processes. The issue at hand, however, demanded that the labelling substance find access to the neurone from the external milieu, making its way (or not) through the putative barrier of the accessory layer. Further, since no information on relevant chemosensory receptors of *T. spiralis* amphids was available, the labelling substance would have to be relatively non-specific. In addition, the absence of any definition of specific activating molecular cues, and their active ligands, precluded the search for, and utilisation of, specific neural receptor mediated probes.

Hedgecock *et al* (1985) described amphid filling in *C. elegans* using FITC applied externally. It has been shown that the dye enters chemosensory neurones through their exposed receptor cilia (Perkins *et al*, 1986). The exact process remains unknown, but, for the purposes of the *T. spiralis* experiments, I suggest that the isothiocyanate group of the molecule binds receptor proteins, which transport the dye throughout the sensory neurone upon being recycled. Further, once bound and internalised, the dye cannot be removed from the neurone. Hedgecock *et al* (1985) present strong evidence that FITC filling of amphidial neurones in *C. elegans* is harmless, since stained worms have normal growth rate, brood size, and mating ability. Since FITC is a hydrophilic substance, it served as an ideal test of the barrier properties of the accessory layer of *T. spiralis* infective larvae.

Autofluorescence control

Checks for autofluorescence under UV excitation were carried out using both fluorescein and rhodamine filters. The larval surface fluoresces noticeably when viewed under a fluorescein filter, with two foci of intense autofluorescence present halfway along the body of the larvae. These autofluorescent spots appear right at the edge of the posterior terminus of the stichocyte region (see Figure 8a, p. 69). What structures these foci correspond to is unknown. No difference in level or pattern of autofluorescence was observed between trypsin and bile treated and

unactivated larvae. No autofluorescence was detected using a rhodamine filter.

Staining of neurones in activated larvae

Incubation of trypsin and bile activated larvae in 1 mM FITC/ PBS solution at 37° C for 20 min resulted in labelling of distinct structures in the anterior region only (see Figure 8b & c, p. 69; Figure 9, p. 70). The majority of examined larvae (65 – 80%) showed the typical migratory behaviour associated with activation, and no other effects of the FITC labelling could be detected. Notably, labelling took place only in larvae which displayed activation-type behaviour.

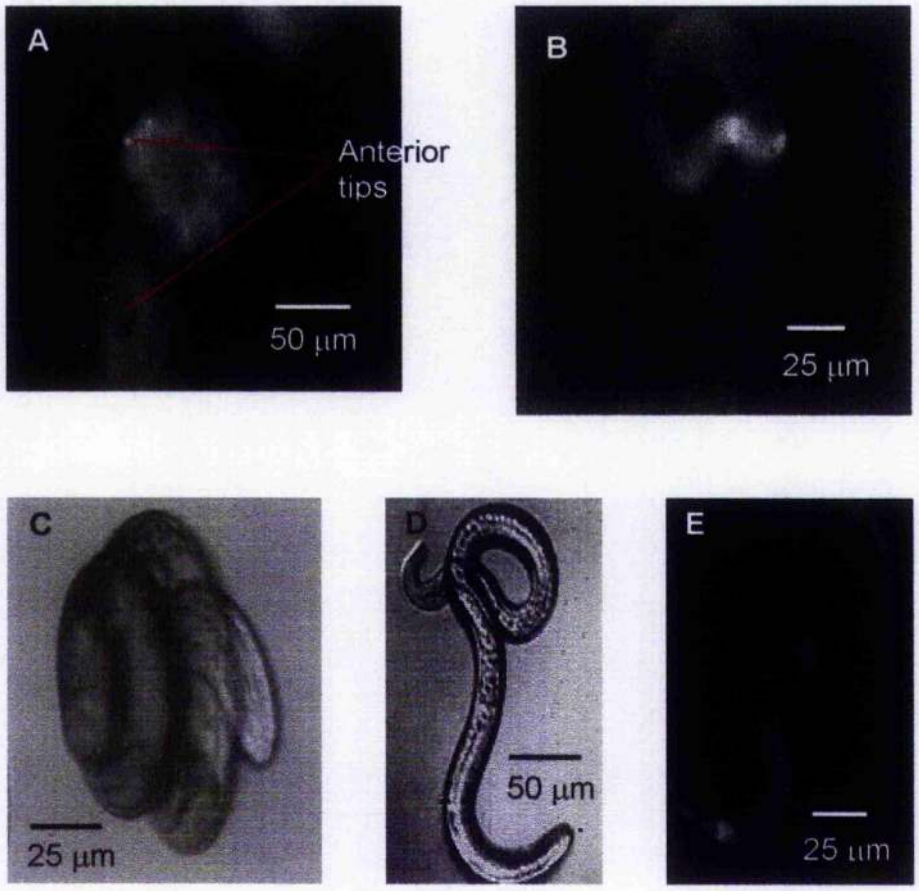
Closer analysis of the labelled structures reveals two, brightly labelled, punctate bodies, which are connected anteriorly to a band-like structure (see Figure 9, p. 70). The labelled structures fit exactly the description of amphidial neurone cell bodies posterior of the anterior nerve ring, with amphidial processes connecting the two types of structures (Bird & Bird, 1991; Hedgecock *et al*, 1985). This result presented the first evidence that amphidial neurones play an important role in chemosensation in *T. spiralis* larvae during this stage of development. It also demonstrated the feasibility of using FITC labelling of amphids in testing the barrier properties of the accessory layer in larvae prior to exposure to trypsin and bile.

The fluorescence at the very anterior tip is more difficult to interpret, but seems to emanate from the buccal cavity. The general surface fluorescence was slightly higher than the observed autofluorescence, and must result from binding of small amounts of FITC to surface proteins.

Staining of neurones in unactivated larvae

Staining of "unactivated" larvae with FITC at 20° C, immediately after the recovery procedure, did not result in neuronal uptake of the dye (see Figure 10, p. 73). Fluorescence was solely detected in the anterior of the buccal cavity, along with slight surface fluorescence, which was attributed to the autofluorescence reported above. This result is not entirely

Figure 10. Staining of "unactivated" larvae with FITC.



(A) Staining of "unactivated" larvae with FITC at 20° C. Notice slight filling of the buccal cavity, but complete lack of amphid labelling. (B & E) Staining of "unactivated" larvae with FITC at 37° C. Notice probing behaviour of the anterior tip, as well as labelled cell bodies in the region of the anterior nerve ring. (C) Light micrograph of "unactivated" larvae incubated with FITC at 20° C. (D) Light micrograph of "unactivated" larvae incubated with FITC at 37° C.

surprising, since it is known that *T. spiralis* larvae are only able to establish infection within a very narrow range of environmental temperatures, between 35° C and 38° C (Despommier, 1983). *C. elegans*, however, will take up FITC into the amphidial neurones at 0° C in the same manner as at 20° C (Perkins *et al*, 1986). There must, therefore, be a fundamental difference in the amphid filling mechanisms of *T. spiralis* and *C. elegans*, unless the accessory layer of the parasite presents a barrier to environmental cues at this temperature (20° C).

Incubation of "unactivated" larvae, immediately after recovery, in FITC at 37° C gave two striking results. Firstly, the amphidial neurones labelled in the same manner as in trypsin and bile treated larvae, and, secondly, the larvae displayed migratory behaviour characteristic of enteral worms. The observed amphid labelling pattern, (see Figure 10b, p. 73), gives strong evidence that the sensory neurone endings are accessible to environmental stimuli under physiological conditions, despite presence of the accessory layer. This brings back the question of why the amphids could not be labelled at 20° C. To elucidate this point, trypsin and bile activated larvae (which have lost the accessory layer), were incubated in FITC at 20° C. The results revealed that the amphidial neurones would not label, and thus indicated that reduced neural activity, (or temperature dependence of FITC – sensory receptor interactions) were responsible for lack of staining at 20° C.

The behavioural change to activation associated migration, stimulated by exposure to FITC, provided a tool to gain further insight into signalling mechanisms which regulate activation. Initially, however, experiments were restricted to confirming this result, and elucidating the mechanism by which FITC affects chemosensory mediated regulation of larval biology. The suspicion, that stock PBS or FITC were contaminated with an unknown substance to which the worms reacted, proved unfounded. Repeated incubations in the stock PBS solutions, (up to 2 h at 37° C), failed to induce any behavioural changes, and utilisation of fresh FITC caused the same activation-related migratory behaviour. Multiple repeats

of the FITC labelling experiments with "unactivated" larvae replicated the result of behavioural activation at 37° C. Notably, the observations of migratory behaviour of the infective larvae took place after the initiating stimulus, (FITC), had been removed by multiple washes with PBS. Labelled larvae continued to show migratory behaviour for up to 25 min after being taken out of the FITC and suspended in fresh PBS (if the temperature was kept at ≈ 37° C). Migrating larvae which were cooled quickly "froze" into extended shapes, while larvae which were cooled slowly would eventually resume a coiled posture.

Once more the difference between *C. elegans* amphidial labelling and the labelling of *T. spiralis* amphids was evident. FITC seems to have no effect on *C. elegans* behaviour or physiology (Hedgecock *et al*, 1985; Perkins *et al*, 1986), yet it stimulates activation-type behaviour in *T.spiralis*. The initial hypothesis, to explain the observed effect on *T. spiralis* larvae, involved a model in which the interactions between the isothiocyanate group of the FITC molecule and the chemosensory receptor protein effected a signalling cascade. A group of FITC related compounds were used to test this hypothesis.

Structural requirements for activation activity of selected compounds

The identification of the mechanism of interaction between the FITC molecule and the sensory receptor would not only give a tool for studying signalling pathways involved in activation, but also give an indication of what environmental biochemical cues infective larvae respond to. As a first step, it was important to identify the structural group of the FITC molecule which mediates the reaction. To this end, derivatives of fluorescein and subunits of the FITC molecule were tested for stimulation of behavioural activation of infective *T. spiralis* larvae.

Activity of fluorescein compounds

Fluorescein

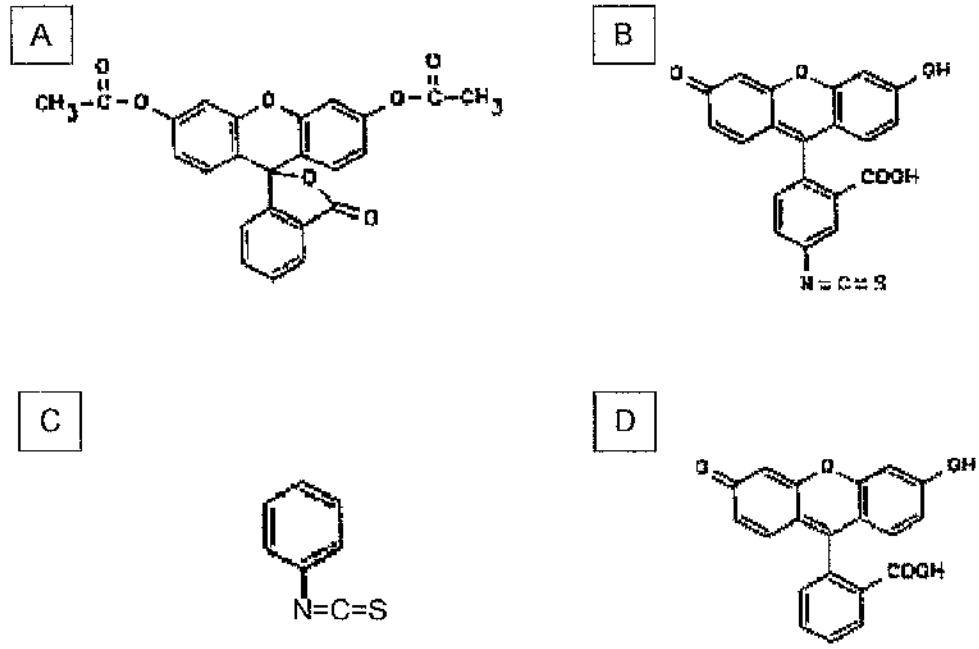
The major molecular substructure of FITC, fluorescein (see Figure 11d, p. 77 for structure), was used to test whether the FITC – sensory receptor interaction, leading to activation-type behaviour, was dependent on the isothiocyanate group of the FITC molecule.

Trypsin and bile activated larvae failed to label with fluorescein, regardless of temperature, and no effect of the compound could be detected. "Unactivated" larvae also failed to label at 37° C, but displayed activation-type migratory behaviour. Incubation of "unactivated" larvae with fluorescein at 20° C had no effect. If larvae, which had been incubated with fluorescein at 20° C, were washed thoroughly with PBS, and subsequently incubated in PBS for a further 20 min, ≈30 % of worms exhibited activation-type behaviour.

These results show that the fluorescein group of the FITC molecule is sufficient to stimulate activation-related behaviour, but that the isothiocyanate group is necessary for amphid labelling. Perhaps, irreversible binding of the sensory receptor, by the isothiocyanate group, is necessary for internalisation of the FITC molecule, a phenomenon which is independent of the behavioural stimulation aspect. Thus bound FITC –receptor complexes persist within the neurones, and become visible upon accumulation. The behavioural stimulation, however, seems to be induced by interaction of the aromatic rings of the fluorescein group with the sensory neurone receptors. To verify this theory, further tests were conducted.

Since fluorescein seems to function as an analogue of a naturally occurring chemosensory cue, activation of larvae with physiologically more relevant concentrations was examined. 5 µM concentration of fluorescein had no effect on larval behaviour, while10 µM fluorescein, however, had a variable effect; 20-45% of larvae showed migratory behaviour. The physical support of 0.05% agar functioned synergistically

Figure 11. Structures of FITC and its derived compounds used for behavioural activation experiments.



(A) Fluorescein diacetate; (B) Fluorescein isothiocyanate; (c) Phenyl isothiocyanate; (D) Fluorescein

with the chemosensory cue of fluorescein, resulting in up to 85% behavioural activation at 5 µM fluorescein concentration. The 0.05% agar solution itself was enough to induce migratory behaviour in 50-80% of larvae. This result could have a variety of causes. It is conceivable that mechanosensation also plays a role in activation (which is not supported by the fact that the chemosensory cues of trypsin and bile, or 1 mM fluorescein, are enough by themselves to induce behavioural change), or the agar may concentrate other chemosensory cues such as pheromones. These possibilities have remained unexplored, but need to be addressed in order to gain further understanding of the biology of these parasites.

Fluorescein diacetate

Fluorescein diacetate (FDA) is a non -fluorescent compound derived from fluorescein by acetylation of two of the aromatic rings of the xanthyl group (see Figure 11a, p. 77). Endogenous esterases within cells will cleave the acetate groups from the fluorescein molecule, restoring fluorescence of the compound (Haugland, 1996). Incubation of trypsin and bile activated larvae with FDA resulted in neither amphid labelling, nor activation-related migratory behaviour. Replication of the same experiment at 20° C showed that FDA had no effect when compared to the acetone/PBS and pure PBS controls. The lack of amphid labelling could result from either failure of the compound to enter, or persist, in the sensory neurones. This result mirrors the result obtained with fluorescein, and it is highly likely that a isothiocyanate group is necessary for entry and persistence of these compounds in amphidial neurones.

"Unactivated" larvae did not respond to FDA incubations at any of the experimental temperatures. FDA incubated larvae were indistinguishable from the controls, and displayed coiling behaviour typical for the unactivated state. These results present strong evidence that the triple aromatic ring structures of the fluorescein molecule is the active group in the interaction with the sensory receptors, leading to activation-type behaviour.

Activity of reducing reagents and natural compounds

Phenyl isothiocyanate

In isolation, the remaining substructure of FITC, phenyl isothiocyanate (PITC), is highly toxic. For the sake of thoroughness, however, this compound was tested for effects on larval behaviour. Incubation with PITC did not affect larval behaviour at either experimental temperature, but proved lethal 25 – 35 min after first exposure to the compound. Larvae were examined every 5 min for 25 min after initial exposure, to detect even the slightest transient change in behaviour, but no activation-related behaviour was observed. In conjunction with the results from the other experiments, these results confirm that the isothiocyanate interaction with neural receptors plays no part in the stimulation of activation-related behaviour.

Dithioerythritol

Although interactions between isothiocyanate and sensory receptor proteins, such as possible disulfide interactions, were tested with PITC, activity of dithioerythritol (DTE) was also tested. No effects of the compound on the larvae could be observed. The actual results of this rather primitive test do not give much scope for interpretations; a positive result, however, would have had greater significance.

5-hydroxy tryptamine

The substructure of fluorescein, active in stimulating behavioural activation in infective larvae, may be analogous to the naturally occurring 5-hydroxy tryptamine (serotonin). The enterochromaffin cells of the intestinal mucosa synthesise and secrete serotonin into the intestinal lumen. The proximal intestine is the major source of serotonin synthesis; the intestinal tract accounts for 60% of total body serotonin content (Mettrick, 1989). Serotonin has been shown to have biological activity in a number of helminths, stimulating glucose uptake by *Hymenolepis diminuta*, *Schistosoma mansoni*, and *Fasciola hepatica*, as well as performing a number of other physiological functions. It is conceivable that serotonin may function as an environmental cue, inducing changes in

parasitic helminths physiologies indirectly through chemosensory pathways (Mettrick, 1989).

Incubation of "unactivated" larvae with 10 μM serotonin had no effect on larval behaviour. The higher concentrations of 1 mM and 10 mM had 15-30% and >80% stimulatory effect, respectively. At these high concentrations, however, it is doubtful that the serotonin acted as a chemosensory cue, but rather probably acted directly on the nervous system of the larvae. It is interesting that putatively direct activity of serotonin should stimulate sinusoidal migratory movements in larvae, since the main excitatory transmitter in the somatic musculature is acetylcholine. Ramisz (1965) has shown the ventral and dorsal cords of *T.spiralis,* as well as their ganglia and commissures, to be cholinergic, indicating that, in this respect, *T. spiralis* is no exception to the general nematode design. Notably, previous research has indicated that serotonin decreases somatic activity (Smart, 1989). The site of action of serotonin in infective larvae must, therefore, be somewhere upstream of the somatic innervation system.

In conclusion, the accessory layer of infective *T. spiralis* larvae does not form a barrier to chemosensation. Even highly hydrophilic cues, such as fluorescein and serotonin, can find access to chemosensory receptors. It has become clear that amphidial neurones are the site of integration of environmental information, and that activation associated migratory behaviour is stimulated via amphid mediated signalling pathways. One environmental cue that can stimulate migratory behaviour is fluorescein, and only the triple aromatic ring structure of the molecule is active in the interaction with the chemosensory receptor protein that elicits stimulation of behavioural activation. What the *in vivo* analogues of the biologically active substructure of fluorescein are, is not known.

As discussed in the last chapter, behavioural changes of infective nematode larvae may not be indicative of, or related to, developmental changes (see *Conclusions* section, p. 50). To further test the effects of stimulating infective larvae with fluorescein, surface properties of infective larvae were examined *post* fluorescein incubation.

Activation-related changes in surface properties

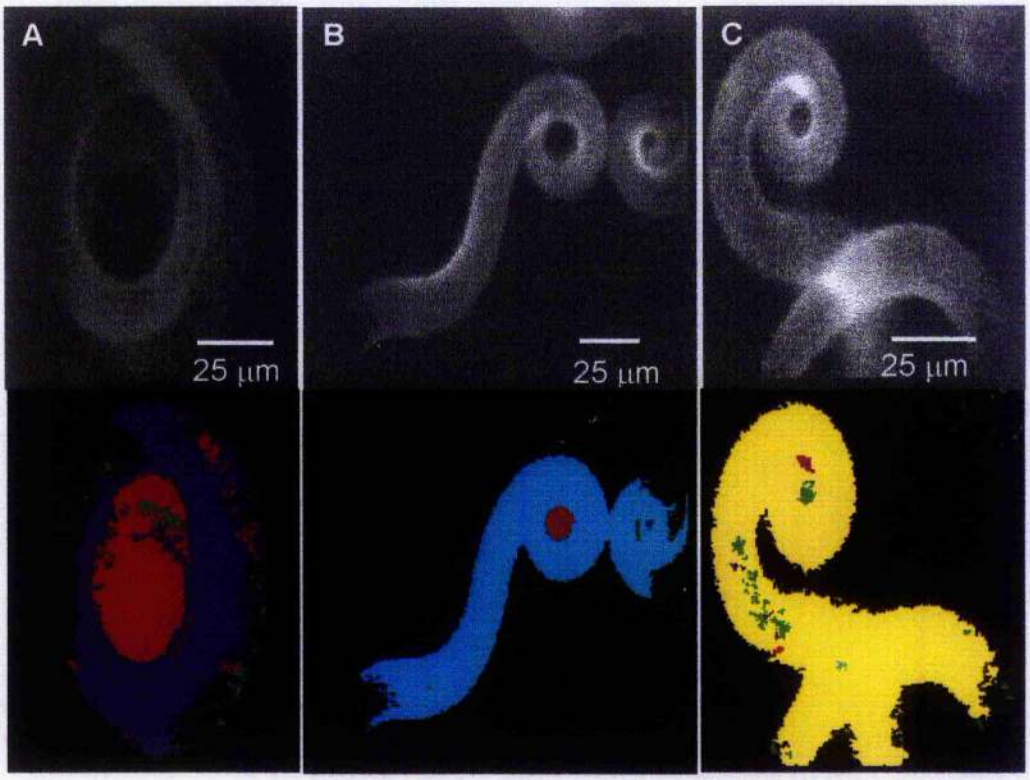
The behavioural activation of infective larvae, observed after exposure to trypsin and bile, has been correlated with physiological and structural changes (Stewart *et al*, 1987; Modha *et al*, 1994). One of these changes, the reported loss of the accessory layer, is associated with alterations in lipophilicity of the larval cuticle, as well as the cuticle's affinity for membrane labels (Modha *et al*, 1994; Modha, Kusel & Kennedy, 1995). The ability to detect these surface alterations using fluorescent lipid probes made it possible to directly examine the nature of the observed activation following exposure of larvae to fluorescein. The issue to be addressed was whether the behavioural change, observed after fluorescein incubation, is an indication of resumption of development. In other words, does fluorescein simply induce a chemotactic reaction, or is it a cue for developmental activation? The question whether loss (or conformational change) of the accessory layer, observed upon activation, is a passive process involving degradation by trypsin and bile, or a reaction effected by the parasite, could thus also be examined.

Insertion of the lipid probe AF18 pre- and post-activation

A rapid change in the surface lipid of a variety of parasitic nematodes, including *T. spiralis*, has been described during the transition to the mammalian host (Proudfoot *et al* 1993a & 1993b). This change involves an alteration in the lipophilicity of the cuticle, as seen by the insertion of the fluorescent lipid analogue 5-*N*-(octadecanoyl)aminofluorescein (AF18) only after exposure to mammalian tissue culture conditions. Modha *et al* (1994) demonstrated that an increase of AF18 insertion into the *T. spiralis* larval cuticle results from loss of the accessory layer. Based on this data, labelling of infective larvae *pre* and *post* fluorescein incubation with AF18 was carried out to detect active restructuring of the larval surface in response to the putative activation stimulus.

Activation of larvae with trypsin and bile resulted in a slight but measurable increase in AF18 insertion (see Figure 12, p. 82). The overall measurable difference varied slightly between samples, and gave

Figure 12. Labelling of the larval surface with 5-*N*-(octadecanoyl) aminofluoresceine (AF18).



Value Range	Mean Value
D	8.14
	17.67
5.00 - 10.00	4.00
10.00 - 15.00	0.10
15.00 - 20.00	17.00
20.00 - 25.00	0.00
25.00 - 30.00	0.00
30.00 - 35.00	0.00

Fluorescence photomicrographs of AF18 labelled larvae, with corresponding false colour gray-level maps below. (A) Un-activated larva; (B) trypsin and bile activat-ed larva; (C) fluorescein activated larva. (D) Key to false colour codes in relation to gray levels are given in the example colour chart. Differences in fluorescence levels are measurable by digital gray level analysis of the gray scale images. The increase in fluorescence observed in activated over unactivated larvae was variable, and only about 25-40% on average.

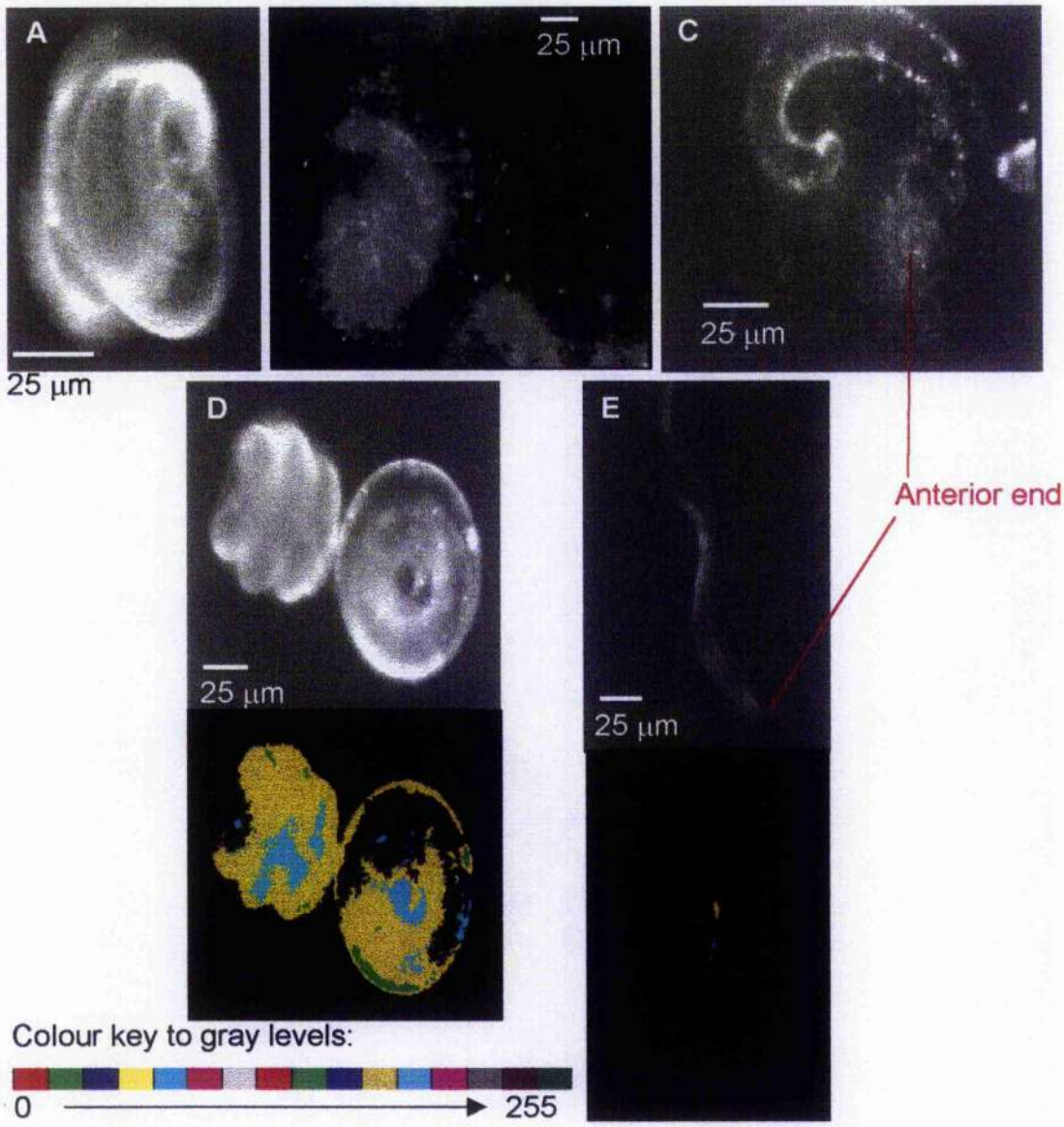
averages from 25-40%. The same result was obtained for larvae exposed to fluorescein at 37° C, but not for larvae incubated in the presence of fluorescein at 20° C. Although image capture was standardised as much as possible, variations in capture conditions did exist, making these results relative and not absolute. Since no internal controls were included within the sample images, which would allow standardised thresholding, no clear comparisons are possible between image series. Since quantitative analyses depend on digital analysis of the fluorescence levels in the images, no statements can be made on absolute fluorescence levels. These results are, therefore, not amenable to rigorous mathematical testing. Nonetheless, these observations gave a basis for the further pursuit of surface investigations.

Labelling of the accessory layer with the lipid probe PKH26

The accessory layer of *T. spiralis* infective larvae can be labelled with the fluorescent lipid probe PKH26 (Modha, Kusel & Kennedy, 1995). This method allows direct visualisation of the surface accessory layer with a fluorescence microscope, and real-time observation of the shedding process. Labelling of unactivated larvae, (when recovered by 2 h acid-pepsin digest), reveals that the accessory layer is constantly shed and replaced in a temperature dependent manner. Larvae chilled to a temperature below 10° C will shed the accessory layer only very slowly, so that the fluorescent labelling is stable for about 25 min. This gives enough time to assess the surface conformation, via PKH26 labelling, of larvae treated with different environmental stimuli.

"Unactivated" larvae (recovered by 2 h acid-pepsin digest) labelled evenly over the entire surface area (see Figure 13, p. 84), while trypsin and bile activated larvae generally failed to label at all. About 10% of trypsin and bile incubated larvae still had remnants of the accessory layer as detectable by PKH26 labelling (see Figure 13, p. 84). These results were expected, and confirm reports of earlier investigations (Modha, Kusel & Kennedy, 1995). Larvae exposed to fluorescein at 37° C completely failed to be labelled with PKH26. If larvae were incubated with fluorescein

Figure 13. Labelling of the larval accessory layer with the fluorescent probe PKH26.



Fluorescence photomicrographs of PKH26 labelled larvae. (A) Unactivated larva; the accessory layer is brightly labelled with PKH26. (B) Shed surface accessory layer material from unactivated larvae, fluorescently labelled as above. (C) Trypsin and bile activated larva with remnants of the accessory layer still present. (D) Unactivated larvae with labelled accessory layers and a digitally produced false colour gray level map of the same image. (E) Combination bright light and fluorescence image of a fluorescein activated larva, which has failed to label with PKH26.

at 20° C, subsequent labelling with PKH26 revealed a fluorescent staining pattern identical with that of the "unactivated" larvae. The incubation controls, in PBS or RPMI1640 medium at 37° C, also resulted in the "unactivated" labelling pattern. Exposure to fluorescein at 37° C clearly stimulates infective larvae to change the conformation of their surface. Whether this alteration in surface properties results from loss of the accessory layer, or a conformational change of the accessory layer, can not be deduced from these observations.

It had already become clear that infective larvae are receptive to environmental cues before entering the host intestine and being exposed to trypsin and bile. Further, larvae not only change their behaviour upon exposure to the environmental stimulus fluorescein, but also actively alter their surface properties. Since the results of the mRNA analyses (see previous chapter) were indicative of an early developmental initiation during the stomach phase of the infectious process, it was important to test whether the acid-pepsin recovery process itself was stimulating larvae to alter their physiological state. Reduction of the acid-pepsin recovery digest time to 35 min resulted in the failure of PKH26 labelling of larval surfaces. If thus recovered larvae were incubated at 37° C in either saline or choline chloride (154 mM) for a further 60 min, the PKH26 labelling pattern, observed in 2 h acid-pepsin digest recovered larvae, was restored. To test which surface properties were detectable in developmentally active intestinal larvae *in vivo,* attempts were made to label rapidly isolated intestinal larvae with PKH26 2 h post infection. The intestinal larvae also completely failed to label with PKH26.

Since the rapidly acid-pepsin isolated muscle larvae, as well as the *in vivo* activated intestinal larvae, fail to label with PKH26, it is fairly certain that the surface property change observed in larvae isolated by a 2 h digestion process, or larvae left in saline after recovery, is a short, transient state. The relevance of this surface alteration to the life cycle of *T. spiralis* is not clear. Nonetheless, the exposure to fluorescein stimulates a response in infective larvae which is analogous to that observed in larvae exposed to the intestinal environment.

Conclusions

The initial aim of the here described studies was to determine whether infective larvae could receive environmental cues before loss of the accessory layer, and thus resume development before entering the intestine of the next host. Since it has been demonstrated that the amphids are the site of integration of environmental information and temporal regulation in *C. elegans* development, it was also of interest to test, if possible, the role of amphidial chemosensation in *T. spiralis* development.

The labelling of the amphidial cell bodies and processes with FITC, in both "unactivated" and activated infective larvae, has given structural as well as functional information. It has also provided some circumstantial evidence for the signalling processes involved in developmental reactivation. Previous research on neurotransmitters of *T. spiralis* has identified the same amphidial structures using the fluorescent glyoxylic acid method (Lee & Ko, 1991). This method, which detects catecholamines (CA), has labelled four neurone cell bodies, which lie anterior to the nerve ring, as well as a corresponding left or right cell body situated slightly posterior to the nerve ring. The cell bodies posterior to the nerve ring are also linked to each other by CA containing processes. The CA containing cell bodies posterior to the nerve ring are the amphidial cell bodies (Bird & Bird, 1991), and correspond to the structures labelled with FITC. Since these cells were shown to be involved in behavioural activation and stimulation of surface alteration by FITC labelling and fluorescein activation, it is highly likely that these putatively developmental processes are under catecholaminergic control. The catecholamines, especially noradrenaline (NA), have been demonstrated to play a part in ecdysis and development in a number of nematodes. Application of NA to the third stage larvae of *Phocanema decipiens* brings about ecdysis, although the moulting process thus induced was observed to be abnormal (Smart, 1989). The effects of various catecholamines and catecholamine antagonists on the third larval moult of *Dirofilaria immitis in vitro* have been investigated (Warbrick & Ward, 1992), and it was demonstrated that

α-type chatecholamine antagonists were effective in disrupting the third stage ecdysis. Exsheathment of *Haemonchus contortus* infective larvae also seems to depend on noradrenaline mediated signalling pathways (Fleming, 1993).

The observation that larvae alter their surface properties within about one hour of being released from the nurse cell gives evidence for two processes. First, larvae constantly monitor their environment, thus detecting degradation of the nurse cell (and perhaps host death) immediately. Second, the larvae react to digestion of the nurse cell (or host death) by changing surface properties after a set time period, in the absence of further cues. This latter process would indicate that either a default programme takes over in the absence of expected cues, or that temporal control of developmental changes is the master regulatory factor in effecting the surface changes. The truth is probably a combination of both possibilities. Fluorescein or the enteral environment can reverse the surface alterations as detectable by PKH26 labelling. Larvae recovered 2 h post infection from mouse intestines cannot be labelled, while larvae incubated for the same amount of time in choline chloride or saline will be labelled. Labelling does not take place, however, until about one hour after recovery from the digest mix (in the case of 35 min digest recovery). Larvae recovered by 2 h acid-pepsin digest can be labelled with PKH26, indicating that temporal control is a more likely overall control mechanism than interruption of a natural sequence of environmental stimuli. The 2 h digest recovery is not so far removed from possible *in vivo* processes, since passage through a pig's stomach, depending on diet, may take up to 4 h. It is, therefore, possible that larvae will alter their surface properties after a set time *post* release from the nurse cell (a process induced by yet unknown factors), and again change their surface conformation upon receiving the next set of environmental cues in the host intestine. One of the intestinal cues is probably analogous, in structure and/ or chemical properties, to the triple aromatic ring structure of fluorescein.

The labelling of amphidial neurones with FITC, despite presence of the surface accessory layer, and the accompanying activation of behavioural and surface changes, gave certainty that infective larvae detect, and react to, the environment prior to reaching the small intestine. It was now important to examine whether larvae react to the liberation from the nurse cell by resuming development. For a further investigation of the timing of developmental initiation, clear biochemical or molecular markers of development were required. The search these markers is the subject of the next chapter.

4. ENVIRONMENTAL CUES AND TIMING OF ACTIVATION: METABOLIC ASPECTS

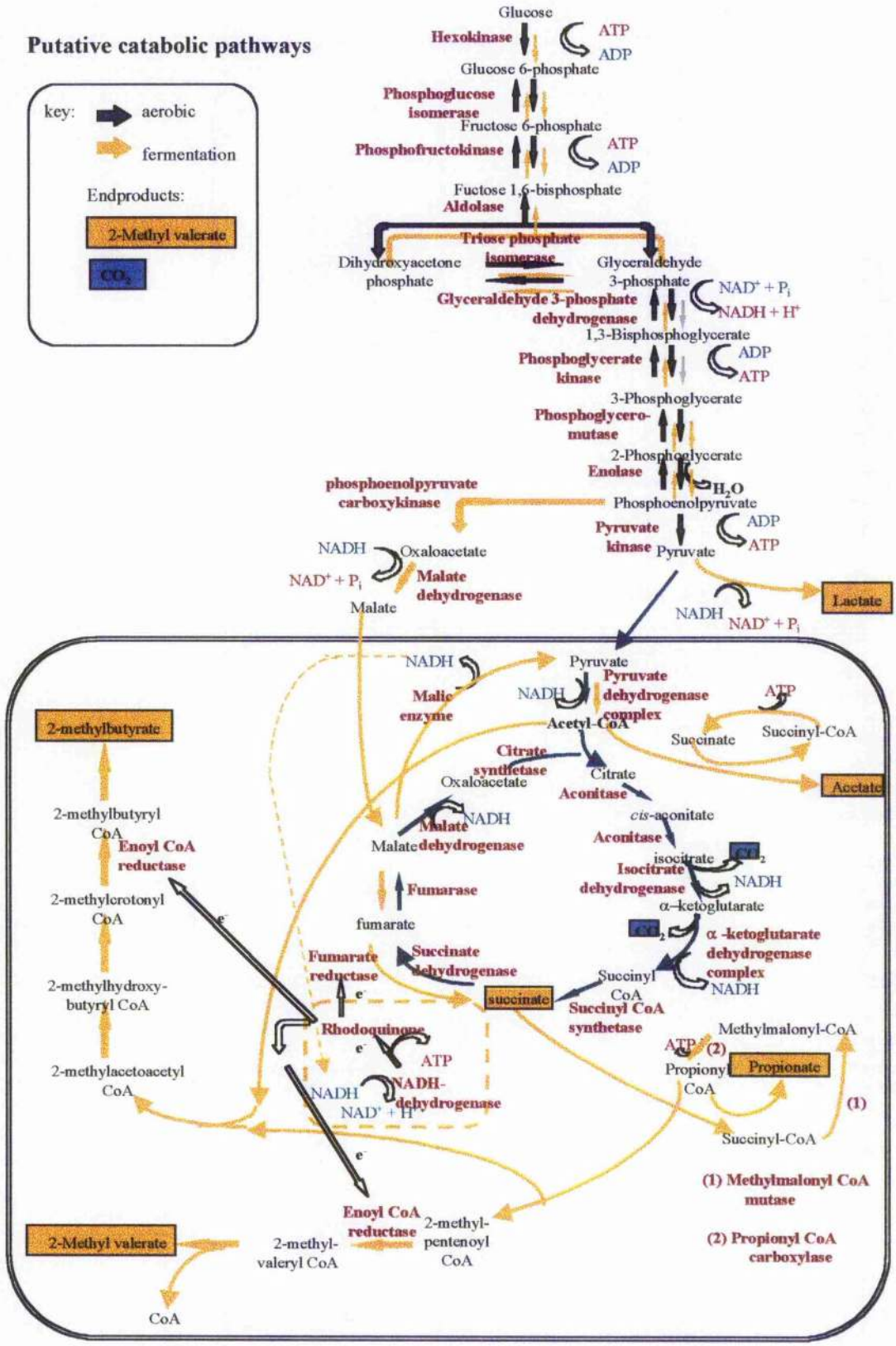
INTRODUCTION

Developmental activation of infective *T. spiralis* larvae has been defined by behavioural, structural, and physiological changes, all occurring within the intestinal environment of the next host (Stewart *et al*, 1987; Modha *et al*, 1994). Observations of the effects of amphidial neurone labelling with FITC on behavioural and surface changes (see previous chapter), of infective larvae prior to exposure to the intestinal environment, have revealed this model of activation may not be correct. The initial surface alteration observed in infective larvae *post* release from the nurse cell, as detectable by PKH26 labelling, seems to take place at a set time after liberation of the larvae and before the reception of intestinal environmental stimuli. This indicates that larvae sense, and react to, their environment prior to entering the host intestine.

In order to further examine the timing and regulation of resumption of development of infective larvae, biochemical and/ or molecular markers of development were required. These developmental markers would permit the assessment of the temporal and environmental requirements for larvae to become committed to the next phase of development, and hopefully avoid the measurement of transient physiological responses of larvae to immediate environmental conditions.

Since *T. spiralis* metabolism has received some attention in the past, and data is available on some of the parasite's metabolic processes (Stewart, 1983), metabolic enzyme activities were considered as developmental markers. A review of the literature on *T. spiralis* metabolism reveals that reports on the catabolic pathways utilised by muscle larvae differ: one set of observations reveals that muscle larvae are anaerobic, while the other shows muscle larvae to be aerobic. Notably, the reports of aerobic muscle larvae are all based on experiments involving a 2 h or longer acid-pepsin digest recovery (Goldberg, 1957;

Figure 14. Proposed catabolic pathways in *T.spiralis*.



Boczon & Michejda, 1978; Boczon, 1986), while those showing that muscle larvae utilise anaerobic metabolic pathways are based on experiments using a 1 h or less acid-pepsin digest recovery time of larvae (Castro & Fairbairn, 1969; Ward, Castro, & Fairbairn, 1969; Ferguson & Castro, 1973). The general consensus, however, is that muscle larvae utilise anaerobic pathways, while intestinal worms are clearly aerobic (and facultatively anaerobic) (Stewart, 1983). Considering that the utilisation of aerobic metabolism is solely dependent on the developmental status of the larvae, since "unactivated" muscle larvae (recovered by < 1 h acid-pepsin digest) only use anaerobic pathways regardless of environmental conditions (Ferguson & Castro, 1973), metabolic enzyme activities might be good markers of developmental status. Further, the switch to the utilisation of aerobic metabolic pathways during the infectious process seems to be irreversible (Ferguson & Castro, 1973; Stewart, 1983).

In this metabolic switch between anaerobic and aerobic (facultative anaerobic) metabolism, phosphoenolpyruvate is an important branch point, leading either via pyruvate kinase (PK) to pyruvate, or else via phosphoenolpyruvate carboxykinase (PEPCK) to oxaloacetate and the reverse citric acid cycle (Barrett, 1981) (see Figure 14, p. 90). In previous work, the PEPCK to PK activity ratio was determined for infective larvae recovered by short digest (Ward, Castro & Fairbairn, 1969). In this work, the short digest of Ward, Castro & Fairbairn (1969) was repeated, and the activity ratio measured during the recovery and post recovery period. Isocitrate dehydrogenase (ICDH) (NADP dependent) and citrate synthase activities were also measured at the same time points, as a further indicator for aerobic metabolism.

To date, only variations of the acid-pepsin digestion method have been described for the recovery of *T. spiralis* muscle larvae from host tissue. Since this form of recovery exposes the infective larvae to some of the same cues as are found in the mammalian host stomach, alternative recovery methods were sought. The aim was to determine, via a process of elimination, which cues, present in the acid-pepsin digest, might play a role in stimulating developmental initiation of infective muscle larvae. To

achieve this aim, plant and fungal proteinases were used at different temperatures and pH ranges to recover infective larvae from host muscle. Selected metabolic enzyme activities of larvae recovered by these alternative methods were to be subsequently compared with activities observed in larvae recovered with acid-pepsin digest.

The present studies were conducted, therefore, to examine the possibility that developmental changes are initiated as a consequence of exposure to the stomach environment. To this end, the larvae recovery times were varied, and the effects of acid-pepsin digest-mix exposure time on changes in metabolic enzyme activities, taking place during post-recovery incubations, examined.

MATERIALS AND METHODS

Since it had become evident that infective larvae are receptive to environmental stimuli much earlier than previously thought, namely upon liberation from the nurse cell in the stomach, it became important to examine the possibility of developmental initiation in the stomach. The acid-pepsin recovery procedure mimics the environmental conditions found in the host stomach, and the possibility that thus recovered larvae are already developmentally activated had to be tested. To this end, an alternative method for recovering infective larvae from muscle tissue was sought. Clear developmental markers were also sought, and activities of selected metabolic enzymes were examined for developmental regulation.

Recovery of muscle stage larvae with alternative methods

As before, laboratory infections of *T. spiralis* were maintained in BALB/c mice, and larvae recovered after 6 weeks post infection for all experiments described below.

Papain recovery

A recovery method was designed using papain to liberate larvae from muscle tissue. 10 g papain (1.7 U/mg) were dissolved in 1 L 0.9% saline, pH 7.4. Homogenised eviscerated mouse carcasses were digested for 1 h (1 carcass/ L digest solution). The digest mix was subsequently filtered with a 250 µm steel mesh sieve and sedimented for 30 min. The sedimented filtrate was further cleared of undigested material and nurse cells with 50% sucrose centrifugation (30 ml) at 1000 ***g*** for 5 min. Larvae were aspirated from the top, and suspended in PBS.

Fungal proteinase recovery

Digest with Aspergillus oryzae *proteinase*

Several ways of recovering larvae using *Aspergillus oryzae* proteinase were explored. The initially successful method involved a digest mix of 25 g protease (0.12 U/mg, type II, Sigma) dissolved in 1 L 0.9% PBS at pH

7.2. Homogenised eviscerated mouse carcasses were digested at 37° C for 20 min to 2 h. The digest was allowed to sediment for 15 min, followed by one wash of the sedimented material with saline, and a further 10 min sedimentation. The parasites were subsequently suspended in PBS.

To increase efficiency, the above method was elaborated. The skinned and eviscerated carcasses were homogenised with 300 ml digest fluid. The homogenate was then diluted at a ratio of 0.5 L per homogenised mouse with digest fluid consisting of 1% w/v crude proteinase from *A. oryzae* (0.12 U/mg, type II, Sigma) in 0.9% w/v saline at pH 7.5, prewarmed to 37° C. The digest was incubated at 37° C under constant stirring with a magnetic stirrer for 2 h. Subsequently, the digest was passed through a 250 µm sieve and allowed to sediment in glass graduated cylinders at 20° C for 25 min. Sedimented larvae were washed into 50 ml falcon tubes and cleaned from mouse debris and nurse cells by 50% sucrose centrifugation (see *Papain recovery*, p. 93) immediately. In the mean time, any undigested tissue remaining in the sieve was further digested with acid-pepsin for 40 min, and recovered larvae were kept separate for further experimentation.

Physical disruption and digestion method

The skinned and eviscerated mouse carcasses were homogenised in 300 ml digest solution (25 g *A. oryzae* proteinase, (0.12 U/mg, type II, Sigma) dissolved in 1 L 0.9% PBS at pH 7.2), and subsequently incubated in aerated digest solution for 10 min at 37° C. The digest was filtered with a 250 µm steel mesh sieve, and the filtrate allowed to sediment while the remaining undigested material was returned to the digest solution. Glass beads (3 mm diameter) were introduced into the digest, and the incubation continued for 20 min at 37° C while shaking at 250 rpm. The digest mix was filtered as above, the filtrate allowed to sediment, and the remaining material returned to the digest solution with glass beads, shaking for a further 10 min at 37° C. The final digest was filtered as above, the sedimented material pooled, and washed three times with saline. The sample was cleaned up with 50% sucrose centrifugation as above.

Release of larvae from nurse cells

High purity *A. oryzae* proteinase (3.6 U/mg type xxiii, Sigma) was used at 40 mg/ml in PBS to release larvae from nurse cells which had been isolated by above procedures. 50 µl to 100 µl incubations, containing 5 nurse cells each, at 37° C were tested for 5 min to 40 min at 5 min intervals.

Metabolic enzyme assays

During the transition from muscle-stage to enteral phase, *T. spiralis* switches from anaerobic to aerobic (facultative anaerobic) metabolism (Stewart, 1983). To determine the timing of this metabolic switch, the activity ratio of pyruvate kinase (PK) to phosphoenolpyruvate carboxykinase (PEPCK) was measured during the recovery and post recovery periods. Isocitrate dehydrogenase (ICDH) (NADP dependent) and citrate synthase activities were also measured at the same time points, as a further indicator of preparation for aerobic metabolism.

Acid-pepsin recovery

Recovery of infective larvae involved digesting homogenised eviscerated carcasses in 0.75% w/v pepsin (1 Anson unit/g, purchased from BDH) in saline/HCl pH 1.6 at 37° C, agitated with a magnetic stirrer. One litre digest solution was used per mouse carcass. Digest times were either 40 min or 60 min, depending on the experiment (see *Experimental incubation conditions* below). The digest mix was strained through a 250 µm sieve, diluted with 1 L saline, and allowed to sediment in glass graduated cylinders for 10 min at 20° C, or at 37° C if subsequent incubation in saline was involved (see *Experimental incubation conditions* below). Sedimented larvae were either used for experimental incubations or washed with sterile PBS, and separated from any remaining debris and nurse cells by sucrose floatation as follows: Larvae were centrifuged in 10 ml 50% w/v sucrose/ sterile distilled water at 1700 ***g*** for 7 min. The top larval layer was aspirated, washed once in ice-cold sterile phosphate

buffered saline (PBS) and three times in sterile 50 mM HEPES (pH 7.5). The larvae were homogenised immediately or frozen in liquid nitrogen.

For the purpose of obtaining carcass derived larvae, infected mice were killed, skinned, eviscerated, and stored in a desiccation jar with water at room temperature for 3 days. In one instance carcasses were incubated at 37° C for 3 days. Larvae were subsequently recovered as described above.

Experimental incubation conditions

Some metabolic enzyme activities were measured from larvae recovered by the fungal proteinase methods described above (see *Fungal proteinase recovery*, p. 93). Due to low parasite yields, however, the fungal proteinase recovery had to be discontinued, and most data were obtained using the acid-pepsin recovery described above.

For acid-pepsin recovered larvae, enzyme activities were measured for larvae recovered from freshly killed mice under the following conditions: 40 min digest, 40 min digest with subsequent 60 min saline incubation at 37° C, and 60 min digest with subsequent 40 min saline incubation at 37° C. The same procedures were carried out for larvae recovered from 3-day carcasses. The pH of the saline used in the post recovery incubations was experimentally varied from pH 2 to pH 7.2.

To further test whether activation with fluorescein is the same as the developmental activation occurring during the early process of infection, enzyme activities of infective larvae were measured after fluorescein incubation. Larvae were recovered by 40 min acid-pepsin digest, incubated in 1 mM fluorescein at 37° C for 20 min, and in saline for a further 40 min at 37° C. For larvae recovered from live hosts, pyruvate kinase activity was measured, and for larvae recovered from carcasses isocitrate dehydrogenase (NADP) activity was measured subsequent to the incubation procedures.

Metabolic enzyme activity assays

Larvae were homogenised with a hand driven homogeniser in 5 volumes 50 mM HEPES pH 7.5 in the presence of proteinase inhibitors (500 mM

PMSF, 5 mM E64, 1 mM pepstatin A and 1 mM 1-10 phenanthroline). The homogenate was centrifuged at 4° C for 15 min at 10000 ***g***, and the supernatant fraction used for assays. PEPCK and PK activities were assayed immediately, with freshly prepared supernatant; ICDH and citrate synthase activity assays were carried out with supernatant that had been stored at -70° C.

For all enzyme assays, reactions were started by addition of soluble parasite extracts. Controls consisted of both reactions lacking substrate and reactions lacking parasite extract. Any activities observed in the control reactions were subtracted from the activity measured in the experimental reaction.

Phosphoenolpyruvate carboxykinase

PEPCK activity assays were conducted as previously described (Ward, Castro & Fairbairn, 1969). The reaction mixture for spectrophotometric PEPCK assays contained (in 1 ml): 50 mM HEPES pH 7.5, 5 mM $MnCl_2$, 20 mM $NaHCO_3$, 5 mM IDP, 0.5 mM NADH, 9 U malate dehydrogenase, 10 mM phosphoenolpyruvate, and 25 mM oxamate. The oxamate was included as a lactate dehydrogenase inhibitor (Chretien *et al*, 1995) to increase the sensitivity and specificity of assays. The change in absorbance at 340 nm was measured during the linear phase of the reaction.

Pyruvate kinase

PK activity assays were conducted as previously described (Ward, Castro & Fairbairn, 1969). The reaction mixture for PK assays contained (in 1 ml): 50 mM HEPES pH 7.5, 7.5 mM $MgCl_2$, 75 mM KCl_2, 0.5 mM NADH, 5 mM ADP, 10 mM phosphoenolpyruvate, and 10 U lactate dehydrogenase. The change in absorbance at 340 nm was measured during the linear phase of the reaction.

Isocitrate dehydrogenase

ICDH assays were conducted as previously described (Denton *et al*, 1996). The reaction mixture contained (in 1 ml): 50 mM HEPES pH 7.5, 2 mM $MnCl_2$, 7 mM isocitrate, and 0.4 mM NADP. The change in

absorbance at 340 nm was measured during the linear phase of the reaction.

Citrate synthase

Citrate synthase was assayed spectrophotometrically in the following reaction mixture: (in a volume of 1 ml at 37° C), 50 mM HEPES, pH 7.5, 1.75 mM acetyl coenzyme A, 3 mM oxaloacetate, and 3 mM 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB). The change in absorbance at 412 nm was measured during the linear phase of the reaction.

Protein concentration

The amount of protein assayed was optimised, and varied between 5 and 20 μg. All assays were carried out with at least three different protein concentrations. Protein concentration was measured using the Pierce Coomassie Protein Assay according to the manufacturers instructions, with bovine serum albumin as a standard.

Statistical analysis

Where statistical significance of data is mentioned, a Wilcoxon-Mann-Whitney rank sum test was applied.

RESULTS AND DISCUSSION

Previous experiments have shown that infective *T. spiralis* larvae change their surface properties, as detected by labelling with the fluorescent lipid probe PKH26, at a set time after liberation from the nurse cell within the host stomach (see previous chapter). It is therefore conceivable that larvae also initiate further development as a result of receiving environmental stimuli in the stomach. Since the acid-pepsin recovery procedure presents the parasites with an environment analogous to that found in the host stomach, alternative muscle larva recovery methods were sought to facilitate the *in vitro* manipulation of developmental activation. To monitor the developmental status of recovered muscle larvae, selected metabolic enzyme activities were measured at set time points *post* recovery.

Recovery of muscle larvae using alternative methods

The design of methods for the recovery of muscle larvae was constrained by several factors. First, a high efficiency was required of the method, to effect the recovery of enough parasites, per infected mouse, to yield suitable amounts of material for biochemical or molecular experiments. Second, recovered larvae must not be affected by the recovery method, to allow the recovery of developmentally inactive parasites, or to enable further *in vitro* manipulation of the larvae. Finally, the method had to be economical, to permit its routine use.

Since chemical methods, such as digestion with KOH, were considered too harsh and certainly harmful to the larvae, enzymatic methods were considered as the only practical alternative. The commercial availability of non-mammalian proteinases, at prices permitting the use of the quantity necessary do digest entire mouse carcasses, turned out to be a limiting factor. Mammalian digestive enzymes were not taken into consideration, since they might act as environmental stimuli for larval activation. From these considerations, papain and two different preparations of *A. oryzae* proteinase were selected for larval recovery experiments.

Although digestion of infected muscle tissue with papain was effective in releasing larvae, the larvae seemed to be adversely affected by this method. About 30% of recovered larvae were dead, while the rest displayed a lack of responsiveness to temperature changes. The larvae were only loosely coiled, and did not exhibit the rapid coiling and uncoiling behaviour observed in healthy larvae at 37° C. The method described in *Materials and Methods* was the most efficient of the devised procedures using this enzyme. Neither lower concentrations of papain, nor shorter recovery periods reduced the negative effect on recovered larvae. Even at its most efficient, the method did yield only 10 to 15 µl packed volume larvae per infected mouse, which is an impractical amount of parasite material.

Initial experiments with fungal proteinase digestion were limited to attempts of breaking down the homogenised tissues using only the *A. oryzae* enzyme, while later experiments included physical methods such as disruption of tissue with glass beads. Digestion of infected carcasses with *A. oryzae* proteinase did not appear to have any adverse effects on larvae. The yield of liberated larvae per mouse, however, was relatively low (< 30 µl packed volume), with an equal amount of purified nurse cells evident in the sample. The *A. oryzae* proteinase digestion used in conjunction with the physical disruption method was the most effective method for maximising larval yield (≈50 µl packed volume larvae per mouse). Although larvae obtained with the fungal proteinase recovery were used for metabolic enzyme activity assays (see below), the total amount of material available was too limiting to carry out the amount of assays required to give significant results. Further, the method ceased to be effective as infections in mice became older than 8 weeks. Whether the observed lack of effectiveness of the recovery procedure merely coincided with the progression of the infection in the mice, or was a direct consequence of the infection age, is not known. Nonetheless, the unexplained cessation of the method's efficacy

prohibited further experiments with fungal proteinase digest recovered larvae.

Although a variety of alternative muscle larva recovery methods, using papain and *A. oryzae* proteinase, was tested, the results did not yield a method of the required efficacy and reliability. All further experiments, therefore, continued to rely on variations of the acid-pepsin digest recovery method.

Metabolic enzyme assays

To monitor the switch from anaerobic to aerobic metabolism in larvae, citrate synthase, isocitrate dehydrogenase (ICDH), pyruvate kinase (PK), and phosphoenolpyruvate carboxykinase (PEPCK) activity levels were measured. The significance of the measured enzyme activities to overall catabolic pathways is based on the general model of aerobic and anaerobic metabolism in parasitic helminths (Köhler, 1985; Tielens, 1994) (see Figure 14, p. 90). Parts of the energy generation pathways in *T. spiralis* have been described in detail (Ferguson & Castro, 1973; Stewart, 1983), with some emphasis on the anaerobic metabolic pathways (Boczon, 1986; Rodriguez-Caabeiro, Criado-Fornelio & Jimenez-Gonzalez, 1985). To date all the data support the relevance of the general model in *T. spiralis*.

Although the definitive proof of which metabolic pathways are utilised can only be given by measurement of metabolic end products, enzyme activities do reveal the potential for flux through a given pathway. It has been shown that changes in flux rates through the PK or PEPCK branches can be correlated with changes in metabolic end products (Barrett, 1981). However, it is notable that the enzyme activities, measured in the present study, can not give any information about actual metabolite flux through the respective pathway branches, but provide information on the potential utilisation of aerobic or anaerobic branches. This is further supported, since the modulation of pyruvate kinase in parasitic helminths is concerned primarily with the regulation of metabolic end products rather than glyconeogenesis (Barrett, 1981). In this study, therefore, the most

relevant results were obtained by measurement of the effects of variation of the recovery time and *post*-release incubations on of the PK to PEPCK activity ratio and ICDH (NADP) activity, using variations of the acid-pepsin digest recovery. Although some data were obtained on citrate synthase activity under the different experimental conditions, including fungal proteinase recovery, difficulties with the assay precluded the collection of complete data sets. In order to focus on the more relevant results, the discussion will be restricted to PK, PEPCK, and ICDH activities measured in larvae obtained by acid-pepsin digest only.

Enzyme assay results are summarised in Table 1, p. 103, and Table 2, p. 104 (All enzyme activities referred to in the text are expressed as nmol min^{-1} mg $protein^{-1}$). Incubation of 40 min recovered larvae in saline at 37°C for 1 h did not cause a change in the PK:PEPCK ratio (0.26) in larvae recovered from freshly killed hosts. Extension of the recovery time to one hour, with subsequent 40 min incubation, caused a major increase in the ratio to 0.59, involving a doubling in the PK activity. ICDH activity followed the same pattern, the only significant ($p < 0.025$) change in activity occurring in response to 1h recovery with 40 min incubation (Table 1). In contrast, larvae recovered from 3-day carcasses by 40 min digest had a ratio of PK to PEPCK of 0.71, and half the ICDH activity of larvae from freshly killed hosts. Three-day carcass derived larvae which had been incubated in saline at 37°C for 1 h subsequent to 40 min digestion did not have significantly altered activities ($p > 0.05$ for PK, $p > 0.1$ for PEPCK and ICDH) (Table 2), although the PEPCK to PK ratio increased to 1.0 due to a slight rise in PK and fall in PEPCK activities. Larvae isolated from 3-day carcasses by 60 min digestion and subsequent 40 min incubation in saline at 37°C had an altered PK to PEPCK activity ratio of 0.49, and an ICDH activity nearly double that of 40 min isolated larvae. The ICDH activity always increased during 60 min recovery with 40 min saline incubation, but failed to respond to saline incubation after 40 min recovery (Tables 1 and 2). The ICDH activity response to host death was a decrease to half the activity found in freshly killed hosts. Conversely, PK activity responded to host death by doubling, and only responded

Table 1. Enzyme activities of larvae recovered by acid-pepsin digest from freshly killed hosts.

	Specific activity (± standard deviation) in nmol/min/mg protein for *n* assays		
Enzyme	40 min recovery	40 min recovery & 1 h saline incubation	1 h recovery & 40 min saline incubation
Pyruvate kinase	266.4 ± 48 *n* = 5	282.7 ± 40.6 *n* = 4	534.7 ± 66.6 *n* = 4
Phosphoenol-pyruvate kinase	983.8 ± 75.8 *n* = 4	1108.2 ± 166.6 *n* = 4	904.3 ± 64.4 *n* = 4
Isocitrate dehydrogenase (NADP)	50.2 ± 6 *n* = 6	42.3 ± 3.1 *n* = 3	78.7 ± 3.6 *n* = 4
Citrate synthase	69.1 ± 2.4 n = 3	67.0 ± 3.6 n = 3	No data
	40 min recovery, 20 min (1 mM) fluorescein incubation, & 40 min saline incubation		
Pyruvate kinase	455.5 ± 48.1 n = 6		

Table 2. Enzyme activities of larvae recovered by acid-pepsin digest from 3-day carcasses.

	Specific activity (± standard deviation) in nmol/min/mg protein for *n* assays		
Enzyme	40 min recovery	40 min recovery & 1 h saline incubation	1 h recovery & 40 min saline incubation
Pyruvate kinase	550.6 ± 96.7 *n* = 6	707.1 ± 126.7 *n* = 3	453 ± 46.4 *n* = 4
Phosphoenol-pyruvate kinase	771.3 ± 116.6 *n* = 3	700 ± 91.1 *n* = 6	926.4 ± 107.6 *n* = 3
Isocitrate dehydrogenase (NADP)	27.6 ± 3 *n* = 5	33.92 ± 2.8 *n* = 5	48.3 ± 5.1 *n* = 4
Citrate synthase	61.0 ± 3.7 n = 4	No data	69.8 ± 11.2 n= 3
	40 min recovery, 20 min (1 mM) fluorescein incubation, & 40 min saline incubation		
Isocitrate dehydrogenase (NADP)	35.3 ± 5.2 n = 6		
	Carcass stored at 37° C		
	40 min recovery		
Isocitrate dehydrogenase (NADP)	26.4 ± 4.9 n = 4		

significantly ($p < 0.025$) to increased recovery time in freshly killed host derived larvae, also by doubling. PEPCK activity fluctuated slightly for all conditions, but did not alter significantly ($p > 0.1$). Carrying out all procedures, and storing carcasses, at 37° C had no effect on isocitrate dehydrogenase activity when compared to activities from carcass larvae stored at 20° C.

The experiments were designed to detect the initiation of developmental programmes rather than transient reactions to immediate environmental conditions. The exposure to the digest mix during the recovery process being the putative environmental stimulus for developmental activation, it was important to limit exposure to this cue, and follow larval responses in a non-stimulatory environment. In order to allow time for the progression of a response to the digest mix, larvae were incubated at 37°C in saline after the recovery process. Activities of larvae obtained by 40 min digest were used as a baseline, and incubation of 40 min recovered larvae in saline for 60 min at 37°C allowed assessment of the effects of saline incubation. The results clearly show that saline incubation at 37°C itself has no effect on the biochemical processes measured. The extension of recovery time to 60 min was complemented with 40 min incubation in saline to allow developmental progression while keeping the overall procedure times equal. The effects of host death on infective larvae were examined by comparing the activities measured from 3-day carcass-derived larvae with those from the initial 40 min baseline digest from freshly killed hosts. Therefore, the measured biochemical changes in the larvae result from processes which are activated by host death or exposure to the digest mix, and which continue even after the latter stimulus is removed.

The measured enzyme activity changes will only be present in a minor percentage of tissues during the initial phase of activation. Thus, the true magnitude of tissue specific enzyme activity is masked by the activities of the unactivated tissues. Taking into consideration that on average only 70-80% of larvae in a sample successfully respond to activation stimuli, if 15% of tissues respond, a measured overall doubling of activity would

mean a specific 8.3 to 9.5 fold increase.

The PK to PEPCK activity ratio of 0.27 that was measured in 40 min recovered larvae is consistent with the previous report of 0.31 by Ward, Castro & Fairbairn (1969). The observed increase in PK activity in response to 60 min-digest / saline incubation, in larvae from freshly killed mice, brings the capacity of the aerobic metabolic pathway more in line with that documented for juvenile and adult intestinal stages (Castro & Fairbairn, 1969; Ferguson & Castro, 1973; Stewart, 1983). In contrast, the PK activity increase in response to host death was unexpected in the anaerobic environment of the carcass since lactate has not been described as a major metabolic end-product in *T. spiralis*. Should compensation for loss of aerobic energy generation be necessary, an increased activity through the PEPCK branch would be expected, but was not observed. However, it is possible that larvae start to produce lactate as a metabolic end-product after host death. An alternative explanation for the increase of PK activity in 3-day carcass larvae might be pre-adaptation to the enteral niche, i.e. production of enzyme without increase in flux through the corresponding pathway. Since it is not known what the actual metabolic flux is in these larvae, these results can only be interpreted in terms of metabolic pathway potential. Immediate compensatory changes in metabolism during environmental transitions are usually not due to changes in enzyme levels or synthesis, but to the plasticity of existing pathways (Barrett, 1987). The enzyme activities that were measured *in vitro* are removed from the context of cellular regulation, and should reflect actual enzyme levels. Thus, it is highly likely that the observed increases in activity reflect changes that are more permanent and are relevant to development. The increase in ICDH activity in response to 60 min-digest / saline incubation for both live host and 3-day carcass derived larvae gives the same indication as the PK activity increase. The significant drop in ICDH (NADP) activity in response to host death contrasts with the corresponding PK activity increase, and may reflect adaptation to the anaerobic conditions in the carcass. The fact that ICDH levels are reduced in the carcass environment, as opposed to the increase in PK levels, argues for an active role of PK in 3-day carcass larva

metabolism, based on the assumption that both pathways would be regulated equally in terms of pre-adaptation or energy conservation. At least as far as ICDH activity is concerned, temperature fluctuations do not affect enzyme levels after host death, as shown by the results obtained from carcasses stored at 37° C.

Since fluorescein had proven itself as a chemosensory cue, stimulating behavioural and surface changes (see previous chapter), it was of interest to test the molecule's effect on metabolic regulation. Since host death was shown to have an effect on PK activity levels, only ICDH activity was measure in carcass larvae which had been exposed to fluorescein. To test if fluorescein exposure had any effect on PK activity levels, larvae were taken only from freshly killed hosts. The results (see Table 1, p. 103, and Table 2, p. 104) indicate that fluorescein exposure had no effect on ICDH activity ($p > 0.1$), but the same effect in elevating PK activity as extended exposure to the digest mix or host death ($p < 0.025$). This observation, taken together with the surface and behavioural changes, gives a strong indication that fluorescein stimulated activation is of a developmental nature. The fact that the ICDH activity changes can not be elicited with fluorescein, indicates that further, unknown cues, must be necessary for the complete resumption of development. Further, there is probably a close integration between temporal (heterochronic) genes and environmental information. Thus, environmental cues must be presented in the right order, and at the correct (pre-programmed) time intervals in order for developmental activation to proceed. Pulse-chase experiments investigating *A. canis* developmental activation have revealed a similar hierarchy in stimuli to which the infective larvae respond (Hawdon & Hotez, 1996).

The evidence given by the metabolic enzyme activity measurements points strongly towards developmental activation of infective larvae in the host stomach. Further, the approximate timing of activation was established as taking place sometime after 40 min *post* entry of the infected tissue into the stomach environment. It now became important to establish the level of gene regulation involved in these changes, which is the subject of the next chapter.

5. CONTROL OF GENE EXPRESSION DURING DEVELOPMENTAL ACTIVATION

INTRODUCTION

A prerequisite, to studying the mechanisms of developmental regulation in infective *T. spiralis* larvae, is the clarification of the interaction between relevant environmental signals and parasite responses. Observations of surface alterations of infective larvae, as detected by PKH26 labelling, in response to liberation form the nurse cell, in an *in vitro* digest environment analogous to the host stomach, have shown that the parasites respond to environmental stimuli early in the infectious process (see chapter 3). This finding is further supported by the observations of FITC labelling of amphidial neurones in stomach-stage larvae, and the behavioural and surface responses accompanying the labelling procedure. Analyses of pyruvate kinase (PK), phosphoenolpyruvate carboxykinase (PEPCK), and isocitrate dehydrogenase (ICDH) activity changes, in response to exposure to the acid-pepsin digest mix, have indicated that developmental activation may be initiated during the stomach phase of the infection (see previous chapter). To further elucidate these putatively developmental changes, it was now important to establish the level of gene regulation involved.

Since the enzyme activity changes had become available as putative developmental markers, it was now possible to test the effects of specific inhibitors, with known sites of action at the level of transcription and translation, in order to dissect the regulatory mechanisms involved in activation. Translation was chosen as a primary target to establish the feasibility of using externally applied inhibitors, since changes in protein synthesis will be more common than changes in transcription. Cycloheximide was selected as the most suitable inhibitor based on its demonstrated efficacy in inhibiting protein synthesis in recovering *C.elegans* dauer larvae (Reape & Burnell, 1991b & 1992).

To get a further indication of changes in gene expression, in response to putative developmental activation, more direct probing methods were developed. Using a novel approach, changes in transcriptional activity in live larvae were observed using the fluorescent nucleic acid dyes SYTO12 and acridine orange. Tissue specific initiation of transcription was examined *in situ* in isolated nurse cells, live larvae during the recovery and post recovery period, and live enteric juveniles 2 h post infection.

The confirmation of the timing of developmental activation in response to the stomach environment, obtained with the nucleic acid labelling techniques, and the concomitantly observed changes in transcription patterns, opened the way to characterisation of developmentally regulated genes. To this end, PCR based RNA fingerprinting techniques were employed. The RAcD and spliced leader PCR methods described in chapter 1 were improved, and kinetic and titration analysis, as has been described for semi-quantitative PCR (Dallman & Porter, 1991; Köhler *et al*, 1995), were integrated into the techniques. As a result of investigating the larval response to the acid-pepsin digest mix, some putatively differentially expressed transcripts were identified, cloned and sequenced. The protein products or functions of the cloned sequence fragments, however, could not be identified to date.

MATERIALS AND METHODS

The changes in larval metabolic enzyme activities, in response to environmental stimuli analogous to those of the host stomach environment, indicated a possible developmental activation. To understand the mechanisms controlling the enzyme activity changes, protein synthesis inhibitors were used in an attempt to inhibit the observed activity increase. Changes in transcription pattern, in response to liberation from the nurse cell in the stomach, were also sought to gain further insight into the nature of the observed activation. To this end, live, *in situ*, fluorescent nucleic acid labelling and a form of differential mRNA display were carried out.

Parasite maintenance and recovery

Parasites were maintained in BALB/c mice and recovered by acid-pepsin digest as described in the previous chapter (see *Acid-pepsin recovery*, p. 95). Briefly, the digest consisted of 0.75% w/v pepsin (1 Anson unit/g, purchased from BDH) in saline/HCl pH 1.6 at 37° C, agitated with a magnetic stirrer. Digest times were either 40 min or 60 min, depending on the experiment (see below). Recovered larvae were cleaned by sucrose centrifugation (see *Acid-pepsin recovery* p. 95), unless they were to be used for live nucleic acid labelling.

For the purpose of obtaining carcass derived larvae, infected mice were killed, skinned, eviscerated, and stored in a desiccation jar with water at room temperature for 3 days. Larvae were subsequently recovered as described above.

Nurse cells were recovered from mice infected for more than 6 weeks by incubating minced eviscerated carcasses in saline/HCl pH 1.0 at 20° C under constant stirring for 1.5 h. The mix was strained through a 250 µm sieve, and sedimented material centrifuged at 1700 ***g*** for 7 min in 40% w/v sucrose/ sterile distilled water. The top nurse cell layer was aspirated and washed three times with saline, and the nurse cells centrifuged out of suspension at 500 ***g*** for 1 min.

Enteric juvenile worms were recovered from mice 2 h after infection with larvae that had been freshly recovered by 40 min digest. The small intestines were removed from the mice, slit open, and rinsed three times with saline. The rinsed intestines were suspended in fine mesh netting and incubated in 50 ml saline at 37° C for 10 min. To ensure that only worms which had successfully invaded the epithelium would be used for experimentation, all sedimented worms were discarded. A subsequent incubation was carried out in fresh saline at 37° C for 25 min, and recovered juveniles washed twice with saline and used immediately for live nucleic acid labelling.

Regulation of protein synthesis

Gel electrophoresis of soluble proteins

To detect changes in larval protein synthesis as a response to exposure to the acid-pepsin recovery digest, soluble extracts from 40 min recovered larvae were compared with extracts from 60 min recovered larvae, which had been incubated in saline for a further 40 min at 37° C (see *Experimental incubation conditions*, p. 96), by polyacrylamide gel electrophoresis. Extracts from larvae recovered with, and incubated in, cycloheximide (see *Effect of translation inhibitors on changes in metabolic enzyme activities*, p. 112) were also analysed.

Larvae were homogenised with a hand driven homogeniser in 5 volumes 50 mM HEPES pH 7.5 in the presence of proteinase inhibitors (500 mM PMSF, 5 mM E64, 1 mM pepstatin A and 1 mM 1-10 phenanthroline). The homogenate was centrifuged at 4° C for 15 min at 10000 ***g***, and the supernatant fraction used for electrophoresis. Protein concentration was measured using the Pierce Coomassie Protein Assay according to the manufacturer's instructions, with bovine serum albumin as a standard.

Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in gels with a 5% to 25% acrylamide gradient. 20 μg and 50 μg of each protein sample were run in separate wells. After electrophoresis,

the gels were stained with 0.1% Coomassie blue R-250 in destain solution (6% methanol, 1% acetic acid, and 0.01% glycerol). Destaining was overnight.

Effect of translation inhibitors on changes in metabolic enzyme activities

Cycloheximide was chosen as the most potentially useful compound for inhibiting the observed increase in ICDH activity in response to the acid-pepsin digest mix. Skinned and eviscerated mice were homogenised in 200 ml saline, and the homogenate was diluted with a further 100 ml saline. 200 ml 5 mM cycloheximide in saline were added to the homogenate, and the mix was incubated on a shaker for 3.5 h. 500 ml digest mix (1.5% pepsin in saline, pH 1.0) were added, and the digest was incubated at 37° C for 40 min with constant stirring. The digest was filtered through a 250 µm sieve, and the larvae allowed to sediment for 15 min. Half of the recovered larvae were cleaned by sucrose centrifugation (see *Acid-pepsin recovery*, p. 95) washed in 50 mM HEPES (pH 7.5) and immediately frozen in liquid nitrogen. The remaining larvae were incubated in 2 mM cycloheximide in saline at 37° C for 1 h, subsequently cleaned by sucrose centrifugation, washed with 50 mM HEPES (pH 7.5), and frozen in liquid nitrogen. Samples from both groups of recovered larvae were examined with the light microscope prior to sucrose centrifugation. As a viability test, some larvae were incubated in 1 mM fluorescein at 37° C for 20 min, and examined with a light microscope.

To test the effect of cycloheximide under activating conditions, mouse carcasses were treated as above, and recovery was carried out as above, but for 60 min. Sedimented larvae were split into two groups. Group 1 was incubated in 2 mM cycloheximide in saline at 37° C for 40 min before purification and freezing as above. Group 2 was cleaned up with sucrose centrifugation and frozen in liquid nitrogen immediately. Viability was tested with fluorescein activation.

In situ nucleic acid labelling

To detect tissue specific changes in transcription patterns during putative developmental activation, in response to liberation form the nurse cell in the stomach environment, *in situ* nucleic acid labelling in live parasites was carried out. Initially, a set of fluorescent nucleic acid dyes of varying properties was tested for suitability. All labelling experiments were repeated three times for each compound, and for three recovery procedures for each experimental condition.

The SYTO dyes (11 to 16) from Molecular Probes Inc. (http://www.probes.com) are lower affinity nucleic acid stains that passively diffuse through cell membranes. The SYTO dyes can stain both RNA and DNA, with the fluorescent wavelength emission intensities being similar for both RNA- and DNA-dye complexes. Exceptions are SYTO12 and SYTO14, which are twice as fluorescent on RNA than on DNA. Stained eukaryotic cells will generally show diffuse cytoplasmic staining as well as nuclear staining. Intense staining of intranuclear bodies is frequently observed (Haugland, 1996). Larvae were recovered by 40 min or 60 min acid-pepsin digest with subsequent saline incubation, and labelled with 50 µM dye (SYTO 11 to 16) in 50 mM HEPES pH 7.5. For labelling purposes, the larvae were incubated at 37° C for 30 min to 1.5 h. Acridine orange was tested at a concentration of 100 µM in 50 mM HEPES pH 7.5 for 30 min at 37° C Labelled larvae were washed three times with 1 ml 50 mM HEPES buffer and viewed with a fluorescence microscope. For all further investigations, the labelling time was reduced to 20 min, and only SYTO12 and acridine orange were used.

Isolated nurse cells (see *Parasite maintenance and recovery* p. 110), containing live larvae, were stained with 50 µM SYTO12 or 100 µM acridine orange in 50 mM HEPES pH 7.5 at 37° C for 25 to 60 min. The nurse cells were subsequently washed twice in HEPES and suspended in 0.25% trypan blue/ saline. Stained nurse cells were viewed immediately with a fluorescence microscope using a fluorescein filter (fluorescence emission maximum for SYTO12 bound to RNA is at 519 nm, for acridine orange the emission wavelength specific for DNA binding is 525 nm), and

images taken with a digital image capture device. Labelling was carried out with freshly isolated nurse cells, and nurse cells stored in saline at 4° C for 3 days.

Larvae and enteric juveniles (see *Parasite maintenance and recovery* p. 110), were labelled with SYTO12 or acridine orange as above, but staining time was limited to 20 min and the trypan blue quenching was omitted. Labelling was carried out with larvae recovered from freshly killed mice under the following conditions: 40 min digest, 40 min digest with subsequent 60 min saline incubation at 37° C, and 60 min digest with subsequent 40 min saline incubation at 37° C. Only the 40 min digest and 40 min digest with subsequent 60 min saline incubation at 37° C procedures were carried out with larvae recovered from 3-day carcasses. Enteric juveniles were labelled immediately after recovery. Labelling was also carried out in PBS as a control, but no differences were detected. Stained parasites were viewed immediately, and images taken as above.

Effects of specific environmental stimuli on transcription patterns

Fluorescein activation

To examine the response to fluorescein at the transcription level, larvae were recovered by 40 min digest, incubated in 1 mM fluorescein at 37° C for 20 min, and subsequently incubated in saline for 40 min at 37° C. Controls were incubated in saline at 37° C throughout. Labelling was carried out with SYTO 12 as described above.

Acid-pepsin

Larvae were recovered by 30 min digest. Immediately after the digest incubation, larvae were allowed to settle in the digest flask for 5 min. 300 ml of the digest mix were removed and set aside. The larvae were recovered from the remaining digest by standard sedimentation. Control larvae were incubated in saline at 37° C for 1 h. The other larvae were returned to 100 ml of the digest mix for 20 min at 37° C, quickly rinsed three times with pre-warmed saline, and incubated in saline for 40 min at

37° C. Both sets of larvae were labelled with the standard SYTO12 protocol.

Trypsin and bile

Larvae were recovered by 60 min acid-pepsin digest and left in saline at room temperature (20° C) for 1 h. The larvae were subsequently incubated in the standard trypsin and bile in medium solution (see *In vitro activation by exposure to components of the host enteric environment*, p. 34) for 40 min. SYTO12 and acridine orange labelling was carried out as described above.

Image analysis

All experiments were documented by capturing live fluorescent or bright light images using a digital image capture device (custom made with a Bosch low light camera).

Image analysis was performed on the captured images using the free UTHSCSA ImageTool program (developed at the University of Texas Health Science Center at San Antonio, Texas and available from the internet by anonymous FTP from ftp://maxrad6.uthscsa.edu).

mRNA expression

In order to characterise the changes in transcription patterns observed in larvae, as a response to exposure to the host stomach environment, a form of differential display was carried out. Reverse transcription of total RNA was carried out with arbitrary primers (18-mers) or "anchored" oligo (dT) primers. The cDNA was then amplified in high stringency PCR using the reverse transcription primer and one primer to the mRNA spliced leader sequence. To increase the sensitivity of the method, titration and kinetic analyses were performed for each set of primers and template.

Larvae were recovered by short digest (40 min) or by 1 h digest followed by 40 min incubation in saline at 37° C as described in *In situ nucleic acid labelling* (p.113).

Total RNA extraction

Larvae were homogenised in 10 volumes 4 M guanidine thiocyanate with a hand driven homogeniser. The homogenate was transferred to 1.5 ml microcentrifuge tubes, and equal volumes of phenol (pH 4.5) were added. The samples were equilibrated at 65° C, incubated for 10 min, and subsequently extracted with 250 µl chloroform per 500 µl phenol (2 to 3 min incubation at room temperature). After centrifugation at 12000 ***g*** at 4° C for 15 min, the aqueous phase was transferred to a fresh tube, and an equal volume of isopropanol was added. The samples were incubated for 10 min at room temperature, and centrifuged at 12000 ***g*** at 4° C for 10 min. The supernatant was discarded and the pellet washed three times in 1 ml 75% ethanol (centrifuged at 7500 ***g*** at 4° C for 5 min). The pellet was air dried at 60° C and dissolved in 400 µl DEPC treated water. An equal volume of 8 M LiCl was added, and the sample incubated for 1 h on ice. After centrifugation at 12000 ***g*** at 4° C for 15 min, the supernatant was discarded and the pellet washed three times in 1 ml 75% ethanol (centrifuged at 7500 ***g*** at 4° C for 5 min). The RNA pellet was dissolved in 100 µl DEPC treated H_2O, and the concentration estimated by measuring the absorbance at 260 nm (see *Total RNA extraction*, p. 34). Purity of the sample was determined by the ratio of absorbance measured at 260 nm to absorbance at 280 nm, 300 nm, and 320 nm. 3 µl of RNA suspension were run on a 1% agarose gel at 100 V for 60 min, stained with 0.5 µg/ml ethidium bromide, and viewed under UV illumination to check the sample for degradation. All RNA samples were stored at -70° C.

First strand synthesis

Initial optimisation of the protocols was carried out using 4.5 µg total RNA. For each 20 µl reaction, the following were added into a nuclease free 0.5 ml microcentrifuge tube: 5.0 µl RNA suspension (appropriately diluted), 2.0 µl (50 pmol) arbitrary primer (see *Primer sequences*, p. 117), and 4.0 µl H_2O. The solution was heated to 70° C for 10 min, then quickly cooled on ice and collected by centrifugation. The following were then added to each reaction: 4.0 µl 5x First Strand Buffer (GibcoBRL), 1.0 µl dNTP (10

mM), 2.0 µl DTT (0.1 M), and 1.0 µl RNasin® Ribonuclease Inhibitor (38 U/µl) (Promega). The reaction was warmed to 42° C for 2 min, after which 1.0 µl (200 U) SuperScript™ II reverse transcriptase (GibcoBRL) was added to each reaction. The reactions were carried out at 42° C for one hour, and stopped by heating to 70° C for 15 min.

Reverse transcriptions carried out with the 'anchored' oligo $(dT)_{12}$ primers (see *Primer sequences*, below) followed the same protocol, except that only 25 pmol of primer were used.

Titration analyses

To estimate the relative abundance of cDNAs amplified during the subsequent PCR, and to counteract some of the artefacts produced by PCR, titration of the RNA was performed during first strand synthesis. Initially 0.5 µg, 2.5 µg and 5.0 µg total RNA were tested in the reverse transcription reaction. Titrations were then changed to 50 ng, 500 ng, and 5 µg RNA reactions.

Primer sequences

Arbitrary primers:

A1	AAT CTA GAG CTC CTC CTC
A2	AAT CTA GAG CTC CAG CAG
A3	AAT CTA GAG CTC TCC TGG
A4	AAT CTA GAG CTC TCC AGC
A5	AAT CTA GAG CTC CCT CCA

Separate, 3' anchored, oligo $(dT)_{12}$ primers were designed for all 16 possible combinations of the two bases at the 3' end. The 5' end consisted of an 8 nucleotide tag with the constant sequence AGG GAA GC.

Four example oligo (dT) primers:

oligo (dT_{12})AA	AGG GAA GCT TTT TTT TTT TTA A
oligo (dT_{12})TA	AGG GAA GCT TTT TTT TTT TTT A
oligo (dT_{12})CA	AGG GAA GCT TTT TTT TTT TTC A

oligo (dT_{12})GA AGG GAA GCT TTT TTT TTT TTG A

Primers to the nematode mRNA 5' *trans*-spliced leaders had the following sequences:

SL1 GGTTTAATTACCCAAGTTTGAG

SL2 GGTTTTAACCCAGTTACTCAAG

PCR of first strand cDNA

PCR amplification of the arbitrarily primed reverse transcription products was carried out under the following reaction conditions: Each 20 µl reaction contained 50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 200 µM dNTPs, 25 pmol spliced leader primer, 25 pmol arbitrary primer, 2 µl cDNA, and 1.0 U *Taq* polymerase. No hot start was carried out, but reactions were assembled and kept on ice until the thermal cycler heating block had pre-heated to 94° C. Controls contained reaction mix and primers, but no cDNA; or reaction mix and cDNA, but no primers. After an initial denaturation at 94° C for 2.5 min, cycling conditions were 35 cycles of 94° C for 30 s, 55° C for 30 s, 72° C for 2 min, with a final extension at 72° C for 3 min.

PCR with the 'anchored' oligo (dT) primed cDNA was carried out under the same conditions as above, including 25 pmol of each spliced leader and anchored oligo (dT) primers. 220 ng anti *Taq* antibody were included to 'hot start' the reaction. The annealing temperature was 56° C (for 30 s).

Titration and kinetic analysis

cDNA products from the titrated first strand reactions were amplified separately and for different numbers of amplification cycles for kinetic analysis. The pilot titration experiment was amplified for 30 cycles, 25 cycles, and 20 cycles. All further experiments were carried out with 28 and 35 cycles.

Gel electrophoresis

PCR products were electrophoresed through 6% polyacrylamide (30:1 *bis*-acrylamide) 0.7 mm thick mini gels (BioRad) at 10 V/ cm, or 16 cm by 0.7 mm gels (Pharmacia), at 28 mA. Glass plates were prepared with bind silane (Pharmacia) and repel silane (Pharmacia). Visualisation of the DNA was by silver staining as described (see *Electrophoresis and visualisation of PCR products*, p. 39).

Purification and cloning of amplified cDNA bands

Recovery of DNA from polyacrylamide gels

Bands of interest were excised from the gels using sterile scalpel blades. The excised band was crushed inside a sterile nuclease free microcentrifuge tube and incubated in 200 µl PCR reaction buffer (final concentration: 50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100) at 94° C for 15 min. The sample was centrifuged at 10000 ***g*** for 1 min, and the supernatant removed to a fresh tube. The concentration of the eluted DNA was measured by reading the absorption at 260 nm of 2 µl sample diluted in 500 µl H_2O, and adjusted to 100 ng/ µl with H_2O for re-amplification or 10 ng/ µl for cloning.

Re-amplification of eluted DNA

The eluted DNA was re-amplified with PCR under the same conditions as the original cDNA (see *PCR of first strand cDNA*, p. 118). 6 µl of the reaction products were separated on 6 % polyacrylamide mini gels and visualised by silver staining. If necessary, the band of interest was re-purified to increase specificity of the cloning reaction.

Cloning of amplified DNA bands

The re-amplified / purified DNA from the band of interest was cloned using a TA cloning vector (TOPO TA Cloning, Invitrogen). 0.5 to 2 µl of the PCR sample (10 ng/µl) were used in the cloning reaction. The reaction was carried out in a final volume of 5 µl, and contained PCR product (0.5 to 2 µl), sterile water (added to a final volume of 4 µl), and pCR®-TOPO vector

(1 µl). The reaction was mixed gently and incubated for 5 min at room temperature (22° C). After a brief centrifugation, the tube was placed on ice, and the transformation reaction initiated immediately. The control reactions contained cloning vector but no insert.

Transformation Protocol

Both TOP10 (Invitrogen) and JM109 (Promega) competent cells were used for transformations.

For TOP10 cell transformation, the following procedures were carried out. 2 µl of 0.5 M β-mercaptoethanol were added to each vial of competent cells (50 µl) and mixed by stirring gently with the pipette tip. 2 µl of the TA-Cloning reaction were added into one vial of competent cells and mixed gently. The reaction was incubated on ice for 30 minutes. The cells were heat shocked for 30 seconds at 42° C without shaking, and then immediately transferred to ice and incubated for a further 2 min. 250 µl of room temperature SOC medium (2% tryptone, 0.5% yeast extracts, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) were added. The tubes were shaken horizontally (about 225 rpm) at 37° C for 1 h, and placed on ice. 80 µl from each transformation were spread on a prewarmed LB medium plate containing 50 µg/ml kanamycin and 40 µl of 40 mg/ml X-gal, and incubated overnight at 37° C. Ten white or light blue colonies were picked for analysis.

JM109 cells were transformed as follows. 100 µl of the competent cells were transferred to a pre-chilled 1.5 ml culture tube for each reaction. 4 µl of the cloning reaction were added, and the reaction was mixed by quickly flicking the tube several times. The tubes were immediately returned to ice for 10 minutes. The cells were heat-shocked for 45-50 seconds in a water bath at exactly 42° C, and then immediately placed on ice for 2 minutes. 900 µl of cold (4° C) SOC medium were added to each transformation reaction, with subsequent incubation for 60 min at 37° C with shaking (approximately 225 rpm). For each transformation reaction, cells were diluted 1:10 and 1:100, and 100 µl of the undiluted, 1:10 and 1:100 dilutions were plated on LB medium plates containing 50 µg/ml

kanamycin, 40 µl of 100 mM IPTG, and 40 µl of 40 mg/ml X-gal. The plates were incubated at 37° C for 14-18 hours. White or light blue colonies were picked for analysis as above.

Controls consisted of transformation reactions set up with the control cloning reaction, a positive pUC18 control, and a H_2O control. For transformations using the pUC18 control DNA, the cells were plated on LB/ ampicillin plates.

Analysis of positive clones

Ten white or light blue colonies were toothpicked from the plates, streaked onto fresh plates, and incubated at 37° C for 16-20 h. Colonies from the streaked plates were then cultured overnight in 1.5 ml LB medium containing 50 µg/ml kanamycin. Plasmid DNA was isolated from the cultured cells using the Promega Wizard Plus Minipreps according to the manufacturer's instructions. The yield of plasmid DNA was measured by reading the absorbance at 260 nm as described (*Total RNA extraction*, p. 116). The plasmids were analysed by either restriction digest with *Eco*RI or by PCR with M13 Forward (-20) and M13 Reverse primers.

Restriction analysis of plasmids was carried out as follows. 1.0 µg plasmid DNA was digested with 1 U *Eco*RI in buffer H (50 mM tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM dithioerythritol, pH 7.5), in a final reaction volume of 20 µl, at 37° C for 60 min. 1 µl 500 mM EDTA was subsequently added to stop the reaction. 15 µl of the reaction were electrophoresed on a 6% polyacrylamide mini gel and visualised by silver staining (see *Electrophoresis and visualisation of PCR products*, p. 39).

For PCR analysis, colonies were toothpicked into 20 µl sterile H_2O and heated to 94° C for 10 min. The sample was then centrifuged at 10000 ***g*** for 5 min, and 2 µl of the supernatant used in the PCR. In the case of purified plasmids, 80 ng of plasmid template was used in the PCR. Reactions were carried out with 5 pmol of M13 forward and reverse primers respectively, reaction conditions being as described previously (see *PCR of first strand cDNA*. p. 118). Gel electrophoresis was carried out as above.

In some cases, subcloning was necessary when clones contained more than one band. In this case, the band of interest was identified after electrophoresis of restriction fragments of the plasmid, excised, eluted, re-amplified, and cloned as above.

Sequencing of plasmid inserts

Cloned DNA was sequenced with fluorescence-based cycle sequencing reactions using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer), with the enzyme AmpliTaq DNA polymerase FS (Perkin Elmer). Samples were run on an ABI 373 Stretch or an ABI 377 automatic sequencer.

Sequencing reactions were carried out with 0.4 µg plasmid DNA and 3.2 pmole pUC/M13 sequencing primers (–40 forward or reverse). Thermal cycling was for 25 cycles of 96° C for 10 s, 50° C for 5 s, and 60° C for 4 min. The reaction products were ethanol precipitated as follows. 2.0 µl 3 M sodium acetate, pH 4.6 and 50 µl 95% ethanol were added to each reaction. The samples were vortexed, placed on ice for 10 min, and subsequently centrifuged at 10000 ***g*** for 20 min. The ethanol solution was aspirated, and the pellet rinsed twice with 250 µl 70% ethanol. The pellet was subsequently air-dried at 60° C, and re-dissolved in gel running buffer prior to electrophoresis.

The four-colour chromatograms, generated by the automated DNA sequencers, showing the results of the sequencing gel, were matched with the text file of sequence data generated by the machine, and ambiguous bases called by eye.

Each clone was sequenced until three consistent sequences were obtained.

Identification of full mRNA transcripts

S1 nuclease protection assay

One 20 µl first strand synthesis reaction (primed with $(dT)_{12-18}$) was precipitated with 2.0 µl 3 M sodium acetate, pH 4.6 and 50 µl 95% ethanol on ice for 10 min, and subsequently centrifuged at 10000 ***g*** for 20 min.

The ethanol solution was aspirated, and the pellet rinsed twice with 250 µl 70% ethanol. The pellet was subsequently air-dried at 60° C, and dissolved in the second strand synthesis reaction solution. Second strand synthesis was performed with 2 pmol gene specific primer at 70° C using *Taq* polymerase. Reaction conditions were 1.5 mM $MgCl_2$, 200 µM dNTPs, 1 U *Taq*, 220 ng anti-*Taq* antibody, and standard PCR buffer (see *PCR of first strand cDNA*, p. 118). The reaction was denatured at 94° C for 2 min, and then cycled twice at 94° C for 30 s and 70° C for 20 min. The reaction products were precipitated as above, and dissolved in S1 nuclease buffer (50 mM sodium acetate (pH 4.5), 280 mM NaCl, and 4.5 mM $ZnSO_4$). The reaction was incubated with 3 U S1 nuclease at 37° C for 10 min. 200 µl H_2O were added to the reaction, and enzymes removed from the sample with Microcon 50 microconcentrators (Amersham) according to the manufacturer's instructions. The concentrated sample was ethanol precipitated as above, dissolved in 6 µl H_2O, and amplified with touchdown PCR using a gene specific primer for a region downstream of the first strand primer. Reaction conditions were as for second strand synthesis, except that 5 pmol gene specific primer and 5 pmol oligo (dT) primer were used. Initial denaturation was at 94° C for 2 min, followed by five cycles of 94° C for 20 s, 62° C for 30 s, and 72° C for 3 min. The annealing temperature was dropped by 2° C for the next five cycles, this pattern being repeated three times to give a final annealing temperature of 56° C for the last five cycles. The PCR products, as well as the second strand products prior to PCR, were analysed by 6% polyacrylamide gel electrophoresis as described (*Gel electrophoresis*, p. 119).

To increase yield and specificity, first strand reaction products from several reactions were pooled and treated with 2 U RNase H prior to second strand synthesis. Wizard Plus DNA Preps (Promega) were used to clean up the reaction after the S1 nuclease digest step according to the manufacturer's instructions.

Reaction products were cloned into TA vectors as described (*Cloning of amplified DNA bands*, p. 119), and used to transform JM109 cells as

described (*Transformation Protocol*, p. 120). Positive clones were analysed as above (p. 121).

RESULTS AND DISCUSSION

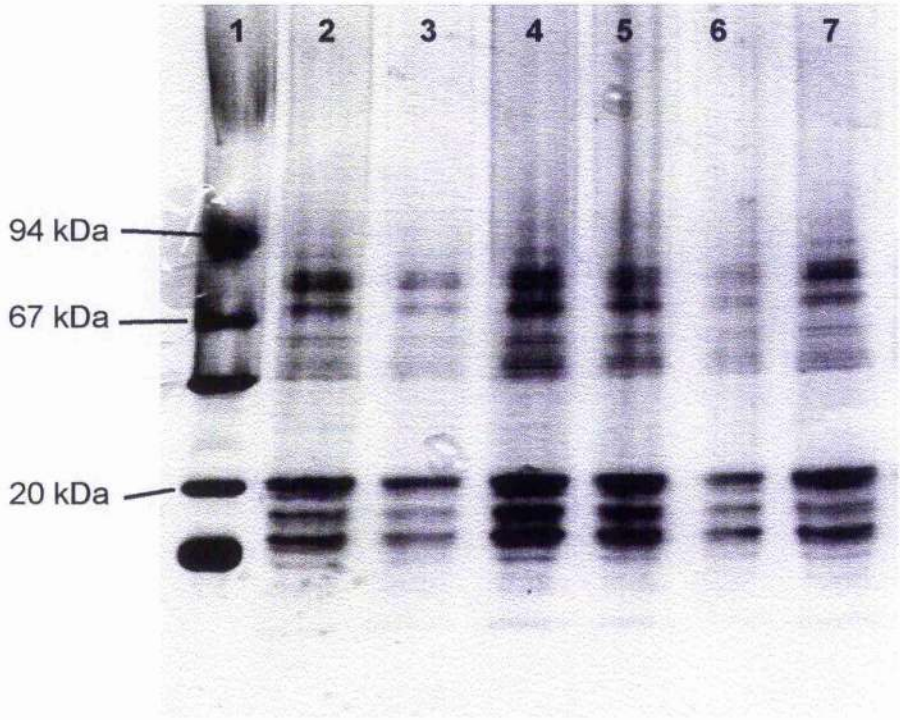
Previous experiments having established that infective *T. spiralis* larvae react to liberation from the nurse cell and exposure to the stomach environment by altering their surface properties and metabolic pathway potential, characterisation of these and any concurrent changes at the protein and nucleic acid level was undertaken.

Translational regulation

As a preliminary step, crude analysis of soluble proteins extracted from larvae, *pre-* and *post-* (putative) developmental activation by exposure to the acid-pepsin digest mix, was performed using SDS-PAGE. Comparisons among 40 min recovered larvae, 60 min recovered larvae (with 40 min saline incubation), and larvae exposed to cycloheximide showed no difference in protein banding patterns (see Figure 15, p. 126; cycloheximide result not shown). It is probable that the Coomassie blue staining method was not sensitive enough to detect the putatively low increase in protein synthesis which should account for the observed enzyme activity increases. Nevertheless, the experiment did demonstrate that there are no major differences in soluble protein profile between larvae recovered with a short digest and putatively developmentally active larvae.

The experiments using cycloheximide were more direct investigations into translational control during larval activation. Due to the complexity of the life cycle, and the ensuing fact that larvae can not be exposed to inhibitors while resident in the live host, only carcass larvae were amenable to inhibitor studies. Thus only ICDH activity could be used as a marker. The increase in ICDH activity observed in response to the exposure to the digest mix (see previous chapter), however, could not be inhibited by cycloheximide. Three-day carcass derived control larvae, recovered in the presence of 2 mM cycloheximide for 40 min, had the same activity as normally recovered carcass larvae (33.0 ± 5.5, $n = 5$; all

Figure 15. SDS-PAGE analysis of soluble proteins from larvae



5% to 25% polyacrylamide gradient gel, stained with Coomassie Blue. Lanes: (1) molecular marker. (2 & 3) 50 μg and 20 μg soluble protein from larvae recovered by 40 min digest. (4 & 5) 50 μg and 20 μg soluble protein from carcass larvae recovered by 40 min digest. (6 & 7) 20 μg and 50 μg soluble protein from larvae recovered by 60 min digest with 40 min saline incubation. Note: the apparent increase of protein in lanes 4 & 5 is due to a pipetting error.

activities expressed in nmol min^{-1} mg^{-1} protein). Larvae, recovered in the presence of 2 mM cycloheximide for 40 min, with a subsequent 1 h 2 mM cycloheximide/ saline incubation also had the same activity (26.0 ± 6.7, *n* = 6) as normally recovered larvae (see Table 2, p.104). Extension of the recovery period to 1 h with 40 min incubation, both in the presence of cycloheximide, failed to inhibit activity increase (47.5 ± 2.9, *n* = 4). Notably, all cycloheximide exposed larvae reacted to the fluorescein exposure by altering their behaviour from coiling to active migration.

The inability to inhibit ICDH activity changes with cycloheximide is likely to be due to failure of the compound to penetrate the larval cuticle. *Trichinella* larvae are notorious for being impermeable to biochemical agents which are in widespread use for signalling/ regulatory pathway studies in cultured mammalian cells (Modha, Kusel & Kennedy, 1995). The effectiveness of cycloheximide in nematodes has been documented in *C. elegans* (Reape & Burnell, 1991b). Unlike *C. elegans* recovering dauer larvae, *T. spiralis* larvae are non-feeding during the early process of infection, and the trans-cuticular route is presumably the only one open for the introduction of inhibitors. This lack of permeability to inhibitors complicates attempts to describe regulatory mechanisms of activation.

In situ nucleic acid labelling

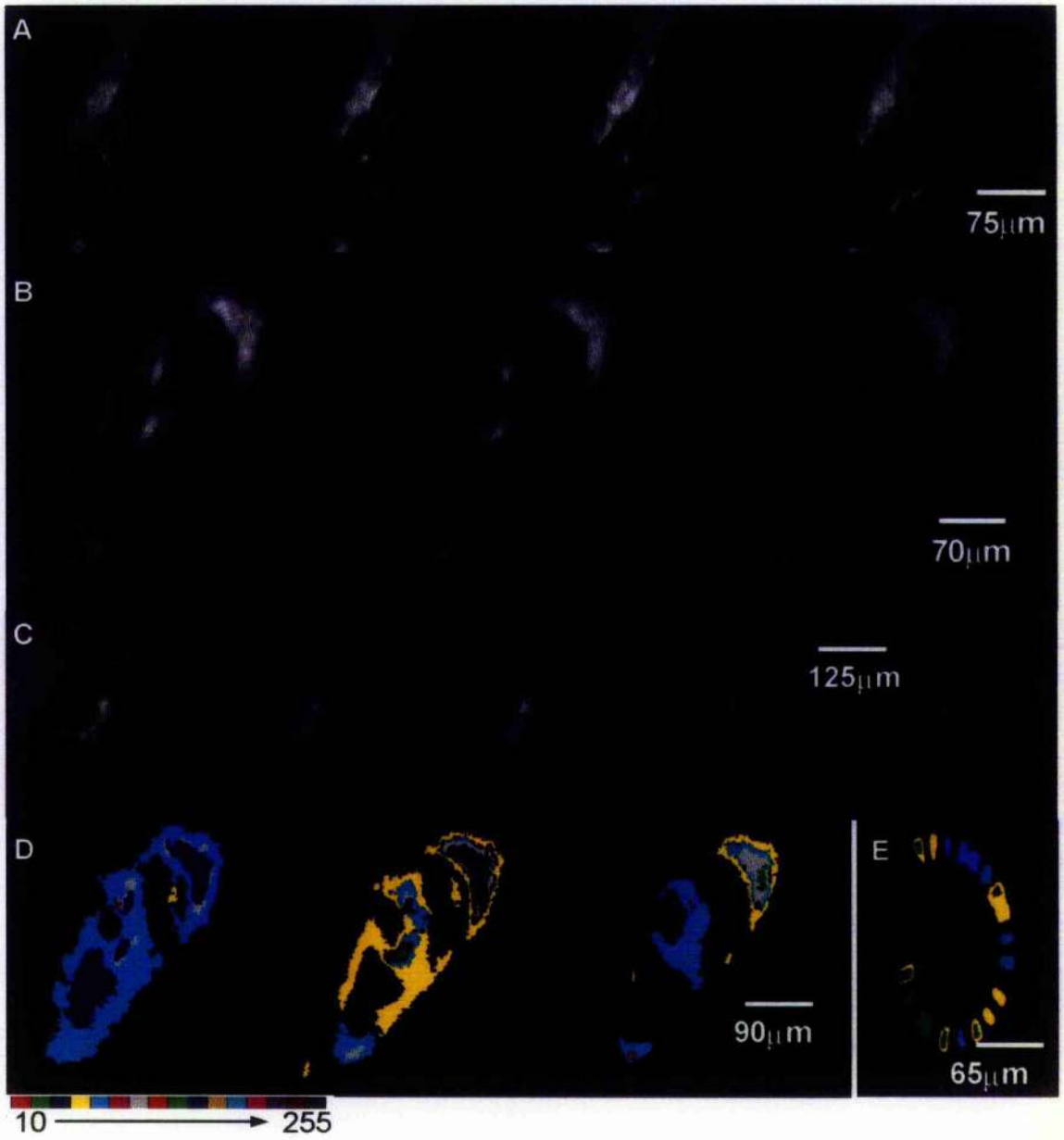
In order to circumvent the difficulties posed to inhibitor studies by the life cycle and impermeability of the cuticle, an unambiguous *in situ* method was sought to enable direct monitoring of gene expression changes. To achieve this, a novel method using fluorescent membrane-permeant nucleic acid dyes was designed. This method permits the tissue-specific measurement of transcriptional activity *in vivo,* and thus the monitoring of transcript levels present in specific tissues at the various life-cycle stages. Only two of the tested probes, SYTO12 and acridine orange, penetrated the larval cuticle and labelled nucleic acids within various tissues.

Nurse cells labelled well with SYTO12, with staining evident throughout the cell body. Trypan blue penetrated to the depth of the collagenous capsule in most instances, effectively cutting out interference

from surface fluorescence. Viability of larvae was judged by motility within the nurse cells, and all examined samples were viable after labelling procedures. Staining of the larvae within the nurse cells with SYTO12 was very weak compared to the surrounding host cytoplasm (Figure 16, p. 129). No difference in labelling pattern was observed between freshly isolated nurse cells and those stored in saline at 4°C for 3 days.

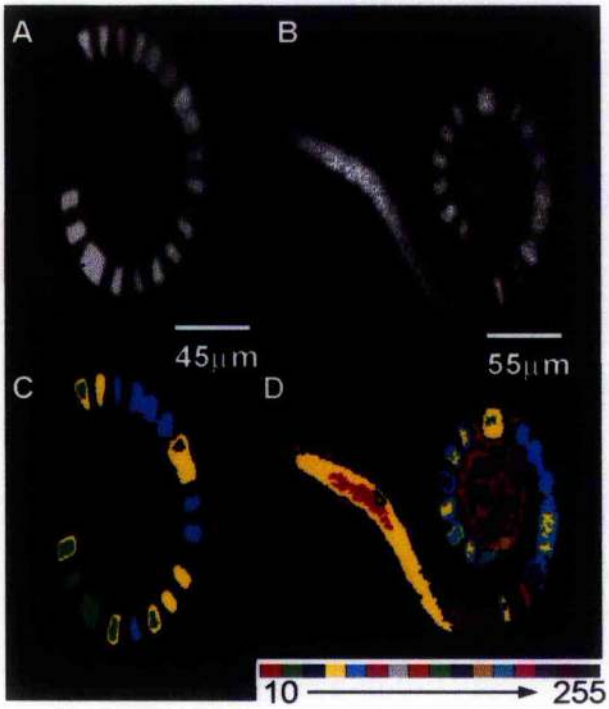
Larvae isolated from freshly killed hosts by 40 min recovery had a distinctive SYTO12 labelling pattern in the stichocyte region. Staining in this region was equal to, or more intense, than that observed in nurse cell cytoplasm (Figure 16, p. 129), and indicated high levels of RNA. The remaining tissues of the larvae labelled only weakly, with the exception of some bright bodies posterior of the stichocyte. Larvae recovered by 40 min digest with subsequent 1 h saline incubation labelled exactly as non-incubated larvae (Figure 18, p. 131). Variability in labelling patterns of live larvae for both experimental groups was restricted to the number of stichocytes fluorescing (18 ± 4, $n = 100$). Extension of the recovery time to 1 h, with subsequent 40 min incubation, effected an increase in labelled tissue regions. The stichocyte region labelled as in 40 min recovered worms, but an additional region posterior to the stichocytes labelled strongly (Figure 17, p. 130). This region maps to what is generally described as part of the genital primordium. Gray level analyses of images shows that labelling in this region has the same or a greater level of fluorescence intensity as the stichocyte region (Figure 17, p. 130 and Figure 18, p. 131). Enteric juveniles labelled with SYTO12 had the same labelling pattern as 60 min digest with 40 min incubation larvae (Figure 18, p. 131). In both experimental groups 70 –80% ($n > 300$) of larvae had the extended transcriptional activity labelling pattern. Labelling posterior of the stichocyte region was linked to motility: actively migrating larvae were usually labelled in the posterior part, while labelling of coiled larvae was usually restricted to the stichocytes. Larvae clearly change their tissue specific transcription patterns during the 60 min digest with 40 min incubation procedure, from those seen in nurse cells and 40 min recovered larvae, to patterns characteristic of enteric juveniles. Three-day

Figure 16. SYTO12 staining of intact nurse cells.



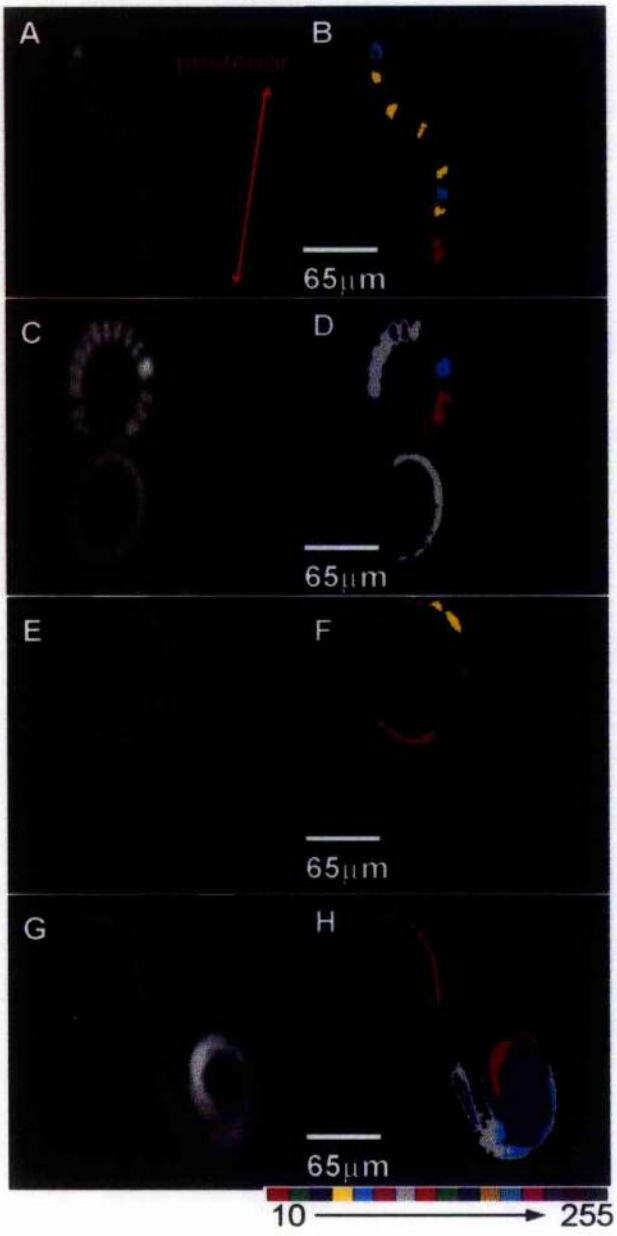
(A) Series of focal planes through a nurse cell in which the coiled larva is seen edge on. The nurse cell cytoplasm fluoresces brightly, while the larva occupies the band-like non-fluorescent patches. The larval coils are seen in cross-section at the top and lower extremities of the nurse cell. (B) Nurse cell in which the larva is viewed obliquely. The focal sections reveal the larva as a loosely coiled non-fluorescent object within the stained cytoplasm of the nurse cell. (C) The larva is seen as a coiled ring around a central fluorescent bright spot in the nurse cell. The first image in this series is a combination fluorescence / brightfield view. In the subsequent focal planes, only very weak staining is evident in the larva. (D) False colour gray level map of (B) to show relative staining intensities. Key to false colour codes in relation to gray levels (10 to 255) is given in the bar diagram below. (E) False colour gray level map of a recovered larva (40 min digest stained with SYTO12 as a comparison to (D).

Figure 17. SYTO12 labelling of larvae recovered from live hosts.



SYTO12 labelling of larvae recovered from live hosts. (A) Larva recovered by 40 min digest. The anterior of the larva is coiled to the centre. The bright fluorescent striped pattern consists of labelled stichocytes. (B) Larva recovered by 60 min digest with subsequent 40 min incubation in saline. The anterior end is coiled. Note increase in labelling posterior to the stichocyte region. (C) & (D) False colour gray level maps of images (A) and (B) respectively. Example key to colour code of values given below (D).

Figure 18. SYTO12 and acridine orange labelling of larvae and enteric juveniles.



SYTO12 and acridine orange labelling of larvae and enteric juveniles. (A) SYTO12 stained larva recovered by 40 min digest from a live host, and incubated in saline at 37°C for 60 min. (B) Gray level map of (A). (C) Enteric juvenile 2 h p.i. labelled with SYTO12, and the corresponding gray levels in (D). Notice the staining posterior to the stichocyte region. (E) Acridine orange staining of larva recovered from a live host by 40 min digest, with gray level analysis in (F). Notice the bead-like staining pattern in the stichocyte region. (G) Acridine orange labelling of an enteric juvenile 2 h p.i. with the corresponding gray levels in (H). Example key to colour code of values given below (H).

carcass derived larvae had the same SYTO12 labelling pattern as enteric juveniles (not shown). Exposure to fluorescein did not result in the extended SYTO12 labelling pattern, and labelling was restricted to the stichocyte region. Trypsin and bile incubation resulted in the same labelling pattern as enteric juveniles in only 1% of larvae (n = 300), all other larvae labelling only in the stichocyte region. Thus neither fluorescein nor trypsin and bile act as stimuli for the initiation of transcription in the region posterior to the stichocyte after interruption of the natural sequence of environmental cues. However, acid-pepsin digest mix (including mouse tissue) also fails to stimulate the transcriptional initiation once the exposure has been interrupted after less than 40 min. Larvae re-introduced into digest mix after having been taken out after 30 min fail to initiate transcription posterior to the stichocyte, as observed in enteral larvae. This observation gives evidence for a complex set of interactions between environmental stimuli and as yet uncharacterised heterochronic regulation within larvae.

DNA binding of acridine orange was selectively assessed by measuring green fluorescence emission (525 nm) patterns (the dye has red fluorescence (≈650 nm) when associated with RNA). Labelling patterns in freshly killed host derived larvae were similar to those observed with SYTO12, although not as clear (Figure 18, p. 131). In 40 min recovered larvae (both incubated and non-incubated), labelling was most intense in the stichocyte region, with minor foci in the posterior. In enteric juveniles and larvae recovered via 60 min digest with 40 min saline incubation, the anterior tip, the stichocyte region, and the region posterior to the stichocytes labelled intensely (Figure 18, p. 131). Three-day carcass derived larvae stained only in the stichocyte region.

The comparison between acridine orange DNA labelling patterns and SYTO12 RNA staining allows conclusions to be drawn about the timing of transcriptional activity. Acridine orange interacts with DNA and RNA by intercalation or electrostatic attractions, which restricts its DNA binding capacity to smooth fibre chromatin or other uncomplexed DNA (Haugland, 1996). This characteristic was used to reveal the transition of chromatin to smooth-fibre chromatin. Most actively transcribed genes, as well as genes

that have recently been, or will shortly become, active, are found in smooth-fibre chromatin (Edmondson & Roth, 1996; Li, Wrange & Eriksson, 1997). Thus, preparation for increased transcriptional activity in developing tissues can be observed with acridine orange, as well as conditions indicating a current increase in RNA production, which would otherwise be masked by the already present RNA. Only when SYTO12 and acridine orange labelling patterns coincide, can active transcription be deduced, and the possibility of exclusively detecting stored RNA be ruled out.

The striking difference in detectable transcription patterns between larvae inside nurse cells and liberated larvae give a clear indication that larvae respond to release from the nurse cell. Although it is possible that the surface properties of larvae inside nurse cells are so different as to make the larvae impermeable to the nucleic acid probe, it is far more likely that there are real differences in transcriptional activity. The stichocytes have been identified as the source of a number of excretory/secretory antigens (ESA) (Arasu *et al,* 1994; Takahashi *et al,* 1992), and it is conceivable that the high transcriptional activity detected in these cells in liberated larvae is connected to ESA production. High rate production of ESA inside the nurse cell would cause a noticeable accumulation of molecules, which has not been confirmed by immunohistochemistry of infected muscle tissue (Arasu *et al,* 1994). The initiation of transcription in the region posterior to the stichocyte, as a response to 60 min-digest / saline incubation, has not been linked to any known developmental or physiological events during the early phase of infection. The main line of evidence for the developmental nature of this *in vitro* change in transcription pattern comes from the *in vivo* observations of the same patterns occurring in enteric juveniles 2 h p.i. Like the PK activity rise in 3-day carcass derived larvae, the RNA labelling pattern observed posterior to the stichocyte in 40 min recovered 3-day carcass larvae is difficult to interpret. Strong evidence for pre-adaptation, in this case, is presented by the fact that acridine orange did not label posterior of the stichocyte in

these larvae. Hence the region is not transcriptionally active, and the observed SYTO12 pattern reflects stored RNA. Should larvae be producing lactate as a metabolic end-product in carcass nurse cells, the nurse cell environment will become quite acid. This low pH might stimulate developmental activation as seen in the transcription initiation posterior to the stichocyte. However, it has not been possible to stimulate transcription initiation of 'unactivated' larvae *in vitro* by exposure to low pH, so other factors must be present in the carcass nurse cell which may work in conjunction with pH.

The tissue specific changes in transcription, which are sequentially initiated during specific time periods during the infectious process, clearly show that activation is a graduated process that affects different tissues at different times. This observation supports the assertion that the measured increases in metabolic enzyme activities reflect tissue specific responses, the magnitude of which is masked by the activity in the remaining tissues. It is not clear whether the enzyme activity changes are directly linked to the changes in transcription patterns.

mRNA expression

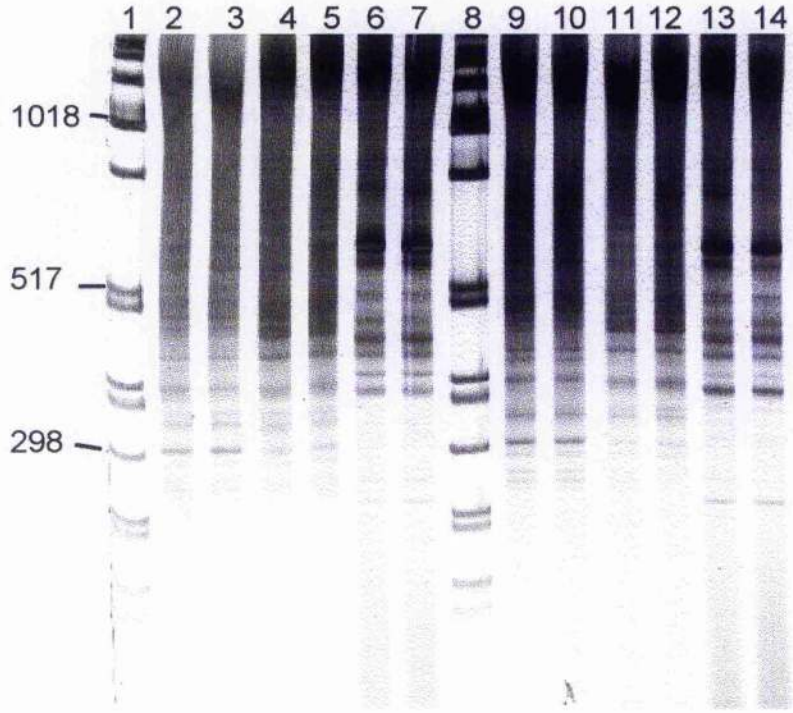
Evidence having been obtained for the tissue-specific initiation of transcription in response to exposure to the stomach environment, an attempt was made to characterise some of the differentially transcribed genes. To achieve this, several important improvements were made to the RNA fingerprinting techniques described in chapter 2. Primer-sequence specific selection was carried out at the reverse transcription level, allowing high stringency conditions during the entire PCR amplification. This measure also increased the sampling of rare sequences, since competition for primer is reduced during both reverse transcription and the initial PCR cycles. Oligo(dT) primers were anchored with 3' twin-nucleotide anchors and a 5' 8-nucleotide stabilising sequence. The 3' anchor sequence was instrumental in selecting sub-populations of mRNA at the reverse transcription stage (Liang & Pardee, 1992; Liang, Averboukh, & Pardee, 1993), while the 5' sequence addition ensured

higher priming specificity during PCR (see *Primer sequences*, p. 117). In the case of arbitrarily primed reactions, selection of mRNA sub-populations also took place at the reverse transcription stage (Welsh *et al*, 1992), and the use of 18-mer primers allowed matched annealing temperature with the spliced leader primer. In order to reduce PCR artefacts, and to establish the relative abundance of transcripts, titration and kinetic analysis techniques, used in semi-quantitative PCR (Dallman & Porter, 1991; Köhler *et al*, 1995), were employed. The results proved the method to yield consistent amplification products (see Figure 19, p. 136). Further, the titration method heightened the chances of rare species to compete with more abundant RNAs for primer (McClelland, Mathieu-Daude & Welsh, 1995). The effects of template concentration-dependent competition for primers can be seen in the variation of PCR products obtained from different starting concentrations of RNA during the reverse transcription (see Figure 19, p. 136).

In general, the results did not yield many differentially amplified bands. Of the five arbitrary primers, only A4 used in combination with the SL1 primer produced one differentially amplified band. Regrettably, time did not allow for utilisation of all the 16 "anchored" oligo(dT) primers, and only the primers with all possible C and G anchor nucleotide combinations were tested. The GC and the CC primers both amplified one differential band in combination with each of the SL1 and SL2 primers, bringing the total of differentially amplified bands that were identified to five (see Table 3, p. 137). If these differentially amplified fragments truly represent differentially expressed transcripts could not be tested due to lack of time. The titration and kinetic analysis would suggest that the likelihood of these transcripts being stage-specific is very high. However, the fragments need to be sequenced, and the sequences used as probes for northern blots or, preferably, *in situ* hybridisation. True quantitative PCR (Köhler *et al*, 1995) should also be carried out once appropriate primers can be designed from the characterised sequences.

Two of the differentially amplified fragments, the SL1 + A4 primed 147 bp (see Figure 20, p. 137) and the SL2 + oligo(dT_{12})GC primed 193 bp

Figure 19. PCR products primed with SL1 and oligo(dT_{12})CG primers



Silver stained 6% polyacrylamide gel. PCR products of 35 amplification cycles. Lanes: (1 & 8) 1 Kb DNA ladder molecular size marker, sizes given in number of base pairs. (2 & 3; 9 & 10) products of 50 ng RNA RT-PCR from 40 min larvae, lanes 2 & 3, and from 60 min + 40 min incubation larvae, lanes 9 & 10. (4 & 5; 11 & 12) products of 500 ng RNA RT-PCR from 40 min larvae, lanes 4 & 5, and from 60 min + 40 min incubation larvae, lanes 11 & 12. (6 & 7; 13 & 14) products of 5 μg RNA RT-PCR from 40 min larvae, lanes 6 & 7, and from 60 min + 40 min incubation larvae, lanes 13 & 14.

Table 3. Primer combinations which amplified bands stage-specifically

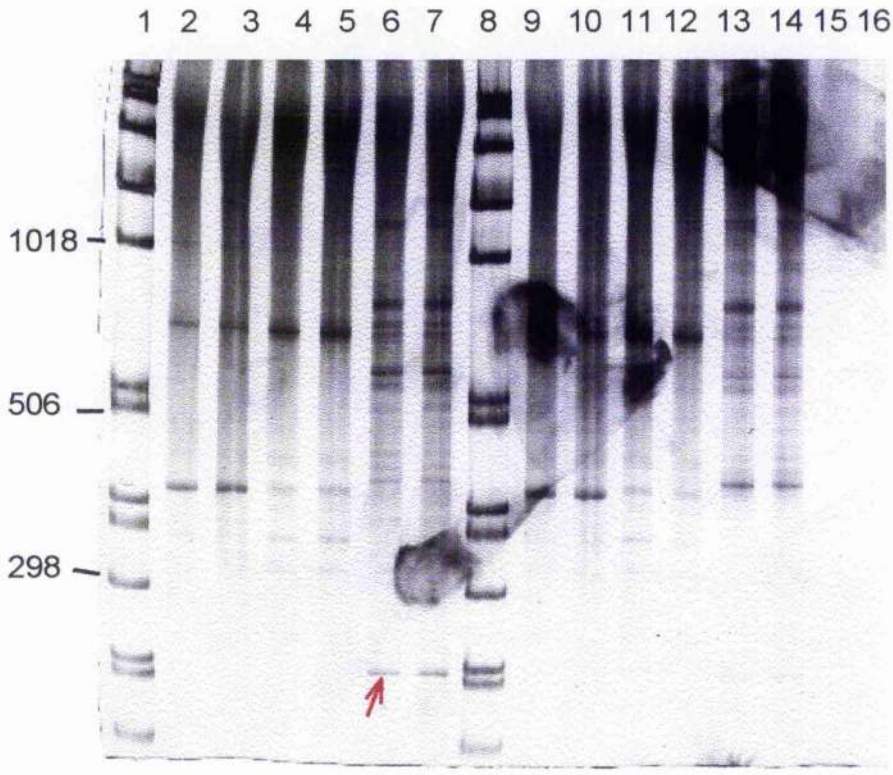
Primer combination	Band size in base pairs (bp)
SL1 + A4	147 bp
SL1 + oligo(dT_{12})GC	≈110 bp
SL1 + oligo(dT_{12})CC	≈260 bp
SL2 + oligo(dT_{12})GC	193 bp
SL2 + oligo(dT_{12})CC	≈320 bp

For primer descriptions, see *Primer sequences*, p. 117.

Figure 20. SL1 + A4 primed PCR products

Silver stained 6% polyacrylamide gel. PCR products of 35 amplification cycles. Lanes: (9 & 18) 1 Kb DNA ladder molecular size marker, sizes given in number of base pairs. (1 & 2; 10 & 11) products of 10 ng RNA RT-PCR from 40 min larvae, lanes 1 & 2, and from 60 min + 40 min incubation larvae, lanes 10 & 11. (3 & 4; 12 & 13) products of 100 ng RNA RT-PCR from 40 min larvae, lanes 3 & 4, and from 60 min + 40 min incubation larvae, lanes 12 & 13. (5 & 6; 14 & 15) products of 1 μg RNA RT-PCR from 40 min larvae, lanes 5 & 6, and from 60 min + 40 min incubation larvae, lanes 14 & 15. (7 & 8; 16 & 17) products of 5 μg RNA RT-PCR from 40 min larvae, lanes 7 & 8, and from 60 min + 40 min incubation larvae, lanes 16 & 17. (19 & 20) controls. Red arrows: 147 bp band

Figure 21. SL2 + oligo(dT_{12})GC primed PCR products.



Silver stained 6% polyacrylamide gel. PCR products of 35 amplification cycles. Lanes: (1 & 8) 1 Kb DNA ladder molecular size marker, sizes given in number of base pairs. (2 & 3; 9 & 10) products of 50 ng RNA RT-PCR from 40 min larvae, lanes 2 & 3, and from 60 min + 40 min incubation larvae, lanes 9 & 10. (4 & 5; 11 & 12) products of 500 ng RNA RT-PCR from 40 min larvae, lanes 4 & 5, and from 60 min + 40 min incubation larvae, lanes 11 & 12. (6 & 7; 13 & 14) products of 5 μg RNA RT-PCR from 40 min larvae, lanes 6 & 7, and from 60 min + 40 min incubation larvae, lanes 13 & 14. (15 & 16) controls. Red arrow: 193 bp band.

(see Figure 21, p. 138) bands, were cloned and sequenced. Cloning of the bands using the described TA cloning method (see *Cloning of amplified DNA bands*, p. 119) was straightforward, and only the 193 bp band needed to be sub-cloned once. This result points to some of the advantages of using polyacrylamide gel silver staining methods. Separation achieved with 6% polyacrylamide can be up to the single base pair range, while the silver staining protocol is extremely sensitive (Sanguinetti, Dias-Neto, & Simpson, 1994). The real advantage over radioactive methods, however, is the ease with which bands can be eluted from the gel. Since bands are directly visible in silver stained gels, they can be excised with far greater precision than from radioactive gels, giving "cleaner" starting material for cloning.

All of the sequenced colonies from one cloning experiment contained the same insert (as determined by sequence), and it was considered sufficient to sequence only three colonies per inserted band since the obtained sequences matched in each case (see Figure 22, p. 140 and Figure 24, p. 142). Database searching using the BLASTN (Altschul *et al,* 1990) and BLASTX (Gish & States, 1993; Altschul *et al,* 1990) algorithms at the National Center for Biotechnology Information, and the FASTA (Pearson & Lipman, 1988) search at the European Bioinformatics Institute, revealed that no significant similarities exist between the sequenced *T. spiralis* transcript fragments and previously described genes (see figures pp. 140, 141 & 143 for details). The best match BLASTN result for the 193 bp band was 42 out of 60 nt (70%) from a human chromosome 17 sequence, and the FASTA search gave 68.8% similarity in a 93 nt overlap with a *Ommastrephes sloanei* omega-crystallin gene (sequence optimised). The sequenced fragments can therefore be considered to belong to as yet uncharacterised genes of unknown function.

The S1 nuclease protection assay was developed to quickly identify the full-length transcripts of the sequenced fragments. Time constraints permitted only processing of the 147 bp fragment. The method was successful in as far as it did yield a single band of an estimated 1000 bp in length, visible in a silver stained polyacrylamide gel. Repeated attempts to

Figure 22. Nucleotide sequence of the SL1 + A4 primed 147 bp band.

```
                    *         20          *
A4SL1a : GGTTTAATTACCCAAGTTTGAGCCAGCTTCA :  31
A4SL1b : GGTTTAATTACCCAAGTTTGAGCCAGCTTCA :  31
A4SL1c : GGTTTAATTACCCAAGTTTGAGCCAGCTTCA :  31
consensus GGTTTAATTACCCAAGTTTGAGCCAGCTTCA

                  40          *          60
A4SL1a : TTCCGACTCTGTAATCTATCTTGATAATATA :  62
A4SL1b : TTCCGACTCTGTAATCTATCTTGATAATATA :  62
A4SL1c : TTCCGACTCTGTAATCTATCTTGATAATATA :  62
consensus TTCCGACTCTGTAATCTATCTTGATAATATA

                *         80          *
A4SL1a : ATAGATCGTAAACAGGAAGTTCCACATCTTG :  93
A4SL1b : ATAGATCGTAAACAGGAAGTTCCACATCTTG :  93
A4SL1c : ATAGATCGTAAACAGGAAGTTCCACATCTTG :  93
consensus ATAGATCGTAAACAGGAAGTTCCACATCTTG

              100         *         120
A4SL1a : ATGGCAAGCAAACAAAAAAGGTGCAACAAAA : 124
A4SL1b : ATGGCAAGCAAACAAAAAANGTGCAACAAAA : 124
A4SL1c : ATGGCAAGCAAACAAAAAAGGTGCAACAAAA : 124
consensus ATGGCAAGCAAACAAAAAAGGTGCAACAAAA

                *         140
A4SL1a : GTTGGGCTGGAGAGCTCTANATT : 147
A4SL1b : GTTGGGCTGGAGAGCTCTAGATT : 147
A4SL1c : GTTGGGCTGGAGAGCTCTAGATT : 147
consensus GTTGGGCTGGAGAGCTCTAGATT
```

A4SL1a, A4SL1b and A4SL1c denote each of the sequenced colonies.

Database search results:

BLASTN at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov, which searches EMBL and GenBank) (Altschul *et al*, 1990): Search of sequence without priming sites returned 12 sequences with better than 10 matches. Best match was 34/45 (75%), *Plasmodium falciparum* unknown function DNA.

FASTA at the European Bioinformatics Institute (http://www2.ebi.ac.uk, which searches EMBL) (Pearson & Lipman, 1988): Search of sequence without priming sites returned best match of 65.657% identity in 99 nt overlap with sequence optimisation, *Homo sapiens* PAC clone DJ0167.

Figure 23. Translation of the SL1 + A4 primed 147 bp band.

```
5'3' Frame 1
G L I T Q V Stop A S F I P T L Stop S I L I I Stop Stop I
V N R K F H I L Met A S K Q K R C N K S W

5'3' Frame 2
V Stop L P K F E P A S F R L C N L S Stop Stop Y N R S
Stop T G S S T S Stop W Q A N K K G A T K V

5'3' Frame 3
F N Y P S L S Q L H S D S V I Y L D N I I D R K Q E V P H
 L D G K Q T K K V Q Q K L

3'5' Frame 1
P T F V A P F L F A C H Q D V E L P V Y D L L Y Y Q D R L
 Q S R N E A G S N L G N Stop T

3'5' Frame 2
Q L L L H L F C L L A I K Met W N F L F T I Y Y I I K I
D Y R V G Met K L A Q T W V I K

3'5' Frame 3
N F C C T F F V C L P S R C G T S C L R S I I L S R Stop
I T E S E Stop S W L K L G Stop L N
```

Database search results:

BLASTX at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov, which searches EMBL and GenBank) (Gish & States, 1993; Altschul *et al*, 1990): Search of sequence without priming sites returned 23 sequences with better than 10 matches. Best match was 11/15 (73%), Frame = -3, E2 Glycoprotein precursor from bovine coronavirus.

This similarity was considered not significant.

Figure 24. Nucleotide sequence of the SL2 + oligo(dT_{12})GC primed 193 bp band.

```
                    *         20          *
SL2-TGCa : GGTTTTAACCCAGTTACTCAAGCTACTAGGC :  31
SL2-TGCb : GGTT-TAACCCAGTTACTCAAGCTACTAGGC :  30
SL2-TGCc : GGTTTTAACCCAGTTACTCAAGCTACTAGGC :  31
Consensus  GGTTtTAACCCAGTTACTCAAGCTACTAGGC

                  40          *          60
SL2-TGCa : TTAGAATCAACCAATATAATAATTCGTAATT :  62
SL2-TGCb : TTAGAATCAACCAATATAATAATTCGTAATT :  61
SL2-TGCc : TTAGAATCAACCAATATAATAATTCGTAATT :  62
Consensus  TTAGAATCAACCAATATAATAATTCGTAATT

                *         80          *
SL2-TGCa : GACTAGCTTAATACAATAACGACTCACTCAA :  93
SL2-TGCb : GACTAGCTTAATACAATAACGACTCACTCAA :  92
SL2-TGCc : GACTAGCTTAATACAATAACGACTCACTCAA :  93
Consensus  GACTAGCTTAATACAATAACGACTCACTCAA

              100         *         120
SL2-TGCa : AATATCACCCAAAACCAAAGATTGACAAACC : 124
SL2-TGCb : AATATCACCCAAAACCAAAGATTGACAAACC : 123
SL2-TGCc : AATATCACCCAAAACCAAAGATTGACAAACC : 124
Consensus  AATATCACCCAAAACCAAAGATTGACAAACC

              *         140         *
SL2-TGCa : ATGGTTACATCAGTAAGGACAAAACAAAATT : 155
SL2-TGCb : ATGGTTACATCAGTAAGGACAAAACAAAATT : 154
SL2-TGCc : ATGGTTACATCAGTAAGGACAAAACAAAATT : 155
Consensus  ATGGTTACATCAGTAAGGACAAAACAAAATT

            160         *         180
SL2-TGCa : TACATGTTACAACTCGGCAAAAAAAAAAAAG : 186
SL2-TGCb : TACATGTTACAACTCGGCAAAAAAAAAAAAG : 185
SL2-TGCc : TACATGTTACAACTCGGCAAAAAAAAAAAAG : 186
Consensus  TACATGTTACAACTCGGCAAAAAAAAAAAAG

             *
SL2-TGCa : CTTCCCT : 193
SL2-TGCb : CTTCCCT : 192
SL2-TGCc : CTTCCCT : 193
Consensus  CTTCCCT
```

SL2-TGCa, SL2-TGCb and SL2-TGCc denote each of the sequenced colonies.

Figure 25. Translation of the SL2 + oligo(dT_{12})GC primed 193 bp band.

```
5'3' Frame 1
L L G L E S T N I I I R N Stop L A Stop Y N N D
 S L K I S P K T K D Stop Q T Met V T S V R T K
 Q N L H V T T R

5'3' Frame 2
Y Stop A Stop N Q P I Stop Stop F V I D Stop L
N T I T T H S K Y H P K P K I D K P W L H Q Stop
G Q N K I Y Met L Q L G

5'3' Frame 3
T R L R I N Q Y N N S Stop L T S L I Q Stop R L
T Q N I T Q N Q R L T N H G Y I S K D K T K F T C
 Y N S A

3'5' Frame 1
C R V V T C K F C F V L T D V T Met V C Q S L V
L G D I L S E S L L Y Stop A S Q L R I I I L V D
S K P S

3'5' Frame 2
A E L Stop H V N F V L S L L Met Stop P W F V N L
W F W V I F Stop V S R Y C I K L V N Y E L L Y W
L I L S L V

3'5' Frame 3
P S C N Met Stop I L F C P Y Stop C N H G L S I F
G F G Stop Y F E Stop V V I V L S Stop S I T N Y
Y I G Stop F Stop A Stop Stop
```

Database search results: .

BLASTX at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov, which searches EMBL and GenBank) (Gish & States, 1993; Altschul *et al,* 1990): Search of sequence without priming sites returned 17 sequences with better than 10 matches. Best match was 11/18 (61%), Frame = +2, *Escherichia coli* DNA.

This similarity was considered not significant.

clone this putative full-length cDNA failed for unknown reasons. Regrettably, time did not permit full optimisation of the methods, and the full-length transcript has not been identified to date.

The results obtained from the RNA fingerprinting experiments can only be considered as preliminary. Nonetheless, the fact that five differentially amplified PCR products were identified, after relatively few experiments, indicates that the method may well be effective in identifying those genes differentially expressed in response to exposure to the stomach environment. The direct response of the PCR product yield to titration of RNA template, and variation in amplification cycles, gives evidence for the sensitivity of the method. The observation that amplification products will consistently appear only at specific RNA template concentrations, in conjunction with a specific number of amplification cycles, supports the reliability of the method in consistently identifying specific RNAs. Thus, although blotting or hybridisation experiments are still to be carried out, it is very probable that the differentially amplified fragments are also differentially expressed. Future work will hopefully be able to characterise the changes in transcription observed in *T. spiralis* infective larvae during the infectious process, and shed some more light on how this remarkable parasite regulates its development.

6. CONCLUSIONS

The first larval stage of *T. spiralis* undergoes a period of developmental arrest upon reaching infectivity within the host muscle tissue. This period of quiescence is abruptly terminated upon entry into the next host, and the parasite is then required to cope with, and adapt to, a radically different and potentially hostile physiological environment.

For infective *T. spiralis* larvae, the process of infecting the next host involves a series of steps that may vary in duration, but never in sequence. Muscle larvae interact with their host cells in a yet not fully understood manner, actively maintaining a de-differentiated state of the "nurse" cells they occupy during their period of developmental arrest (Despommier, 1993; Jasmer, 1995). The intriguing questions of what activity muscle larvae maintain during developmental dormancy, and how this relates to the infectious process, remain to be answered. Nevertheless, the first step in the infectious process involves the death of the current host, and a termination of the interactions between nurse cell and larva. The larvae must then respond to being ingested, or, more usually, the anoxic environment and deteriorating conditions of a carcass before ingestion by scavengers or opportunistic feeders (Campbell, 1983a; Campbell, 1988). In either case, the larvae face a dramatic change in environment and must respond appropriately to reactivate development at the right time in order to successfully establish infection.

Until now, the resumption of development of infective larvae was thought to take place in the intestinal environment of the next host (Despommier, 1983). The reaction of *T. spiralis* to intestinal stimuli has been noted to be accompanied by a change in the lipophilicity of the surface (Proudfoot *et al*, 1993a & 1993b), loss of the surface accessory layer; (Stewart *et al*, 1987; Modha *et al*, 1994), and physiological changes (Stewart *et al*, 1987), all occurring before the next molt. How the larvae regulate their developmental transition, and how gene expression is controlled during development, has remained undescribed until now. The original aim of the present research, therefore, was to determine at which levels gene expression is regulated during the initiation of development of

infective *T. spiralis* larvae, and to identify genes associated with, or regulating, developmental initiation.

The results, presented in the previous chapters, give evidence for a complex set of interactions between environmental stimuli and larval temporal regulation during the infectious process, eventually leading to resumption of differentiation and growth. The SYTO12 labelling of nurse cells indicates that infective muscle larvae have reduced transcriptional activity to a minimum, and do not appear to possess any detectable quantity of stored mRNA. This is in keeping with observations made in other nematodes to date, which indicate that the resumption of development of arrested larval stages involves changes in gene expression at the transcriptional level. It has been documented that recovery of *C.elegans* larvae from the dauer state is accompanied by an ordered temporal sequence of gene expression which involves initiation of mRNA transcription (Dalley & Golomb, 1992). Further, studies using transcription inhibitors, such as actinomycin D and α-amanitin, have demonstrated that full resumption of development depends on transcription in infective larvae of *Haemonchus contortus* and *Nippostrongylus braziliensis* (Petronijevic & Rogers, 1983; Bonner & Buratt, 1976). *T. spiralis* larvae inside nurse cells, which have been stored for three days, fail to label with SYTO12 in the same way as larvae inside freshly recovered nurse cells. But, some undetectable changes must be taking place, since these larvae recovered from 3-day carcasses will have a PK activity twice that of their counterparts recovered from freshly killed hosts, and label with SYTO12 (but not acridine orange) in the region posterior to the stichocyte after only 40 min digest recovery. Thus, it appears that muscle larvae may be developmentally dormant, but certainly very reactive to any environmental changes, well before entering the digestive tract of the next host. Further, larvae are able to modulate their reactions to environmental stimuli very specifically. Upon liberation from the nurse cells, larvae initiate transcription only in specific cells in the stichocyte region. Whether this transcriptional activity detected in these cells is connected to ESA production is not clear, but the stichocytes have

been identified as the source of a number of ES antigens which are produced by liberated larvae (Arasu *et al,* 1994; Takahashi *et al,* 1992). Without the presentation of further environmental cues, no additional changes in tissue specific transcription patterns or selected metabolic enzyme activities can be detected. This does not mean that larvae cease to proceed with any physiological or structural alterations, however, since changes to the surface properties can be detected via PKH26 labelling within the following hour. This surface alteration seems to proceed at a set time, regardless of the environment in which the larvae find themselves after 40 min post liberation from the nurse cells. At this juncture, temporal regulation of larval development appears to become evident. In *C.elegans* larval development, the integration of temporal and environmentally stimulated developmental regulation has been mapped to specific amphidial neurones (Bargmann, Thomas & Horvitz, 1990; Bargmann & Horvitz, 1991; Vowels & Thomas, 1992). In *T. spiralis* larvae liberated from nurse cells, FITC labelling has also demonstrated that at least environmental information is integrated into the developmental regulation via the amphids. Using FITC or fluorescein as an environmental stimulus, it was shown that amphidial chemosensation mediates alterations in behaviour, surface properties as detectable by PKH26 labelling, and PK activity. Further, it is very probable that these processes are under catecholaminergic control, since Lee & Ko (1991) demonstrated that these amphidial neurones, which were labelled with FITC, contain catecholamines. Development and ecdysis have been demonstrated to be under catecholaminergic control in a number of nematodes, including *Phocanema decipiens, Dirofilaria immitis* and *Haemonchus contortus* (Smart, 1989; Warbrick & Ward, 1992; Fleming, 1993). The fact that ICDH activity increase and transcription posterior to the stichocyte could not be stimulated with fluorescein indicates that probably more than one environmental cue is necessary to stimulate full resumption of development in infective larvae. However, the as yet undefined stimuli present in the acid-pepsin digest, an environment analogous to the stomach, suffice to induce the switch in metabolic pathway potential, as measured by PK and ICDH activity increases, as well as initiation of

transcription posterior to the stichocyte region. Both these alterations are characteristic of actively developing intestinal juveniles, and give strong evidence that the cues presented in the stomach suffice to stimulate full developmental activation. As growth and differentiation continue, after the resumption of development, a continual change in gene expression can be expected. The genes involved in the structural and physiological changes documented to take place in response to exposure to trypsin and bile in previous research (Stewart *et al*, 1987; Proudfoot *et al*, 1993a; Modha *et al*, 1994), however, appear to be transcribed before exposure to these stimuli takes place. Notably, transcriptional activation (as detected by SYTO12) posterior to the stichocyte could not be stimulated by exposure to trypsin and bile, and RNA fingerprinting techniques did not detect any changes in mRNA profile in response to these cues (the latter being, however, not a conclusive result). RNA fingerprinting did detect putatively differentially transcribed genes in response to 20 min extended exposure to the acid-pepsin digest. Regrettably, the genes which are differentially transcribed in response to exposure to an environment analogous to the stomach, as evidenced by SYTO12 and acridine orange labelling, could not be identified to date.

In light of the evidence presented in the previous chapters, and above conclusions, I propose that *T. spiralis* infective larvae begin to resume development in response to liberation from the nurse cell in the stomach, and commit to development after a critical time of exposure to the stomach environment. For development to continue to adulthood, however, other research has shown that further environmental cues are needed, since no *in vitro* culture system could be developed for the intestinal stages of the parasite to date (ManWarren *et al*, 1997, Judy Appleton, personal communication). Further, I propose that regulation of development is under transcriptional control, and that tissue specific transcription is initiated early during the infectious process, perhaps immediately after release from the nurse cell. Development is under both environmental and temporal control, and environmental stimuli must be presented in a set order for development to continue. Hopefully, future

research will lead to the characterisation of the genes involved in resumption of development of infective *T. spiralis* larvae.

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