ABSTRACT

TRIVEDI, SHWETA. Host cytokines and immune responses in pregnancy associated transmission of arrested hookworm larvae (Under the direction of Dr. Prema Arasu)

Over one billion people worldwide are infected with the hookworms, Necator and Ancylostoma spp. Upon entry into the host, infective larvae (third stage L3 which are free-living and non-feeding) typically mature into blood-feeding adults in the small intestines. An important aspect of the life cycle for A. duodenale (humans) and A. caninum (dogs) is the propensity for L3 to undergo a temporary state of developmental arrest in the host. In female hosts, these tissue-arrested L3 reactivate during pregnancy and are transmitted to the neonates through milk. During pregnancy transforming growth factor (TGF)-β is apparently upregulated in host tissues including the mammary gland. Studies from the free-living nematode Caenorhabditis elegans show that TGF-β and insulin-like signaling pathways regulate larval arrest and resumption of development. Similar signaling pathways are proposed in the pregnancy-associated reactivation of arrested Ancylostoma larvae. We have previously used an in vitro assay to demonstrate that recombinant human TGF-β can stimulate a feeding response in tissue-arrested A. caninum L3 larvae. We speculate that host factors like TGF-β and pregnancy hormones such as estrogen and prolactin signal arrested L3 larvae to resume development. To facilitate analyses of mechanisms of reactivation and transmission in vivo, we have utilized a mouse model of A. caninum infection; mice serve as an excellent model because infective L3 do not develop into adults but migrate to different somatic tissues.
and arrest, later reactivating during the periparturient period to transmit through milk. Skeletal muscle and mammary gland are the major tissues of interest during this process of arrest, reactivation and transmission. We investigated TGF-β1, TGF-β2 and IGF-1 serum and transcript cytokine profiles during late pregnancy, early lactation and mid-lactation in mice infected with *A. caninum* to correlate their levels with the transmammary transmission of the larvae to the nursing pups. An *in vitro* co-culture system was also developed in an attempt to mimic *in vivo* conditions for assessing the effects of TGF-β and, estrogen and prolactin on larval reactivation. *A. caninum* L3 were co-incubated with primary skeletal muscle and mammary epithelial cells in a Transwell® setup and larval reactivation was measured utilizing the *in vitro* feeding assay. Additionally, the immune responses during concurrent pregnancy and helminthic infection were assessed given that both conditions are known to be biased towards a T helper (Th)-2 type of response. Serum and transcript levels of IFN-γ (representative of the Th1 arm of the immune response) and IL-4 (for Th2) were measured in skeletal muscle, mammary gland and spleen during pregnancy and *A. caninum* infection in the mouse. These findings which are based upon serum and transcript levels suggest that host-derived TGF- β1 and IGF-1 may play roles in the reactivation and transmission of arrested *A. caninum* larvae; levels of TGF- β2 did not however, show a correlation with the timepoints of pregnancy and lactation associated with larval reactivation and transfer. Also, a Th2-like response characterized by elevation in IL-4 transcript levels was observed in skeletal muscle while a mixed Th1/Th2 profile was observed in mammary gland when comparing the different permutations of infection with *A. caninum* versus pregnancy/lactation in BALB/c mice.
HOST CYTOKINES AND IMMUNE RESPONSES IN PREGNANCY
ASSOCIATED TRANSMISSION OF ARRESTED HOOKWORM LARVAE

by

SHWETA TRIVEDI

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APPROVED BY:

Dr. Prema Arasu
(Chair of Advisory Committee)
Dr. William Miller

Dr. Scott Laster
Dr. Paul Mowdziak
Dedication

To my parents: Dr. Amresh Kumar and Mrs. Mridula Trivedi
Thanks to you, I never needed to search for other role models in life.
BIOGRAPHY

Shweta Trivedi was born on 10\textsuperscript{th} June, 1975 in Moradabad, Uttar Pradesh, India. She finished her primary schooling until high school from Campus School, Pantnagar in 1993. It was her ambition to become a veterinary surgeon like her father. She joined the College of Veterinary Medicine at Gobind Ballabh Pant University of Agriculture and Technology, Pantnagar in 1993 and graduated with a degree in Bachelor of Veterinary Science and Animal Husbandry in 1998. She successfully competed in a national exam for Junior Research Fellowship awarded by the Indian Council of Agricultural Research and joined Indian Veterinary Research Institute, Izatnagar in 1998. For her Master’s work, she characterized and tested the short-term culture filtrate proteins from \textit{Mycobacterium bovis} as potential diagnostic reagents for tuberculosis testing. After completing her postgraduate degree in Master’s of Veterinary Immunology in 2000, she got admission in the Immunology Program at College of Veterinary Medicine, North Carolina State University and in the Microbiology department at University of Tennessee, Knoxville. She joined the Immunology program at CVM, NCSU in 2001 where she worked on her PhD under the direction of Dr Prema Arasu. The major focus of her graduate work was on host-parasite interactions involved in arrest and reactivation in canine hookworm, \textit{Ancylostoma caninum}. She will join the National Institute of Allergy and Infectious Diseases at Rockville, Maryland as a visiting fellow in the lab of Dr. Andrea Keane-Myers studying the role of T regulatory cell in the development of allergic diseases.
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1. Introduction

The World Health Organization estimates that over one billion people in most tropical and subtropical regions of the world are infected with blood-feeding intestinal hookworms, predominantly *Necator americanus* and *Ancylostoma duodenale* (Chan, et al., 1997). In addition, approximately 44 million women are infected with hookworms during pregnancy with the potential for transmammary transmission of *A. duodenale* to their nursing infants (Bundy, et al., 1995; Schad and Page, 1982). Studies have correlated retarded physical and cognitive development with early childhood parasitic diseases (Watkins and Pollitt, 1997; Drake, et al., 2000; Dickson, et al., 2000). Hookworms are also prevalent in other hosts; *A. caninum* is a major parasite of dogs in the U.S. and other parts of the world (Blaghburn, et al., 1996) and can cause moderate to severe iron deficiency anemia, hypoproteinemia and bloody diarrhea that can be fatal to puppies and immunosuppressed dogs (Georgi and Georgi, 1991). *A. caninum* is also a zoonosis to humans due to skin penetration by the soil-dwelling infective larvae resulting in cutaneous larva migrans (Miller, et al., 1991) and eosinophilic enteritis due to the potential, albeit rare, of larval development to the adult stage in the human gut (Prociv and Croese, 1990; Croese et al., 1994).

The lifecycle of nematodes is relatively simple involving development from egg, through different larval stages to reach adulthood. With parasitic nematodes, the later stages of development have an obligate requirement of a host. Interestingly, *A. duodenale* and *A. caninum*, like a number of other parasitic nematodes, also have the capacity to infect a host, abort their normal maturation pathway and undergo developmental arrest in somatic tissues. Tissue-arrested larvae are metabolically quiescent and resilient to host immune responses as well as the chemotherapeutic agents typically used to eliminate the intestinal adult stage.
(Lee, et al., 1975; Schad, 1991; Arasu, 1998). This reservoir of larvae can however respond to host signals and reactivate during pregnancy or stress (Stone and Girardeau, 1968). Once reactivated, the third stage larvae (L3) either resume development to become egg-producing adults in the intestines or are transmitted to the newborns via milk. Vertical transmission of infection is a relatively common route of passage of parasites to a new generation of hosts but little is understood about the molecular or immuno-physiological mechanisms facilitating the process in the host/pathogen relationship. While a generalized immunosuppressive state is associated with gestation, previous studies have shown no correlation between the immune responses and reactivation/transmission of \textit{A. caninum} larvae (Arasu and Heller, 1999). In separate studies, the hormonal fluxes during pregnancy have been implicated to directly or indirectly mediate the reactivation of the arrested larval population (Stoye and Krause, 1976); in the proposed 'indirect' role, estrogen and prolactin are known to regulate the expression of various developmental and immunomodulatory cytokines such as transforming growth factor beta (TGF-\(\beta\)) and insulin-like growth factor (IGF). Recent studies have shown that these mammalian signaling molecules are also present and critical to the development of nematodes (Crook, et al., 2005; Brand and Hawdon, 2004).

The free-living nematode, \textit{Caenorhabditis elegans}, has been used as a model to draw parallels for understanding the signaling mechanisms responsible for the phenomena of arrest and reactivation in parasitic nematodes (Hotez, et al., 1993). At least three signaling pathways, the TGF-\(\beta\) pathway, insulin-like pathway and a cGMP pathway, have been implicated to control the dauer or arrested form in \textit{C. elegans} (Riddle and Albert, 1998). \textit{C. elegans} and hookworms fall in Clade V of Phylum Nematoda (Blaxter, 1998). Given their close phylogenetic relationship, it is likely that signaling pathways involved in regulating
development might be commonly shared. We and others have hypothesized that tissue-arrested L3 larvae of *A. caninum* could receive signals from the host resulting in similar signaling through these known pathways in *C. elegans* eventually leading to reactivation (Hawdon and Schad, 1991).

One objective of this study was to use an *in vivo* system to examine the host’s circulating and tissue-specific profile of expression of TGF-β and IGF during different phases of pregnancy and lactation in the presence and absence of *A. caninum* infection, and to correlate these levels with larval transmission. The second objective of this study was to establish an *in vitro* system to examine the direct effects of TGF-β and IGF on *A. caninum* larvae cultivated in the environment of muscle (site of larval arrest) versus mammary cells (site of larval transmission).

Using mice, an experimental model for studies of tissue-arrest, reactivation and transmammary transmission of *A. caninum* larvae was previously established (Arasu and Kwak 1998). Mice serve as a paratenic host for *A. caninum* in that the L3 larvae enter the host (by ingestion or skin penetration) and persist in various somatic tissues but are unable to mature into adults (Arasu and Kwak, 1999). These tissue-arrested L3 larvae however, display pregnancy-associated transmammary transmission of infection to the nursing pups providing an encapsulated model for studies of this process of vertical transmission.

For the *in vitro* analyses, primary skeletal and mammary epithelial murine cell cultures were established and the conditions were defined for propagation of *A. caninum* L3s. The co-culture system was used to examine the effect of normal dog serum and recombinant mammalian TGF-β as well as the indirect effects of estrogen and prolactin on larval reactivation using an established feeding/activation assay.
The third objective of this study was to examine the immunological effects of *A. caninum* infection on the viability of pregnancy. Pregnancy and helminthic infection are both known to evoke Th2-biased immune responses (Wegmann, et al., 1993; Pearce and Reiner, 1995). However, there are no reports to date on the concurrent effects of pregnancy and helminthic infection or the inflammatory responses associated with larval arrest and reactivation.

The fourth aspect of this thesis concerns a relatively new and powerful analytical tool. Real time polymerase chain reaction (PCR) analysis is extensively used, including in the above described studies, to compare gene expression levels between different treatments and experimental groups. The validity of relative comparisons is however highly dependent on the use of a suitable endogenous reference gene for normalization of the measured transcript levels. This section describes the validation of six different commonly used endogenous reference genes in real time PCR analyses of gene expression in different developmental stages, strains and treatments of *A. caninum*. These studies also serve as a template for validation studies in any given experimental system of relative expression using real time PCR analyses.
2. Literature Review

Despite their health significance and global importance, little is known about how hookworm parasites interact with their hosts to establish a chronic infection. The review will outline the biology, normal lifecycle and public health significance of hookworm infection. Additionally, hookworms undergo developmental arrest during one of their environmental life-stages (as free-living infective larvae) as well as inside the host (hypobiosis). Hypobiotic hookworm larvae can reactivate in response to host signals received during pregnancy / lactation and resume their development resulting in the transmammary transmission of infection to the newborns. This phenomenon of arrest and reactivation has been well studied in Caenorhabditis elegans, a free-living nematode in which distinct signaling pathways have been discovered. The immuno-physiological changes occurring during pregnancy have also been implicated in facilitating the reactivation process and is also addressed below, followed by a discussion of the host immune responses during acute and chronic parasitic infections and during pregnancy.

2.1 Life cycle of Ancylostoma caninum

Ancylostoma caninum is one of the two most common parasitic infections of domestic dogs (Blagburn, et al., 1996) and is a good model for studies on human hookworm disease. The life cycle of A. caninum is similar to that for A. duodenale and begins with shedding of embryonated eggs in the feces of an infected host (Kassai, 1999). The hatched first-stage larva develops through two molts to the infective third-stage (L3), which is still ensheathed in the outer cuticle from the second larval stage. By positioning itself on top of a grass blade or other objects, this non-feeding L3 maximizes its chances of penetrating the
skin of a host. Infective L3s can persist in the environment under warm moist conditions for extended periods of time (~6 months or more). While infection by skin penetration is prevalent, larvae can also be orally ingested. After entering the host through skin the maturing fourth stage larvae find their way to the lungs via blood vessels or lymphatics where they are coughed up and swallowed. They become blood feeding adults in the intestines by 14-21 days post-infection. In contrast, larvae that enter by ingestion may either follow the above mentioned path or bypass the lungs and mature directly in the intestines to become sexually mature male and female worms (Figure 1). While attached to the intestinal mucosa, adult worms secrete a range of molecules including proteases and anti-coagulants to prevent clotting in order to receive a blood meal (Williamson, et al., 2003; Stassens, et al., 1996). A single female worm can produce about 10,000 eggs per day resulting in large numbers of infective larvae in the environment; by contrast, only 50-100 adult worms are sufficient to cause life threatening anemia in a neonatal puppy (Loukas, et al., 2005).

The currently available approach for controlling hookworm infection in humans or animals is targeted at elimination of the intestinal adult stages of the parasite with various classes of anthelmintics such as the macrolides (e.g. ivermectin) and benzimidazoles (Bungiro and Cappello, 2004). However, the requirement of repeated doses, the development of drug-resistant strains and the risk of reinfection are the main limitations of chemotherapeutic intervention (Quinnell et al., 1993; Hotez and Pritchard, 1995). Besides the use of anthelmintics, other factors like sanitation, use of footwear and health education can prevent or reduce the burden of infection (Burke and Roberson, 1979a,b) as occurred in the hookworm eradication era of the 1920s-40s in many parts of the southern U.S. (Ciesielski et
al., 1992). Efforts are also underway to develop an effective vaccine against hookworms (Brooker, et al., 2005; Goud, et al., 2005).

### 2.2 Developmental Arrest and Reactivation in Hookworms

An interesting aspect of the *A. caninum* and *A. duodenale* life cycles is the capacity of the parasite to undergo developmental arrest at two stages (Figure 2). The first one occurs when the non-feeding third larval stage is in the environment waiting to infect a suitable host. Unless it infects the host, further development cannot occur as it only reactivates upon receiving host cues and resumes development within the host. The second stage occurs when the infecting third stage larvae enter a host; instead of continuing to develop into a blood-feeding adult in the intestines, a portion of the entering larvae remain within the somatic tissues as developmentally arrested L3s; these L3s have been found throughout the body in different organs and especially in skeletal muscle (Lee, et al., 1975; Schad, 1990). The developmentally arrested larvae can persist in the tissues for long periods of time. They display reduced susceptibility to drugs that effectively eliminate adult stage infections (Schad, 1991; Arasu, 1998). These arrested larvae possess the capacity to reactivate and resume normal development thereby serving as a reservoir of infection in the same host i.e. being able to reactivate and re-establish an active infection (Schad and Page, 1982). Secondly, in a female host during pregnancy/lactation, the arrested larvae apparently receive specific cues to reactivate and get transmitted through the mammary glands to the newborn pups via milk (Stone and Smith, 1975; Burke and Roberson, 1985).

Vertical transmission has been also observed in other helminths including *Toxocara canis*, the other major intestinal parasite of dogs (Shoop, 1991; Swerczek et al., 1971).
mechanisms by which developmentally arrested parasitic stages are cued to reactivate during pregnancy and transmit to a new generation of hosts, either via milk or the uterus (as in the case of *Toxocara*), are unknown but serve as a major route of transmission of infection. Previous studies have shown that host humoral responses (Arasu and Heller, 1999) and the generalized immunosuppression associated with pregnancy are not directly involved in the process of parasite reactivation.

### 2.2.1 Developmental arrest/reactivation and signaling pathways in *Caenorhabditis elegans*

*Caenorhabditis elegans*, the free-living soil nematode, is a valuable model system for parasitic nematodes of both plants and animals. *C. elegans* has a relatively short life cycle (~4 days) which also involves development through four larval stages (L1-L4) to the adult stage. Normal development and hermaphroditic reproduction occurs in the presence of adequate food (soil bacteria), optimal temperatures and low population density (Riddle and Albert, 1998). However, when the population density or temperatures are very high and food resources are limited, an alternate developmental pathway occurs resulting in a non-feeding dauer larva which can persist for several weeks without feeding (but also with the capacity for motility, albeit only when necessary e.g. prodding). With the return of favorable environmental and food conditions, the dauer larva begins feeding and resumes development to an adult. *C. elegans* perceives the environmental signals via chemosensory neurons located in its head, namely ASI, ASJ, ADF and ASG (Riddle and Albert, 1998). From extensive genetic and molecular studies, at least three signaling pathways have been shown to regulate
the entry and exit from the dauer stage, namely, a TGF-β-like, an insulin-like, and a cGMP-regulated pathway (Massague, et al., 2000; Braeckmann, et al., 2001; Birnby, et al., 2000).

**TGF-β pathway**

The TGF-β pathway in *C. elegans* comprises a series of genes including a ligand encoding *daf-7*, and the type I and type II receptors (*daf-1* and *daf-4* respectively). By analogy to TGF-β signaling in the mammalian system, it was hypothesized that *daf-7* binds to and activates the receptor *daf-1/daf-4* complex. Genetic analysis has shown that *daf-1* acts as the type I receptor in the signaling cascade and has been localized to the amphidial chemosensory neurons in the head of the worm (Gunther, et al., 2000). The second receptor is encoded by *daf-4* and shows 30% amino acid identity with the conserved kinase domain in *daf-1*, and 40% and 34% identity to the mouse activin type II receptor and human type II TGF-beta receptor, respectively (Estevez, et al., 1993). It was also shown by the authors that DAF-4 binds to the mammalian bone morphogenetic protein BMP-2 and its expression at the L1 stage is enough to rescue the dauer constitutive phenotype (Estevez, et al., 1993). Since DAF-4 can act in a cell non-autonomous way (Inoue and Thomas, 2000), it has been suggested that DAF-7 might be binding to DAF-4 on neurons to generate a secondary signal that acts to promote differentiation of tissue involved in development to the adult stage (Beall and Pearce, 2002).

A second TGF-β pathway, involving the *dbl-1* TGF-β like ligand, and the receptors *sma-6* (another Type I receptor) and *daf-4*, has been shown to be involved in the regulation of body size and male tail formation (Patterson and Padgett, 2000). Other genes with
homology to the TGF-β ligands have been identified in *C. elegans* but have not been characterized.

**Insulin-like growth factor pathway**

The insulin-like *daf-2* receptor associated pathway acts synergistically with the pathway activated by the *daf-7* TGF-β-type signal (Ogg, et al., 1997) in dauer arrest/reactivation. There are 38 insulin-like proteins that have been identified in *C. elegans* and can act as potential ligands (Pierce, et al., 2001; Li, et al., 2003). Of these, DAF-28 is expressed in the ASI and ASJ chemosensory neurons and its expression is downregulated by dauer-inducing conditions (Li, et al., 2003). These findings suggest that DAF-28 is the functional ligand for DAF-2 and necessary for prevention of dauer formation. The transmembrane DAF-2 receptor is a tyrosine kinase that facilitates the formation of a dauer under favorable conditions (Kimura, et al., 1997). Upon ligand binding, DAF-2 gets autophosphorylated and activates AGE-1, a phosphatidylinositol-3-OH kinase (Morris, et al., 1996; Malone et al., 1996). Subsequently, activated AGE-1 produces secondary messengers phosphatidylinositol bis and tris- phosphate phospholipids, PIP$_2$ and PIP$_3$. These secondary messengers activate other kinases like Protein kinase B (PKB/Akt) and 3-phosphoinositide-dependent kinase (PDK). Two PKB/Akt kinases, AKT-1 and AKT-2 have been found to mediate this signaling in *C. elegans* (Paradis and Ruvkun, 1998). Later, PDK-1 was identified and shown to be involved in DAF-2 signaling (Paradis, et al., 1999). These activated kinases phosphorylate DAF-16, a forkhead transcription factor, which presumably regulates long-lived dauer larva (McElwee, et al., 2003).
Cyclic GMP signaling pathway

The cGMP pathway includes a membrane guanylyl cyclase that binds an undefined ligand and catalyses the synthesis of the secondary messenger cGMP from GTP. ‘Loss-of-function’ mutants of the membrane guanylyl cyclase constitutively enter the dauer stage and have been shown to be rescued by membrane permeable analogues of cGMP (Birnby, et al., 2000). It appears that the cGMP signaling pathway might be involved in dauer development upstream of daf-7 pathway. There is evidence that a guanylyl cyclase, DAF-11, is necessary to stimulate expression of DAF-7 (Murakami, et al., 2001). Expression of daf-11 cDNA by cell specific promoters suggests that daf-11 acts cell autonomously in ASI chemosensory neurons for daf-7 expression (Murakami, et al., 2001).

2.2.2 Signaling Pathways in Parasitic Nematodes

Over the years evidence has emerged that similar signaling pathways might be present in parasitic helminths for the purpose of regulating the resumption of development upon receiving the appropriate signals from the host. Components of both the TGF-β and insulin-like pathways have been investigated in several different parasites to understand how they may regulate the entry into and exit from the arrested state. In parasitic nematodes components of the TGF-β signaling pathway were first identified in the filarial nematodes, Brugia malayi and Brugia pahangi, which cause lymphatic filariasis and elephantiasis in humans and domestic pets like cats and dogs.

The first type I TGF-beta receptor, Bp-trk-1, from a parasitic helminth was cloned from Brugia using degenerate primers (Gomez-Escobar, et al., 1997). EST sequencing efforts of the Washington University Nematode Sequencing Project subsequently showed the
presence of TGF-β receptor homologs in both animal and plant parasitic nematodes

Identification of the Type II receptor daf-4-like gene has been more difficult; to date it has been identified only by random EST sequence analyses of Strongyloides stercoralis (GenBank Accession number BE029357).

Two genes, tgh-1 and tgh-2, encoding ligands from the TGF-beta superfamily have been also identified in Brugia malayi (Gomez-Escobar, et al., 1998; Gomez-Escobar, et al., 2000). The tgh-1 gene shows greatest homology to the bone morphogenetic protein subfamily of BMP1 and has been shown to be maximally expressed at the first (L1 to L2) and second (L2 to L3) molts, and is completely absent in the stages associated with developmental arrest, i.e. microfilaria (similar to an L1) (Gomez-Escobar, et al., 1998). Expression of tgh-1 at a time when the parasites are maturing and molting and its homology to BMP subfamily is suggestive of a role in growth and development of the parasite. The second ligand encoding gene, tgh-2, was identified the B. malayi EST database (Washington University Nematode Sequencing Project) and is most similar to the C. elegans daf-7 gene. Expression of tgh-2 is greatest in microfilariae (L1 larvae) and also in adult male and female parasites (Gomez-Escobar, et al., 2000), therefore, coinciding with developmental arrest as well as terminal development of the parasite.

Components of the TGF-β signaling pathway have also been identified in other classes of helminthic parasites including Schistosoma mansoni which is a trematode or flatworm. The type I TGF-β receptor, Smrk-1, shares up to 58% homology with the conserved kinase domain of other type I TGF-β receptors but is considered a divergent member of this family as it has an atypical GS domain that is involved in regulation of type I receptor kinase activity (Davies, et al., 1998). A chimeric receptor containing the
extracellular domain of SmRK1 joined to the intracellular domain of the human type I TGF-β receptor. The chimeric receptor bound radiolabeled TGF-β and activated a luciferase reporter gene in response to both TGF-β1 and TGF-β3 but not BMP7 (Beall and Pearce, 2001). This study suggests that a host ligand may directly stimulate parasite-associated receptors of the TGF-β pathway. Recently, a type II TGF-β receptor was isolated from *S. mansoni* and was found to be most closely related to the Activin type II receptor family (Forrester, et al., 2004).

In 2001, an insulin-like homolog was cloned and sequenced in *Strongyloides stercoralis* (GenBank Accession number BG224639) but a *daf-2* like insulin receptor has not been identified. Regardless, a forkhead transcription factor gene, *fktf-1*, proposed to be orthologous to the *C. elegans* dauer-regulatory gene *daf-16* was discovered in *S. stercoralis*. Discovery of *fktf-1* indicates the presence of an insulin-like signaling pathway in *S. stercoralis* similar to that known to regulate dauer development in *C. elegans* (Massey, et al., 2003). Ablation of neuron pairs ASF and ASI in *S. stercoralis* larvae, an intestinal nematode of humans and dogs, caused the larvae to develop directly into the dauer-like infective resting stage and prevented development of the soil-dwelling adult worms (Ashton, et al., 1998). Loss of the sensory neurons presumably interfered with generation of signals needed for normal development from a larva to an adult.

### 2.2.3 Signaling Pathways in Hookworms

The hookworm parasites and *C. elegans* fall within the same clade in the phylum Nematoda ie Clade V (Blaxter, 1998). It is therefore plausible to speculate that many developmental control mechanisms are shared between *A. caninum* and *C. elegans*. Recently,
two *A. caninum* TGF-β-like ligands, *Ac-dbl-1* and *Ac-daf-7* were cloned and characterized (Freitas and Arasu, 2005). *Ac-dbl-1* showed 60% amino acid identity to the *C. elegans dbl-1* while *Ac-daf-7* showed 46% amino acid identity to *C. elegans daf-7*. Since *C. elegans daf-7* mutants are constitutive dauers, Rajan (1998) hypothesized that parasitic nematodes behave as if they were *daf-7* mutants to ensure developmental arrest at the L3 stage. He proposed that these parasites then utilize the *daf-7* gene product from their host to reenter the developmental pathway.

In support of this hypothesis, an *in vitro* assay was used to show that physiological concentrations of recombinant mammalian TGF-β isoforms, TGF-β1 and TGF-β2, had significant stimulatory effects on tissue-arrested larvae resulting in the resumption of a feeding response; additionally, the stimulatory effect of normal dog serum could be blocked by preincubation with anti-TGF-β antibodies (Arasu, 2001). These *in vitro* feeding/reactivation analyses also showed that the pregnancy/lactation associated hormones estrogen and prolactin did not have a stimulatory effect on infective or tissue-arrested *A. caninum* larvae (Arasu, 2001); insulin was also shown to not have a direct effect but as mentioned above, TGF-β isoforms 1 as well as 2 had a direct effect on the feeding or reactivation response of tissue-arrested larvae.

To further investigate the role of insulin signaling in hookworm larval activation, the phosphatidylinositol-3-OH kinase inhibitor LY294002 was tested for its effect on *in vitro* activation using the resumption of feeding as a marker for activation. LY294002 prevented feeding in *A. caninum* infective larvae stimulated with host serum filtrate and a glutathione-analogue, the muscarinic agonist arecoline, or the cell permeable cGMP-analogue 8-bromo-cGMP (Brand and Hawdon, 2004). Similar results were seen with the congeneric hookworm
*A. ceylanicum*. These data suggest that an insulin-signaling pathway mediates activation in hookworm larvae, as in *C. elegans*, and that the phosphatidylinositol-3-OH kinase inhibitor acts downstream of the cGMP and muscarinic signaling steps in the pathway. In *A. caninum*, LY294002 had no effect on the release of excretory/secretory products associated with activation, suggesting that the secretory pathway diverges from the activation pathway upstream of the phosphatidylinositol-3-OH kinase step (Brand and Hawdon, 2004). These results provide additional support for the insulin-signaling pathway as one of the primary pathway for activation to parasitism in hookworm larvae.

The *in vitro* feeding assay has been used in several studies above as a direct method to evaluate the activation status of larval stages with feeding being equated to resumption of development (Hawdon and Schad, 1990, 1992; Hawdon, et al., 1993). Over the years many factors such as 10% normal canine serum (Hawdon and Schad, 1990), reduced glutathione (Hawdon and Schad, 1992), muscarinic antagonists (Tissenbaum, et al., 2000) and cyclic GMP (Hawdon and Datu, 2003) have been shown to stimulate similar feeding behavior in *A. caninum* iL3. Using fluorescein isothiocyanate (FITC)-labeled bovine serum albumin it was shown that in the presence of 10% normal canine serum *A. caninum* iL3 displayed significant feeding behavior (a classic *C. elegans* dauer reactivation response) at 37°C in presence of 5% CO₂ (Hawdon and Schad, 1990). Reduced glutathione stimulated larval feeding in greater than 90% of the iL3 population, in a specific and concentration-dependent manner with highest at 5-10 mM, and reaching a plateau at 25-50 mM (Hawdon and Schad, 1992). Using increasing concentrations of oxotremorine and other muscarinic agonists, it was shown that muscarinic pathway regulates the recovery of *A. caninum* developmental arrest (Tissenbaum, et al., 2000). Recently, a membrane permeable analogue
of cyclic GMP, 8-bromo-cyclic GMP, was tested for its ability to stimulate feeding in iL3. Populations of iL3 reached maximum feeding at 3.5-5.0 mM and secreted Ancylostoma secreted protein 1 suggesting reactivation (Hawdon and Datu, 2003).

2.2.4 TGF-β and insulin in the mammalian system

Different isoforms of mammalian TGF-β are closely related structurally and functionally but have diverse tissue-specific signaling effects on growth and differentiation. TGF-β1 isoform predominates in many tissue locations including skeletal muscle which is the preferred site of arrest for the A. caninum larvae (Lee, et al., 1975). It is known to be released by major inflammatory cells like eosinophils and mast cells that respond to parasitic infections (Wong, et al., 1991; Baumgartner, et al., 1996). TGF-β1 has also been suggested to play a role in the regeneration of skeletal muscle tissue (Husmann, et al., 1996). TGF-β2 is the dominant isoform in the uterus and mammary gland and has been shown to be specifically upregulated by estrogen and prolactin, the hormones associated with pregnancy/lactation (Cheng, et al., 1993; Schneider, et al., 1996). Plasma levels of TGF-β2 show a transient increase during the peri-parturient period as compared to levels during normal non-pregnant state (Schneider, et al., 1996). IGF-1 has been shown to be present in myoepithelial cells of mammary gland of non-pregnant, pregnant and lactating rats. IGF-1 synthesized in mammary gland is reported to play a role in development of this organ during pregnancy as mammary epithelial cells also express specific IGF-1 receptors (Marcotty, et al., 1994). During lactation, IGF-1 can be transferred from serum into milk (Marcotty, et al., 1994).
2.3 Host Response to Arrest and Reactivation of Parasites

During developmental arrest or hypobiosis parasites persist inside the host in a quiescent manner and reactivate in response to unknown stimuli. Upon reactivation the parasite resumes development thereby leading to reinfection of the host, spread into the environment and transmission into the newborn (Schad and Page, 1982; Schad, 1990; Shoop, 1991). It has been well documented in *A. caninum* infection of dogs that developmentally arrested L3 larvae reactivate during pregnancy and are transmitted to the neonatal puppies via suckling of milk from infected dams (Stone and Girardeau, 1968; Stoye, 1973; Burke and Roberson, 1985). A similar phenomenon of arrest and reactivation has been suggested for *A. duodenale* infection of humans (Arasu and Kwak, 1999). Reactivation of arrested larvae has been hypothesized to occur, directly or indirectly, due to influence of maternal hormones during pregnancy and lactation (Stone and Smith, 1973; Stoye and Krause, 1976). It was shown that repeated exogenous administration of pregnancy-associated hormones, such as estrogen, progesterone and prolactin, to ovariectomized post-partum *A. caninum* infected dogs harboring tissue arrested L3 led to gradual resurgence of L3 larvae in milk by induction with oxytocin for milk let down (Stoye and Krause, 1976).

Previous work has shown that expression and secretion of TGF-β2 is tightly regulated by estrogen and prolactin which are critical factors in the tissue-specific regulation of the local production of TGF-β2 in the mammary gland and uterine tissues (Schneider, et al., 1996). Furthermore, elevated levels of TGF-β2 were detected in late pregnant maternal plasmas (> 100 pM), and in the milk (> 500 pM) during early lactation (Schneider, et al., 1996) whereas normal endogenous levels of TGF-β1 and TGF-β2 in sera of mouse are 125
ng/ml and 5 ng/ml (Emax Immunoassay kit, Promega, Madison, WI). These findings suggest that host-derived TGF-β might play a role in reactivation of tissue-arrested larvae.

Furthermore, studies in rats and pigs have demonstrated that IGF-1 is also upregulated during pregnancy (Marcotty, et al., 1994; Lee, et al., 1993). In rats, maternal serum IGF-I concentration rose during the first half of pregnancy while in the second half of pregnancy, the mean serum IGF-I concentration fell sharply from 1140 +/- 150 ng/ml at seven days of pregnancy to 470 +/- 85 ng/ml at 20 days. Using RNase protection assay it was determined that from the onset of pregnancy to term, IGF-I gene expression in the mammary gland diminished (Marcotty, et al., 1994). Similarly in mammary tissue of pregnant pigs, steady-state levels of the mRNAs encoding IGF-I, IGF-II and type-I IGF receptor as well as the levels of the membrane-associated type-II IGF receptor were higher during the early phase of mammogenesis (≤ or = day 45) than during the subsequent stages of mammary development. Mammary IGF-I, IGF-II and type-I receptor mRNAs were expressed at their lowest levels around day 90 of pregnancy (20-40% of those for day 30 of pregnancy) (Lee, et al., 1993).

To understand the underlying mechanisms of tissue-arrest and subsequent reactivation, a murine model of infection was developed (Stoye and Krause, 1976; Arasu and Kwak, 1999). The mouse serves as a paratenic host for A. caninum in that infective larva does not develop into mature adults but instead distributes throughout the body persisting for extended periods in a developmentally arrested state (Lee, et al., 1975). These tissue-arrested larvae do however have the capacity to reactivate and display the same transmammary transmission as seen in dogs and humans. In previous studies, 2-4 % (Steffe and Stoye, 1984)
and up to 8% (Arasu and Kwak, 1999) of tissue-arrested larvae in the dam reactivated and got transmitted to the nursing pups during post-partum lactation.

From an immunological perspective, BALB/c and C57BL/6 mice are known to typically display divergent immune responses to infection with BALB/c displaying a Th2 biased response and C57BL/6 mice predominantly produce Th1 cytokines (Brenner, et al., 1994; Nabors, et al., 1995; Honore, et al., 1998). However, when these two strains were compared there was no difference in tissue larval burden or in numbers transferred to pups (Arasu and Kwak, 1999). Initial comparisons of BALB/c versus C57BL/6 mice showed that both the strains mounted strong Th2 biased IgG1 and IgE antibody responses to *A. caninum* infection (Arasu and Heller, 1998) and that the immune response was not directly correlated with the phenomenon of larval reactivation/transmission.

### 2.4 Helminthic infection and Host Immune Responses

Mosmann and others defined the two distinct CD4+ T cell subsets by differential secretion of cytokines (Mosmann et al., 1986), which has revolutionized the understanding of regulatory mechanisms underlying resistance and susceptibility to helminth infection. The T helper type 1 (Th1) cells produce type 1 cytokines IFN-γ, lymphotoxin, and IL-2 stimulating immunoglobulin (Ig) G2a production and cell mediated effector responses. The T helper 2 (Th2) secrete type 2 cytokines IL-4, IL-5, IL-6, IL-9 and IL-13 and promote mastocytosis, eosinophilia, and the production of IgE and IgG1 (Mosmann and Sad, 1996; O’ Garra, 1998). Traditionally, immune responses to extracellular helminth parasites have been considered to be Th2 in nature (Pearce and Reiner, 1995). This inference is based primarily on animal models of helminthic infections. Human studies also suggest that Th2 cytokines dominate the
immune responses seen in chronic, longstanding helminth infections (King and Nutman, 1991). Where helminths inhabit a tissue environment, the situation is considerably more complex. One explanation for this is that, although Th1 responses may be more effective at parasite clearance, they are also more likely to cause more pathology (Pearce, et al., 1996). The skew to a Th2 response may reflect the classic compromise of minimizing host pathology at the expense of accepting some level of continuing infection.

2.4.1 Immune Responses to Parasitic Helminths

Recently an in vitro system was used to study the early immune responses to infective L3 from *Brugia malayi* by co-culturing with peripheral blood mononuclear cells (PBMCs) from previously unexposed individuals. After 24 h of culture, the frequency of T cells expressing Th1 cytokines (IFN-γ, TNF-α) was significantly increased in comparison to ones expressing Th2 cytokines (IL-4, IL-5, IL-10) (Babu and Nutman, 2003). This data suggests that the initial primary immune response to infective L3s of *B. malayi* is not predominantly Th2 but rather dominated by a proinflammatory Th1 response. This finding in conjunction with similar findings from study of immune responses in the schistosome trematodes reveals an emerging pattern of dominant Th1 responses during the early phase of helminthic infections (Pearce, et al., 1991). The main adaptive immune response against Schistosomes is mediated by CD4 T cells (Hernandez, et al., 1997a). An initial pro-inflammatory Th1-polarized response lasts around five weeks post-infection, at which point granulomatous inflammation gets underway. However, within the next one or two weeks, granuloma formation rises amid a dramatic change in the cytokine environment which become dominated by anti-inflammatory Th2-type cytokine (Pearce, et al., 1991). IL-4 along with T-
cell co-stimulatory systems (B7-CD28, CD40-CD40L) contribute to this conversion (Brunet, et al., 1997; Hernandez, et al., 1999; MacDonald, et al., 2002). The evolving cellular response is gradually accompanied by abundant production of mainly non-complement fixing IgG and IgE antibodies (Hernandez, et al., 1997b). A Th1 to Th2 conversion is vital for the host because it has been demonstrated that failure to convert is associated with a lethal disease characterized by severe hepatic inflammation with hepatocellular injury and necrosis (Hernandez, et al., 1999; MacDonald, et al., 2002).

A spectrum of responses develops against *Trichuris muris*, whipworm nematode infection in genetically different strains of mice. Cellular responses range from a strong Th2 response associated with worm expulsion (BALB/c mouse), to a mixed Th1 and Th2 response and delayed expulsion (C57BL/6 mouse), to finally a Th1 response resulting in a chronic infection (AKR mouse) (Deschoolmeester, et al., 2002; Anderson, 2000). Additional studies demonstrated that blockade of the IL-4 receptor in the C57BL/6 resistant mouse strain resulted in the production of a Th1 response with the development of a chronic unresolved infection (Else, et al., 1994). Conversely, administration of IL-4 to susceptible mouse strain BALB/c resulted in the expansion of Th2 response and clearance of infection (Else, et al., 1994). The *T. muris* system provides clear evidence that it is the interplay between host response and parasite survival strategies that lead to the observed infection levels. It is clear that a host genotype that leads to development of a strong and dominant Th2 response infection will lead to resistance (Grencis, 2001).
2.4.2 Immune Responses to Hookworms

The complexity of the hookworm life cycle offers numerous opportunities for the parasite and host to interact at the molecular level. Extensive antibody responses are mounted against larval and adult hookworms, but their effect on the parasite remains unclear. Furthermore, it is difficult to distinguish between anti-larval and anti-adult responses, given that L3s and adults share many antigens (Carr and Pritchard, 1987; Behnke J, 1991). In many respects, hookworms are typical gastrointestinal nematodes in the types of immune responses they generate in their definitive hosts (hosts in which the life cycle is completed i.e. infective larvae can mature to reach the adult stage). Antibody isotypes and subclasses as well as cellular responses loosely fit within the framework of a Th2 immune response. The common features of helminth-induced Th2 responses have long been noted as IgE production, eosinophilia and mastocytosis. While much of the IgE response in helminth infection is not directed against the parasite, the detection of IgE antibodies against Necator hookworm L3s proved to be highly specific and sensitive in diagnosing infections and, furthermore, IgE was observed to be the least cross-reactive isotype (Ganguly et al., 1988; Pritchard and Walsh, 1995).

Humoral responses (antibody production) to hookworms have been well documented; little is known about the role of adaptive T cell responses (Loukas and Prociv, 2001). While hookworm infection exhibits some of the hallmarks of a Th2 response (IgE and local and systemic eosinophilia), the immune responses clearly fail to protect most infected individuals. Recently, several studies have described T helper cell responses and susceptibility to hookworm infection (Quinnell et al., 2004; Pit, et al., 2000 and 2001). Observations from endemic regions in China and Brazil have shown profound cellular
hyporesponsiveness induced by chronic hookworm infection (Loukas et al., 2005). In a re-infection study in Papua New Guinea, cytokine and proliferative responses to *Necator* were measured. Most subjects produced detectable Th1 (IFN-γ) and Th2 (IL-4 and IL-5) cytokines in response to crude adult worm extract before anthelmintic treatment. Pre-treatment IFN-γ responses were negatively associated with hookworm burden and increased significantly after anthelminthic treatment (Quinnell et al., 2004). In a separate study, peripheral blood mononuclear cells (PBMCs) from *N. americanus* infected school children, who had recently received chemotherapy, had reduced proliferative capacity against the phytohemagglutinin mitogen and adult worm antigen extract compared to controls (Geiger et al., 2004). These individuals also produced higher levels of IL-10 and lower levels of both Th1 (IL-12 and IFN-γ) and Th2 (IL-5 and IL-13) cytokines. Such mixed Th1-type and Th2-type immune responsiveness associated with persisting gastrointestinal parasitic nematodes may reflect a state of infection where a permissive Th1-type cytokine profile favors parasite persistence and the chronicity of infection (Pit, et al., 2001).

Over the years immune responses to hookworms have been well-studied in animal models as well. The Syrian Golden hamster (*Mesocricetus auratus*) has been used to model infections with the *Ancylostoma ceylanicum* hookworm (Garside and Behnke, et al., 1989). To determine the impact of *A. ceylanicum* hookworm infection on host cellular responses, cytokine production and lymphoproliferation were measured (Mendez, et al., 2005). Initial larval infection with 100 third-stage *A. ceylanicum* larvae resulted predominantly in Th1 responses characterized by upregulation of IL-2, IFN-γ and TNF-α mRNA levels which occurred during larval migration and continued up to 14 days postinfection or prepatency (period before production of eggs). Subsequently, development of larvae into egg-laying
adult hookworms or patency coincided with a switch to Th2 predominant responses with a marked increase in IL-4 and IL-10 production. This switch also concurred with reduced host lymphoproliferative responses to hookworm antigens (Mendez, et al, 2005).

2.5 Immune responses during pregnancy

It is well-understood that pregnancy is associated with changes in local and peripheral immune responses which appear necessary for a successful implantation of a semiallogeneic graft, which is the fetus. Tom Wegmann and others suggested that there was a bidirectional cytokine interaction between the maternal immune system and reproductive system during pregnancy and, it appeared that successful pregnancy was a Th2 phenomenon (Wegmann, et al., 1993). Based on different studies it has been a generally accepted idea that Th1 cytokines involved in cellular responses are deleterious to pregnancy whereas Th2 cytokines involved in humoral responses are protective for the fetus (Wegmann, et al., 1993; Nieuwenhoven, et al., 2002). However, in the light of evidence from studies showing the requirement of IFN-γ for implantation of blastocyst this would be an oversimplification (Ashkar and Croy, 2001). Uterine natural killer (uNK) cells were shown to produce 90% of pregnancy-induced uterine IFN-γ. Implantation sites in uNK cell-deficient and IFN-γ signal-disrupted mice displayed anomalies in decidua and its spiral arteries which could adversely affect the establishment of pregnancy (Ashkar and Croy, 2001).

It has also been shown in humans that during pregnancy the ratio of production of Th1 and Th2 cytokines from peripheral lymphocytes and natural killer cells was decreased (Nieuwenhoven, et al., 2002). It was previously demonstrated in rats that peripheral lymphocytes, monocytes and granulocytes shown an activated phenotype in the last week of
pregnancy (Faas, et al., 2000). A recent study evaluated the species difference between humans and rats for lymphocyte cytokine production during pregnancy (Faas, et al., 2005). The study revealed that during human pregnancy, the percentage of lymphocytes producing IFN-\(\gamma\) was decreased but the percentage of IL-4 producing lymphocytes was not affected. In contrast, the rat immune system adapted to pregnancy by decreasing the total number of various lymphocytic populations but not by affecting the percentage of IFN-\(\gamma\) and IL-4 producing lymphocytes (Faas, et al., 2005).

2.5.1 Effect of Pregnancy Hormones on Immune Responses

There is a large body of data implicating the effect of pregnancy levels of estrogen on the Th1/Th2 cytokines. Previously published data suggests that female hormones may act to bias T cells towards a Th2 phenotype (Krishnan, et al., 1996a and b). Support for estrogen’s role in enhancement of Th2 responses is provided by studies in experimentally-induced allergic encephalomyelitis (EAE) and collagen induced arthritis (CIA) models; which are believed to be instructive models for multiple sclerosis (Kim, et al., 1999; Gilmore, et al., 1997). Treatment of EAE mice with estrogen led to reduction of IFN-\(\gamma\)- dependent anti-myelin basic protein IgG2a and increased production of IL-10 (Kim, et al., 1999). In the CIA model, estradiol caused a reduction in IFN-\(\gamma\) expression whereas Th2 cytokine production was not increased (Gilmore, et al., 1997). Clinical signs in both the diseases improved after estrogen treatment.

Besides estrogen, progesterone is also likely to contribute to pregnancy being a Th2-dominant effect (Kidd, 2003). When mice made arthritic with *Borrelia burgdorferi*, the causative agent for Lyme disease, were either impregnated or injected with progesterone,
arthritic signs ameliorated and IL-4 was markedly increased (Moro, et al., 2001). Joachim and others showed that dydrogesterone (6-dehydro-retroprogesterone), a progesterone derivative, dramatically increased the percentage of IL-4 positive decidual immune cells in stressed mice. Their data suggested that dydrogesterone abrogated stress-triggered abortion by inducing a Th2 biased local immune response (Joachim, et al., 2003).

Very little data is available to implicate prolactin, the pituitary-derived hormone responsible for milk letdown after parturition, as a factor affecting Th1/Th2 responses during pregnancy. It is well known that prolactin has an immunoregulatory function and receptors for prolactin are present on T and B lymphocytes (Gunes and Mastro, 1997). Prolactin production increases 10-fold at full-term pregnancy and 30-fold during suckling in the post-partum period (Ostensen, 1999). Prolactin has also been found elevated in Systemic Lupus Erythematosus (SLE) patients of both sexes and correlated to disease activity in several studies (Ostensen, 1999).

2.5.2 Effect of Parasitic Infection on Pregnancy

Relatively few studies have been focused on the effect of infection concurrent with pregnancy. Infection with apicomplexan parasite *Plasmodium falciparum*, which causes malaria, has been recognized to have adverse effects on pregnancy (Brabin and Brabin, 1992). Pregnant women suffer higher incidences of infection, more severe pathology and higher mortality than any other group of the population (Menendez, 1995). In a report on cytokine concentrations in placentas collected from women delivering in urban hospitals in malaria-holoendemic or nonendemic areas of Kenya, normal placentas displayed a bias toward Th2 cytokines; Th1 cytokines IFN-γ and IL-2 were absent in placentas not exposed to
malaria but present in a large proportion of placentas from the holoendemic areas. TNF-α and
TGF-β concentrations were significantly higher in placentas from the holoendemic area
(Fried, et al., 1998). Other studies have also suggested that malaria infection induces a
potentially harmful proinflammatory response in the placenta with a significantly increased
mRNA expression of IL-1β, IL-8, and TNFα (Moormann, et al., 1999).

The protozoal parasite, *Neospora caninum*, causes neuromuscular disease, abortion,
stillbirth and congenital infection in livestock and companion animals (Dubey and Lindsay,
1996). Reactivation of latent *N. caninum* is often associated with pregnancy (Quinn, et al.,
2002a). Numerous studies in mice and cattle have shown that immunity to *N. caninum*
infection involves a predominantly Th1-type immune response as would be expected for
However, a variety of studies on the immune system during pregnancy along with a
concurrent *N. caninum* infection have demonstrated that immunity during pregnancy is
biased towards a Th2 type response and away from a Th1 response (Quinn, et al., 2002b;
Kano, et al., 2005). Spleen cells from both infected/non-pregnant and infected/pregnant mice
produced IFN-γ, TNF-α and IL-12 (Th1) and IL-10 (Th2); however the levels of Th1 (IFN-γ,
TNF-α and IL-12) cytokines were significantly lower. Infected/pregnant mice exclusively
produced higher levels of IL-4 and it appeared to be responsible for decline in Th1 cytokine
production (Quinn, et al., 2004). Another study conducted in BALB/c mice to examine the
relationship between occurrence of vertical transmission and Th1 /Th2 type of immune
responses suggested that mice infected during pregnancy may acquire a weaker immune
response against *N. caninum* than mice infected before pregnancy (Kano, et al., 2005).
Results also suggested that mice infected during pregnancy may show an enhanced Th2 immune response during recurrence of infection (Kano, et al., 2005).

*Leishmania major* infection in mice is an excellent model for illustrating the importance of Th1 response for the control of intracellular protozoan infections (Louis, et al., 1998; Scott and Farrell, 1998). C3H and C57BL/6 strains of mice mount a strong Th1 response to *L. major*, which controls parasite multiplication, whereas BALB/c mount a strong Th2 response and are incapable of resolving the infection (Sadick, et al., 1986; Heinzel, et al., 1989; Locksley and Louis, 1992). The combination of pregnancy and *L. major* infection in resistant mice (C57BL/6) can be predicted to have two outcomes. Firstly, pregnancy may compromise resistance to *L. major* infection and secondly, a dominant Th1 response to the parasite may in turn compromise pregnancy (Krishnan, et al., 1996a; Krishnan, et al., 1996b). In the first scenario where pregnancy was suggested to compromise resistance to infection, parasite burden was increased when compared with non-pregnant infected mice. This was seen in association with enhanced expression of cytokines such as IL-4, IL-5 and IL-10 as well as reduced production of IFN-γ by lymph node and spleen cells (Krishnan, et al., 1996a). In the second scenario where a Th1 response was implicated for compromising pregnancy, frequency of viable pregnancies in infected mice was much less than in pregnant non-infected mice. This corresponded with a relatively low placental production of IL-4 and IL-10, and an increase in IFN-γ and TNF-α production by placental cell (Krishnan, et al., 1996b). It was suggested that increased implantation failure or fetal resorption was probably due to beneficial anti-parasite Th1 responses which were adversely affecting the pregnancy outcome (Krishnan, et al., 1996b).
**Figure 2.1** Life cycle of *Ancylostoma caninum* in the host and environment.

Egg - L1/L2 - L3 - L4 - Adult (male and female)
Figure 2.2 Model of larval arrest and reactivation.
3. Transcript and serum levels of TGF-β and IGF-1 during pregnancy and *Ancylostoma caninum* infection in BALB/c mice

### 3.1 Introduction

*Ancylostoma caninum* is a blood feeding intestinal nematode found in dogs and is one of the major parasitic infections in the US pet population (Blagburn, et al., 1996). It is closely related to human hookworms, *A. duodenale* and *Necator americanus*, which are estimated to infect over 1.2 billion people worldwide (De Silva, et al., 2003). Like several other parasitic nematodes, *A. caninum* also has the capacity to undergo developmental arrest within the infected host (Schad, 1979, 1990; Schad and Page, 1982). Developmental arrest or hypobiosis is a strategy used by parasites to evade the host responses, persist for long periods and subsequently reactivate upon receiving appropriate but unknown stimuli for resuming development. Reactivation of this latent reservoir of infection can lead to reinfection as well as transmission of relevant parasitic stages to the surrounding environment or to the neonates (Shoop, 1991). It has been well documented in dogs that developmentally arrested third-stage larvae of *Ancylostoma* sp. reactivate during pregnancy and get transmammarily transmitted to the suckling newborn puppies via milk (Stone and Girardeau, 1968; Burke and Roberson, 1985). A similar phenomena has been suggested for *A. duodenale* infection in humans and is of considerable importance as of the 1.2 billion people infected worldwide with hookworm disease (*A. duodenale* and *N. americanus*), 44 million are pregnant women (Wang, 1988; Schad, 1991; Bundy, et al., 1995). The reactivation of arrested larvae during pregnancy has long been hypothesized to be under the influence of hormonal fluctuations associated with different stages of pregnancy and lactation (Stoye and Krause, 1976). Experiments in which
pregnancy associated hormones- estrogen, progesterone and prolactin- were administered exogenously into ovariectomized, post-partum bitches, a gradual resurgence of L3 stage larvae were observed in oxytocin-induced milk (Stoye and Krause, 1976).

_Caenorhabditis elegans_, a well-studied free-living nematode, undergoes similar arrested development in response to environmental cues like food, temperature and pheromones. Extensive studies in _C. elegans_ have identified genes that control entry into and exit from the arrested larval state (Georgi, et al., 1990; Estevez, et al., 1993; Ren, et al., 1996). One of these genes is _daf-7_ which has sequence homology to members of the TGF-β superfamily and is expressed on one of the chemosensory neurons located in the amphidial region at the anterior end of the worm. Environmental cues such as food exposure triggers _daf-7_ expression and correlate with larval recovery from the arrested state (Ren, et al., 1996). Recently, two TGF-β-like genes, _Ac-dbl-1_ and _Ac-daf-7_, were cloned and characterized from _A. caninum_ suggesting the existence of similar signaling machinery (Freitas and Arasu, 2005). Additionally an _in vitro_ assay was used to show that at physiological concentrations, recombinant mammalian TGF-β isoforms, TGF-β1 and TGF-β2, had significant stimulatory effects on tissue-arrested larvae which could be blocked by preincubation with anti-TGF-β antibodies (Arasu, 2001). However, _in vitro_ analyses also showed that estrogen, prolactin and insulin did not have a direct effect on the feeding/reactivation response of infective or tissue-arrested _A. caninum_ larvae (Arasu, 2001). Other studies have shown that expression and secretion of TGF-β2 is regulated by estrogen and prolactin which are critical factors in the tissue-specific regulation of the local production of TGF-β2 in the mammary gland and uterine tissues (Schneider, et al., 1996). These findings suggest host-derived TGF-β might
play a role in reactivation of tissue-arrested larvae during pregnancy resulting in transmammary transmission of infection to the newborn mice puppies.

Another signaling mechanism that has been shown to regulate dauer formation in *C. elegans* is *daf*-2 which is an insulin-like receptor gene (Kimura, et al., 1997). IGF-1 which is also upregulated during pregnancy might mediate a separate signaling pathway in larval reactivation (Lee, et al., 1993). Since phosphatidylinositol-3-OH kinase (PI3K) is component of the insulin-like signaling pathway, role of insulin-like signaling in hookworm larval activation was investigated *in vitro* using the PI3K inhibitor, LY294002, and resumption of feeding was considered as a marker for activation (Brand and Hawdon, 2004). Muscarinic agonists like arecoline have been shown to initiate dauer recovery in *C. elegans* and *A. caninum* (Tissenbaum, et al., 2000). LY294002 prevented feeding in *A. caninum* infective larvae stimulated with host serum filtrate, a glutathione-analogue and the muscarinic agonist arecoline. These results suggest that an insulin-like signaling pathway mediates the reactivation of hookworm larvae similar to *C. elegans*. However, it has been shown that direct stimulation of *A. caninum* larvae with physiological concentrations of insulin did not elicit a stimulatory response (Arasu, 1999).

A third signaling pathway in *C. elegans* for dauer arrest and reactivation involves cGMP. The cGMP pathway includes a membrane associated guanylyl cyclase and loss-of-function mutants of this guanylyl cyclase enter into the dauer stage constitutively (Birnby, et al., 2000). The cell permeable cGMP-analogue, 8-bromo-cGMP, has been shown to mediate recovery of *C. elegans* dauers as well as *A. caninum* L3 larvae from arrested state to feeding state (Birnby, et al., 2000; Hawdon and Datu, 2003; Brad and Hawdon, 2004). Similar results were seen with the congeneric hookworm *A. ceylanicum* (Brand and Hawdon, 2004). Taken
together, these studies suggest that several signaling pathways may be similarly relevant during post-partum lactation in the reactivation and transmission of hookworm larvae.

To facilitate in vivo analyses of mechanisms mediating the pregnancy-induced reactivation and transmammary transmission, our lab has defined the mouse as an experimental model of infection. Mice serve as normal paratenic hosts for *A. caninum* as it has been previously shown that the larvae distribute and arrest in different tissues throughout the body without maturing into the adult intestinal stage (Nichols, 1956; Lee, et al., 1975; Arasu and Kwak, 1999). Furthermore, we along with others have shown that similar to dogs, pregnancy in mice triggers the reactivation and transmammary transmission of larvae to the neonatal pups (Steffe and Stoye, 1984; Burke and Roberson, 1985; Arasu and Kwak, 1999). In this study we investigated TGF-β1, TGF-β2 and IGF-1 cytokine profiles during late pregnancy, early lactation and mid-lactation in the mouse model of *A. caninum* infection to correlate their levels with the transmammary transmission of the larvae to the nursing pups. We measured the changes in serum levels of TGF-β1, TGF-β2 and IGF-1 as well as mRNA transcript levels in skeletal muscle (site of arrest), mammary gland (site of transmammary transmission) and spleen as the indicator organ of immune responses to the parasitic infection.

### 3.2 Materials and Methods

#### 3.2.1 Larval Parasite cultures

Infective *A. caninum* L3 were harvested from charcoal co-cultures of feces from male laboratory beagle dogs (Marshal Farms, North Rose, NY) orally infected at 8-10 weeks of age with 150 L3 as previously described (Burke and Roberson, 1979; Arasu, 1997). The use
of dogs in this study was approved by the Institutional Animal Care and Use Committee at North Carolina State University. Feces containing *A. caninum* eggs (range of 500-3000 eggs/g depending on stage of infection) were mixed with activated charcoal and deionized water in Pyrex® baking trays and placed in a humidified incubator, in the dark, at 25°C for 7-10 days. The L3 were harvested utilizing a modified Baermann funnel assembly (Garcia and Ash, 1979), repeatedly washed in phosphate-buffered saline (PBS) medicated with 20 mg/L gentamicin (Sigma Chemical Co., Missouri) and 20mg/L lincomycin (Sigma Chemical Co., Missouri) and maintained at room temperature for 5-7 days prior to use.

3.2.2 Mice breeding

Nine to ten-week-old female BALB/c mice were purchased from Charles River Laboratories, and maintained at the Laboratory Animal Resources facility in accordance with Institutional Animal Care and Use Committee guidelines. Mice were mated at a ratio of 2 females: 1 male for a period of 7 days and examined twice daily for the presence of a vaginal impregnation plug. Observation of a plug was designated as day 0 of the pregnancy. On day 5 post-impregnation (observation of vaginal plug), the female mice were injected subcutaneously in the dorsal cervical interscapular region with PBS (control) or with 1000 larvae in PBS. Mice were killed by decapitation at three different time points: time point 1 corresponding to day 19 of gestation or day 14 post-infection (pi), and time points 2 and 3 corresponding to day 1 and 10 of postpartum lactation or day 15-16 pi and day 25-26 pi, respectively (Figure 3.1). The tissue samples collected immediately after killing were sections of the gastrocnemius skeletal muscle, mammary gland, and spleen as well as blood. Approximately 200mg of muscle, mammary tissue or spleen was stored in RNA-STAT 60
(Tel-Test, Texas) at -80°C for RNA extraction. From the blood, serum was collected after incubation at 37°C for 1 h, 4°C for 4 h and subsequently stored at -20°C until further use. The female mice were grouped as infected or not infected and bred or not bred (n = 5 mice per group). The resulting four groups were uninfected/unbred (UN or normal controls), infected/unbred (IN), uninfected/bred (UB) and infected/bred (IB).

3.2.3 Harvesting tissue L3

For harvesting tissue-arrested larvae, carcasses were skinned, minced, wrapped in eight layers of cheesecloth and incubated in a modified Baermann assembly for 4 h at 37°C in medicated PBS (Arasu, 1998). Migrating larvae that collected at the bottom of the cups were repeatedly washed with medicated PBS and gently spun at 2000 rpm for 10 minutes. Larval numbers in dams versus each litter of pups were counted with the aid of a dissecting microscope.

3.2.4 TGF-β1 and –β2 ELISA

Biologically active TGF-β1 and β2 levels were determined using the TGF-β1 and β2 E_max® Immunoassay system (Promega, Madison, WI), a sandwich enzyme linked immunosorbent assay (ELISA) kit. The assays were performed by coating flat-bottom 96-well ELISA plates (Corning Costar® Acton, MA) with 100 µl of TGF- β1 and TGF- β2 coat antibody dissolved in carbonate coating buffer. The plates were incubated at 4°C overnight. Non-specific binding sites were blocked using 270 µl of TGF- β1 and TGF –β2 blocking buffer at 37°C for 35 minutes. Serum samples diluted 1:4 using PBS and for each 50 µl of diluted sample, 1µl of 1N HCl was added and the pH was verified to be 3.0 or lower. Serum
samples were mixed, incubated for 15-20 minutes at room temperature and neutralized by adding 1µl of 1N NaOH; the pH was verified to be approximately 7.6. According to the manufacturer, TGF-β1 standard curve was linear between 15.6 and 1000 pg/ml of the TGF-β1 standard whereas the TGF-β2 standard curve was linear between 32 and 1000 pg/ml. The TGF-β1 and TGF-β2 standard were supplied at the concentration of 1µg/ml and were diluted 1:1000 in TGF-β sample buffer. For each plate (100µl/well), six 1:2 serial dilutions of the standards (starting with 1 ng/ml) and acidified serum samples (starting with 1:20) were analyzed. The TGF-β1 and TGF–β2 controls were set at 100 pg/ml and 500 pg/ml for each plate. The 96-well plate was incubated on a plate shaker at 500 rpm for 1.5 hours at room temperature. After washing five times with TBST (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% Tween-20) wash buffer, anti-TGF-β1 and TGF-β2 polyclonal antibody were respectively added at 1:1000 and 1:2000 dilutions and incubated with shaking for an additional 2 hours at room temperature. The plates were washed five times with TBST buffer followed by 100 ul of the TGF-β/horseradish peroxidase (HRP) conjugates diluted 1:100 with sample buffer. The plates were incubated with shaking for 2 hours at room temperature and subsequently washed five times at the end of the incubation. For color development 100µl of 3, 3', 5, 5'-Tetramethylbenzidine (TMB) One was added to each well using a multichannel pipettor and the plates were incubated for 15 minutes without shaking. The color reaction was stopped by adding 100µl of 1N HCl in the same order in which substrate was added to the wells. The blue color changed to yellow as the pH decreased. The absorbance was recorded at 450nm on a plate reader within 30 minutes of stopping the reaction.
3.2.5 IGF-1 Enzyme ImmunoAssay (EIA)

IGF-1 levels in mouse sera were determined using the Active® Mouse/Rat IGF-1 EIA kit based on a competitive binding enzyme immunoassay (Diagnostic Systems Laboratory, Inc., Webster, Texas). The kit contains microtitration strips coated with rabbit anti-goat gamma globulin immobilized on the inside of each well. Pretreatment of samples involved addition of 140 µl of the provided Sample Buffer I to 10 µl of mouse serum and incubation at room temperature (~25ºC) for 30 minutes. Subsequently, 150 µl of Sample Buffer II was added and mixed thoroughly. The mouse/rat IGF-1 standards were used at 0, 150, 300, 650, 1250 and 3600 ng/ml. The two controls, Level I and Level II, contained low (600 ± 175 ng/ml) and high (1100 ± 325 ng/ml) levels of mouse/rat IGF-1 in a protein-based buffer, respectively. In the assay, 50 µl of standards, controls and pretreated sera samples were incubated on an orbital microplate shaker at 500 rpm for 1 h at room temperature with 100 µl of biotin-labeled mouse/rat IGF-1 and 100 µl of goat anti-mouse/rat IGF-1 antiserum in microtitration wells allowing the unlabeled and biotin-labeled antigens to compete for the limited number of anti-mouse/rat IGF-1 binding sites. Wells were washed five times with the wash solution and incubated with streptavidin-horseradish peroxidase (HRPO), which binds to the biotinylated mouse/rat IGF-1. The unbound streptavidin-HRPO was washed, followed by incubation with 100 µl of substrate TMB chromogen solution. An acidic stopping solution was then added to each well with a multichannel pipettor and the plate was shaken by hand for 5-10 seconds. The degree of enzymatic turnover of the substrate was determined by dual wavelength absorbance measurement at 450 with background wavelength correction set at 600 or 620 nm.
3.2.6 RNA extraction and cDNA synthesis

Total RNA from all the tissues (skeletal muscle, mammary gland and spleen) was extracted using RNA-STAT (Tel-Test, Inc., Friendswood, TX) according to Chomczynski and Sacchi method (Chomczynski and Sacchi, 1987). Residual genomic DNA was removed from RNA by treating with DNase (20U per 100 µg of RNA). RNA quantity and quality was checked by spectrophotometric measurements at 260 and 280 nm (Pharmacia) and by analyzing 1µg RNA for rRNA bands on a 1% ethidium bromide agarose gel.

For reverse transcription, first strand cDNA was synthesized by adding 10µg of total RNA to a 40µl reaction mix containing a final concentration of 2.5nM dNTPs, 0.1M, dithiothreitol, 1ug Oligo d (T) primer (Promega, Madison, WI), 24U RNase inhibitor and incubation with 200U Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) at 37°C for 1 hour. To confirm absence of contaminating genomic DNA, RT negative reactions with 5µg total RNA were setup. RT-plus (cDNA)/minus reactions were subjected to RT-PCR (reverse transcription-polymerase chain reaction) using GAPDH primers as it was abundantly expressed in all tissues of interest. PCR reactions were run on a 2% agarose gels to check for product formation.

3.2.7 Primer design

Primer sets for genes of interest, TGF-β1, -β2, IGF-1, IL-4 and IFN-γ, and reference gene 60S acidic ribosomal protein were designed from mouse-specific Genbank sequences listed in Table 3.1 using Primer 3 software (Rozen and Skaletzky, 2000) (http://www-genome-wi.mit.edu/cgi-bin/primer/primer3_www.cgi). In order to minimize primer-dimer formation, the maximum self-complementarity was 6 and the maximum 3’ self-
complementarity was 0. The targets amplified by primer pairs were characterized using the Mfold program (SantaLucia, 1998) ([http://bioinfo.math.rpi.edu/~mfold/dna/form1.cgi](http://bioinfo.math.rpi.edu/~mfold/dna/form1.cgi)) for predicting the nature of any secondary structures which may form at the site of primer binding. Primer pairs that bound at the site of predicted loop were discarded. Primer sets were synthesized by Integrated DNA Technologies (Coralville, IA) and primers were reconstituted at 100 pM/ul in nuclease-free water prior to use.

3.2.8 Real-time PCR

The real-time PCR reactions were carried out using the iCycler™ iQ PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). In each 25µl reaction, 12.5µl of iQ™ SYBR green supermix (Bio-Rad) was added to 300nM of each primer along with 250ng of cDNA. PCR amplification was performed in duplicate for each sample using the following cycle conditions: 3 min at 95°C followed by 45 repeats of 1 min at 95°C, 30s at 55°C and 30s at 72°C. Temperature optimization was carried out for all the primer sets to be amplified simultaneously. Annealing temperatures were tested from the 50-65°C range; all the primer sets amplified optimally at 55°C. A melt curve analysis step was included at the end of cycles to check for primer-dimer and non-specific product formation. Efficiency of the PCR reactions was derived by doing a standard curve of 10-fold serially diluted mouse spleen cDNA and was consistently in the 95-98% range. Non-template controls were used to detect any genomic DNA contamination and amplified products were also examined on a 2% agarose gel to verify that the amplified products were of the expected sizes. Raw Ct values were analyzed using Relative Expression Software Tool-384 (REST-384) to generate a fold increase or decrease in the transcript levels (Pfaffl, et al., 2002). The 60S acidic ribosomal
protein was used as the endogenous reference gene for normalizing transcript levels among tissues of interest. 60S ribosomal protein was previously shown to be the least variable from comparative analyses of various genes including β-actin, cyclophilin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) all of which were tested as potential candidate reference genes similar to the ones described in Chapter 6.

3.2.9 Statistics

Data reported for larval burden and serum levels of cytokines as mean ± standard deviation was analyzed by two-way ANOVA using the General Linear Models (GLM) procedure in SAS (Cary, North Carolina) and $p < 0.05$ was considered significant. For adjustment of multiple comparisons, Tukey’s test was used to detect significant differences between uninfected controls, infected, not bred and bred groups of mice. For comparing variation in transcript levels of cytokine genes, Pair-Wise Fixed Reallocation Randomization Test© in the REST-384 was used (Pfaffl, et al., 2002). The mathematical model used to compute the relative expression ratio of a target gene relies on its real-time PCR efficiencies (E) and the threshold cycle difference ($\Delta Ct$) of an unknown sample versus a control ($\Delta Ct_{control\,\,-\,sample}$). The target gene expression is normalized to a reference gene (ref) in the Equation I mentioned below:

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta Ct_{\text{target\,control\,\,-\,sample}}}}{(E_{\text{ref}})^{\Delta Ct_{\text{ref\,control\,\,-\,sample}}}}$$

In our experimental setup control was the uninfected/unbred (UN) mice and samples were the infected/unbred, uninfected bred (UB) and infected/bred (IB). For evaluating fold changes in transcript levels, analyses were also done by comparing IN, UB and IB group
each to the UN at respective timepoints. For example, Equation II mentioned below shows
calculations for changes in transcript levels of IN when compared to UN:

\[
\text{Ratio} = \frac{(E_{\text{target}})^{\Delta Ct_{\text{target}}(UN - IN)}}{(E_{\text{ref}})^{\Delta Ct_{\text{ref}}(UN - IN)}}
\]

For evaluating fold upregulation or downregulation of transcripts between timepoints, early-lactation (timepoint II) and mid-lactation (timepoint III) were compared to late-gestation (timepoint I). For example, Equation III mentioned below shows calculations for changes in transcript levels of early lactation (timepoint II) when compared to late-gestation (timepoint I):

\[
\text{Ratio} = \frac{(E_{\text{target}})^{\Delta Ct_{\text{target}}(\text{timepoint I} - \text{timepoint II})}}{(E_{\text{ref}})^{\Delta Ct_{\text{ref}}(\text{timepoint I} - \text{timepoint II})}}
\]

### 3.3 Results

#### 3.3.1 Assessment of larval burden

For comparing larval distribution in dams, eight week old female BALB/c mice were subcutaneously injected with 1000 *A. caninum* L3 larvae. A comparison of larval burden during the course of *A. caninum* infection was made between infected/not bred (IN) and infected/bred (IB) mice at the three different timepoints corresponding to late gestation and early to mid lactation (Figure 3.2). The total number of larvae recovered from IN mice was not significantly different from the IB mice at late gestation or mid lactation. However, there was a significant difference \((p < 0.005)\) in the larval burden of IN mice \((368 \pm 64)\) and IB mice \((249 \pm 31)\) at timepoint II, during the initial 24 hours of post-partum lactation.

To correlate the level of transmammary transmission from dam to pups, larval counts were also assessed in pup litters from IB dams: \(5 \pm 4\) and \(9 \pm 2\) larvae were respectively obtained during the timepoints for early and mid lactation (for timepoints II and III,
respectively). The larval burden in the feti (timepoint I) was not assessed as previous studies have shown that there is no in utero transmission of *A. caninum* larvae from dam to fetus during pregnancy (Steffe and Stoye, 1984; Arasu and Kwak, 1998).

3.3.2 Serum levels of TGF-β1, TGF-β2 and IGF-1 during *A. caninum* infection and breeding

**TGF-β1 serum levels**

To compare the TGF-β1 levels during different phases of breeding, sera was collected at the three time points indicated above spanning gestation and post-partum lactation. TGF-β1 levels were assessed in uninfected/not bred (UN) controls and compared to the levels in infected/not bred (IN), uninfected/bred (UB) and infected/bred (IB) group of mice. At timepoint I (late gestation), the circulating levels of TGF-β1 were not significantly different between UN, IN and UB groups; however, there was a significantly higher level in IB mice when compared with normal controls (*p* < 0.0001) (Figure 3.3a). At timepoint II, TGF-β1 levels were significantly lower among bred groups, UB (*p* < 0.0001) and IB (*p* < 0.05), when compared to normal (Figure 3.3b). At timepoint III, all three IN, UB and IB groups were significantly different from controls; TGF-β1 levels were significantly elevated in IN mice (*p* < 0.0001) whereas the levels were significantly lower in both UB (*p* < 0.0001) and IB (*p* < 0.0001) (Figure 3.3c). In figure 3.3d, comparison of TGF-β1 levels among different mouse groups was done over 3 timepoints of interest (I, II and III). In summary, TGF-β1 dropped significantly at timepoints II and III in the UB and IB groups as compared to controls suggesting that TGF-β1 levels decline after parturition and remains so until mid-lactation. Also, the significant increase in TGF-β1 levels at timepoint III in the IN groups suggests a time-dependent role for TGF-β1 during *A. caninum* infection.
TGF-β2 serum levels

Circulating TGF-β2 levels remained unchanged and did not vary significantly between IN, UB and IB when compared to the UN controls at timepoint I (Figure 3.4a). At timepoint II, TGF-β2 levels decreased significantly in UB ($p \leq 0.0001$) and IB ($p \leq 0.0001$) when compared to the UN mice. However, TGF-β2 levels did not vary significantly in the IN mice compared to the UN mice (Figure 3.4b). At timepoint III, significantly lower TGF-β2 levels were observed in all the groups (IN, UB, IB) when compared to UN mice ($p < 0.0001$) (Figure 3.4c). In figure 3.4d, comparison of TGF-β2 levels among different mouse groups was done over 3 timepoints of interest (I, II and III). Therefore, an overall significant drop in TGF-β2 levels was observed at timepoints II and III in the UB and IB groups when compared to UN mice corroborating with previous findings where higher TGF-β2 levels were documented during pregnancy and significantly lower levels after parturition and beginning of lactation (Schneider, et al., 1996).

IGF-1 serum levels

Serum IGF-1 levels decreased significantly in IN ($p < 0.0001$) and UB ($p < 0.0001$) compared to UN at timepoint I (Figure 3.5a). IGF-1 levels in all groups were similar at timepoint II (Figure 3.5b). At timepoint III, IGF-1 levels were significantly lower in IN ($p < 0.0001$), UB ($p < 0.0001$) and IB ($p < 0.0001$) groups when compared to UN mice (Figure 3.5c). In figure 3.5d, comparison of IGF-1 levels among different mouse groups was done over 3 timepoints of interest (I, II and III). In summary, a significant drop in IGF-1 levels in the IN group was observed at two weeks and continued to remained so at the later timepoint.
of *A. caninum* infection (timepoint III). Similarly, serum IGF-1 levels decreased below normal levels at the end of gestation (timepoint I) and during mid-lactation (timepoint III) in bred and infected/bred groups.

3.3.3 Transcript levels of TGF-β1, TGF-β2 and IGF-1 in muscle, mammary gland and spleen

**TGF-β1 transcript levels**

TGF-β1 transcripts levels were evaluated during the course of pregnancy and infection with *A. caninum* using real-time PCR. REST software was used to analyze the fold changes in expression of transcript levels (Pfaffl, et al., 2002) in skeletal muscle, mammary tissue and spleen. Transcript levels of TGF-β1 were evaluated using equation II and III (Materials and Methods). Data presented in Figures 3.6 a-i mentioned in the discussion section of this chapter was calculated by comparing IN, UB and IB with UN controls (Equation II). Data presented in Figures 3.9 a-i was calculated by comparing timepoint II and III with timepoint I (Equation III) as discussed in this section in more detail. In skeletal muscle, TGF-β1 transcripts levels were significantly downregulated at timepoint III in IN (*p* < 0.001), UB (*p* < 0.01) and IB (*p* < 0.05) groups with 37, 13 and 22-fold decrease compared to timepoint I (Figure 3.9a, 3.9b and 3.9c). In mammary gland, TGF-β1 transcripts levels were significantly upregulated by 11-fold at timepoint III in the IN (*p* < 0.05) group compared to timepoint I (Figure 3.9d). A significant upregulation by 29 and 17-fold in TGF-β1 transcripts levels was observed in the mammary gland of UB (*p* < 0.001) and IB (*p* < 0.01) mice respectively during early lactation i.e. timepoint II (Figure 3.9e and 3.9f). In spleen, TGF-β1 transcripts levels were significantly downregulated at timepoint II in IN (*p* < 0.05) (Figure 3.9g). However, TGF-β1 transcripts levels were not significantly different in UB and IB
groups in spleen (Figure 3.9h and 3.9i). In summary, an overall downregulation of TGF-β1 transcripts was observed in skeletal muscle 24 days into *A. caninum* infection in the IN group and during mid-lactation in UB and IB groups. However, in mammary gland significant upregulation of TGF-β1 transcripts was detected 24 days into *A. caninum* infection in IN group and during early lactation in UB and IB groups.

**TGF-β2 transcripts levels**

TGF-β2 transcripts levels were evaluated during the course of *A. caninum* infection and different stages of pregnancy and lactation in skeletal muscle, mammary gland and spleen. Previous studies have documented the upregulation of TGF-β2 in mammary gland during late pregnancy and this level of expression is maintained during the lactational phase (Schneider, et al., 1996). Transcript levels of TGF-β2 were evaluated using equation II and III (Materials and Methods). Data presented in Figures 3.7 a-i mentioned in the discussion section of this chapter was calculated by comparing IN, UB and IB with UN controls (Equation II). Data presented in Figures 3.10 a-i was calculated by comparing timepoint II and III with timepoint I (Equation III) as discussed in this section in more detail. In skeletal muscle, TGF-β2 transcripts levels were significantly downregulated at timepoint III in IN (*p* < 0.001), UB (*p* < 0.001) and IB (*p* < 0.001) groups with 18, 5 and 61-fold decrease compared to timepoint I (Figure 3.10a, 3.10b and 3.10c). In mammary gland, there was a significant downregulation of TGF-β2 transcripts in the IN groups at timepoint I (*p* < 0.01), II (*p* < 0.001) and III (*p* < 0.01) (Figure 3.10d). During early lactation, there was a significant upregulation of TGF-β2 transcripts in mammary gland with an 85 -fold increase in the UB group (*p* < 0.05) at timepoint II (Figure 3.10e). There was no significant change in TGF-β2 transcripts in
mammary gland of IB group (Figure 3.10f). In spleen, TGF-β2 transcripts were significantly upregulated with a 35-fold increase at timepoint I ($p<0.01$) in the IN group (Figure 3.10g). Similarly, at timepoint I ($p<0.05$) significant upregulation with a 37-fold increase was also observed in the UB group (Figure 3.10h) but no significant changes were observed in spleen of IB group (Figure 3.10i). In summary, TGF-β2 transcripts levels were significantly downregulated in the skeletal muscle at 24 days of *A. caninum* infection in the IN group and mid-lactation in the UB and IB bred groups. In mammary gland, there was a significant downregulation in TGF-β2 transcripts levels at all the timepoints of *A. caninum* infection in the IN group. A significant upregulation observed in TGF-β2 transcripts during early lactation in the UB group was similar to previously published studies done in mammary gland (Schneider, et al., 1996). However, no upregulation of TGF-β2 transcripts was observed in the IB group at any of the timepoints during pregnancy or lactation.

**IGF-1 transcripts levels**

IGF-I mRNA content in mammary glands has been shown to be upregulated during pregnancy (Lee, et al., 1993). Transcript levels of IGF-1 were evaluated using equation II and III (Materials and Methods). Data presented in Figures 3.8 a-i mentioned in the discussion section of this chapter was calculated by comparing IN, UB and IB with UN controls (Equation II). Data presented in Figures 3.11 a-i was calculated by comparing timepoint II and III with timepoint I (Equation III) as discussed in this section in more detail. In skeletal muscle, IGF-1 transcript levels were significantly upregulated at timepoint III ($p<0.05$) in the IN groups compared to timepoint I (Figure 3.11a). No significant changes were observed in skeletal muscle of UB group (Figure 3.11b). IGF-1 transcript levels were also significantly
upregulated in skeletal muscle at timepoint II in the IB group compared to timepoint I ($p<0.05$) (Figure 3.11c). In mammary gland, IGF-1 transcript levels were significantly downregulated at the timepoints I ($p<0.01$) and II ($p<0.001$) but upregulated at timepoint III ($p<0.01$) in the IN groups (Figure 3.11d). No significant changes were observed in IGF-1 transcript levels in mammary gland of UB group (Figure 3.11e). However, in the IB group IGF-1 transcript levels were significantly upregulated in the mammary gland at timepoint II ($p<0.01$) and timepoint III ($p<0.01$) with a 9 and 112-fold increase compared to timepoint I (Figure 3.11f). IGF-1 transcript levels did not vary significantly in spleen of IN, UB and IB mice at the timepoints of interest (Figures 3.11g, 3.11h and 3.11i). In summary, IGF-1 transcript levels were upregulated in skeletal muscle and mammary gland at 24 days of *A. caninum* infection. IGF-1 transcript was upregulated in skeletal muscle and mammary gland of IB mice during early to mid-lactation.

### 3.4 Discussion

Mice serve as an excellent model to study host-parasite interactions of *A. caninum* as they are paratonic hosts and encapsulate the phenomena of arrest and pregnancy-associated reactivation leading to subsequent transmammary transmission as observed in dog and human *Ancylostoma* hookworm infection. As reported previously, infection of mice with *A. caninum* results in the gradual migration of L3s throughout the body particularly to the skeletal muscles (Arasu and Kwak, 1999) with stable distribution by 10-14 days post-infection (Lee, et al., 1975; Steffe and Stoye, 1984). Due to previously observed low breeding efficiencies with early infection (Arasu and Kwak, 1999), we followed the infection protocol of sub-cutaneous injection of 1000 L3 at 5 days post-impregnation which allows for
larval migration/distribution during the remaining time of the 19-21 day gestational period.

In the studies reported here, the total number of tissue larvae harvested was not significantly different at day 19 of gestation between bred and unbred groups of infected mice which is consistent with the absence of pre-partum transmission of infection. However, there were significant differences observed in the total number of larvae between bred and unbred infected groups at the timepoint corresponding to day 1 of lactation suggesting that transmammary transmission starts within the first 24 hours of suckling. At day 10 of lactation, even though the total number of larvae was lower in bred versus unbred infected mice, no significant difference was observed. Previous studies have shown that in experimentally infected dogs, only a subpopulation of arrested larvae are transmitted during lactation leaving a residual population (re-entering a phase of arrest) and available for transmission in following pregnancies (Stoye, 1973).

Host factors that facilitate the transition of arrested larval stages to a reactivated state for transmammary transmission during hookworm infection have not yet been clearly defined. Though the precise nature of these host factors remains unknown, it appears to involve specific components of serum (Hawdon and Schad, 1990, 1991, 1992). Parallels have also been drawn between the arrested dauer larval stage of C. elegans and the third-stage larvae from parasitic nematodes (Hotez, et al., 1993; Rajan, 1998). TGF-β and insulin-like signaling have been shown to regulate dauer formation in C. elegans (Georgi, et al., 1990; Estevez, et al., 1993; Ren, et al., 1996; Kimura, et al., 1997). Studies utilizing an in vitro feeding assay have shown that both TGF-β1 and TGF-β2 are able to stimulate tissue-arrested A. caninum larvae (Arasu, 1999). In the host, TGF-β1 appears to be required for regeneration of skeletal muscle tissue and is produced by eosinophils and mast cells.
associated with the inflammatory response to parasites (Wong, et al., 1991; Baumgartner, et al., 1996; Maizels, et al., 1993; Cooper, et al., 1991). It is also an important cytokine detectable at high levels in feto-placental tissues during pregnancy (Thompson, et al., 1989). In these studies, TGF-β1 serum levels were significantly higher in the infected bred IB group (Fig. 3.3a) group at day 19 of gestation relative to all other groups, and immediately after parturition relative to the uninfected bred group (Fig. 3.3b), suggesting that infection as well as pregnancy/lactation may play a role in sustained production of TGF-β1 and a potential role in reactivation of arrested larvae in muscle sites. Differences in circulating level of TGF-β1 were found in UN control mouse during 3 timepoints (Fig. 3.3d). At present, we are unable to explain such changes in serum levels. However, it is possible that TGF-β1 levels fluctuate during stress which can be caused by loud noise or change in environment in the housing facility. With infection, an increase in TGF-β1 levels was seen at timepoint III in the IN group which could suggest an increase in cells secreting TGF-β1 that respond to infection such as eosinophils and mast cells (Figure 3.3d). Comparison of mRNA levels revealed that TGF-β1 transcripts were significantly downregulated in skeletal muscle at ~24 days of *A. caninum* infection in both IN and IB groups (timepoint III) which would be consistent with the period of stabilized dispersion and diminished migration of the larvae; however, a similar decline was also seen in uninfected bred UB mice (Fig. 3.9b). When UB was compared to UN, TGF-β1 transcripts were significantly downregulated at timepoint III in skeletal muscle (Figure 3.6b). In mammary gland, TGF-β1 mRNA levels were significantly elevated during early lactation but eventually declined during mid-lactation in both uninfected and infected bred groups of mice (Fig. 3.9e and 3.9f). When UB and IB were compared to UN, TGF-β1 transcripts showed a significant downregulation it was not as dramatic as observed during IN
and UN comparison during timepoint II (Fig. 3.6d, e and f). Also, during timepoint III there was a significant upregulation in TGF-β1 transcript when IN was compared to UN (Fig. 3.6d). These findings suggest that dramatic suppression of TGF-β1 transcript seen during early infection is overcome by ~24d of infection and pregnancy appears to offset such suppression as observed by less dramatic downregulation in UB and IB groups (Fig. 3.6 e and f). Similarly, a significant downregulation is also observed in spleen of IN mice during timepoint II (Fig. 3.6g).

In these studies, the infected bred IB group of mice is of greatest interest when assessing the fluctuations in TGF-β isoforms as this group encompasses the phenomena of larval arrest and transmission in arrested *A. caninum* larvae. We expected TGF-β1 levels to be elevated during the peri-parturient period as this is the timepoint when arrested larvae are assumed to `awaken’ to resume migration. Serum levels were indeed elevated in the IB group of mice compared to all other groups at late gestation (Fig. 3.3a). However, TGF-β1 transcript levels in skeletal muscle were generally not elevated (Figs. 3.9a, 3.9b and 3.9c) except for showing a slight increase at timepoint II (day 16-17 pi/day 1 lactation) of the infected groups, IN and IB (Fig. 3.9a and 3.9c). Taken together with previous *in vitro* feeding/reactivation results on tissue-arrested larvae (Arasu, 1999), these results suggest that TGF-β1 may potentially be playing a role in larval reactivation in the *in vivo* situation but would require further confirmatory analyses.

With TGF β2, elevated levels were detected in late pregnant maternal plasma in the rat model which declined immediately after parturition (Schneider, et al., 1996). In contrast to TGF-β1, TGF-β2 levels in the UN groups remained constant over time. Our results from the mouse show a similar trend with no significant differences in serum levels amongst any
of the groups at late gestation followed by a significant decrease in UB and IB groups relative to UN subsequent to parturition (Fig. 3.4a). TGF-β2 levels declined sharply in the IN group at timepoint III during which larvae are expected to undergo arrest (Figure 3.4d). This decline suggests the possibility that initiation of arrest might lead of secretion of a TGF-β2 receptor or cells making TGF-β2 in response to A. caninum infection get inhibited. With the TGF-β2 transcripts, levels were significantly elevated in the mammary gland and uterine tissues during late gestation and lactation in rats (Schneider, et al., 1996). Our findings corroborate these studies as a similarly significant upregulation was observed in TGF-β2 transcripts in mammary gland during early lactation in the uninfected bred group (Fig. 3.10e). TGF-β2 transcript levels were significantly downregulated in skeletal muscle at timepoint III ie mid-lactation in UB mice when compared to UN controls which correlate with decline in circulating levels also (Fig. 3.7b and 3.4d). However, it should also be noted that numerous studies have shown that mRNA transcript levels are not always reflective of protein levels (Anderson and Seilhamer, 1997; Gygi, et al., 1999). Hence, there was a significant upregulation of TGF-β2 transcript in the in mammary gland of UB group at timepoint II coinciding with sustained expression of TGF-β2 during early lactation whereas serum levels had started to decline sharply (Fig. 3.7e). In the IB group relative to other groups, we had accordingly expected TGF-β2 transcript levels to be elevated in mammary gland during the lactational period as reflective of high localized levels of the expressed cytokine in order to sustain a state of larval reactivation and facilitate transmission of larvae via milk. Serum levels of TGF-β2 were not remarkable (as mentioned above) and consistent with the well-documented tissue-specific localization of TGF-β2 (Cheng, et al., 1993; Schneider, et al., 1996). Transcript levels of TGF-β2 were also not significantly different in
the IB group. From these observations, there does not appear to be a correlation between host-derived TGF-β2 and larval reactivation.

In studies from rats and pigs, IGF-1 mRNA and protein levels are differentially regulated during pregnancy (Marcotty, et al., 1994; Lee, et al., 1993); in rats, maternal serum IGF-I levels were high during the first half of pregnancy and fell sharply during the second half of pregnancy (Marcotty, et al., 1994). Using RNase protection assay, IGF-I gene expression in the mammary gland diminished during the course of pregnancy (Marcotty, et al., 1994). In our studies, serum IGF-1 levels in IB mice were not significantly different from normal UN mice during the peri-parturient period (timepoints I and II; Figures 3.5a, 3.5b) but showed a gradual decrease from ~800 pg/ml at late gestation to ~100 pg/ml at day 10 lactation. Infection at timepoint II ie early lactation had an effect on IGF-1 serum levels as observed by an increase in IN and IB groups (Figure 3.5d). No significant differences were observed in IGF-1 levels in skeletal muscle of any group of mice when compared with the UN (Fig. 3.8a, b, c). However, there was a significant upregulation in IGF-1 transcript in the IB group at parturition when comparing day 19 gestation and day 1 lactation timepoints in both skeletal muscle (Fig. 3.11c) and mammary gland (Fig. 3.11f). These in vivo analyses of IGF-1 levels show a potential correlation between infection and pregnancy/lactation with a potential role in the reactivation/transmission of arrested larvae. In previous in vitro studies to identify factors associated with signaling and reactivation of tissue-arrested A. caninum larvae, insulin did not appear to have a direct effect on the feeding response (Arasu, 1999); IGF-1 was not assessed in these studies. However, the IGF-1 signaling inhibitor LY294002 was shown to prevent a feeding response in A. caninum infective larvae stimulated with dog
serum (Hawdon and Datu, 2003) supporting the hypothesis that an insulin-like signaling pathway involving IGF-1 may be involved in larval reactivation during pregnancy.

In order to directly implicate the involvement of host-derived TGF-β and IGF-1 signaling in larval reactivation and transmission, binding kinetics studies between the parasite encoded TGF-β receptors with mammalian TGF-β should be performed as have been done with *C. elegans* and the parasitic fluke, Schistosoma (Ren et al., 1996; Davies and Pearce, 1999). *A. caninum* TGF-β like receptor sequences as well as two TGF-β like ligands have been cloned in our laboratory (Freitas and Arasu, 2005; data not shown).

It is well known that anthelminthic drugs are inefficacious in eradicating tissue-arrested larval stages (Burke and Roberson, 1983; Arasu, 1998). Therefore, elucidating the host responses during different phases of pregnancy and lactation during a concurrent *A. caninum* infection may help in a better understanding of the phenomenon of tissue-arrest, reactivation and subsequent transmammary transmission of the parasite. Effective control strategies can be aimed at triggering the reactivation of arrested larvae followed by anthelminthic therapy.
Table 3.1 Primer sequences used for cytokine and reference gene transcript quantification by real-time RT-PCR

<table>
<thead>
<tr>
<th>Primer pairs</th>
<th>Gene</th>
<th>Accession #</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GAPDH</td>
<td>BC098095</td>
<td>ccactacatgtctacatgg</td>
<td>ctcgctctgggaagatg</td>
<td>122</td>
</tr>
<tr>
<td>2</td>
<td>60S ribosomal protein</td>
<td>BC011291</td>
<td>gattccggatatgtcctg</td>
<td>aaagccctggaagaaggag</td>
<td>132</td>
</tr>
<tr>
<td>3</td>
<td>TGF-β1</td>
<td>NM_011577</td>
<td>tgtcctcaccatacgagaa</td>
<td>tgggttaggggcaaggac</td>
<td>182</td>
</tr>
<tr>
<td>4</td>
<td>TGF-β2</td>
<td>NM_009367</td>
<td>aatgacaaacgagacacca</td>
<td>gatgagactaacgcctcc</td>
<td>198</td>
</tr>
<tr>
<td>5</td>
<td>IGF-1</td>
<td>AF440690</td>
<td>tggatgtctcagtcg</td>
<td>tctgagcttggcatgc</td>
<td>225</td>
</tr>
</tbody>
</table>
Figure 3.1 Experimental design. Nine to ten week old BALB/c female mice were mated and injected with 1000 *A. caninum* L3 larvae on day 5 post-impregnation. Mice were sacrificed at three different timepoints during pregnancy and lactation and divided into four groups based on infection and breeding. Serum, skeletal muscle, mammary gland and spleen were collected from each timepoint in each group for ELISA and real-time PCR analyses.
**Figure 3.2** Comparison of total larval burden in unbred versus bred BALB/c mice infected at times corresponding to day 19 gestation, day 1 and day 10 of postpartum lactation. Mice were infected with 1000 *A. caninum* L3 larvae subcutaneously. n = 5 mice per group.

* indicates significant difference at $p < 0.005$ between unbred and bred groups.
Figure 3.3 Comparison of serum TGF-β1 levels in UN, IN, UB and IB mouse. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation).

a. TGF-β1 levels at Timepoint I
* indicates significant difference at $p<0.0001$ between uninfected unbred and infected bred groups.

b. TGF-β1 levels at Timepoint II
* indicates significant difference at $p<0.0001$ between uninfected unbred and uninfected bred groups.
** indicates significant difference at $p<0.05$ between uninfected unbred and infected bred groups.

c. TGF-β1 levels at Timepoint III
* indicates significant difference at $p<0.0001$ between uninfected unbred and infected unbred, uninfected bred and infected bred groups.

d. Comparison of TGF-β1 levels in UN, IN, UB and IB groups over time
Figure 3.4 Comparison of serum TGF-β2 levels in UN, IN, UB and IB mouse. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation).

a. TGF-β2 levels at Timepoint I

b. TGF-β2 levels at Timepoint II

* indicates significant difference at $p < 0.0001$ between uninfected unbred and uninfected bred and between uninfected unbred and infected bred groups.

c. TGF-β2 levels at Timepoint III

* indicates significant difference at $p < 0.0001$ between uninfected unbred and infected unbred, uninfected bred and infected bred groups.

d. Comparison of TGF-β2 levels in UN, IN, UB and IB groups over time
Figure 3.5 Comparison of serum IGF-1 levels in UN, IN, UB and IB mouse. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation).

a. IGF-1 levels at Timepoint I
* indicates significant difference at $p<0.0001$ between uninfected unbred and infected unbred and uninfected bred groups.

b. IGF-1 levels at Timepoint II

c. IGF-1 levels at Timepoint III
* indicates significant difference at $p<0.0001$ between uninfected unbred, uninfected bred and infected bred groups.

d. Comparison of IGF-1 levels in UN, IN, UB and IB groups over time
Figure 3.6a
No significant differences were found between UN and IN at given timepoints.

Figure 3.6b
* indicates significant difference at $p < 0.05$ between UN and UB at timepoint III.

Figure 3.6c
No significant differences were found between UN and IB at given timepoints.

Figure 3.6a, b, c TGF-β1 transcript levels in skeletal muscle of IN (a), UB (b) and IB (c) mice. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation). RNA was extracted from skeletal muscle collected from each timepoint and cDNA was synthesized for real-time PCR analyses. For evaluating fold upregulation or downregulation of transcripts in IN, UB and IB groups, comparisons were made relative to UN controls.
Figure 3.6d, e, f TGF-β1 transcript levels in mammary gland of IN (d), UB (e) and IB (f) mice. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation). RNA was extracted from mammary gland collected from each timepoint and cDNA was synthesized for real-time PCR analyses. For evaluating fold upregulation or downregulation of transcripts in IN, UB and IB groups, comparisons were made relative to UN controls.

* indicates significant difference at $p<0.01$ between UN and IN at timepoint I, II and III.

* indicates significant difference at $p<0.01$ between UN and UB at timepoint II.

* indicates significant difference at $p<0.01$ between UN and IB at timepoint II.
Figure 3.6g
* indicates significant difference at \( p < 0.05 \) between UN and IN at timepoint II.

Figure 3.6h
No significant differences were found between UN and UB at given timepoints

Figure 3.6i
No significant differences were found between UN and IB at given timepoints

Figure 3.6g, h, i TGF-β1 transcript levels in spleen of IN (g), UB (h) and IB (i) mice. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation). RNA was extracted from spleen collected from each timepoint and cDNA was synthesized for real-time PCR analyses. For evaluating fold upregulation or downregulation of transcripts in IN, UB and IB groups, comparisons were made relative to UN controls.
Figure 3.7a
No significant differences were found between UN and IN at given timepoints.

Figure 3.7b
* indicates significant difference at $p < 0.005$ between UN and UB at timepoint III.

Figure 3.7c
No significant differences were found between UN and IB at given timepoints.

**Figure 3.7a, b, c** TGF-β2 transcript levels in skeletal muscle of IN (a), UB (b) and IB (c) mice. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation). RNA was extracted from skeletal muscle collected from each timepoint and cDNA was synthesized for real-time PCR analyses. For evaluating fold upregulation or downregulation of transcripts in IN, UB and IB groups, comparisons were made relative to UN controls.
Figure 3.7d
* indicates significant difference at $p < 0.01$ between UN and IN at timepoints I, II and III.

Figure 3.7e
* indicates significant difference at $p < 0.001$ between UN and UB at timepoint II.

Figure 3.7f
No significant differences were found between UN and IB at given timepoints.

**Figure 3.7d, e, f** TGF-β2 transcript levels in mammary gland of IN (d), UB (e) and IB (f) mice. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation). RNA was extracted from mammary gland collected from each timepoint and cDNA was synthesized for real-time PCR analyses. For evaluating fold upregulation or downregulation of transcripts in IN, UB and IB groups, comparisons were made relative to UN controls.
Figure 3.7g
* indicates significant difference at $p < 0.005$ between UN and IN at timepoint I.

Figure 3.7h
* indicates significant difference at $p < 0.05$ between UN and UB at timepoint I.

Figure 3.7i
No significant differences were found between UN and IB at given timepoints.

Figure 3.7g, h, i TGF-β2 transcript levels in spleen of IN (g), UB (h) and IB (i) mice. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation). RNA was extracted from spleen collected from each timepoint and cDNA was synthesized for real-time PCR analyses. For evaluating fold upregulation or downregulation of transcripts in IN, UB and IB groups, comparisons were made relative to UN controls.
Figure 3.8a
No significant differences were found between UN and IN at given timepoints.

Figure 3.8b
No significant differences were found between UN and UB at given timepoints.

Figure 3.8c
No significant differences were found between UN and IB at given timepoints.

**Figure 3.8a, b, c** IGF-1 transcript levels in skeletal muscle of IN (a), UB (b) and IB (c) mice. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation). RNA was extracted from skeletal muscle collected from each timepoint and cDNA was synthesized for real-time PCR analyses. For evaluating fold upregulation or downregulation of transcripts in IN, UB and IB groups, comparisons were made relative to UN controls.
Figure 3.8d, e, f  IGF-1 transcript levels in mammary gland of IN (d), UB (e) and IB (f) mice. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation). RNA was extracted from mammary gland collected from each timepoint and cDNA was synthesized for real-time PCR analyses. For evaluating fold upregulation or downregulation of transcripts in IN, UB and IB groups, comparisons were made relative to UN controls.

**Figure 3.8d**
* indicates significant difference at $p<0.01$ between UN and IN at timepoints I and II.

**Figure 3.8e**
No significant differences were found between UN and UB at given timepoints.

**Figure 3.8f**
* indicates significant difference at $p<0.01$ between UN and IB at timepoint II.
Figure 3.8g
No significant differences were found between UN and IN at given timepoints.

Figure 3.8h
No significant differences were found between UN and UB at given timepoints.

Figure 3.8i
No significant differences were found between UN and IB at given timepoints.

Figure 3.8g, h, i IGF-1 transcript levels in spleen of IN (g), UB (h) and IB (i) mice. Mice were sacrificed at three different timepoints during pregnancy and lactation (I = late gestation, II = early lactation and III = mid-lactation). RNA was extracted from spleen collected from each timepoint and cDNA was synthesized for real-time PCR analyses. For evaluating fold upregulation or downregulation of transcripts in IN, UB and IB groups, comparisons were made relative to UN controls.
Figure 3.9a, b, c Comparison of TGF-β1 transcript levels in skeletal muscle of IN (a), UB (b) and IB (c) mice over time. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation). RNA was extracted from skeletal muscle collected from each timepoint and cDNA was synthesized for real-time PCR analyses. For evaluating fold upregulation or downregulation of transcripts between timepoints, timepoint II and timepoint III were compared to timepoint I using equation III.

Figure 3.9a  
* indicates significant difference at $p < 0.001$ between timepoint I and timepoint III.

Figure 3.9b  
* indicates significant difference at $p < 0.01$ between timepoint I and timepoint III.

Figure 3.9c  
* indicates significant difference at $p < 0.05$ between timepoint I and timepoint III.
Figure 3.9d, e, f Comparison of TGF-β1 transcript levels in mammary gland of IN (d), UB (e) and IB (f) mice over time. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation). RNA was extracted from mammary gland collected from each timepoint and cDNA was synthesized for real-time PCR analyses. For evaluating fold upregulation or downregulation of transcripts between timepoints, timepoint II and timepoint III were compared to timepoint I using equation III.

**Figure 3.9d**  
* indicates significant difference at $p<0.05$ between timepoint I and timepoint III.

**Figure 3.9e**  
* indicates significant difference at $p<0.001$ between timepoint I and timepoint II.

**Figure 3.9f**  
* indicates significant difference at $p<0.01$ between timepoint I and timepoint II.
Figure 3.9g
* indicates significant difference at \( p < 0.05 \) between timepoint I and timepoint II.

Figure 3.9h
No significant differences were found between timepoints.

Figure 3.9i
No significant differences were found between timepoints

Figure 3.9g, h, i Comparison of TGF-β1 transcript levels in spleen of IN (g), UB (h) and IB (i) mice over time. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation). RNA was extracted from spleen collected from each timepoint and cDNA was synthesized for real-time PCR analyses. For evaluating fold upregulation or downregulation of transcripts between timepoints, timepoint II and timepoint III were compared to timepoint I using equation III.
Figure 3.10a, b, c Comparison of TGF-β2 transcript levels in skeletal muscle of IN (a), UB (b) and IB (c) mice over time. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation). RNA was extracted from skeletal muscle collected from each timepoint and cDNA was synthesized for real-time PCR analyses. For evaluating fold upregulation or downregulation of transcripts between timepoints, timepoint II and timepoint III were compared to timepoint I using equation III.

Figure 3.10a
* indicates significant difference at $p<0.001$ between timepoint I and timepoint III.

Figure 3.10b
* indicates significant difference at $p<0.001$ between timepoint I and timepoint III.

Figure 3.10c
* indicates significant difference at $p<0.001$ between timepoint I and timepoint III.
Figure 3.10d
* indicates significant difference at $p < 0.001$ between timepoint I and timepoint III.

Figure 3.10e
* indicates significant difference at $p < 0.05$ between timepoint I and timepoint II.

Figure 3.10f
No significant differences were found between timepoints

Figure 3.10d, e, f Comparison of TGF-β2 transcript levels in mammary gland of IN (d), UB (e) and IB (f) mice over time. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation). RNA was extracted from mammary gland collected from each timepoint and cDNA was synthesized for real-time PCR analyses. For evaluating fold upregulation or downregulation of transcripts between timepoints, timepoint II and timepoint III were compared to timepoint I using equation III.
Figure 3.10g, h, i Comparison of TGF-β2 transcript levels in spleen of IN (g), UB (h) and IB (i) mice over time. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation). RNA was extracted from spleen collected from each timepoint and cDNA was synthesized for real-time PCR analyses. For evaluating fold upregulation or downregulation of transcripts between timepoints, timepoint II and timepoint III were compared to timepoint I using equation III.
Figure 3.11a
* indicates significant difference at $p<0.05$ between timepoint I and timepoint III.

Figure 3.11b
No significant differences were found between timepoints.

Figure 3.11c
* indicates significant difference at $p<0.05$ between timepoint I and timepoint II.

Figure 3.11a, b, c Comparison of IGF-1 transcript levels in skeletal muscle of IN (a), UB (b) and IB (c) mice over time. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation). RNA was extracted from skeletal muscle collected from each timepoint and cDNA was synthesized for real-time PCR analyses. For evaluating fold upregulation or downregulation of transcripts between timepoints, timepoint II and timepoint III were compared to timepoint I using equation III.
Figure 3.11d, e, f Comparison of IGF-1 transcript levels in mammary gland of IN (d), UB (e) and IB (f) mice over time. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation). RNA was extracted from mammary gland collected from each timepoint and cDNA was synthesized for real-time PCR analyses. For evaluating fold upregulation or downregulation of transcripts between timepoints, timepoint II and timepoint III were compared to timepoint I using equation III.

**Figure 3.11d**
* indicates significant difference at $p<0.01$ between timepoint I and timepoint II.
** indicates significant difference at $p<0.001$ between timepoint I and timepoint III.

**Figure 3.11e**
No significant differences were found between timepoints.

**Figure 3.11f**
* indicates significant difference at $p<0.01$ between timepoint I and timepoint II, and timepoint I and timepoint III.
Figure 3.11g, h, i Comparison of IGF-1 transcript levels in spleen of IN (g), UB (h) and IB (i) mice over time. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation). RNA was extracted from spleen collected from each timepoint and cDNA was synthesized for real-time PCR analyses. For evaluating fold upregulation or downregulation of transcripts between timepoints, timepoint II and timepoint III were compared to timepoint I using equation III.

Figure 3.11g
No significant differences were found between timepoints.

Figure 3.11h
No significant differences were found between timepoints.

Figure 3.11i
No significant differences were found between timepoints.
4. Development of an *in vitro* co-culture system to study the effects of TGF-β and pregnancy hormones on reactivation of hookworm larvae

4.1 Introduction

After infecting a host parasitic nematodes may display developmental arrest where larvae have the option to resume further development upon receiving appropriate stimuli from the host. In hookworms, reactivation of the arrested larval population has a tremendous biological and economic significance as it results in active intestinal infection as well as transmammary transmission of the parasite (Schad, 1990). Molecular signals mediating such resumption of development have been well-studied in developmentally arrested dauer stage of the free-living soil nematode *Caenorhabditis elegans* (Riddle and Alberts, 1998). Genetic and molecular analyses in *C. elegans* have shown that transforming growth factor (TGF)-β and insulin-like growth factor (IGF)-1 signaling pathways mediate neuroendocrine responses leading into dauer formation as well as exit from the dauer stage (Georgi, et al., 1990; Estevez, et al., 1993; Ren, et al., 1996; Kimura, et al., 1997). Parallels have been drawn from these signaling pathways in studying the mechanisms of larval arrest and reactivation in hookworms (Hawdon and Schad, 1993). It has also been speculated that hormonal changes occurring during pregnancy potentially provide a stimulus for larval reactivation. Exogenous administration of estrogen and prolactin in *A. caninum* infected dogs resulted in resurgence of larvae in milk (Stoye and Krause, 1976). Since estrogen and prolactin have been shown to upregulate TGF-β2 isoform in uterus and mammary gland (Schneider et al., 1996), it is likely that a combination of hormones and cytokines in a timely manner causes the reactivation of the latent larval reservoir for re-infection and transmission.
Cell culture systems have been previously used in studies on helminth biology mainly, to culture the infective-stage L3 larvae to promote molting into the fourth stage in *Brugia* (Smith, et al., 2000), develop a cell line derived from viable L3 to evaluate antigen-specific humoral immune response in *Haemonchus* (Coyne and Brake, 2001), and nematode establishment, molting and reproduction in intestinal epithelial cells by *Trichinella* (ManWarren, et al., 1997; Gagliardo, et al., 2002). One of the primary goals of *in vitro* culture systems is to mimic the *in vivo* environment, thus providing the parasite with host-like conditions.

*In vivo*, the reactivation of arrested larvae leads to resumption of development with growth and morphological changes which require energy and therefore, intake of food. In *C. elegans*, dauer larvae start to feed within 2-3 hours of exposure to bacterial food and exit from the arrested stage (Ren, et al., 1996). In hookworms, a simple *in vitro* assay was developed in which larval reactivation results in activation of the feeding response and the ingestion of fluorescein-labeled albumin leading to fluorescent intestinal tracts in the larvae that can be easily scored under a fluorescent microscope (Hawdon, et al., 1993; Kumar and Pritchard, 1994). Besides hookworms, the *in vitro* feeding assay has been used as a direct method for measuring the reactivation of larvae in other parasitic nematodes also as it is speculated that resumption of feeding is associated with resumption of development (Hawdon, et al., 1992, 1993; Gamble and Mansfield, 1996). It was used in the present study to evaluate the candidate cytokines and hormones that may be responsible for triggering reactivation of *A. caninum* larvae. It appears that the amphids which contain axon endings of chemosensory neurons at the anterior end of the larvae are exposed to the environment and are therefore, critical for signaling during entry into and exit from the arrested stage.
(Bargmann and Horvitz, 1991; Ashton, et al., 1998, 1999). Using immunofluorescence studies, it was recently shown that anti-TGH-2 (A. caninum TGF-β isoform) antibodies identified native protein in the anterior region of the larvae resembling areas corresponding to the chemosensory region of the amphidial pores in C. elegans (Freitas and Arasu, 2005).

With the objective to compliment our in vivo studies on the influence of pregnancy hormones and cytokines associated with reactivation of tissue-arrested hookworm larvae, an in vitro cell culture system was developed. We focused on establishing primary skeletal and mammary epithelial cell cultures as upon reactivation larvae migrate from skeletal muscle, which is the favored site of arrest, to mammary gland leading to transmammary transmission to the newborn. We successfully established both types of primary cell cultures in our laboratory and co-cultured these cells with infective A. caninum L3 larvae. Previous in vitro feeding/reactivation assays had shown that A. caninum infective L3 were not susceptible to the effects of TGF-β while ‘tissue arrested’ L3s recovered from the carcasses of infected mice were stimulated to resume feeding (albeit with no evidence of morphological change or development to the 4th larval stage; Arasu, 2001). This experimental setup was designed to pre-condition or transform infective L3 larvae by the mammalian cell co-culture to mimic ‘tissue-arrested’ larvae and to then test the hypothesis that mammalian host-derived TGF-β and pregnancy hormones result in reactivation and resumption of development.

4.2 Materials and Methods

4.2.1 Primary skeletal muscle cell culture

For starting a primary culture of skeletal muscle, 4-5 of <1 day-old neonatal Cr:NIH (S) mouse pups (National Cancer Institute, Frederick, MD) were killed. After skinning,
hindlimbs and forelimbs were collected in Hank’s Balanced Salt Solution (HBSS) (Mediatech, Herndon, VA) and minced finely with scissors. The minced muscle mass was subjected to digestion using an enzymatic solution of 0.17% Trypsin (T-8128, Sigma-Aldrich, St. Louis, Missouri) and 0.085% Collagenase (C-6885, Sigma-Aldrich, St. Louis, Missouri) in a beaker incubated at 37°C for 25 minutes to obtain a free cell suspension. After incubation the contents in the beaker were transferred into a 15 ml sterile disposable tube and centrifuged at 2500 rpm for 5 minutes. Supernatant was removed by a Pasteur pipette and the digested muscle mass along with cells were resuspended in 5 ml of complete Dulbecco’s Modified Eagles Medium (cDMEM) containing 5% Fetal Bovine Serum (FBS) (Mediatech, Herndon, VA) and 1% antibiotic/antimycotic solution (Mediatech, Herndon, VA) to neutralize Trypsin. The muscle mass was washed 3 times as mentioned above and then filtered through a Nytex™ (TETKO, Kansas City, MO) membrane filter into a fresh tube. The cell suspension was diluted to 5 ml of cDMEM and cell concentration was determined with a hemocytometer. Cells were plated at a density of 100,000 cells per well in a 24-well cell culture plate (Costar). The monolayer typically became 75-80% confluent after 72 hours.

4.2.2 Primary mammary epithelial cell culture

Timed pregnant full-term Cr:NIH (S) female mice (National Cancer Institute, Frederick, MD) were killed by cervical dislocation. Mammary glands were excised, collected in HBSS and minced into a fine paste with scissors and scalpel. The minced mammary gland tissue was subjected to digestion using an enzymatic solution of 0.35% Collagenase Type 3 (C-6885, Sigma-Aldrich, St. Louis, Missouri) and 0.65% Hyaluronidase (H-3506, Sigma-Aldrich, St. Louis, Missouri) in a beaker shaking at 200 rpm at 37°C for 2 hours for a free
cell suspension. After incubation the mammary gland was mixed well with the help of a 10 ml pipette and filtered through a Nytex membrane into a 15 ml sterile disposable tube and centrifuged at 1500 rpm for 5 minutes. The supernatant containing milk, fat globules and tissue debris was removed carefully without disturbing the cell pellet. The cell pellet was resuspended in cDMEM and washed three times at 2500 rpm for 5 minutes each at room temperature. After the final washing the cell pellet was resuspended in cDMEM, filtered again through the Nytex membrane and collected in 5 ml cDMEM for cell enumeration using a hemocytometer. Cell were plated at a density of 50,000 cells per well in a 24-well Collagen I-coated cell-culture plate (Greiner Bio-One, Kremsmünster, Austria). The monolayer typically became 75-80% confluent around 72 hours.

4.2.3 Immunohistochemistry

Immunohistochemistry was performed on primary skeletal muscle cells and mammary epithelial cells to confirm the presence/prevalence of the respective cells of interest. For preparing a cytospin, cells were detached using 50 µl trypsin/EDTA at 37ºC for 4 minutes and suspended in trypsin inhibitor (DMEM with 10% FBS). Cells were dissociated by pipetting up and down ten times. After adding cytospin fluid, the cell suspension was attached to the glass slides by centrifuging at 1000 rpm for 1 minute in Shandon cytospin-3 (Labtics, Helsingborg, Sweden). Cytospins were fixed using 2% ice-cold paraformaldehyde for 10 minutes and rinsed well with PBS. Histostain®-SP kit (Zymed, San Francisco, CA) was used for immuno-histological staining. Peroxidase quenching solution consisting of 30% hydrogen peroxide and 9 parts of absolute methanol was used to flood the whole slide for 10 minutes. After rinsing the slide with PBS, one drop of serum blocking solution was applied
per cytospin for 10 minutes. Anti-rabbit c-met (Receptor tyrosine kinase) antibody (Zymed, San Francisco, CA) was used at a 1:100 dilution for staining skeletal muscle cells overnight at 4°C. Anti-rabbit cytokeratin antibody (Novocastra, NewCastle, UK) was used at 1:50 dilution for staining mammary epithelial cells for 1 hour and incubated in a moist chamber. After rinsing the slides with PBS, 100 µl of biotinylated secondary antibody was added to each slide and incubated for 10 minutes. After the incubation step, the rinsing step was repeated to remove any remaining biotinylated secondary antibody. Two drops or 100 µl of enzyme-conjugate solution was added to each slide for 10 minutes and rinsed off with PBS. Two drops or 100 µl of substrate-chromogen mixture was added to each slide and incubated for 15 minutes and rinsed well with distilled water. The slide were counterstained with two drops or 100 µl of hematoxylin for 3 minutes and washed under tap water. Two drops of mounting solution was put on the slides before applying the coverslip over the cell pellet. The slide was observed under microscope for the development of a red color indicating positive staining. For negative controls, primary antibody was not added and C2C12 cells served as a positive control for skeletal muscle cells whereas HC11 cells served as a positive control for mammary epithelial cells.

4.2.4 L3 larvae

Infective A. caninum L3 were harvested from charcoal co-cultures of feces from male laboratory beagle dogs (Marshal Farms, North Rose, NY) orally infected at 8-10 weeks of age with 150 L3 as previously described (Burke and Roberson, 1979a; Arasu, 1998). The use of dogs in this study was approved by the Institutional Animal Care and Use Committee at North Carolina State University. Feces containing A. caninum eggs (range of 500-3000
eggs/g depending on stage of infection) were mixed with activated charcoal and deionized water in Pyrex® baking trays and placed in a humidified incubator, in the dark, at 25°C for 7-10 days. The L3 were harvested utilizing a modified Baermann assembly (Garcia and Ash, 1979), repeatedly washed in phosphate-buffered saline (PBS) medicated with 20 mg/L gentamicin (Sigma Chemical Co., Missouri) and 20 mg/L lincomycin (Sigma Chemical Co., Missouri) and maintained at room temperature for 5-7 days prior to use.

To prepare the larvae for co-culture, infective L3 were exsheathed using 0.1% Clorox bleach (Oakland, CA) to 5000 L3 larvae suspended in 10 ml of PBS in a 50 ml polypropylene tube and kept on a shaker (Fisher Scientific, Pittsburgh, PA) at 150 rpm for 5 minutes. After filling the tube with excess PBS up to 45 ml, the larvae were washed at 1000 rpm for 3 minutes by centrifugation. The washing step was repeated 4-5 times prior to use.

4.2.5 Primary cells and Larval Co-culture

The monolayer of cells was washed 3 times with 750 µl of cDMEM to remove media and residual serum as serum can activate a feeding response in the L3 larvae. DMEM-Serum Replacement II (SRII) (Sigma Aldrich, St. Louis, Missouri) was prepared the same way as cDMEM but by replacing serum with 1% SRII. After adding 1000 µl of DMEM-SRII to each well, Transwell® polycarbonate membrane with 3.0 µm pore size (Costar, Corning Inc., Corning, NY) were placed into each well. The Transwell® membranes split the cell culture wells into a lower well and an upper well. Negative controls were setup in triplicate by adding 5% SRII to the final volume of 1.25 ml. Similarly, positive controls were setup in
triplicate by adding 5% FBS to the same final volume. To each well, 1000 exsheathed larvae were added for setting up the co-culture.

4.2.6 In vitro Feeding Assay

Briefly, 250 exsheathed L3 larvae harvested from the co-culture system were washed at 1000 rpm for 15 seconds three times with PBS prior to use. Larvae were placed in 96-well microtiter plate and each treatment or control was assayed in triplicate. To each well 100 µl of 5 mg/ml fluorescein-isothiocyanate-conjugated bovine serum albumin (FITC-BSA; Sigma-Aldrich, St. Louis, Missouri) was added and the plate was incubated at 37°C, 5% CO₂ for 1.5-2 hours (Hawdon and Schad, 1990). The larvae were washed three times with PBS to remove residual FITC prior to examination by UV microscopy at 400X magnification. At least 50-100 larvae were enumerated for fluorescent intestinal tracts as positive (reactivated) or negative (arrested). Percentage feeding was determined by 100 X (number positive)/ (number positive + number negative).

For each experiment, the positive control was normal dog serum and the negative control was cDMEM. Test reagents included TGF-β1 and TGF-β2 (recombinant human; R&D Systems, Inc., Minneapolis, Minnesota), Estrogen (Sigma-Aldrich, St. Louis, Missouri) and Prolactin (mouse; National Hormone and Pituitary Program, Rockville, Maryland). For functional activation, TGF-β1 and TGF-β2 were acidified in 4 mM HCl, 0.1% BSA at a stock concentration of 1 µg/ml prior to use, control wells were similarly treated with HCl/BSA. Estrogen was dissolved in sterile distilled water at stock concentration of 25 mg/ml and prolactin was dissolved in PBS at a stock concentration of 1 µg/100 µl. All experiments were repeated two or more times.
4.3 Results

4.3.1 Staining of primary cell cultures

Primary skeletal muscle cells and primary mammary epithelial cells showed positive staining with anti-c-met antibody and anti-cytokeratin antibody respectively, using the Histostain kit (Zymed laboratories, San Francisco, CA). After evaluating the stained cells under the microscope it was found that more than 80% of the cells in the primary skeletal muscle cell culture were of skeletal muscle origin (Figure 4.1a, 4.1b, 4.1c). The other major cell type was fibroblast which did not stain with anti-c-met antibody. Based on staining with anti-cytokeratin, more than 90% of the cells in the primary culture were mammary epithelial cells (Figure 4.1d, 4.1e, 4.1f). Negative controls stained blue indicating absence of antigen recognition by the primary antibody. Positive controls (C2C12, skeletal muscle cells and HC11, mammary epithelial cells) for respective cultures stained and were used for comparison when identifying positive staining in test cells.

4.3.2 Effect of serum on larval feeding

Preparasitic *A. caninum* L3 larvae were co-incubated in the presence or absence of primary skeletal muscle cells to test the effect of DMEM media supplemented with 5% SRII instead of serum. No differences were observed in the basal levels of reactivation between the larvae that were co-incubated with cells or in the absence of cells (Figure 4.2a). Normal dog serum had previously been shown to stimulate feeding behavior in infective *A. caninum* L3 larvae (Schad, 1991; Arasu, 2001). Based on these findings, 5% normal dog serum was used as positive control and absence of serum served as a negative control. As shown in Fig. 4.2a, L3s co-cultured with primary skeletal muscle cells showed a significant
reactivation/feeding response when exposed to serum (Figure 4.2b). Similar results were observed with L3 obtained from co-cultures with mammary epithelial cells (Figure 4.2c).

4.3.3 Effect of TGF-β on larval feeding

TGF-β has been shown to be exhibit a dose-dependent stimulatory effect on the tissue-arrested larvae that were harvested from the carcass of infected BALB/c mice but not on infective, pre-parasitic L3 (Arasu, 2001). In these analyses, TGF-β had no stimulatory effect on the A. caninum L3 larvae co-incubated with primary skeletal muscle cells for 24 h, 48 h and 72 h; the background levels of percent reactivated L3 larvae were similar to the negative control (Figure 4.3a, 4.3b, 4.3c, respectively). Similar results were obtained with co-incubation of A. caninum larvae with primary mammary epithelial cells (Figure 4.4a, 4.4b and 4.4c). The experiment was repeated three times.

4.3.4 Effect of pregnancy hormones on larval feeding

In a previously published report, pregnancy-associated hormones, estrogen and prolactin, had no effect on the tissue-arrested L3 or infective larvae at physiological (0.1-1 µg/ml) or at high concentrations of 1 mg/ml (Arasu, 2001). To determine if hormones might have a modulatory effect on the mammalian cells with subsequent excretion of stimulatory cytokines such as TGF-beta, estrogen (100 ng/ml) and/or prolactin (100 ng/ml) were added to the co-cultures for 24 h, 48 h and 72 h (Figure 4.5a, 4.5b and 4.5c). There was no effect on the percentage feeding response of L3 larvae co-cultured with primary skeletal muscle cells as compared to that of serum alone (positive control). Similarly, no effect of hormones was observed on the L3 larvae co-cultured with primary mammary epithelial cells (Figure 4.6a,
4.6b and 4.6c). Supernatants were not tested to evaluate if TGF-β was released by exposure of the cells to the hormones.

### 4.4 Discussion

Mice have been used as normal paratenic hosts for *in vivo* studies on *A. caninum* larval arrest and reactivation (Arasu and Kwak, 1999). Similar to the dog, pregnancy/lactation in the mouse triggers the transmammary transmission of larvae to the nursing neonates (Steffe and Stoye, 1984; Arasu and Kwak, 1999). It is known from studies in *C. elegans* that TGF-β signaling is one of the pathways involved in arrest and reactivation (Georgi, et al., 1990; Ren, et al., 1996). In mammals, TGF-β2 is apparently upregulated in uterine and mammary tissue during pregnancy (Schneider, et al., 1996). It was therefore hypothesized that *A. caninum* larvae have evolved to utilize signaling via host-derived TGF-β resulting in reactivation, migration through somatic tissues and subsequent transmission to the newborn. In a previously published report, physiological concentrations of TGF-β stimulated a feeding response which was comparable to the stimulatory effect induced by 10% normal dog serum on tissue-arrested *A. caninum* L3 larvae (Arasu, 2001).

Pregnancy-associated hormones such as estrogen and prolactin have been implicated in larval reactivation and transmission. When estrogen and prolactin were administered exogenously to post-partum dogs with latent (arrested) infections of *A. caninum*, a resurgence of larvae was seen in their milk if let-down was induced by administration of oxytocin (Stoye and Krause, 1976). However, hormones did not have any direct stimulatory effect on tissue-arrested larvae as mentioned above. There might be indirect effects of hormonal fluctuations observed during pregnancy. TGF-β2 tissue mRNA and serum levels were elevated during
late pregnancy (Schneider, et al., 1996). It was further shown that TGF-β2 mRNA levels were elevated in the same tissues (uterine and mammary gland) as a result of exogenous estrogen treatments in ovariectomized rats (Das, et al., 1992; Cheng, et al., 1993; Schneider, et al., 1996). Taken together, these findings suggest that host-derived cytokine and hormonal cues may be involved in specific reactivation of tissue-arrested *A. caninum* larval stages during pregnancy and lactation.

In an attempt to test our hypothesis that host factors like cytokines and hormones stimulate reactivation of tissue-arrested *A. caninum* larvae, we developed a primary cell/larvae co-culture system. Our major focus was to use the primary skeletal muscle and mammary epithelial cell cultures for co-incubation with pre-parasitic *A. caninum* L3 to mimic tissue-arrest outside the body of the host. As mentioned before, skeletal muscle is the favored site of larval arrest and mammary gland is the site where the larvae get transmitted to the neonates via suckling. Larvae were co-incubated with cells for 24 hours before adding the cytokines (TGF-β1 and TGF-β2) and hormones (estrogen and prolactin) of interest. We did not observe any significant feeding behavior in the co-cultured L3 larvae that were treated with TGF-β isoforms and hormones. One of the reasons for such observations could be the inability of the co-culture setup to induce tissue-arrest as it occurs inside a host.

In order to closely match the *in vivo* conditions, a variety of host factors and molecules have been added to parasitic cultures in different studies. Certain factors like ascorbic acid are critical in the morphogenesis and development of parasitic helminths like *Brugia* (Rajan, et al., 2003). Similarly, a simple reproducible method has been developed for protozoa i.e. short-term *ex-vivo Plasmodium vivax* culture in which glucose, ascorbic acid, thiamine, hypoxanthine, and 50% human AB+ serum are added to the standard *P. falciparum*
in vitro culture medium (Chotivanich, et al., 2001). This simple method of culturing P. vivax ex vivo is suitable for anti-malarial susceptibility and immunoparasitology studies. It is therefore possible that besides the supplementation of media with serum replacement media II (Sigma Aldrich), we would have to add other factors to simulate in vivo conditions closely. Since host molecules important in A. caninum larval arrest are not known, it would laborious and time-consuming to test candidate reagents. Transwell® filters keep the L3 larvae physically separate from the cell monolayer. It is possible that the larvae have to be in close physical contact with the cells to receive signals for undergoing arrest. However, it is very difficult to do so in case of A. caninum L3 larvae as they are constantly moving thereby, disrupting the cell monolayer within hours of physical contact.

We have proposed that larval reactivation can be assessed in independent ways besides the in vitro co-culture setup. Firstly, PCR amplification can be used to evaluate the upregulation of Ac-daf-7, which is the C. elegans daf-7 homologue recently cloned and characterized in A. caninum, in reactivated versus arrested L3 larvae. However, recent studies by Freitas and Arasu (2005) show that expression of daf-7 is maximal at the arrested infective L3 as well as reactivated (serum-stimulated) L3 larval stages which differs from that of Ce-daf-7 expression and may be unique to parasitic nematodes that have an obligate requirement to undergo developmental arrest (Freitas and Arasu, 2005). Secondly, reactivation status can be tested by using protein binding studies i.e. whether Ac-DAF-7 can bind to and stimulate signaling from mammalian TGF-β receptors. Similar studies in Schistosoma mansoni have shown that a chimeric receptor containing the extracellular domain of type I TGF-β receptor, SmRK1, that is joined to the intracellular domain of the human type I TGF-β receptor, could activate a luciferase reporter gene in response to TGF-β
(Beall and Pearce, 2001). The efforts to express Ac-DAF-7 are under way in our lab. Similar studies of *B. malayi* have suggested an immunomodulatory role for recombinant Bm-TGH-2 which can bind to and stimulate signaling from mammalian TGF-β receptors (Gomez-Escobar et al., 2000). Further studies are required to examine the host-parasite interaction in *A. caninum* infection utilizing ligand and receptor interaction studies. Finally, larval reactivation status can be checked by utilizing antibodies to *Ancylostoma* secreted protein (ASP). It has been previously demonstrated that preparasitic L3 larvae stimulated by serum start releasing excretory-secretory products such as ASPs which appear to facilitate in larval penetration and migration (Hawdon, et al., 1996b). A better understanding of the mechanisms associated with larval arrest and reactivation in *A. caninum* can contribute to development of chemotherapeutic and vaccine-based strategies for elimination of latent infections.
Figure 4.1 Immunohistochemistry. Cytospins of primary skeletal muscle were stained with c-met antibody overnight. A, C2C12 skeletal muscle cell line, positive control; B, no primary antibody, negative control; C, skeletal muscle cells stained positive with c-met antibody (20X).
Figure 4.1 Immunohistochemistry. Cytospins of primary mammary epithelial cells were stained with anti-cytokeratin antibody overnight. A, HC11 mammary epithelial cell line, positive control; B, no primary antibody, negative control; C, mammary epithelial cells stained positive with anti-cytokeratin antibody (20X).
**Figure 4.2a** Effect on percent feeding response of L3 larvae co-cultured in the presence or absence of primary skeletal muscle cells. L3 larvae were co-incubated in DMEM supplemented with 5% Serum Replacement media II in Transwells®.
Figure 4.2b Effect of serum on percent feeding response of L3 larvae co-cultured with primary skeletal muscle cells. L3 larvae were co-incubated in DMEM supplemented with 5% Serum Replacement media II as negative control and larvae were stimulated with 5% normal dog serum as positive control. The asterisk indicates significant difference at p< 0.05.
Figure 4.2c Effect of serum on percent feeding response of L3 larvae co-cultured with primary mammary epithelial cells. L3 larvae were co-incubated in DMEM supplemented with 5% Serum Replacement media II as negative control and larvae were stimulated with 5% normal dog serum as positive control. The asterisk indicates significant difference at p<0.05.
Figure 4.3a Effect of TGF-β2 on feeding response of L3 larvae. L3 larvae were co-cultured with primary skeletal muscle cells in DMEM supplemented with 5% Serum Replacement media II as negative control, larvae were stimulated with 5% normal dog serum as positive control and TGF-β2 (10 ng/ml) for 24 h in triplicate. The asterisk indicates significant difference at p< 0.05.
**Figure 4.3b** Effect of TGF-β2 on feeding response of L3 larvae. L3 larvae were co-cultured with primary skeletal muscle cells in DMEM supplemented with 5% Serum Replacement media II as negative control, larvae were stimulated with 5% normal dog serum as positive control and TGF-β2 (10 ng/ml) for 48 h in triplicate. The asterisk indicates significant difference at p< 0.05.
**Figure 4.3c** Effect of TGF-β2 on feeding response of L3 larvae. L3 larvae were co-cultured with primary skeletal muscle cells in DMEM supplemented with 5% Serum Replacement media II as negative control, larvae were stimulated with 5% normal dog serum as positive control and TGF-β2 (10 ng/ml) for 72 h in triplicate. The asterisk indicates significant difference at p< 0.05.
Figure 4.4a Effect of TGF-β2 on feeding response of L3 larvae. L3 larvae were co-cultured with primary mammary epithelial cells in DMEM supplemented with 5% Serum Replacement media II as negative control, larvae were stimulated with 5% normal dog serum as positive control and TGF-β2 (10 ng/ml) for 24 h in triplicate. The asterisk indicates significant difference at p< 0.05.
Figure 4.4b Effect of TGF-β2 on feeding response of L3 larvae. L3 larvae were co-cultured with primary mammary epithelial cells in DMEM supplemented with 5% Serum Replacement media II as negative control, larvae were stimulated with 5% normal dog serum as positive control and TGF-β2 (10 ng/ml) for 48 h in triplicate. The asterisk indicates significant difference at p< 0.05.
Figure 4.4c Effect of TGF-β2 on feeding response of L3 larvae. L3 larvae were co-cultured with primary mammary epithelial cells in DMEM supplemented with 5% Serum Replacement media II as negative control, larvae were stimulated with 5% normal dog serum as positive control and TGF-β2 (10 ng/ml) for 72 h in triplicate. The asterisk indicates significant difference at p< 0.05.
Figure 4.5a Effect of pregnancy-associated hormones on feeding response of L3 larvae. L3 larvae were co-cultured with primary skeletal muscle cells in DMEM supplemented with 5% Serum Replacement media II as negative control, larvae were stimulated with 5% normal dog serum as positive control and estrogen (100 ng/ml), prolactin (100 ng/ml) and estrogen/prolactin for 24 h in triplicate. The asterisk indicates significant difference at p<0.05.
Figure 4.5b Effect of pregnancy-associated hormones on feeding response of L3 larvae. L3 larvae were co-cultured with primary skeletal muscle cells in DMEM supplemented with 5% Serum Replacement media II as negative control, larvae were stimulated with 5% normal dog serum as positive control and estrogen (100 ng/ml), prolactin (100 ng/ml) and estrogen/prolactin for 48 h in triplicate. The asterisk indicates significant difference at p<0.05.
Figure 4.5c Effect of pregnancy-associated hormones on feeding response of L3 larvae. L3 larvae were co-cultured with primary skeletal muscle cells in DMEM supplemented with 5% Serum Replacement media II as negative control, larvae were stimulated with 5% normal dog serum as positive control and estrogen (100 ng/ml), prolactin (100 ng/ml) and estrogen/prolactin for 72 h in triplicate. The asterisk indicates significant difference at p<0.05.
Figure 4.6a Effect of pregnancy-associated hormones on feeding response of L3 larvae. L3 larvae were co-cultured with primary mammary epithelial cells in DMEM supplemented with 5% Serum Replacement media II as negative control, larvae were stimulated with 5% normal dog serum as positive control and estrogen (100 ng/ml), prolactin (100 ng/ml) and estrogen/prolactin for 24 h in triplicate. The asterisk indicates significant difference at p<0.05.
Figure 4.6b Effect of pregnancy-associated hormones on feeding response of L3 larvae. L3 larvae were co-cultured with primary mammary epithelial cells in DMEM supplemented with 5% Serum Replacement media II as negative control, larvae were stimulated with 5% normal dog serum as positive control and estrogen (100 ng/ml), prolactin (100 ng/ml) and estrogen/prolactin for 48 h in triplicate. The asterisk indicates significant difference at p<0.05.
Figure 4.6c Effect of pregnancy-associated hormones on feeding response of L3 larvae. L3 larvae were co-cultured with primary mammary epithelial cells in DMEM supplemented with 5% Serum Replacement media II as negative control, larvae were stimulated with 5% normal dog serum as positive control and estrogen (100 ng/ml), prolactin (100 ng/ml) and estrogen/prolactin for 72 h in triplicate. The asterisk indicates significant difference at p<0.05.
5. IL-4 and IFN-γ serum protein and transcript levels during pregnancy and

*Ancylostoma caninum* infection in BALB/c mice

### 5.1 Introduction

More than one billion people in the tropics and sub-tropics are known to be infected with hookworms (Chan, 1997). Thus, hookworm infection remains a major contributor to iron-deficiency anemia as adult parasites feed on blood from the intestines. Since hookworms are known to exhibit latency and reactivation, they interact with their hosts at an intimate level and have been reported to 'counter' the complex immune responses generated against them (Behnke, 1991; Loukas and Prociv, 2001). Immune responses to hookworms have been studied in both humans and experimental animal hosts in attempts to understand host protection as well as parasite survival particularly with regard to the development of hookworm vaccines. Eosinophilia, mastocytosis and IgE production are the most prominent immune alterations observed during a hookworm infection (Loukas and Prociv, 2001). In many ways, hookworms are typical gastrointestinal nematodes based on types of immune responses generated in definitive hosts. Humoral and cellular responses fit loosely within the framework of a T helper 2 (Th2) type of response (Loukas, et al., 2005). Humans infected with *Necator* hookworms produce high levels of parasite-specific and total IgE (Pritchard and Walsh, 1995; Pritchard, et al., 1995) which is accompanied by peripheral and local eosinophilia (White, et al., 1986; Maxwell, et al., 1987). While humoral responses to hookworms have been well documented; little was known about the role of adaptive T cell response until recently (Quinnell et al., 2004; Pit, et al., 2000 and 2001).
There is a notable lack of suitable animal models for human hookworm infection, and extrapolating from immunological models of an abnormal host can be unreliable. *N. americanus* matures in hamsters; however, there is wide variability in the number of L3 that develop into intestinal adult worms (Rose and Behnke, 1990; Jian, et al., 2003). Similarly, *A. duodenale* has been shown to develop in dogs, but only with exogenous administration of steroids (Leiby, et al., 1987). Syrian Golden hamsters (*Mesocricetus auratus*) have also been used to model infections with *Ancylostoma ceylanicum* (Garside, et al., 1990). In a recent study, impact of *A. ceylanicum* hookworm infection on host cellular responses, cytokine production and lymphoproliferation were measured (Mendez, et al., 2005). Initial larval infection with 100 third-stage *A. ceylanicum* larvae resulted predominantly in Th1 responses (upregulation of proinflammatory cytokines such as IFN-γ and TNF-α) which occurred during larval migration and continued up to 14 days post-infection or prepatency. Subsequently, development of larvae into egg-laying adult hookworms or patency coincided with a switch to Th2 predominant responses with a marked increase in IL-4 and IL-10 production. This switch also concurred with reduced host lymphoproliferative responses to hookworm antigens (Mendez, et al, 2005).

*A. caninum* infection in dogs serves as a good model for understanding the host-parasite interactions occurring during human hookworm infection. However, canines are not a realistic option due to high animal costs as well as ethical concerns with euthanasia for these types of studies. Our lab has utilized the mouse as a circumscribed model for specifically assessing pregnancy-associated transmammary transmission of *A. caninum* infection in the neonate (Arasu and Heller, 1999; Arasu and Kwak, 1999). The murine model was used to compare the responses of infected versus uninfected animals that were either
bred or not bred to ascertain whether suppression of *A. caninum*-specific antibody responses during pregnancy facilitated the reactivation and transmammary transfer of hookworm larvae. Initial comparisons of genetically divergent BALB/c versus C57BL/6 mice showed that both the strains mounted strong Th2 biased IgG1 and IgE antibody responses to *A. caninum* infection (Arasu and Heller, 1999). It was also confirmed that larval transfer to the mouse pups occurred during the post-partum lactational period as there was only one previously published report using mice to assess the transmammary transmission of *A. caninum* larvae (Steffe and Stoye, 1984). In dams, levels of total and antigen-specific IgG1 and total IgE (Th2 response) were highly correlated with parasite burden (Arasu and Heller, 1999). During most phases of pregnancy and lactation, infected dams had lower total IgG1, IgG2a and IgE levels as compared to unbred mice at comparable times post-infection which supported the established dogma of a generalized immunosuppression associated with pregnancy (Arasu and Heller, 1999). However, correlative studies showed that the parasite-specific antibody responses did not play a major role in the pregnancy-associated transmammary transmission of *A. caninum* larvae (Arasu and Heller, 1999). These studies do not rule out the possibility of underlying fluctuations in the levels of Th1 and Th2 cytokines associated with pregnancy and infection that may be involved in the process of larval reactivation and transmission.

We report here the immunological profile of IL-4 and IFN-γ mRNA transcripts during pregnancy and *A. caninum* infection in skeletal muscle (the favored site of latent infection), mammary gland (where the larvae get transmitted) and spleen (major site to monitor immune responses during parasitic infections). Interestingly, in our model of breeding and infection we found that during early lactation IL-4 transcript levels predominate
over IFN-\(\gamma\) in skeletal muscle but were concurrently downregulated in mammary gland in infected bred mice. We also found that both IL-4 and IFN-\(\gamma\) transcripts were upregulated at 14 days post-infection in mammary gland of infected unbred mice which support the findings that acute helminthic infections are often associated with mixed Th1/Th2 responses. We were unable to correlate mRNA transcript levels with serum levels as IL-4 was undetectable in the sera of majority of the mice.

5.2 Materials and Methods

5.2.1 Mice breeding and infection

Nine to ten-week-old female BALB/c mice were purchased from Charles River Laboratories, and maintained at the Laboratory Animal Resources facility in accordance with Institutional Animal Care and Use Committee guidelines. Mice were mated at a ratio of 2 females: 1 male for a period of 7 days and examined twice daily for the presence of a vaginal impregnation plug. Observation of a plug was designated as day 0 of the pregnancy. On day 5 post-impregnation (observation of vaginal plug), the female mice were injected subcutaneously in the dorsal cervical interscapular region with 100 ul PBS (control) or with 1000 larvae in PBS. Mice were killed by decapitation at three different time points: time point I corresponding to day 19 of gestation or day 14 post-infection (pi), and time points II and III corresponding to day 1 and 10 of postpartum lactation or day 15-16 pi and day 25-26 pi, respectively. The tissue samples collected were sections of the gastrocnemius skeletal muscle, mammary gland, and spleen as well as blood. Approximately 200mg of each tissue sample (except blood) was stored in RNA-STAT 60 (Tel-Test, Texas) at -80°C for RNA extraction. Serum was collected after incubating whole blood at 37°C for 1 h and
subsequently at 4°C for 4 h and subsequently stored at -20°C until further use. The female mice were grouped as infected or not infected and bred or not bred (n = 5 per group). The resulting four groups were uninfected/ unbred (UN or normal controls), infected/ unbred (IN), uninfected/ bred (UB) and infected/ bred (IB).

5.2.2 Harvesting tissue L3

For harvesting tissue-arrested larvae, carcasses were skinned, minced, wrapped in eight layers of cheesecloth and incubated in a modified Baermann assembly for 4 h at 37°C in PBS medicated with 20 mg/L gentamicin (Sigma Chemical Co., Missouri) and 20mg/L lincomycin (Sigma Chemical Co., Missouri). Migrating larvae that collected at the bottom of the cups were repeatedly washed at 2000 rpm for 10 minutes with medicated PBS. Larval numbers in dams versus each litter of pups were counted with the aid of a dissecting microscope.

5.2.3 Mouse IL-4 ELISA

IL-4 levels were determined using a Mouse IL-4 ELISA kit (Pierce Endogen, Rockford, IL) which is based on the principle of an indirect ELISA. The assays were performed using anti-mouse IL-4 pre-coated 96-well strip plates. The standards (0, 15, 75 and 375 pg/ml) were prepared according to the manufacturer’s protocol. Using a multichannel pipettor 50 µl of plate reagent was added to each well before adding 50 µl of reconstituted standards and test sera samples from different groups of mice. The plate was covered with an adhesive tape and incubated for 3 hours at 37°C in a humidified incubator. After washing five times with wash buffer, 100 µl prediluted conjugate reagent was added to
each well and the plate was incubated for 1 hour 37°C in a humidified incubator. After repeating the washing step five times, 100 µl of TMB substrate solution was added to each well. Enzymatic color reaction was allowed to develop at room temperature in the dark for 30 minutes. The substrate reaction yielded a blue solution that turned yellow when Stop Solution containing hydrochloric acid was added. Absorbance was measured on an ELISA plate reader set at 450 nm and 550 nm. The standard curve was used to determine IL-4 in pg/ml in the unknown samples.

5.2.4 RNA extraction and cDNA synthesis

Total RNA from all the tissues (skeletal muscle, mammary gland and spleen) was extracted using RNA-STAT (Tel-Test, Inc., Friendswood, TX) according to Chomczynski and Sacchi (1987). Residual genomic DNA was removed from RNA by treating with DNase (20U per 100 µg of RNA). RNA quantity and quality was checked by spectrophotometric measurements at 260 and 280 nm (Pharmacia) and by analyzing 1µg RNA for rRNA bands and integrity on a 1% ethidium bromide agarose gel.

For reverse transcription, first strand cDNA was synthesized by adding 10µg of total RNA to a 40µl reaction mix containing a final concentration of 2.5nM dNTPs, 0.1M dithiothreitol, 1 µg Oligo d (T) primer (Promega, Madison, WI), 24U RNase inhibitor and incubation with 200U Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) at 37°C for 1 hour. To confirm absence of contaminating genomic DNA, RT negative reactions with 5µg total RNA were setup. RT-plus (cDNA)/minus reactions were subjected to RT-PCR (reverse transcription-polymerase chain reaction) using GAPDH primers. PCR reactions were run on a 2% agarose gels to check for product formation and DNA contamination. The
thermal cycler program was one cycle of 94°C for 5 min, 40 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1 min, followed by one cycle of 72°C for 1 min.

5.2.5 Primer design

Primer sets for genes of interest, IL-4 and IFN-γ and reference gene 60S acidic ribosomal protein were designed from mouse-specific Genbank sequences listed in Table 1 using Primer 3 software (Rozen and Skaletzky, 2000) (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). In order to minimize primer-dimer formation, the maximum self-complementarity was 6 and the maximum 3’ self-complementarity was 0. The targets amplified by primer pairs were characterized using the Mfold program (SantaLucia, 1998) (http://bioinfo.math.rpi.edu/~mfold/dna/form1.cgi) for predicting the nature of any secondary structures which may form at the site of primer binding. Primer pairs that bound at the site of a predicted loop were discarded. Primer sets were synthesized by Integrated DNA Technologies (Coralville, IA) and primers were reconstituted at 100 pM/ul in nuclease-free water prior to use.

5.2.6 Real-time PCR

The real-time PCR reactions were carried out using the iCycler™ iQ PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). In each 25µl reaction, 12.5µl of iQ™ SYBR green supermix (Bio-Rad) was added to 300nM of each primer along with 250ng of cDNA. PCR amplification was performed in duplicate for each sample using the following cycle conditions: 3 min at 95°C followed by 45 repeats of 1 min at 95°C, 30s at 55°C and 30s at 72°C. Temperature optimization was carried out for all the primer sets to be amplified.
simultaneously. Annealing temperatures were tested from the 50-65°C range; all the primer sets amplified optimally at 55°C. A melt curve analysis step was included at the end of cycles to check for primer-dimer and non-specific product formation. Efficiency of the PCR reactions was derived by doing a standard curve of 10-fold serially diluted mouse spleen cDNA and was consistently in the 95-98% range. Non-template controls were used to detect any genomic DNA contamination and amplified products were also examined on a 2% agarose gel to verify that the amplified products were of the expected sizes. Raw C<sub>t</sub> values were analyzed using Relative Expression Software Tool-384 (REST-384) to generate a fold increase or decrease in the transcript levels (Pfaffl, et al., 2002). The 60S acidic ribosomal protein was used as the endogenous reference gene for normalizing transcript levels among tissues of interest. 60S ribosomal protein was previously shown to be the least variable from comparative analyses of various genes including β-actin, cyclophilin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) all of which were tested as potential candidate reference genes (using methodology as described in Chapter 6 of this dissertation).

5.2.7 Statistical analysis

Data reported for larval burden as mean ± standard deviation was analyzed by two-way ANOVA using the General Linear Models (GLM) procedure in SAS (Cary, North Carolina) and <i>p</i>&lt;0.05 was considered significant. For comparing variation in transcript levels of cytokine genes, Pair-Wise Fixed Reallocation Randomization Test© in the REST-384 was used (Pfaffl, et al., 2002). The mathematical model used to compute the relative expression ratio of a target gene relies on its real-time PCR efficiencies (E) and the threshold cycle
difference ($\Delta Ct$) of an unknown sample versus a control ($\Delta Ct_{\text{control - sample}}$). The target gene expression is normalized to a reference gene (ref) in the equation mentioned below:

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta Ct \text{ target (control - sample)}}}{(E_{\text{ref}})^{\Delta Ct \text{ ref (control - sample)}}}$$

In our experimental setup control was the uninfected/unbred (UN) mice and samples were the infected/unbred, uninfected bred (UB) and infected/bred (IB). For evaluating fold upregulation or downregulation of transcripts between timepoints, early-lactation (timepoint II) and mid-lactation (timepoint III) were compared to late-gestation (timepoint I) expression of which was relative to control (UN). For evaluating fold changes in transcript levels, analyses were also done by comparing IN, UB and IB group each to the UN at respective timepoints (See Appendix II).

### 5.3 Results

#### 5.3.1 Breeding efficiency and larval burden assessment

Breeding efficiency, measured by dividing bred mice by total number of mice mated, was used to evaluate the effect of *A. caninum* infection on the outcome of pregnancy. A total of 82 female BALB/c mice were infected with 1000 *A. caninum* larvae post-impregnation, of which 16 gave birth to live pups. Thus, breeding efficiency was 20% in the infected/bred (IB) mice. Compared to the IB group, uninfected/bred (UB) mice had breeding efficiency of 45% as 22 of 49 resulted in live litters. These results suggest that *A. caninum* infection has an adverse effect on pregnancy, as shown by lower breeding efficiencies.

To compare larval distribution in dams, eight week old female BALB/c mice were subcutaneously injected with 1000 *A. caninum* L3 larvae. A comparison of larval burden during the course of *A. caninum* infection was made between infected/not bred (IN) and IB
mice at the three different timepoints corresponding to late gestation and early to mid lactation (Figure 5.1). The total number of larvae recovered from IN mice was not significantly different from the IB mice at late gestation or mid lactation. However, there was a significant difference ($p < 0.005$) in the larval burden of IN mice (368 ± 64) and IB mice (249 ± 31) at timepoint II, immediately postpartum.

To correlate the level of transmammary transmission from dam to pups, larval counts were also assessed in litters from IB dams: 5 ± 4 and 9 ± 2 larvae were respectively obtained during the timepoints for early and mid lactation, for timepoints II and III, respectively. The larval burden in the feti (timepoint I) was not assessed as previous studies have shown that there is no in utero transmission of *A. caninum* larvae from dam to fetus during pregnancy (Steffe and Stoye, 1984; Arasu and Kwak, 1999).

### 5.3.2 Serum IL-4 levels

To compare the IL-4 levels during different phases of breeding, serum was collected at late gestation (timepoint I), early lactation (timepoint II) and mid-lactation (timepoint III). IL-4 levels were assessed in uninfected/not bred (UN) controls and compared to IL-4 levels from IN, UB and IB mice at timepoints I, II and III. IL-4 was not detectable in serum samples from any group except for two individual mice in the IN group. Mouse # 4 and mouse #5 had serum IL-4 levels of 210 pg/ml and 302 pg/ml respectively at timepoint III, which is the last timepoint included in this study. Serum levels of IFN-γ could not evaluated due to insufficient quantities of serum remaining as 50µl/well was required for the IFN-γ ELISA (Pierce Endogen, Rockford, IL).
5.3.3 Transcript levels of IL-4 and IFN-γ in skeletal muscle and mammary gland

IL-4 mRNA transcripts levels were evaluated during the course of *A. caninum* infection at different stages of pregnancy and lactation in skeletal muscle, mammary gland and spleen using real-time PCR. REST software was used to analyze the fold changes in expression of transcripts (Pfaffl, et al., 2002). Skeletal muscle was assessed as it is the favored site of arrest for L3 larvae.

In skeletal muscle, IL-4 transcript levels were significantly upregulated during early lactation at timepoint II (*p* < 0.05) in the IB group compared to the timepoint I (Figure 5.2a). No significant changes in IL-4 levels were observed skeletal muscle of IN and UB groups (Figures 9.3a, 9.3b in Appendix II). In mammary gland, IL-4 transcript levels were significantly upregulated at timepoint I (*p* < 0.005) corresponding to two weeks (timepoint II for IN group) of *A. caninum* infection in the IN group when compared to UN (Figure 5.2b). However, IL-4 transcript levels were significantly downregulated at timepoint II (*p* < 0.05) in the mammary gland of IB group as compared to timepoint I (Figure 5.2c). No significant changes were observed in IL-4 transcripts in the UB group (Figure 9.3c in Appendix II). In the spleen, no significant changes in expression of IL-4 were identified in the IN, UB or IB groups (Figures 9.3d, 9.3e, 9.3f in Appendix II). In summary, IL-4 was predominantly expressed in skeletal muscle but downregulated in mammary gland of the IB group.

IFN-γ transcript levels were also evaluated during the course of *A. caninum* infection and different stages of pregnancy and lactation in skeletal muscle, mammary gland and spleen using real-time PCR. No significant changes in IFN-γ transcripts were observed in any of the groups in skeletal muscle (Figures 9.4a, 9.4b, 9.4c in Appendix II). In mammary gland, IFN-γ transcript levels were significantly upregulated at timepoint I (*p* < 0.05) in the IN group.
compared to the UN controls (Figure 5.3). This is consistent with the recent reports that during the early phase of hookworm infection Th1 responses predominate over Th2. No significant changes in IFN-γ transcripts were observed in mammary gland of UB and IB groups. Similarly, no significant differences were noted in IFN-γ transcript levels in skeletal muscle or spleen of the IN, UB and IB groups at the three timepoints of interest (Figures 9.4d-h in Appendix II).

Discussion

Hookworm infection has been globally recognized as one of the most common persistent infections of humans, with majority of cases occurring in the tropics and subtropics (de Silva, et al., 2003). Clinical symptoms of hookworm infection include anemia, eosinophilic gastroenteritis, weight loss and retardation of growth. Latency and reactivation is a common phenomenon in the life cycle of the hookworm which leads to chronicity of infection that is well documented in dogs (Stoye and Krause, 1976). Developmentally arrested third stage larvae (L3) of Ancylostoma species of hookworms have the capacity to reactivate and mobilize during pregnancy which leads to transmission of L3 to the immunologically naïve offspring via milk (Stone and Smith, 1973; Schad, 1979). We have developed a mouse model of A. caninum infection that includes arrest and reactivation to study the immunological changes occurring inside somatic tissues where larvae arrest and migrate after reactivation, i.e. skeletal muscle and mammary gland, respectively. Classically, a helminth infection generates a Th2 dominant response in the host defined by the production of interleukin-4 (IL-4), IL-5, IL-9, IL-10 and IL-13 and consequently the development of
strong IgE, eosinophil and mast cell responses. Similarly, pregnancy has also been suggested to model the parameters of a Th2 response (Wegmann, et al., 1993).

In this report we show that infected mice have a breeding efficiency of 20% as compared to 45% in normal uninfected mice. It was also observed that some female mice had a post-implantation vaginal plug but did not become pregnant. In those cases scarring in the uterus was observed (personal observation, S. Trivedi), which suggested that fetal resorption might have occurred. Immune responses generated against parasitic infections have been implicated to have deleterious effects on pregnancy ultimately leading into its termination. Studies in the protozoal parasite, *Leishmania major*, suggest that although a Th1 response is protective against the parasitic infection, it can adversely affect pregnancy outcome. Th1 cytokines may be deleterious not only for placental maintenance but also for preimplantation events (Krishnan, et al., 1996b).

Since little is known about cytokine patterns associated with hookworm infection; especially at the site of larval arrest (skeletal muscle) and the site to which larvae migrate after resuming development (mammary gland), we chose to evaluate two characteristic cytokines which promote Th1 (IFN-γ) and Th2 (IL-4) cell expansion, respectively. We measured IL-4 levels in serum because it was not feasible to isolate peripheral blood mononuclear cells (PBMCs) or T cells from a relatively large sampling group of small animals. We utilized serum samples collected for measuring TGF-β and IGF-1 levels (S. Trivedi Dissertation, Chapter 3) to detect IL-4 levels from the different groups. However, we were unable to detect IL-4 levels in the majority of the serum samples except for two *A. caninum* infected mice. Cytokine production is measured directly by either isolating PBMCs
or specific immune cells such as T helper (CD4⁺) and T cytotoxic (CD8⁺), and comparing cytokine production between basal and mitogen-stimulated levels.

Since larvae arrest more commonly in skeletal muscle one would expect an ensuing inflammatory response against the parasite. Using histochemistry we have previously demonstrated a zone of eosinophils surrounding the larvae in the skeletal muscle (Arasu and Kwak, 1999). It is likely that once the larvae have reactivated during pregnancy, a similar inflammation would be caused by the traversing of the larvae during migration from skeletal muscle to other parts of the body. Using real-time RT-PCR, we found that IL-4 was prominently expressed in skeletal muscle (Figure 5.2a) but downregulated in mammary gland of the infected bred mice immediately after parturition i.e. the early lactation (timepoint II) (Figure 5.2c). Establishment of a Th2 response by an increase in IL-4 production has been reported to favor worm expulsion in other gastrointestinal helminths (Finkelmann, et al., 1997).

We also found that both IL-4 and IFN-γ transcripts were upregulated at two weeks post-infection with *A. caninum* L3 (timepoint I) in mammary gland of infected mice (Figure 5.2b and 5.3). This mixed Th1/Th2 response, which could be caused by larvae traversing throughout the body before arrest, has also been shown to occur in filarial nematodes. Immune responses to larval stages during acute infections in residents of filarial-endemic areas, travelers and transmigrants from a non-endemic to an endemic have been found to be associated with a mixed Th1/Th2 cytokine profile (Klion, et al., 1991; Elson, et al., 1995; Cooper, et al., 2001; Henry, et al., 2001). Observations from human subjects in endemic regions in China and Brazil have shown profound cellular hyporesponsiveness induced by chronic hookworm infection (Loukas et al., 2005). In a re-infection study in Papua New
Guinea, cytokine and proliferative responses to *Necator* were measured. Most subjects produced detectable Th1 (IFN-γ) and Th2 (IL-4 and IL-5) cytokines in response to crude adult worm antigen extract before anthelminthic treatment. Pre-treatment IFN-γ responses were negatively associated with hookworm burden and increased significantly after anthelminthic treatment (Quinnell et al., 2004). In a separate study, peripheral blood mononuclear cells (PBMCs) from *N. americanus* infected school children, who had recently received chemotherapy, had reduced proliferative capacity against phytohemagglutinin and adult worm antigen extract compared to controls (Geiger et al., 2004). These individuals also produced higher levels of IL-10 and lower levels of both Th1 (IL-12 and IFN-γ) and Th2 (IL-5 and IL-13) cytokines. Such mixed Th1-type and Th2-type immune responsiveness associated with chronic gastrointestinal parasitic nematode infections may reflect a state of infection where a permissive Th1-type cytokine profile favors parasite persistence (Pit, et al., 2001). These reports suggest that the mixed cytokine profile observed in mammary gland of *A. caninum* infected mice may represent a typical cytokine profile associated with hookworm infection. In conclusion, our results indicate that a Th2-like response characterized by elevation in IL-4 levels predominates at the site of larval arrest in the infection and pregnancy model. However, a mixed Th1/Th2 profile is observed in mammary gland of the *A. caninum* infection mouse model.
Table 5.1 Primer sequences used for cytokine and reference gene transcript quantification by real-time RT-PCR

<table>
<thead>
<tr>
<th>Primer pairs</th>
<th>Gene</th>
<th>Accession #</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Product size</th>
</tr>
</thead>
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<tr>
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<td>GAPDH</td>
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<td>ctcgctcctgaagagttc</td>
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</tr>
<tr>
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<td>BC011291</td>
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<td>aaagctgguaggaagagg</td>
<td>132</td>
</tr>
<tr>
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<td>IL-4</td>
<td>NM_021283</td>
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<td>atcgaaagctggaaagagt</td>
<td>155</td>
</tr>
<tr>
<td>4</td>
<td>IFN-γ</td>
<td>K00083</td>
<td>gttgcatcctccctca</td>
<td>gtcaccatcttttgcagt</td>
<td>162</td>
</tr>
</tbody>
</table>
Figure 5.1 Comparison of total larval burden in unbred versus bred BALB/c mice infected at times corresponding to day 19 gestation, day 1 and day 10 of postpartum lactation. Mice were infected with 1000 *A. caninum* L3 larvae subcutaneously. *n = 5 mice per group.*

* indicates significant difference at *p* < 0.005 between unbred and bred groups.
Figure 5.2a IL-4 transcript levels in skeletal muscle of infected bred mice. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation). RNA was extracted from skeletal muscle collected from each timepoint and cDNA was synthesized for real-time PCR analyses. * indicates significant difference at $p < 0.05$ between uninfected unbred and infected bred groups.
Figure 5.2b IL-4 transcript levels in mammary gland of infected unbred mice. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation). RNA was extracted from mammary gland collected from each timepoint and cDNA was synthesized for real-time PCR analyses. * indicates significant difference at $p< 0.005$ between uninfected unbred and infected unbred groups.
Figure 5.2c IL-4 transcript levels in mammary gland of infected bred mice. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation). RNA was extracted from mammary gland collected from each timepoint and cDNA was synthesized for real-time PCR analyses. * indicates significant difference at $p < 0.05$ between uninfected unbred and infected bred groups.
Figure 5.3 IFN-γ transcript levels in mammary gland of infected unbred mice. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation). RNA was extracted from mammary gland collected from each timepoint and cDNA was synthesized for real-time PCR analyses. * indicates significant difference at $p<0.05$ between uninfected unbred and infected unbred groups.
6. Evaluation of endogenous reference genes for real-time PCR quantification of gene expression in *Ancylostoma caninum*

Shweta Trivedi, Prema Arasu*

Department of Molecular Biomedical Sciences, North Carolina State University, 4700 Hillsborough Street, Raleigh, NC 27606, USA.

Keywords: *Ancylostoma* hookworms, real-time PCR, validation, reference gene

*Corresponding author:

Prema Arasu

NC State University, 4700 Hillsborough Street, Raleigh, NC 27606, USA.

Tel: 1-919-513-6530; Fax: 1-919-513-6465; Email: prema_arasu@ncsu.edu
Real-time PCR is a powerful technique for analyzing gene expression especially in parasitic systems where samples are typically limited. As compared to conventional PCR, it provides a dynamic range of absolute and relative quantification, greater precision, technical sensitivity, and lower risk of sample contamination [1]. In relative quantification, expression levels are normalized to endogenous reference genes or housekeeping genes which must be carefully selected since high variability has been noted with commonly used genes such as actin and glyceraldehyde-3-phosphate dehydrogenase [2-4]. The aim of this report was to identify a reference gene(s) that showed minimal variability for gene expression analyses across various life stages, strains and experimental conditions of the intestinal blood feeding hookworm, *Ancylostoma caninum*. *A. caninum* is a major parasite of dogs and can cause moderate to severe iron deficiency anemia, hypoproteinemia and bloody diarrhea that can be fatal to puppies and immunosuppressed dogs [5]. *A. caninum* can also contribute to cutaneous larva migrans and eosinophilic enteritis in man [6]. Related hookworms in tropical and subtropical regions of the world also cause malnutrition and anemia in humans; in fact children infected as infants show retarded cognitive and physical development [7]. For this study, egg, larval stages 1 and 2 (L1/L2 combined), and infective larval stage 3 (iL3) were derived from an *A. caninum* strain from a naturally infected dog in North Carolina (strain ‘N’) that has been passaged in laboratory Beagles since 2000 [8]. Adult worms were recovered from the intestines of infected dogs that had been euthanized by the local animal shelter and from each batch, intact male and female worms were separately picked and stored at -70°C. For inter-strain comparisons, *A. caninum* iL3s were obtained from T. Nolan, University of Pennsylvania, from a strain that has been propagated since the early 1970s by infecting steroid immuno-suppressed dogs (strain ‘P’). For the studies reported here, both *A.
*caninum* strains were maintained in non-steroid treated Beagles. To study the effect of
treatment, L3s from N and P strains were incubated in the absence or presence of normal dog
serum which stimulates the infective pre-parasitic larvae to resume feeding, thereby
simulating the early phases of transition to the parasitic mode of development [9]. Total
RNA was extracted from the parasites using RNA Stat-60 as per manufacturer’s instructions
(TelTest, Friendswood, TX) and residual genomic DNA was removed by DNase I treatment.
Reverse transcription was done using oligo-d(T) priming and RT-PCR was performed with
intron-spanning actin primers to confirm the absence of contaminating genomic DNA (data
not shown). Based on previous reports [10 -12], genes for actin, 18S rRNA, 60S acidic
ribosomal protein, cAMP-dependent protein kinase A (cAMP), β-tubulin and RNA
polymerase II subunit (RNA pol II) were selected as candidate references from the *A.
caninum* database. While 18S rRNA does not have a polyA tail, it was included in the
analyses because sufficient internal priming by oligo d(T) can apparently occur [13]. The 5’
and 3’ gene-specific primers (presented 5’ to 3’) designed using the Primer3 software [ 14]
were respectively: Actin, GenBank Accession Number BQ667158, GAT CTG GCA CCA
CAC CTT CT and TCT CTG TTG CTC TTG GGG TTC (99 bp product), 18S rRNA,
BI773318, GCC CTC CAA TAG ATC CTC GT and CGC GCA AAT TAC C CA CTC (125
bp), 60S acidic ribosomal protein, BF250585, GTC GGA ATC GTC GGA AAG TA and
GTC TTG TTG CAT TTC GAG CA (167 bp), cAMP, U15983, ATG GGA GAA TCC AGC
AGA and TCC AAA ATC TTC ATG GCA AA (138 bp), β-tubulin, AF077870, CTG TTG
TCC CCT CAC CAA AG and TTT CAA GGT TCG GAA GCA AA (146 bp), and RNA pol
II, AW588389, TCT TGG TAC TCG TGC GCT TC and AGC GGA TCC GTC TCT CCT T
(75 bp). Primer design with the Primer 3 software was based on melting temperatures in the
57-63°C range; actual optimization by test runs in the 50-65°C range showed optimal amplification at 55°C. Conventional RT-PCR confirmed that none of the primer sets amplified products from dog or *E. coli* (data not shown). The real-time PCR reactions were carried out simultaneously on all candidate genes using the iCycler™ iQ PCR detection system (Bio-Rad Laboratories, Hercules, CA). Amplified product was detected with the SYBR Green I DNA binding dye which has been shown to provide similar sensitivity and reproducibility as sequence-specific fluorescent probes [15, 16]. In each 25µl reaction, 12.5µl of iQ™ SYBR green supermix (Bio-Rad) was added to 300nM of each primer with 250ng of cDNA. PCR amplification was performed in duplicate for each sample using the following cycle conditions: 3 min at 95°C followed by 45 repeats of 1 min at 95°C, 30s at 55°C and 30s at 72°C. A melt curve analysis step, included at the end of each run, verified the absence of primer-dimers and non-specific products. Efficiency of the reactions, derived from a standard curve of 10-fold serially diluted iL3 cDNA, was consistently in the 95-98% range. For variation across stages, cDNAs from egg, L1/L2, iL3, and adult worms were assessed and for variation among N and P strains, iL3 cDNA was used. The effect of serum treatment was tested with iL3 and ssL3 cDNA from both strains. Non-template controls were used to verify the absence of genomic DNA contamination. Each cDNA was analyzed in duplicate and each experiment was repeated twice. The amplified products were also examined on a 2% agarose gel for verification of the expected sizes (data not shown). In real-time PCR, the threshold cycle number (*C*_t) at which the amplification-associated fluorescence exceeds a specific threshold level of detection (background noise) is inversely correlated with the amount of nucleic acid present in the sample [17]; *C*_t values were therefore used to compare the variability in transcript levels of the reference genes and are
displayed as "box and whisker" plots with medians (as lines), 25th percentile to the 75th percentile (as boxes) and ranges (as whiskers). Determination of coefficients of variation (CV) and one-way ANOVA were performed using the Prism 4.0 software (GraphPad Software Inc., San Diego, CA). For stage-specific analyses across egg, L1/L2, iL3, and adult male and female worms, actin and cAMP showed the highest variability with CV of 27.4% and 16.4%, respectively, as compared to CV of 12.4%, 6.6% and 6.6% for the 60S, β-tubulin and RNA pol II genes (Figure 6.1). The highest transcript levels were noted for 60S (median Ct = 15.3) followed by β-tubulin (median Ct = 17.6) and RNA pol II (median Ct = 19.1). For comparison between the N and P geographical strains (Figure 6.2), actin, β-tubulin and RNA pol II showed highest variability with CVs > 10% as compared to CVs of 3.5% and 1.5% for 60S and cAMP respectively. As with the analyses across stages, the 60S transcript level was the most abundant with median Ct = 15.9. Finally, to assess the effect of serum treatment on transcript levels of the reference genes, evaluations were done with iL3 and ssL3 from the N as well as the P strain of A. caninum. With the N strain, all reference genes had CVs < 5% except for actin (CV of 17.6%); with the P strain, CVs were <7% for all genes except for actin and 18S with CVs of 13.2% and 17% respectively. From a combined analysis of the threshold cycle values from amplifications with iL3 and ssL3 cDNAs from both N and P strains (Figure 6.3), the 60S (median Ct =15.9) and cAMP (median Ct =21) genes emerged as the most suitable references with the lowest CVs of 4.3% and 3.4%, respectively, as compared to CV > 10% for all the other genes. From these results, RNA pol II and β-tubulin are suitable reference genes for analysis of transcript differences across developmental stages of A. caninum but are highly unsuitable for comparisons between different strains or treatment effects. Similarly, cAMP-dependent protein kinase A would be
an appropriate reference for less abundantly expressed genes in analyses of strains and treatments but showed a high CV in stage-specific analyses. Overall, the 60S acidic ribosomal protein was the best reference gene for analyses across stages, strains and treatments especially for genes that are expressed at equally high abundance [4, 11]. Interestingly, the 18S rRNA non-polyadenylated transcript which is not expected to be reproducibly reverse transcribed by oligo-d(T) primers showed lower CVs than the more commonly used actin (Figure 6.1) or β-tubulin (Figures 6.2, 6.3) cytoskeletal proteins. While this might suggest using the 18S rRNA as a reference gene for templates generated by reverse transcription with random primers, randomly primed cDNA has been reported to overestimate mRNA copy number in real time PCR analyses by up to 19-fold [18]. In a recent report of gene expression in adult male and female worms of the Brugia malayi filarial nematode, actin 2B, histone H3 and NADH dehydrogenase subunit I were all shown to be at approximately equivalent transcript levels resulting in the use of actin-2B as the reference gene for real-time PCR analyses [21]. In this study with A. caninum adult worms, Ct levels for actin-1 were 29+/-1 and 16+/-0.3 for adult male and females, respectively, indicating a wide discrepancy and its unsuitability as a reference gene. In conclusion, this study confirms that reference genes should always be validated for real time gene expression analyses and that the 60S acidic ribosomal protein is a suitable choice for studies on A. caninum and related nematodes.
Acknowledgements

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References


Figure Legends

**Figure 6.1** Expression levels of candidate reference genes across different developmental stages of *A. caninum*. Box plots for each reference gene represent a compilation of the threshold cycles (Ct) for cDNAs from egg, L1/L2, iL3, and adult male and female worms (n = 20 from duplicate analyses of each cDNA in 2 independent experiments). Eggs were harvested via sucrose flotation as previously described [19]. L1/L2 stage larvae were obtained by hatching eggs on agar plates for 24-36 hrs, sucrose centrifugation to remove remaining eggs and repeated washing of the larvae in medicated PBS before freezing at -70C. L3 stage larvae were harvested from fecal/charcoal co-cultures [20] and adult worms were recovered from the intestines of infected dogs at necropsy.

**Figure 6.2** Expression levels of candidate reference genes in two different strains of *A. caninum*. The ‘N’ strain was derived from a naturally infected dog in North Carolina and the ‘P’ strain was originally derived from Maryland and has been propagated in laboratory dogs for more than 25 years. Box plots for each reference gene include threshold cycles (Ct) for cDNAs from infective iL3 (n = 8).

**Figure 6.3** Effect of combination of serum treatment and strain of *A. caninum* on expression levels of candidate reference genes. Box plots for each reference gene include threshold cycles (Ct) for cDNAs from infective L3 (n = 4) and serum-stimulated L3 (n = 4). Infective iL3 from N and P strains of *A. caninum* were incubated for 20-24 hrs at 37C, 5% CO₂ with or without 5% normal dog serum; larvae were then incubated with an equal volume of 5 mg/ml
flourescein isothiocyanate-labeled bovine serum albumin (Sigma). Serum-stimulated larvae (80-90% positive) were scored for reactivation by examination for fluorescent intestinal tracts using UV microscopy [22].
Figure 6.1

The diagram illustrates the distribution of threshold cycles (Ct) for various reference genes. The x-axis represents the reference genes, and the y-axis shows the threshold cycles. Each boxplot represents the distribution of Ct values for a specific gene, with the central line indicating the median value, the box representing the interquartile range, and the whiskers showing the range of data points excluding outliers.
Figure 6.2

![Box plot showing threshold cycles (Ct) for different reference genes.](image)
Figure 6.3

[Box plot showing the threshold cycles (Ct) for different reference genes: Actin, 18S rRNA, 60S, PKA, β-tubulin, RNA-pol II.]
7. References


Pfaffl, M. W., G. W. Horgan and L. Dempfle. (2002). Realtime Expression Software Tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. 30(9):e36


8. APPENDIX

For comparing variation in transcript levels of cytokine genes, Pair-Wise Fixed Reallocation Randomization Test\textsuperscript{©} in the REST-384 was used (Pfaffl, et al., 2002). The mathematical model used to compute the relative expression ratio of a target gene relies on its real-time PCR efficiencies (E) and the threshold cycle difference (ΔCt) of an unknown sample versus a control (ΔCt\textsubscript{control - sample}). The target gene expression is normalized to a reference gene (ref) in the equation mentioned below:

\[
\text{Ratio} = \frac{(E_{\text{target}})^{\Delta C_t \text{target (control - sample)}}}{(E_{\text{ref}})^{\Delta C_t \text{ref (control - sample)}}}
\]

In our experimental setup control was the uninfected/unbred (UN) mice and samples were the infected/unbred, uninfected bred (UB) and infected/bred (IB). For evaluating fold upregulation or downregulation of transcripts between timepoints, early-lactation (timepoint II) and mid-lactation (timepoint III) were compared to late-gestation (timepoint I) expression of which was relative to control (UN). For fold upregulation or downregulation of TGF-β isoforms and IGF-1 in IN, UB and IB groups, comparisons were also made relative to UN controls during early-lactation (timepoint II) and mid-lactation (timepoint III) were compared to late-gestation (timepoint I).
Figure legends

Figure 8.1a IL-4 transcript levels in skeletal muscle of infected unbred mice. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation). RNA was extracted from skeletal muscle collected from each timepoint and cDNA was synthesized for real-time PCR analyses. Comparisons were made relative to UN.

Figure 8.1b IL-4 transcript levels in skeletal muscle of uninfected bred mice. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation). RNA was extracted from skeletal muscle collected from each timepoint and cDNA was synthesized for real-time PCR analyses. Comparisons were made relative to UN.

Figure 8.1c IL-4 transcript levels in skeletal muscle of infected bred mice. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation). RNA was extracted from skeletal muscle collected from each timepoint and cDNA was synthesized for real-time PCR analyses. Comparisons were made relative to UN.

Figure 8.1d IL-4 transcript levels in mammary gland of infected unbred mice. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation). RNA was extracted from mammary gland collected from each timepoint and cDNA was synthesized for real-time PCR analyses. Comparisons were made relative to UN.

Figure 8.1e IL-4 transcript levels in mammary gland of uninfected bred mice. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation). RNA was extracted from mammary gland collected from each timepoint and cDNA was synthesized for real-time PCR analyses. Comparisons were made relative to UN.

Figure 8.1f IL-4 transcript levels in mammary gland of infected bred mice. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation). RNA was extracted from mammary gland collected from each timepoint and cDNA was synthesized for real-time PCR analyses. Comparisons were made relative to UN.

Figure 8.1g IL-4 transcript levels in spleen of infected unbred mice. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation). RNA was extracted from spleen collected from each timepoint and cDNA was synthesized for real-time PCR analyses. Comparisons were made relative to UN.

Figure 8.1h IL-4 transcript levels in spleen of uninfected bred mice. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early...
lactation and III= mid-lactation). RNA was extracted from spleen collected from each timepoint and cDNA was synthesized for real-time PCR analyses. Comparisons were made relative to UN.

**Figure 8.1i** IL-4 transcript levels in spleen of infected bred mice. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation). RNA was extracted from spleen collected from each timepoint and cDNA was synthesized for real-time PCR analyses. Comparisons were made relative to UN.

**Figure 8.2a** IFN-γ transcript levels in skeletal muscle of infected unbred mice. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation). RNA was extracted from skeletal muscle collected from each timepoint and cDNA was synthesized for real-time PCR analyses. Comparisons were made relative to UN.

**Figure 8.2b** IFN-γ transcript levels in skeletal muscle of uninfected bred mice. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation). RNA was extracted from skeletal muscle collected from each timepoint and cDNA was synthesized for real-time PCR analyses. Comparisons were made relative to UN.

**Figure 8.2c** IFN-γ transcript levels in skeletal muscle of infected bred mice. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation). RNA was extracted from skeletal muscle collected from each timepoint and cDNA was synthesized for real-time PCR analyses. Comparisons were made relative to UN.

**Figure 8.2d** IFN-γ transcript levels in mammary gland of infected unbred mice. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation). RNA was extracted from mammary gland collected from each timepoint and cDNA was synthesized for real-time PCR analyses. Comparisons were made relative to UN.

**Figure 8.2e** IFN-γ transcript levels in mammary gland of uninfected bred mice. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation). RNA was extracted from mammary gland collected from each timepoint and cDNA was synthesized for real-time PCR analyses. Comparisons were made relative to UN.

**Figure 8.2f** IFN-γ transcript levels in mammary gland of infected bred mice. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation). RNA was extracted from mammary gland collected from each timepoint and cDNA was synthesized for real-time PCR analyses. Comparisons were made relative to UN.
**Figure 8.2g** IFN-γ transcript levels in spleen of infected unbred mice. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation). RNA was extracted from spleen collected from each timepoint and cDNA was synthesized for real-time PCR analyses. Comparisons were made relative to UN.

**Figure 8.2h** IFN-γ transcript levels in spleen of uninfected bred mice. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation). RNA was extracted from spleen collected from each timepoint and cDNA was synthesized for real-time PCR analyses. Comparisons were made relative to UN.

**Figure 8.2i** IFN-γ transcript levels in spleen of infected bred mice. Mice were sacrificed at three different timepoints during pregnancy and lactation (I= late gestation, II= early lactation and III= mid-lactation). RNA was extracted from spleen collected from each timepoint and cDNA was synthesized for real-time PCR analyses. Comparisons were made relative to UN.

**Figure 8.3a** IL-4 transcript levels in skeletal muscle of infected unbred mice. Comparisons were made relative to timepoint I.

**Figure 8.3b** IL-4 transcript levels in skeletal muscle of uninfected bred mice. Comparisons were made relative to timepoint I.

**Figure 8.3c** IL-4 transcript levels in mammary gland of uninfected bred mice. Comparisons were made relative to timepoint I.

**Figure 8.3d** IL-4 transcript levels in spleen of infected unbred mice. Comparisons were made relative to timepoint I.

**Figure 8.3e** IL-4 transcript levels in spleen muscle of uninfected bred mice. Comparisons were made relative to timepoint I.

**Figure 8.3f** IL-4 transcript levels in spleen muscle of infected bred mice. Comparisons were made relative to timepoint I.

**Figure 8.4a** IFN-γ transcript levels in skeletal muscle of infected unbred mice. Comparisons were made relative to timepoint I.

**Figure 8.4b** IFN-γ transcript levels in skeletal muscle of uninfected bred mice. Comparisons were made relative to timepoint I.

**Figure 8.4c** IFN-γ transcript levels in skeletal muscle of infected bred mice. Comparisons were made relative to timepoint I.
**Figure 8.4d** IFN-γ transcript levels in mammary gland of uninfected unbred mice. Comparisons were made relative to timepoint I.

**Figure 8.4e** IFN-γ transcript levels in mammary gland of uninfected bred mice. Comparisons were made relative to timepoint I.

**Figure 8.4f** IFN-γ transcript levels in mammary gland of infected bred mice. Comparisons were made relative to timepoint I.

**Figure 8.4g** IFN-γ transcript levels in spleen of infected unbred mice. Comparisons were made relative to timepoint I.

**Figure 8.4h** IFN-γ transcript levels in spleen of uninfected bred mice. Comparisons were made relative to timepoint I.

**Figure 8.4i** IFN-γ transcript levels in spleen of infected bred mice. Comparisons were made relative to timepoint I.
Figure 8.1g

Figure 8.1h

Figure 8.1i
Figure 8.2d

Figure 8.2e

Figure 8.2f
Figure 8.2g

Figure 8.2h

Figure 8.2i
Figure 8.3d

Figure 8.3e

Figure 8.3f
Figure 8.4a

Figure 8.4b

Figure 8.4c
Figure 8.4d

Figure 8.4e

Figure 8.4f