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Brugia phangi: effects of third stage larvae ES immunization on early migration and parasite establishment in Mongolian gerbils (Meriones unguiculatus)

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BRUGIA PAHANGI: EFFECTS OF THIRD STAGE LARVAE ES IMMUNIZATION ON EARLY MIGRATION AND PARASITE ESTABLISHMENT IN MONGOLIAN GERBILS (MERIONES UNGUICULATUS)

A Thesis

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Master of Science

in

The Interdepartmental Program in Veterinary Medical Sciences through the Department of Pathobiological Sciences

by

Ginger Ann Robertson
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**Abstract**

*Brugia* infections occur via the bite of an infected mosquito. Third stage infective larvae (L3) deposited on the skin during feeding migrate into the bite wound, through skin and into the lymphatic system. It is hypothesized that L3 excretory/secretory products (ES) are important in this initial phase of the infection. A model for these early migrations has been established by inoculating L3s into the dermis (ID) of the permissive gerbil host. In this model, most L3s injected ID in the lower hind limb travel to the popliteal lymph node by 3 days post infection. Adult parasites are located primarily in the spermatic cord lymphatics by 28 days post infection. L3s injected into the peritoneal cavity (IP) do not migrate, thus ES may play a different role in these infections. Knowledge is lacking on the role of L3 ES in *B. pahangi* migration and establishment. Proteins in 24 hour L3 ES may facilitate early L3 migration and antibodies to ES may inhibit migration and/or worm establishment. Migration inhibition was assayed *in vivo* by immunizing gerbils with either 24 hour L3 ES in RIBI adjuvant or RIBI alone. Gerbils were subsequently challenged either ID or IP with 100 L3s and euthanized at 3 and 106 days post infection. Western blot analysis indicates that antibodies in prechallenge sera are produced against ES and share homology with antigens in other *B. pahangi* stages. ES immunization increased L3 recovery in both ID and IP infected animals at 3DPI. No difference was noted at 106DPI. ES immunization also reduced L3 migration in ID infected gerbils at 3DPI. At 106DPI, immunized animals showed fewer circulating microfilaria and intralymphatic thrombi. At 3DPI, the increase in worm recoveries following immunization may be associated with a decrease in larval migration. The results also suggest that antibody to ES is insufficient to provide protection at both 3DPI and 106DPI.
Nonetheless, this response appears to limit the fecundity of adult worms and subsequent formation of intralymphatic thrombi.
Chapter 1: General Introduction

Lymphatic filariasis (LF) affects approximately 128 million people in mainly tropical and subtropical regions of the world and is caused by the lymphatic dwelling filarial nematodes *Brugia malayi*, *B. timori*, and *Wuchereria bancrofti* (Scott, 2000). According to the Global Alliance to Eliminate Lymphatic Filariasis (GELF), a partnership and control program created to raise political, financial, and technical support for LF, more than 1 billion people in approximately 80 countries are at risk of contracting LF. One way GELF provides support is by distributing anti-filarial medications (albendazole and ivermectin or albendazole and diethylcarbamizine). This chemotherapy restores filarial antigen-specific immune responsiveness which is usually downregulated during infection (Michael, 2002). In addition, the medications also kill microfilariae, the sheathed offspring produced by adult female worms. This is vital to preventing transmission, which is dependent on the ingestion of microfilariae by mosquito vectors during feeding. The chemotherapy has been successful in some areas but minimal in others because of compliance issues in endemic communities. Everyone in an area should be treated multiple times for transmission inhibition to be effective (Kazura, 2002). GELF also provides support by educating infected patients on hygiene practices benificial to chronic filariasis. For example, washing the feet of infected people minimize secondary bacterial infections and decrease lymphedema by promoting lymph flow.

LF can present as a broad spectra of disease manifestations. These manifestations may be related to the different immune responses of infected individuals (Nutman, 1995). The establishment of distinct categories of this clinical spectrum was needed to compare the manifestations of disease with immunological and other physiologic measurements.
In regions with endemic LF, infections are divided into three groups. Most individuals are microfilaremic and asymptomatic. They are typically hyporesponsive to filarial antigens and serve as a reservoir for continued transmission of lymphatic dwelling filaria. Another group of infected individuals exhibit hyperresponsiveness to filarial antigens and show symptoms and pathology associated with infection such as elephantiasis, adenolymphangitis, and tropical pulmonary eosinophilia (TPE). These individuals exhibit acute and/or chronic lymphatic pathology. Acute disease is characterized by adenolymphangitis and episodic fevers. Chronic disease is associated with elephantiasis, lymphatic obstruction, and lymphedema (Kumaraswami, 2000). These individuals are typically free from circulating microfilariae (Nutman, 1995). A third group includes patients with occult infections that are asymptomatic and amicrofilaremic. These individuals, termed “endemic normals” (EN) or “putatively immune” (PI), are either immune to infection or are not sufficiently exposed to the parasite to become infected.

The life cycles of filarial nematodes require two hosts: an arthropod intermediate host which serves as the vector and a terrestrial vertebrate as a final host (Bain and Babayan, 2003). Initial entry into a host is preceded by trauma to the epithelium made by an agent other than the parasite (Lewert, 1958). In humans, mosquito-derived B. malayi infective third stage larvae (L3s) are deposited onto skin in mosquito hemolymph during feeding (Ewert and Ho, 1967). Tissue migration is required before establishment in the definitive host can occur. It has been suggested that the larvae secrete proteases and other enzymes such as elastases and collagenases, allowing penetration through local connective tissue into the lymphatics (Scott, 2000). Nine to fourteen days post infection (DPI), the molt from the L3 to fourth stage larvae (L4) occurs (Scott, 2000). The L4s then molt to
become immature adult parasites. Mature adults mate and females produce microfilariae, which travel to the bloodstream and can be ingested by a feeding mosquito. These microfilariae enter the midgut of the mosquito (Bartholomay and Christensen, 2002). Those that escape mosquito defenses exsheath within a few hours and migrate to the thoracic flight musculature where they further develop to the first larval stage (L1). L1s molt to the second stage larvae (L2) followed by a subsequent molt to the infective L3 stage. Ten to fifteen days after microfilarial ingestion, L3s migrate through the hemocoel to the proboscis tip where they are transmitted to a host upon feeding of the mosquito (Bartholomay and Christensen, 2002). During this process, the L3s are deposited onto the skin in a drop of hemolymph. The larvae then migrate presumably into the bite wound created by the mosquito (Ewert, 1967).

As previously stated, during this life cycle, it has been hypothesized that biologically active molecules produced and secreted by the parasite facilitate tissue penetration. These molecules are hypothesized to be in the excretory and secretory products (ES) of the larvae. Adult parasite ES has been well studied in nematodes, including filariae (Kaushal et. al., 1982; Allen and McDonald, 1998; (Tezuka et al., 2003). Information regarding ES from larval stages of filaria is lacking due to difficulty in obtaining sufficient parasite material (Yenbutr and Scott, 1995). Multiple functions for larval and adult nematode ES have been suggested, including parasite migration in the host (Tsuji et al., 2003; Tsuji et al., 2004) parasite establishment (Sen et al., 2000) and host immunomodulation (Harnett et al., 1999b; Gomez-Escobar et al., 2005) including macrophage and granulocyte function modification (Lightowlers and Rickard, 1988), suppression of B and T cell proliferation (Harnett and Harnett, 1993; Hartmann et al.,
1997; Harnett and Harnett, 1999) and surface-bound antibody shedding (Selkirk et al., 1993). In addition, immunity to ES products have been suggested to be involved in immunopathology and host protection (Maizels et al., 1999).

Two of the few studies aimed at assessing the influence of ES product(s) on larval migration were performed in BALB/c mice (Tsuji et al., 2003) and swine (Tsuji et al., 2004) using the nematode *Ascaris suum*. Usually, pigs are infected with *A. suum* after ingesting eggs containing the L2 stage. The eggs hatch in the small intestine and penetrate the intestinal wall where the L2 to L3 molt takes place. L3s then migrate to the liver and lungs, where they break out of the alveolar septa and are subsequently swallowed. The L3 molts to the L4, then to the adult stage in the small intestine.

BALB/c mice and pigs were immunized with the recombinant form (rAS16) of a 16 kDa protein produced and secreted by embryonated eggs, L3s, and adult parasites (AS16). Antibodies produced in mice to rAS16 were used to localize native AS16 to the hypodermis, cuticle, intestine and ovary of *A. suum* adult females. These antibodies also recognized AS16 in the 48 hour ES products of infective L3s, lung stage L3s, adult females and males. In infective and lung stage L3s, pig anti-rAS16 antibodies bound to the esophagus, intestine, and hypodermis. The predominant antibody profile to rAS16-specific antibodies in mice included IgA, IgG, and IgE. A similar response was seen in swine. Murine IgG isotypic responses included elevated IgG1, IgG2a, and IgG3, indicating a mixed Th1/Th2 immune response. These results were supported by significantly higher levels of IFN-γ, IL-2 (type I cytokines) as well as IL-10 (a type II cytokine) in supernatants from cultures of mice splenocytes stimulated with rAS16. Supernatants from peripheral blood mononuclear cells (PBMC) of swine displayed
elevated IgG1 antibodies along with IL-4 and IL-10, indicating a Th2 dominant immune response.

A reduction in larval migration was reported in both mice and pigs immunized with rAS16. In mice, there was a 50% reduction in larval migration from the small intestine to the lung, while 58% fewer larvae were recovered from immunized swine. In addition, the L3 to L4 molt was inhibited in immunized swine. Furthermore, AS16 has low similarity to mammalian proteins (Tsuji et al., 2003). This is important because the immune responses to these proteins may limit the possibility of autoimmune reactions (Gregory et al., 2000).

There have been multiple proteins, glycoproteins, enzymes, and enzyme inhibitors identified in lymphatic filarial ES and known to be specifically secreted by microfilarial and adult stages, and to a much lesser extent, infective larvae. The following four proteins will be discussed in more detail: abundant larval transcripts (ALTs), ES-62, acetylcholinesterases (AchE) and serine protease inhibitors (serpins).

The most notable proteins of *B. malayi* L3 ES are abundant larval transcript proteins –1, and –2 (ALT-1,-2, respectively). ALT-1 is produced exclusively by L3s. ALT-2 is also produced by L3s although lower amounts of *alt-2* mRNA are present in other life cycle stages. Although no known mammalian homologues of these proteins exist, the two proteins are 79% identical to *D. immitis* abundant immunogen Di-20/22L (Frank et al., 1995) and similar to Ov-ALT-1 and –2 in *Onchocerca* spp. (Joseph et al., 1998). The lack of mammalian homologues and thus the low risk of crossreactivity with host proteins have made the ALTs favorable targets for immunization in host protection studies. Recombinant ALT-1 (rALT-1) is a 20kDa protein that is intensely stained in the
L3 glandular esophagus upon hybridization with anti-Bm-rALT-1 antibody (Gregory et al., 2000) and is secreted into its environment (Gomez-Escobar et al., 2005). Antibodies to rALT-1 have also been observed in human sera from amicrofilaremic and microfilaremic patients both without parasites and with subpatent infections. The isotypic responses in these patients indicated elevated IgG1 and IgG3 with no IgG4. Gerbils immunized with rALT-1 were found with 76% fewer parasites four weeks post infection when compared to controls (Gregory et al., 2000).

A subsequent experiment to study the function(s) of alt-1 and -2 used alt-transfected Leishmania mexicana in mice (Gomez-Escobar et al., 2005). Leishmania mexicana infection is initiated when an infected sandfly bites a host and injects the infective promastigote stage into the host. Promastigotes invade circulating macrophages, differentiate and reproduce as amastigotes before lysing the host cell. A sandfly then ingests amastigotes upon feeding on the host, which then differentiate into promastigotes in the sandfly.

Murine macrophages infected with alt-transfected amastigotes contained more parasites compared to wild-type (WT) controls at 24 hours post infection. Larger and accelerated (8-10 weeks post infection) lesion development was also observed in mice infected with alt-transfected amastigotes compared to infections with WT parasites (12-15 weeks post infection). In addition, alt-transfected parasites were more resistant to IFN-Γ induced killing by macrophages. These observations support the role of ALTs in host immunomodulation. The Th2 associated transcripts GATA-3 and SOCS-1 were upregulated in alt-transfected macrophages when compared to WT controls at 7 DPI. Similar results were observed in vivo, with mice showing upregulation of the same genes
in lymphocytes derived from the peritoneal cavity following IP infection with *B. malayi* L3.

rALT-2 is a 14kDa immunogenic protein as determined by protein extracts of L3s hybridized with sera from mice immunized with Bm-rALT-2 as well as sera from human patients with chronic lymphatic obstruction (Ramachandran et al., 2004). Significant reductions in viable larvae were observed in CBA mice immunized with Bm-rALT-2 (74%) following implantation of L3s in a chamber in the peritoneal cavity when compared to PBS controls. Sera from mice immunized with the recombinant protein indicated elevated IgG1, IgG2a, IgG2b and lower titers of IgM and IgA. Predominant antibody responses in those immunized with an alt-2 DNA vaccine were IgG2a, IgG2b, IgM and IgA as opposed to IgG1, unlike the response to the recombinant protein.

ES-62 is a 62kDa phosphorylcholine (PC) containing glycoprotein suggested to be secreted by all filarial nematodes (Stepek et al., 2004) including adult stages of *B. pahangi* and *B. malayi* (Stepek et al., 2002). ES-62 induces Th2 responses by suppressing IL-12, IL-6, and TNF-α signaling by macrophages (Goodridge et al., 2001). In *Acanthocheilonema vitae*, this glycoprotein accounts for more than 95% of all protein released from adult parasites *in vitro* (Harnett et al., 1999b). *In vivo* secretion has also been demonstrated, supported by its detection in the bloodstream of infected gerbils (Harnett et al., 1990). Therefore, ES-62 has the potential to interact with cells of the immune system (Harnett et al., 1999a). ES-62 has been shown to downregulate lymphoproliferative responses in infected animal models by modulating signal transduction pathways associated with the antigen receptor of B cells (Harnett and Harnett, 2001). It has been shown that ES-62 incubated with resting splenic murine B
cells at a concentration similar to that found in the bloodstream of parasitized humans (Lal et al., 1987), partially prevents B cell proliferation associated with ligation of the B cell antigen receptor. This effect is likely due to PC, as the same effect has been demonstrated using PC conjugated to BSA as well as PC alone. These results were also demonstrated in vivo after giving mice injections of PC-BSA or BSA alone. Cells from mice given PC-BSA were less able to proliferate in response to antigen receptor ligation (Harnett et al., 1999b). In addition, ES-62 is able to induce anergy to cellular activation through the T-cell antigen receptor (Harnett et al., 1998).

 Constituents of B. malayi adult and microfilarial ES, although less well described, include acetylcholinesterases (AchEs) and serine protease inhibitors (serpins). Acetylcholinesterases (AchEs) have been hypothesized to function in neuromuscular transmission. These enzymes may interfere with the host immune system by degrading acetylcholine. Acetylcholine enhances mast cell degranulation and cytotoxicity upon activation by lymphocytes and neutrophils. Rathaur et. al., 1987 has shown a 100kDa Brugian AchE is recognized by sera from humans with B. malayi and W. bancrofti infections (Rathaur et al., 1987). In addition, AchE activity has been localized to excretory and anal vesicles, and amphid and phasmid structures at the cephalic and caudal ends of W. bancrofti microfilariae (Omar and Kuhlow, 1977). It remains unclear whether nematode AchEs influence parasite survival, although acetylcholine may release T cells from dependence on IL-2 for IFN-Γ production (Johnson et al., 1982).

 Parasite serpins regulate a wide variety of biological functions including blood coagulation (Cappello et al., 1995) and antigen processing (Bennett et al., 1992). A stage-specific serpin, Bm-SPN-2, has been described in B. malayi microfilarial ES. This
47.5 kDa protein inhibits enzymatic activity of human neutrophil cathepsin G and neutrophil elastase (Zang et al., 1999). In addition, Bm-SPN-2 is a prominent T cell antigen that induces an intense, but short-lived Th1 response in mice, as shown by the in vitro production of IFN-Γ but not IL-4 or IL-5 14 DPI (Zang et al., 2000).

Additional ES constituents have been extensively studied in other nematodes. *Ancylostoma* secreted proteins-1 and –2 (ASP-1, -2) are abundantly produced by L3 of the skin-penetrating hookworms *Ancylostoma caninum* and *Necator americanus* (NaASP-2) (Goud et al., 2005). They are cysteine-rich proteins (Hawdon et al., 1996) belonging to the pathogenesis related (PR) protein superfamily (Asojo et al., 2005). In order to assess the capacity of NaASP-2 to influence parasite migration, an in vitro migration assay using hamster skin was performed (Goud et al., 2005). Infective stage larvae were incubated with sera from rats immunized with Na-ASP-2 and Alhydrogel adjuvant or with Alhydrogel alone and applied to hamster skin to assess the number of larvae that were able to migrate through skin. It was shown that rat anti-NaASP-2 antibodies inhibited migration by 90 ± 7%, while Alhydrogel controls showed only 17 ± 7% inhibition.

ASP-2 has been shown to be secreted as determined by sera from rabbits immunized with recombinant ASP-2 on western blots using 24hr. activated L3 ES (Hawdon et al., 1999). Dogs vaccinated with ASP-2 showed elevated IgG1 and IgG2 isotypes, with moderate IgE titers (Bethony et al., 2005). Sera from these dogs recognized ASP-2 in L3 extracts as demonstrated in western blots. The protein was immunolocalized to the glandular esophagus of *A. caninum* L3s as well as the channels that connect the glandular esophagus to the L3 surface, the cuticle and epicuticle. After percutaneous inoculation of
L3s, a 26% reduction in gastrointestinal (GI) worm burdens were observed in vaccinated animals relative to controls. Moreover, a 69% reduction in fecal egg counts were recovered in vaccinated animals when compared to controls, suggesting the protein is involved in adult worm fecundity. Using the in vitro migration assay, antibodies to ASP-2 were shown to inhibit tissue penetration by 60% compared to control sera. This indicates that the immune response induced by ASP-2 vaccination interferes with early host invasion. As a result, fewer larvae would reach the GI tract resulting in reduced numbers of adult parasites and blood loss (Bethony et al., 2005).

Several animal models have been employed to study the immunology and pathogenesis of human filarial infections. A number have proven useful in the laboratory including rodents, cats, dogs, ferrets, and primates infected with B. malayi or B. pahangi (Scott, 2000). Most experimental filarial research has been performed in rodents, specifically mice and the Mongolian gerbil using Brugia spp. inoculated subcutaneously or intraperitoneal. Immunologically intact mice generally do not support larval maturation past the L3 to L4 molt, but infections of nude and SCID mice do develop to the adult stage (Babu et al., 1998).

Although all of these animal models have provided much knowledge regarding host-parasite dynamics, none of them require the larvae to pursue a more natural route of invasion by migrating through layers of skin prior to lymphatic establishment (Porthouse et al., 2006; Chirgwin et al., 2006). Using the permissive Mongolian gerbil, our laboratory has developed a B. pahangi intradermal (ID) model in order to mimic a more natural course of filarial infection (Chirgwin et al., 2006; Porthouse et al., 2006). Upon injection of larvae ID in the hindleg, larvae can migrate away from the injection site
within hours, with the majority leaving the site by 3 DPI. At this timepoint, the largest number of L3 are found in the popliteal lymph node (POP), followed by the subiliac and inguinal lymph nodes (SUB), renal lymph node (RLN), and skin and muscle surrounding the injection site. Approximately 7 DPI the L3 molt to L4 and at approximately 28 DPI adults are present in the lymphatics. By 60 DPI, adults have produced microfilariae that reach the peripheral blood (Ash and Riley, 1970). Brugian infections in gerbils result in the development of an infection by 90 DPI characterized by lymphatic lesions including lymphatic thrombi, lymphadenitis, lymphangitis and a persistent microfilaremia (Klei et al., 1988). Furthermore, although *B. malayi* and *B. pahangi* both develop fully to fecund adults, *B. pahangi* has attracted more attention for laboratory use because it is easier to maintain in the laboratory. In addition, gerbils infected with *B. malayi* have a lower microfilaremia when compared to infections with *B. pahangi* (Ash and Riley, 1970). Nonetheless, the kinetics of lesion development and immune responses of *B. pahangi* and *B. malayi* infections of gerbils are similar, thereby justifying the use of this nonhuman parasite model for LF.

Though the described research using ES has identified some of its components, detailed information regarding early L3 ES produced by *B. pahangi* is lacking. The aim of this study was to assess the significance of *B. pahangi* L3 ES on early parasite migration and development in Mongolian gerbils. The working hypothesis to be tested was that proteins in *Brugia* spp. L3 ES are essential in early parasite migration, development, and establishment within a permissive host, therefore antibodies to this ES will alter the protein function and subsequently inhibit or alter early larval migration, development, and establishment.
Chapter 2: *Brugia pahangi*: Effects of Third Stage Larvae ES Immunization on Early Migration and Parasite Establishment in Mongolian Gerbils (*Meriones unguiculatus*)

2.1. Introduction

Lymphatic filariasis, caused by the filarial nematodes *Brugia malayi*, *B. timori*, and *Wuchereria bancrofti*, affects approximately 128 million people in mainly tropical and subtropical regions of the developing world and it has been estimated that 1 billion people are at risk (Scott, 2000). These infections are initiated when an infected mosquito feeds on a host and infective third stage larvae (L3) emerge from the mosquito proboscis onto the skin surface in a drop of hemolymph (Ewert and Ho, 1967). The L3 then migrates presumably into the bite wound created by the mosquito. L3 migrate through connective tissues of the skin, into the lymphatics and subsequently to the lymph nodes (Scott, 2000). Earlier studies by Ah and his coworkers (1973) demonstrated that vector derived *B. pahangi* L3 are capable of extensive rapid migration through a variety of tissue types. In order to quantitatively study this early migration an *in vivo* intradermal inoculation model has been established using *B. pahangi* infections in gerbils (Chirgwin et al., 2006; Porthouse et al., 2006). In this model, L3s are required to move through the connective tissues of the skin prior to establishment in the lymphatics mimicking more closely the natural early L3 migration. Although it is presumed that molecules secreted by the L3 facilitate this migration and are involved in parasite establishment (Scott, 2000) little is known regarding the parasite factors involved.

During this parasitic life cycle of *Brugia* spp., it is presumed that all stages produce and secrete molecules into the environment (Kaushal et al., 1982; Zang et al., 2000; Scott, 2000). These components, termed excretory/secretory products (ES) contain many
proteins including collagenases (Petralanda et al., 1986), immunomodulators (Harnett et al., 1999a), and unique proteins of unknown functions (Gregory et al., 2000; Ramachandran et al., 2004). ES has been hypothesized to function in many ways to benefit the parasite. In *Brugia* spp. and other nematodes, these include aiding in parasite migration and establishment (Sen et al., 2000; Tsuji et al., 2003; Tsuji et al., 2004), host immune modulation (Harnett et al., 1999a), and other unknown functions (Gregory et al., 2000; Ramachandran et al., 2004).

Previous studies have identified various proteins in *B. malayi* L3 ES including collagenases (Petralanda et al., 1986) and perhaps most notably abundant larval transcript proteins -1 and -2 (ALT-1, -2) (Gregory et al., 2000; Ramachandran et al., 2004). These parasite-specific proteins have no mammalian homologues and no known function aside from immunomodulation (Gomez-Escobar et al., 2005). The ALT proteins are L3-specific and represent promising candidates for vaccine studies. Gerbils immunized with ALT-1 harbored 76% fewer adult *B. malayi* in the peritoneal cavity at 4 weeks post infection when compared to controls. Mice immunized with ALT-2 showed comparable reductions of viable larvae at 48 hours post infection. Immunizing mice with homologues of ALTs secreted by *Onchocerca volvulus*, the secreted larval acidic proteins (SLAPs), have also produced significant protection against subcutaneous challenge (Wu et al., 2004).

Although these previous reports have provided some knowledge concerning specific components of ES, none of them have focused on the role of total ES on L3 migration. The hypothesis to be tested in these studies was that ES proteins secreted by early L3s are essential to early L3 migration and establishment and that antibody to these proteins
would inhibit or alter this migration. A profile of all these proteins was created.

Antibody responses from ES immunized gerbils were examined.

It is shown here that an immune response, presumably antibody, to vector derived L3 ES reduces the rate of early larval migration. But, this response does not induce a significant protection against migrating L3s or the subsequent establishment of adult parasites. Despite these results, adult worm fecundity as measured by the production of microfilariae and the development of granulomatous lymphatic lesions, intralymphatic thrombi, were significantly reduced at the time of patency.

2.2. Materials and Methods

2.2.1. Parasites

Third stage *B. pahangi* infective larvae (L3) were collected from infected *Aedes aegypti* mosquitos using techniques as previously described (Klei, et al., 1990). In brief, 12 days following an infected blood meal, mosquitos were crushed in Roswell Park Memorial Institute medium (RPMI) (Hyclone, Logan, UT) and L3 collected in a Baermann apparatus.

2.2.2. Excretory/Secretory Products (ES)

Seven hundred fifty viable L3s were aliquoted into 15ml conical tubes and washed 3 times with RPMI. Final aliquots were resuspended in 2.5ml RPMI in 24 well tissue culture plates (BD Falcon, Franklin Lakes, NJ) at a concentration of 750 L3/well. Plates were incubated for 24 hours at 37°C in an atmosphere of 5% CO₂. The culture medium containing ES (ES) was collected using pipets and a stereomicroscope, to ensure larvae were not collected. Less than 10 L3 per culture were nonmotile or inviable at this time. ES was stored at -20°C.
ES was thawed on ice and concentrated 100X using YM-3 Centriprep centrifugal filter units (Millipore, Billerica MA) with a 3kDa molecular weight cutoff. ES was centrifuged at 4°C at 2850 X g until approximately 700µl ES in RPMI was retained. The RPMI was exchanged with cold phosphate buffered saline (PBS) containing 1% general protease inhibitor cocktail (Sigma, St. Louis, MO) by centrifuging as above. The ES was collected and supplemented with an additional 1% protease inhibitor. Protein concentration was determined by Lowry's method using the DC Protein Assay (BioRad, La Jolla, CA). LPS activity in concentrated ES was measured using the Pyrotell Limulus Amebocyte Lysate (LAL) assay (Associates of Cape Cod, East Falmouth, MA). The minimum LPS concentration considered as positive for the presence of LPS was 0.25 endotoxin units/ml.

2.2.3. Animals, Experimental Design and Necropsy

Male Mongolian gerbils (*Meriones unguiculatus*) were purchased from Charles River Laboratories (Wilmington, MA) at 8 weeks of age and maintained on standard rodent chow and water *ad libitum*. Two *in vivo* experiments were conducted to test the effect of anti-ES on early larval migration and survival (Experiment 1) and parasite maturation within gerbils (Experiment 2). The experimental design is shown in Table 1. In both experiments, 60 gerbils were divided into 8 groups. Four groups each containing 10 gerbils were used for infections. Four groups each containing 5 gerbils were used for media controls. Gerbils were immunized with ES in RIBI adjuvant (Sigma) or adjuvant alone. Gerbils in each immunization group designated for infection received multiple injections until 95-100 *B. pahangi* L3s were inoculated ID within the dermis of the lower leg or into the peritoneal cavity (IP) as previously described (Chirgwin et al., 2006;
Table 1. Summary of Experimental Design to Test the Effects of ES Immunization on Early Larval Migration (Experiment 1)\(^a\) and Parasite Establishment (Experiment 2)\(^b\)

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Immunization</th>
<th>Inoculation</th>
<th>Location of Inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunized and challenged intradermally (ES+BP+ID)</td>
<td>ES in AJ(^c)</td>
<td>BP(^d) ID(^e)</td>
<td></td>
</tr>
<tr>
<td>Immunized and challenged intraperitoneally (ES+BP+IP)</td>
<td>ES in AJ</td>
<td>BP</td>
<td>IP(^f)</td>
</tr>
<tr>
<td>Immunized only (ES+MC+ID)</td>
<td>ES in AJ</td>
<td>MC(^g) ID</td>
<td></td>
</tr>
<tr>
<td>Immunized Only (ES+MC+IP)</td>
<td>ES in AJ</td>
<td>MC</td>
<td>IP</td>
</tr>
<tr>
<td>Control and challenged intradermally (AJ+BP+ID)</td>
<td>AJ(^h)</td>
<td>BP</td>
<td>ID</td>
</tr>
<tr>
<td>Control and challenged intraperitoneally (AJ+BP+IP)</td>
<td>AJ</td>
<td>BP</td>
<td>IP</td>
</tr>
<tr>
<td>Control only (AJ+MC+ID)</td>
<td>AJ</td>
<td>MC</td>
<td>ID</td>
</tr>
<tr>
<td>Control only (AJ+MC+IP)</td>
<td>AJ</td>
<td>MC</td>
<td>IP</td>
</tr>
</tbody>
</table>

\(^a\) Necropsies were performed in each treatment group at 3DPI
\(^b\) Necropsies were performed in each treatment group at 106DPI
\(^c\) ES in AJ: Gerbils were immunized with ES in RIBI adjuvant
\(^d\) BP: 95-100 *Brugia pahangi* L3s
\(^e\) ID: intradermal challenge
\(^f\) IP: intraperitoneal challenge
\(^g\) MC: RPMI media controls
\(^h\) AJ: Gerbils were immunized with RIBI adjuvant alone
Porthouse et al., 2006). The syringe was flushed with RPMI to check for any remaining L3. The numbers of larvae injected were recorded. The ID inoculations serve as a more natural challenge in that L3s are required to migrate through the tissues of the skin prior to establishment in the lymphatics. Also, the pattern of L3 migration following this injection route has been established in gerbils (Chirgwin et al., 2006; Porthouse et al., 2006). The IP inoculations, in which the majority of parasites do not migrate and remain in the peritoneal cavity, served to determine if any anti-ES effects seen in the ID infected groups were related only to migration. Gerbils receiving similar immunizations but challenged with RPMI from the Baermann apparatus served as uninfected controls.

2.2.3.1. Experiment 1

In Experiment 1, necropsies were performed at 3 to 5 DPI in order to measure the effects of anti-ES immune responses on early migration and parasite survival. Previous studies have shown that at 3 DPI, most larvae are located in the left popliteal lymph node following ID inoculation, indicating that larvae are actively migrating away from the injection site (Chirgwin et al., 2006; Porthouse et al., 2006). All necropsies were not performed 3 DPI due to time constraints. Any subsequent citations pertaining to 3-5 DPI are termed 3 DPI for convenience.

On days 3, 24, and 71, gerbils were immunized with 50μg LPS-free ES in PBS suspended in 300μl of 1:1 RIBI adjuvant:LPS-free physiological saline. Controls were immunized with 300μl of 1:1 RIBI adjuvant:LPS-free physiological saline, according to the manufacturer's protocol. Each inoculation was given in multiple intramuscular and subcutaneous sites. Animals were bled for sera on days 0 and 86.
Gerbils were challenged with 100L3s on day 88 either ID in the left hind limb above the knee or 100L3 IP. Control gerbils were injected ID or IP with RPMI from the Baermann apparatus.

At 3 DPI, all gerbils were euthanized and worm recoveries performed. The skin and muscle at the injection site of all ID infected gerbils were removed. Muscle biopsies were taken from the removed tissue near the injection site with a 6mm biopsy punch and preserved in 10% formalin for histological examination. Right and left popliteal lymph nodes (RPOP and LPOP), right and left renal lymph nodes (RRLN and LRLN), ilio-lumbar vessels (ILV), right and left spermatic cord lymphatics (RSPCD and LSPCD), right and left sub-inguinal and iliac nodes (RSUB and LSUB) and right and left testes (RTEST and LTEST) were gently teased in PBS under a stereomicroscope. Following teasing, all tissues were left to soak for 1 hour to allow larvae to emerge. Tissues were examined twice, with hourly soaks in between, and all visible L3s recovered.

Necropsies of gerbils with IP infections were similarly performed except the skin and muscle were not removed from the leg. In addition, for these treatment groups, the peritoneal cavity was washed with RPMI as well as the viscera and the carcass soaked for 1 hour in PBS.

2.2.3.2. Experiment 2

The second experiment (Experiment 2) was performed to assess the role of anti-ES immune responses on parasite establishment and lesion formation. The design, immunization and challenge groups in this experiment were similar to those of Experiment 1. Sera were collected on days 0 and 154. Fifteen microgram ES immunizations and control immunizations were given on days 75, 96 and 144. Challenge
was performed on day 157. Microfilariae were counted in 0.5ml blood at 101 DPI by the modified Knott’s method (Chirgwin et al., 2003). Necropsies on infected animals were performed at 106-110 DPI. All necropsies were not performed at 106 DPI due to time constraints. Any subsequent citations pertaining to 106-110 DPI are termed 106 DPI. ID infected animals were injected with approximately 0.1ml 1% Evan’s blue dye in saline in each hind footpad and testes prior to necropsy to better visualize spermatic cord intralymphatic thrombi (ILT), adult parasites, and lymphatic dilation (Klei et al., 1982). ILT were counted in the SPCD and categorized as small, medium or large in size as previously described (Jeffers et al., 1987). Lymphatic dilation scores ranging from 0-4 were determined for each ID infected animal (Klei et al., 1982). The criteria for scoring was as follows: 0, no change; 1, 10-20% of the spermatic cord length of infected animals dilated; 2, 30-50% dilation; 3, 50-80% dilation; 4, >80% dilation. Tissues were teased for adult worms, which were subsequently sexed. In addition to the tissues examined in Experiment 1, the heart and lungs were teased in both IP and ID infected animals. For ID and IP infected animals, the peritoneal cavity was washed with RPMI as well as the viscera and the carcass soaked for 1 hour in PBS.

2.2.4. CBC Analysis

For all animals in both experiments, 400µl blood was collected into EDTA tubes for Complete Blood Counts (CBC) immediately prior to euthanasia. The total numbers of cells were counted per microliter. Cells were stained with a modified Wright's Giemsa stain (Hema-Tek Stain Pak; Bayer Corp., Tarrytown, N.Y.). One hundred cells were counted and identified as neutrophils, eosinophils, basophils, monocytes, or lymphocytes.
2.2.5. ES Characterization and Antibody Response to ES

SDS-PAGE was performed to profile ES proteins and to compare the profile with that of somatic adult worm antigen (SAWA) and somatic *B. pahangi* L3 antigen (SL3A). Protein electrophoresis was conducted using a 4-20% Tris-HCL polyacrylamide gradient gel (Biorad) and a Mini Protean II apparatus (BioRad) as previously described (Edmonds, 2001). Eight micrograms of ES, SAWA or 25 live *B. pahangi* L3s were mixed with loading buffer and denatured as previously described (Laemmli, 1970; Edmonds, 2001). Electrophoresis was carried out at 20 mAmp. Gels were stained with Coomassie Blue and destained as previously described (Edmonds, 2001). Additional staining using the ProteoSilver system (Sigma) was carried out according to the manufacturer's protocol. Photoimaging was carried out on a FluorChem 8800 photoimager (Alpha Innotech, San Leandro, CA).

Preimmunized, ES immunized but prechallenge, and AJ immunized but prechallenge sera from both experiments, pooled sera from *B. pahangi* infections of > 1yr (chronic) and from infections of 35 days duration (acute) were analyzed for anti-ES antibodies using Western blots and ELISAs. Antigens used in Western blots included ES, SAWA, and SL3A to assess immunogenic proteins in each extract and those shared with ES. ES and SAWA were used in ELISAs to assess the relative level and isotypic character of the antibody response.

2.2.5.1. ELISAs

Immulon I B flat bottomed, 96 well polystyrene microwell plates (Dynex Tech., Chantilly, VA) were coated with 50µl ES or SAWA diluted to 15 µg/ml in coating buffer (0.02M sodium carbonate buffer, pH 9.6). After antigen was bound overnight at 4ºC,
antigen and coating buffer were removed and plates washed 3X for 5min with 200µl of PBS-0.05% Tween 20 (PBST). Plates were blocked for 1.5 hours at room temperature with 100µl of 3% fish gelatin-PBS and washed again. Primary antibody was diluted 1:100 in 1% fish gelatin-PBST (Ab buffer) and incubated in triplicates with ES or SAWA for 1hr at 37ºC. Five pools of sera (preimmunized, ES and AJ prechallenge, chronic, and acute infection sera) were screened against SAWA. For ES coated plates, three pools of sera (preimmunized, chronic and acute infection sera) and sera from 10 randomly chosen individual samples (either ES immunized or adjuvant immunized) were used as the primary antibody. After washing, 50µl of goat anti-mouse IgG antibody conjugated to alkaline phosphatase (IgG-AP) (Jackson Immunoresearch, West Grove, PA), or rabbit anti-mouse IgG1, IgG2a, IgG2b, or IgG3 -AP (Rockland, Philadelphia, PA) diluted 1:1000 in Ab buffer were added to plates and incubated 1hr at 37ºC. After washings, 100µl of BluePhos microwell phosphatase substrate was added to each well according to the manufacturer's protocol (KPL, Gaithersburg, MD). Reactions were stopped after 20min with 100 µl of 1X BluePhos Stop Solution (KPL, Gaithersburg, MD). OD values were read on a MRX TC Revelation ELISA reader standardized with PBST. The minimal OD value considered accurate was either 2.5% of the coefficient of variance or 0.005 OD, whichever is greater, as determined by the manufacturer.

2.2.5.2. Western Blot Analysis

Eleven and a half micrograms of ES, SAWA, and SL3A from 80 live *B. pahangi* L3s were separated using SDS-PAGE as described above. Unstained electrophoretically-resolved proteins were transferred to nitrocellulose using a Mini Trans Blot electrode (BioRad). Protein transfer was carried out at 25 mAmp overnight followed by 225
mAmp for 1 hour at 4°C. Western blot hybridization was carried out as previously described (Edmonds, 2001). In Experiment 1, all sera were diluted 1:1000 in 1% fish gelatin-TTBS (Ab buffer). Various dilutions of sera from Experiment 2 were used. Chronic, acute, preimmunized, and adjuvant prechallenge sera were diluted 1:250 for each type of antigen. Anti-ES was diluted 1:1000 for ES and 1:250 for SL3A and SAWA. IgG-AP was diluted 1:10,000 in Ab buffer and incubated for 1 hr at 37°C. Mouse reagents were used due to a lack of suitable commercial gerbil reagents. These reagents recognize gerbil antibodies although isotype matching of gerbil immunoglobulins remains undescribed. Conjugates were developed with 3 ml of BCIP/NBT 1-component phosphatase substrate (KPL, Gaithersburg, MD). Double-distilled water was used to stop the reaction development at appropriate times. Photoimaging was carried out on a FluorChem 8800 photoimager (Alpha Innotech).

2.2.6. Statistical Analysis

Statistical analysis was performed using SigmaStat software (Chicago, IL). When data passed normality tests, pairwise multiple comparisons were made using the Student-Newman-Keuls (SNK) method. One Way Analysis of Variance on Ranks was used when normality tests failed. Differences were considered statistically significant at \( p < 0.05 \). Because all infected gerbils did not receive exactly 100 L3s, total parasite recovery means were calculated as percentages from number of larvae injected. As for distributions and other recovery calculations, percentages from the number of parasites recovered were calculated.
2.3. Results

2.3.1. SDS-PAGE Gels

SDS-PAGE was used to demonstrate the protein profile of ES and compare it to that of other parasite extracts. Some small differences in the protein profiles were observed between ES used in Experiment 1 and ES used in the Experiment 2 (Fig 1). Fewer distinct bands were observed in Experiment 2 ES although more bands were observed in the lower molecular weight range. Nonetheless, the general pattern of major bands appeared to be the same. The protein profile of SL3A and SAWA were quite different from that of the ES used in both experiments (Fig 1).

2.3.2. ELISAs

In both experiments, a marked IgG response to ES was seen in ES immunized gerbils and those with acute and chronic infections (Table 2). Antibody responses to ES from immunized animals were significantly greater than in AJ immunized animals and those with acute and chronic infections. Marked IgG1, IgG2a and IgG3 responses to ES were seen in immunized animals indicating a mixed Th1/Th2 immune response to ES. While greater than adjuvant controls, the responses were less in animals with both acute and chronic infections than in those immunized with ES. The mean OD values of acute and chronic sera suggest, like that of anti-ES, that the responses are a mixed Th1/Th2 response.

Measurable OD values to SAWA detected in ES immunized animals consisted of an IgG and IgG1 isotypic response, especially in Experiment 2 (Table 3). The acute and chronic responses were, as expected, greater than the ES immunized, in most instances. Also, OD values were significantly higher in chronic than in acute infection sera. These
Fig 1. Silver stained SDS-PAGE gel of antigens used in both experiments. Lanes show Experiment 1 ES (ES1), Experiment 2 ES (ES2), somatic L3 antigen (SL3A), and somatic adult worm antigen (SAWA).
Table 2. ELISAs using ES from both experiments. Values are expressed as mean OD values ± S.D. according to isotype from sera collected from different immunizations and infections.

<table>
<thead>
<tr>
<th>Sera</th>
<th>IgG</th>
<th>IgG1</th>
<th>IgG2a</th>
<th>IgG2b</th>
<th>IgG3</th>
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<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
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<tr>
<td>A+ES</td>
<td>1.074 ± 0.261&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.057 ± 0.020&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.047 ± 0.013&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.026 ± 0.004&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.070 ± 0.016&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Chronic</td>
<td>0.452 ± 0.009&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.028 ± 0.002&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.024 ± 0.006&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.020 ± 0.001&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.027 ± 0.002&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Acute</td>
<td>0.331 ± 0.060&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.038 ± 0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.016 ± 0.003&lt;sup&gt;b&lt;/sup&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.020 ± 0.001&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.025 ± 0.001&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>A</td>
<td>0.163 ± 0.031&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.017 ± 0.002&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.012 ± 0.004&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.020 ± 0.003&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.020 ± 0.002&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
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<tr>
<td>A+ES</td>
<td>1.026 ± 0.283&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.097 ± 0.024&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.054 ± 0.022&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.011 ± 0.003&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.102 ± 0.050&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Chronic</td>
<td>0.426 ± 0.032&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.073 ± 0.008&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.021 ± 0.001&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.011 ± 0.001&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.034 ± 0.001&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Acute</td>
<td>0.338 ± 0.051&lt;sup&gt;b&lt;/sup&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.067 ± 0.004&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.006 ± 0.001&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.006 ± 0.0005&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.029 ± 0.004&lt;sup&gt;b&lt;/sup&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>A</td>
<td>0.268 ± 0.075&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.055 ± 0.004&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.005 ± 0.001*&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.009 ± 0.002&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.023 ± 0.004&lt;sup&gt;c&lt;/sup&gt;</td>
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</table>

Letters indicate significance between sera type within each experiment at p<0.05. Types of sera include that from individual animals immunized with ES in RIBI adjuvant (A + ES), pooled sera from a chronic infection (Chronic) and from an acute infection (Acute), and from individual animals immunized with RIBI adjuvant (A).

* Value falls below the threshold for accuracy.
Table 3. ELISAs using SAWA from both experiments. Values are expressed as mean OD values ± S.D. according to isotype from sera collected from different immunizations and infections.

<table>
<thead>
<tr>
<th></th>
<th>Sera</th>
<th>IgG</th>
<th>IgG1</th>
<th>IgG2a</th>
<th>IgG2b</th>
<th>IgG3</th>
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<tr>
<td><strong>Experiment 1</strong></td>
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<tr>
<td>Chronic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.062 ± 0.073\textsuperscript{a}</td>
<td>0.246 ± 0.019\textsuperscript{a}</td>
<td>0.021 ± 0.002\textsuperscript{a}</td>
<td>0.049 ± 0.003\textsuperscript{a}</td>
<td>0.056 ± 0.002\textsuperscript{a}</td>
<td></td>
</tr>
<tr>
<td>Acute</td>
<td>0.362 ± 0.017\textsuperscript{b}</td>
<td>0.075 ± 0.005\textsuperscript{b}</td>
<td>0.014 ± 0.001\textsuperscript{b, c}</td>
<td>0.021 ± 0.001\textsuperscript{b}</td>
<td>0.019 ± 0.001\textsuperscript{b}</td>
<td></td>
</tr>
<tr>
<td>ES pool</td>
<td>0.094 ± 0.006\textsuperscript{c}</td>
<td>0.024 ± 0.001\textsuperscript{c}</td>
<td>0.019 ± 0.005\textsuperscript{a, b, c}</td>
<td>0.014 ± 0.001\textsuperscript{c}</td>
<td>0.020 ± 0.001\textsuperscript{b}</td>
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<tr>
<td>AJ pool</td>
<td>0.040 ± 0.003\textsuperscript{d}</td>
<td>0.014 ± 0.002\textsuperscript{d}</td>
<td>0.016 ± 0.000\textsuperscript{b, c}</td>
<td>0.014 ± 0.003\textsuperscript{c}</td>
<td>0.024 ± 0.003\textsuperscript{b}</td>
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<td><strong>Experiment 2</strong></td>
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<tr>
<td>Chronic</td>
<td></td>
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<tr>
<td></td>
<td>1.062 ± 0.073\textsuperscript{a}</td>
<td>0.246 ± 0.019\textsuperscript{a}</td>
<td>0.021 ± 0.002\textsuperscript{a}</td>
<td>0.049 ± 0.003\textsuperscript{a}</td>
<td>0.056 ± 0.002\textsuperscript{a}</td>
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<tr>
<td>Acute</td>
<td>0.362 ± 0.017\textsuperscript{b}</td>
<td>0.075 ± 0.005\textsuperscript{b}</td>
<td>0.014 ± 0.001\textsuperscript{b}</td>
<td>0.021 ± 0.001\textsuperscript{b}</td>
<td>0.019 ± 0.001\textsuperscript{b}</td>
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<tr>
<td>ES pool</td>
<td>0.308 ± 0.010\textsuperscript{c}</td>
<td>0.051 ± 0.001\textsuperscript{c}</td>
<td>-0.003 ± 0.005\textsuperscript{* c}</td>
<td>-0.003 ± 0.001\textsuperscript{* c}</td>
<td>0.003 ± 0.002\textsuperscript{* c}</td>
<td></td>
</tr>
<tr>
<td>AJ pool</td>
<td>0.078 ± 0.008\textsuperscript{d}</td>
<td>0.003 ± 0.004\textsuperscript{* d}</td>
<td>0.003 ± 0.004\textsuperscript{* b, c}</td>
<td>-0.003 ± 0.001\textsuperscript{* c}</td>
<td>0.002 ± 0.001\textsuperscript{* c}</td>
<td></td>
</tr>
</tbody>
</table>

Letters indicate significance between sera type within each experiment at \( p < 0.05 \). Types of sera include pooled sera from a chronic infection (Chronic), from an acute infection (Acute), from ES immunized animals (ES pool), and from adjuvant immunized animals (AJ pool).

*Values fall below the threshold for accuracy.
responses to SAWA were dominated by IgG1.

2.3.3. Western Blots

ES used in Experiment 1 and Experiment 2, SAWA and SL3A were transferred to nitrocellulose after separation by SDS-PAGE and hybridized with pooled prechallenge sera from Experiment 1 and Experiment 2, pooled preimmunized sera as well as pooled sera from gerbils with acute and chronic infections.

Some nonspecific binding of antibody to ES was observed when hybridized with all types of sera. This is particularly distinct in bands at 62kDa and 48kDa (Fig 2). Prechallenge sera from ES immunized animals in both experiments detected multiple similar protein bands in homologous ES. However, there are also some differences. Increased multiple band recognition at 27-46kDa, 9-20kDa, and <6.9kDa was observed in ES used in Experiment 1. There were 2 bands observed in Experiment 1 ES at 53 and 97kDa not visible in Experiment 2 ES. In both experiments, anti-ES recognized heavy bands at 14kDa and 20kDa, suggesting the presence of antibody to abundant larval transcript proteins 1 and 2 (ALT-1, -2) respectively (Gregory et al., 2000; Ramachandran et al., 2004). Some bands detected in ES with prechallenge sera were also recognized weakly by acute and chronic infection sera.

In ES used in Experiment 2, chronic sera recognized bands in predominantly lower molecular weight ranges between <6.9kDa to 110 kDa (Fig 2). Fewer bands were observed in ES from the same experiment when hybridized with acute infection sera. Identical reactions were seen using control sera (adjuvant and preimmunized sera) hybridized to both Experiment 1 and Experiment 2 ES. Therefore, one blot using each type of sera is shown.
Fig 2. Western blots using ES used in Experiments 1(A1, B1, C1, D1, & E1) and 2 (A2, B2, & C2). Blots were hybridized with various pooled sera including anti-ES prechallenge sera from Experiment 1 (A1) and Experiment 2 (A2), acute infection sera (B1, B2), chronic infection sera (C1, C2), adjuvant sera (D1), and preimmunized sera (E1).
The same 62kDa nonspecific band was present in SAWA and in ES on all membranes (Fig 3, 4). As expected, acute and chronic sera recognized different proteins in both SAWA and SL3A. In SAWA hybridized with acute infection sera, bands recognized were in the lower molecular weight region as opposed to those recognized by chronic infection sera. In SL3A, nonspecific reactions were not seen with any sera (Fig 4). Anti-ES, acute, and chronic infection sera recognized a 14kDa band, perhaps indicating the presence of ALT-2 (Ramachandran et al., 2004).

2.3.4. Hematology

Complete Blood Counts (CBCs) were employed to determine the role of ES immunization on leukocyte production following challenge. The total numbers and types of leukocytes were enumerated for each treatment group. Differences between groups were not seen for either total leukocytes or any cell type enumerated in Experiment 1. However, a trend was noticed with higher basophil and eosinophil numbers in infected animals irrespective of their immunizations (ES+BP+ID, ES+BP+IP, AJ+BP+ID, AJ+BP+IP) (see appendix-Table A). In Experiment 2, circulating leukocytes were compared as in Experiment 1. Although not significant, higher numbers of eosinophils were seen in all infected animals as in Experiment 1 compared to media controls (see appendix-Table B). Significantly more eosinophils were found in ES+BP+IP animals when compared to ES+MC+IP animals.

2.3.5. Experiment 1 Worm Recoveries

Total L3 recoveries were significantly greater in animals immunized with ES and challenged ID (ES+BP+ID) as compared to ID controls which were immunized only with
Fig 3. Western blots using somatic adult worm antigen (SAWA) and sera from both experiments. Blots were hybridized with anti-ES prechallenge sera from Experiment 1 (A1) and 2 (A2), acute infection sera (B1), chronic infection sera (C1), adjuvant sera from Experiment 1 (D1), and preimmunized sera from Experiment 1 (E1).
Fig 4. Western blots using somatic L3 antigen (SL3A) and sera from both experiments. Blots were hybridized with anti-ES prechallenge sera from Experiment 1 (A1) and 2 (A2), acute infection sera (B1), chronic infection sera (C1), adjuvant sera from Experiment 1 (D1), and preimmunized sera from Experiment 1 (E1).
adjuvant (AJ+BP+ID) (Fig 5). Although not significant, greater numbers of L3s were also found in ES immunized animals challenged IP (ES+BP+IP) as compared to IP controls (AJ+BP+IP). Recoveries were significantly higher in IP infected animals of both treatment groups (ES+BP+IP, AJ+BP+IP) when compared to ID inoculated animals (ES+BP+ID, AJ+BP+ID).

In both ID groups (ES+BP+ID, AJ+BP+ID), the total percent recovery of L3s from different sites showed that a majority of larvae migrated away from the injection site of the left leg by 3 DPI (Fig 6). Most of these larvae were located on the left side of the animals’ bodies in both treatment groups. At 3 DPI, tissues that harbored the most larvae in ES immunized and ID challenged animals (ES+BP+ID) were the LPOP, ILV, skin, and muscle. In the ID controls (AJ+BP+ID), the larvae were found in higher numbers in the LPOP, LRLN, muscle, LSUB and LSPCD. Significantly more larvae were recovered from LSUB in AJ + BP + ID animals and from the ILV in ES + BP + ID animals.

Larval recoveries from different tissues following ID challenge (ES+BP+ID, AJ+BP+ID) were also calculated according to proximity to the intradermal challenge site (Fig 7). Necropsy sites proximal to the site included the skin and muscle surrounding the site and LPOP which is the initial lymph node that filters lymph from the challenge site. Other lymphatic sites considered distal to the injection site included RRLN, LRLN, RSUB, LSUB, RSPCD, LSPCD, RTEST, and LTEST. The RPOP was not considered since no larvae were recovered in this location in either treatment group. In addition, the ILV was not considered due to ambiguity as to whether its location is proximal or distal to the challenge site. As expected, based on previous observations (Chirgwin et al., 2006;
Fig 5. Mean larval recoveries by treatment group in Experiment 1. Animals were immunized with either ES in RIBI adjuvant (ES immunized) or with RIBI adjuvant alone (AJ immunized). Animals were challenged ID or IP. Three DPI, larvae were recovered and reported as mean percent recoveries. Letters indicate significance ($p < 0.05$) between experimental and control groups within and between each type of challenge.
Fig 6. Mean larval recoveries from ES immunized animals challenged ID (ES+BP+ID) and AJ immunized animals challenged ID (AJ+BP+ID) from specific sites following ID challenge in Experiment 1. Sites included the skin and muscle surrounding the injection site. Lymph nodes examined were the left popliteal (LPOP), left and right subiliac and inguinal (LSUB and RSUB), and left and right renal (LRLN and RRLN) nodes. Other lymphatic sites included the left and right spermatic cord (LSPCD and RSPCD), ileolumbar vessels (ILV), and the left and right testes (LTEST and RTEST). The RPOP was not included because no larvae were found in this site in either immunization group. Letters indicate significance between treatment groups within each necropsy site ($p<0.05$).
Fig 7. Mean larval recovery percentages according to proximity to injection site in ES immunized animals challenged ID (ES + BP + ID) and AJ immunized animals challenged ID (AJ + BP + ID) in Experiment 1. Proximal necropsy sites include skin, muscle, and LPOP. Distal sites are RRLN, LRLN, RSUB, LSUB, RSPCD, LSPCD, RTEST, and LTEST. Letters indicate significance ($p<0.05$) between experimental and control groups within each site grouping as well as between groupings.
Porthouse et al., 2006) in both treatment groups, the majority of larvae were found proximal to the challenge site at 3 DPI (Fig 7). However, there was a significantly greater percentage of larvae recovered proximally in ES immunized animals challenged ID (ES+BP+ID) compared to ID controls (AJ+BP+ID). Conversely, in ID controls (AJ+BP+ID), significantly more larvae were recovered distally to the injection site. These results indicate that immunization with ES limits L3 migration and suggests that ES may facilitate early larval migration through host tissues.

IP challenged animals were analyzed according to larval presence in the peritoneal cavity compared to other tissues outside the peritoneal cavity. The majority of larvae in both treatment groups were confined to the peritoneal cavity with 97.8 ± 2.1% in ES immunized animals challenged IP (ES+BP+IP) and 94.3 ± 5.7% in IP controls (AJ+BP+IP) (see appendix-Fig. A).

No cell infiltration was observed in the histological sections.

2.3.6. Experiment 2 Worm Recoveries

A similar number of adult parasites were recovered in ID challenged animals in both treatment groups (ES+BP+ID, AJ+BP+ID) (Fig 8). As for IP infected animals, 19% fewer adult parasites were recovered in ES immunized animals (ES+BP+IP) compared to controls (AJ+BP+IP).

The majority of adult parasites recovered were located in the heart and lungs, SPCDs, and ILV of both ID infected groups (ES+BP+ID, AJ+BP+ID) (Fig 9). A preponderance of worms was recovered on the right side of the body (RPOP, RSUB, RRLN, and RSPCD) in ES+BP+ID gerbils. Furthermore, increased worm recoveries from AJ+BP+ID controls were noted on the left side of the body (LPOP, LSUB, LSPCD, and
Fig 8. Mean adult parasite recoveries by treatment group for Experiment 2. Animals were immunized with either ES in RIBI adjuvant (ES immunized) or with RIBI adjuvant alone (AJ immunized). Animals were challenged ID or IP with *B. pahangi* L3s. At 106 DPI, adult parasites were recovered and reported as mean percent recoveries. Error bars represent SEM.
Fig 9. Mean adult parasite recoveries from specific tissues following ID challenge in Experiment 2. Animals were immunized with ES in RIBI adjuvant and challenged ID with *B. pahangi* L3s (ES + BP + ID) or with RIBI adjuvant alone and challenged similarly (AJ + BP + ID). At 106 DPI, adult parasites were recovered and reported as mean percent recoveries. Sites examined included lymph nodes: left and right popliteal (LPOP and RPOP), left and right subiliac and inguinal (LSUB and RSUB), and left and right renal (LRLN and RRLN) nodes. Other lymphatic sites included the left and right spermatic cord (LSPCD and RSPCD), ileo-lumbar vessels (ILV), and the left and right testes (LTEST and RTEST). Non-lymphatic sites were the heart and lungs and the peritoneal cavity (Per Cavity). Letters indicate significance ($p<0.05$) between experimental and control groups within each necropsy site. Error bars represent SEM.
The only significant difference was observed in the LTEST. Because of these trends, the mean numbers of parasites for each treatment were grouped according to the right and left side of the gerbil body (Fig 10). Significantly more parasites were recovered on the left side compared to the right side in AJ+BP+ID controls as expected. The difference between recoveries of the left vs right sides in ES+BP+ID animals is much less pronounced although more adults were recovered on the right side of the body.

As in Experiment 1, we examined the mean percent recoveries according to proximity to the intradermal injection site. The only proximal site examined in this experiment was the LPOP. Distal sites were identical to those examined in Experiment 1. For each ID treatment group (ES+BP+ID, AJ+BP+ID), significantly fewer parasites were recovered proximally (1.2 ± 2.7) (see appendix-Fig. B). Most were located in distal sites (92.1 ± 21.1). No differences were observed between treatment groups.

Like Experiment 1, most parasites were confined to the peritoneal cavity in IP infected gerbils with very few migrating to other tissues (see appendix-Fig. C). The majority of larvae were confined to the peritoneal cavity with 87.31 ± 12.9 in ES immunized animals (ES+BP+IP) and 89.81 ± 14.3 in AJ immunized animals (AJ+BP+IP).

2.3.7. Experiment 2 ILT

The role of ES immunization on the development of ILT was examined. There were significantly more ILT produced per parasite in AJ+BP+ID control animals compared to ES+BP+ID animals (Fig 11). In order of frequency of occurrence, small, medium, and large sized ILT were observed for both treatment groups. No differences were detected in degree of lymphatic dilation between treatment groups.
Fig 10. Mean numbers of adult parasites recovered in Experiment 2 when the gerbil body is divided sagitally. Animals were immunized with ES in RIBI adjuvant and challenged ID with *B. pahangi* L3s (ES + BP + ID) or with RIBI adjuvant alone and challenged similarly (AJ + BP + ID). Tissues located on the left side of the body include LPOP, LRLN, LSUB, LTEST, and LSPCD. Tissues on the right side include RPOP, RRLN, RSUB, RTEST, and RSPCD. Letters indicate significance (*p*<0.05) between the left and right sides of the body within each treatment group. Error bars represent SEM.
Fig 11. Total intralymphatic thrombi (ILT) produced per worm in the SPCDs in Experiment 2. Animals were immunized with either ES in RIBI adjuvant and challenged ID with *B. pahangi* L3s (ES + BP + ID) or RIBI adjuvant alone and challenged similarly (AJ + BP + ID). At 106 DPI, adult parasites and ILT were counted in the SPCD lymphatics. Letters indicate significance ($p<0.05$) between experimental and control groups. Error bars represent SEM.
2.3.8. Experiment 2 Microfilaremia

Although there were no differences in the total numbers of microfilariae between treatment groups, more microfilariae were noticed in AJ+BP+ID (413 ± 430) and AJ+BP+IP (110 ± 218) controls when compared to ES+BP+ID (172 ± 314) and ES+BP+IP (57 ± 115) groups (see appendix-Fig. D). But, significantly more microfilariae produced per female parasite recovered were noticed in AJ+BP+ID controls (20.17 ± 17.37) compared to ES+BP+ID animals (9.64 ± 14.12) (Fig 12).

2.4. Discussion

ES is likely to serve multiple vital roles in the early migration and establishment of tissue migrating nematode L3s. These include but are not limited to, aiding in parasite migration (Ghosh and Hotez, 1999; Tsuji et al., 2003; Tsuji et al., 2004; Bethony et al., 2005), immunomodulation perhaps by modulating cytokine signaling (Gomez-Escobar et al., 2005), and/or suppressing B and T cell proliferation (Harnett and Harnett, 1993). Anti-ES immunity has been demonstrated to be protective in animals with other nematode infections (Gregory et al., 2000; Tsuji et al., 2003; Tsuji et al., 2004; Bethony et al., 2005). Antibody to ES has also been demonstrated to inhibit migration of Necator americanus L3 through hamster skin in vitro (Goud et al., 2005). Therefore, functional inhibition of essential components in ES by antibody can be expected to alter the outcome of infection. This experiment was designed to test this hypothesis by immunizing gerbils with ES followed by ID challenge with infective B. pahangi larvae. The ID inoculation model has been characterized in the gerbil, a permissive laboratory host (Chirgwin et al., 2006; Porthouse et al., 2006). This system allows for the in vivo investigations of factors important to the early migration and establishment of lymphatic
Fig 12. Mean numbers of microfilariae (MFs) produced per female adult recovered in Experiment 2. Animals were immunized with either ES in RIBI adjuvant and challenged ID with *B. pahangi* L3s (ES + BP + ID) or with RIBI adjuvant alone and challenged similarly (AJ + BP + ID). At 101 DPI, 500µl blood was collected and microfilariae were counted. Letters indicate significance ($p<0.05$) between experimental and control groups. Error bars represent SEM.
infections. The effects of anti-ES on tissue migration and early establishment were detected by performing necropsies at 3 days following ID inoculations. Necropsies at a later time measured the significance of L3 ES on later migrations and development of a patent infection. Any effects of the immunizations on IP infections could be related to inhibitions of parasite factors not important to tissue migration.

Some differences in the protein profiles of the ES used in Experiment 1 and Experiment 2 were seen in silver stained SDS-PAGE gels and differential band recognition by acute and chronic sera. Nonetheless, ES immunized gerbils in both experiments were shown to produce a strong, mixed Th1/Th2 antibody response to multiple similar proteins in ES at the time of challenge. In spite of the presence of these antibodies, no significant worm reductions were noted in immunized gerbils challenged either ID or IP in either experiment, indicating that the induced immune response and these antibodies to multiple ES proteins do not provide protection.

The lack of protection observed is unlike that shown in previous experiments using ES components such as abundant larval transcripts (ALT-1, and 2) and other secreted proteins (Ghosh and Hotez, 1999; Gregory et al., 2000; Sen et al., 2000; Tsuji et al., 2003; Tsuji et al., 2004; Ramachandran et al., 2004; Bethony et al., 2005). *B. malayi* L3s produce and secrete ALT-1 and –2. ALT-1 immunized gerbils have been shown to harbor reduced parasite burdens (76%) when challenged IP (Gregory et al., 2000). Similarly, ALT-2 immunization reduced viable parasite loads by 74% in mice implanted with a chamber containing L3s (Ramachandran et al., 2004). Although the presence of ALT proteins in the ES used in the current experiments was not confirmed with known
antibody, heavy protein bands of similar sizes to the ALTs (14kDa and 20kDa) were demonstrated in the ES used in Experiments 1 and 2.

The reasons for the lack of protection induced by ES in the current experiments are unknown. In previous studies (Ghosh and Hotez, 1999; Gregory et al., 2000; Sen et al., 2000; Tsuji et al., 2003; Tsuji et al., 2004; Ramachandran et al., 2004; Bethony et al., 2005), protection was dependent upon immunizing animal hosts with a single recombinant ES protein. Challenges occurred in mice orally (Ghosh and Hotez, 1999; Sen et al., 2000; Tsuji et al., 2003) or by chamber implantation (Ramachandran et al., 2004), in pigs orally (Tsuji et al., 2004), in dogs percutaneously (Bethony et al., 2005), or in gerbils IP (Gregory et al., 2000). In the current study, a mixture of unknown ES proteins was used for the immunizations and challenges were performed using both the IP and ID routes of inoculation. It is possible, although unlikely, that ALT proteins were not present in the ES preparations used in the current experiments. It is also possible that these proteins were present but altered during the collection process destroying the relevant epitopes required for protection. Alternatively, other proteins present in the ES could have interfered with the protection induced by ALTs on their own. By inducing an antagonistic immune response that negated the protective response provided by ALT-induction, a protective response may not have been initiated (Abraham et al., 2001).

It is also possible that the larvae migrate too quickly from the injection site to the lymphatics for any protective response to have an effect on the tissue migration process. Anti-ES immunity may minimize the down regulatory effect on protective responses, but the response could have an effect later on. If this is true, then antibody-mediated neutralization of the ES produced by the migrating larvae may not be initiated in time for
effective protection at this stage. In addition, a cellular response may also be limited, if an antibody-dependent cell mediated cytotoxicity mechanism is involved in protection. Alternatively, protection may be based on a different mechanism of cellular response and it is possible that the immunization protocol did not induce the appropriate cellular immune response required for protection. This seems unlikely as cellular responses are necessary to induce the antibody responses, which were demonstrated in immunized gerbils. Therefore, it is logical to assume that the immunization must have produced a strong cellular response. Finally, it must be considered that even if sufficient immune responses were produced to ES, protection against migrating L3 may not be central to protection in this system. In this regard it is important to note that protection was also not seen in IP infected animals, in contrast to what has been previously reported using ALT in this model system (Gregory et al., 2000).

Although no significant protection was observed in either experiment, it is interesting to note that larval recoveries in Experiment 1 were actually increased in immunized animals, particularly in the ID infected individuals. This may be due to decreased larval migration, facilitating the recovery of L3 that concentrated in the area around the injection site. In control animals, the larvae were more scattered and therefore more difficult to recover. It is known that larvae also migrate to tissues other than those examined at necropsy, such as the heart and lungs (Ah and Thompson, 1973). Since larvae in control animals migrated faster than in immunized animals, it is possible that more larvae migrated to those tissues not examined at necropsy.

While protection was not observed in immunized animals, alterations in migration patterns were seen in ID infected individuals. Since Brugia spp. requires extensive tissue
migration prior to parasite establishment, it is probable that proteins involved in processes such as tissue destruction are produced and secreted by invading L3s (Scott, 2000). ES components of other nematodes have been implicated in migration of larvae in vitro including *B. malayi* (Petralanda et al., 1986) and *Strongyloides stercoralis* (McKeprow et al., 1990) and in vivo including *A. suum* (Tsuji et al., 2003; Tsuji et al., 2004) and *A. caninum* (Sen et al., 2000; Bethony et al., 2005). In vitro studies with *S. stercoralis* and *B. malayi* L3 secretions have isolated metalloproteases with collagenase (Petralanda et al., 1986; Williamson et al., 2006) and elastase (McKerrow et al., 1990) activities. It is known that these larvae must pass through the collagen and elastin-containing extracellular matrix of connective tissue prior to establishment. In an in vitro assay, an L3-secreted metalloprotease with elastase activity of *S. stercoralis* was able to degrade a model of the dermal extracellular matrix of connective tissue. Similarly, collagenase activity was detected in L3 secretions of *B. malayi*, which was shown to degrade collagen fibrils in vitro. In addition, the collagenase described shares antigenic epitopes with that of a bacterial collagenase of *Clostridium histolyticum*. Sera from human patients infected with Brugian filariasis recognized the *C. histolyticum* collagenase as a 97kDa protein on Western blots. This is the same molecular weight of an immunogenic protein found in ES used in Experiment 2 when hybridized with anti-ES (Fig 2). Although not visualized in Experiment 1 ES, the 97kDa protein may still be present but at lower concentrations. It is possible that antibody-mediated neutralization of this protein could partially explain the reduced migration rates found in immunized animals at 3 DPI.
In vivo demonstration of proteins involved in larval migration include those produced by *A. suum* (Tsuji et al., 2003; Tsuji et al., 2004) and *A. caninum* (Sen et al., 2000; Bethony et al., 2005). Mice and swine immunized with a protein secreted by *A. suum* L3s (rAS16) show significant reductions in larval migration from the small intestine to the lung. In *A. caninum* ES, abundant secreted proteins-1 and –2 (ASP-1, -2) were also shown to reduce larval migrations in a similar manner.

A decrease in fecundity of *B. pahangi* adults was noted in ES immunized animals in Experiment 2. The levels of circulating microfilariae were lower in immunized gerbils challenged ID and IP although this reduction was not significant. More importantly, significantly fewer microfilariae were produced per female worm recovered following ID infections.

Immunizations with ES proteins have been demonstrated to reduce female nematode fecundity in other host parasite systems. Dogs immunized with ASP proteins show reduced adult worm fecundity as determined by a reduction in egg shedding (Bethony et al., 2005). Similarly, significant reductions in parasite fecundity can also be seen in hamsters immunized with ASP-2 produced by *Ancylostoma ceylanicum* (Mendez et al., 2005). Cathepsin-L cysteine proteases (CPLs) have been identified in the ES of *B. malayi* and *B. pahangi* L3s. These proteases have been implicated in eggshell remodeling (Guiliano et al., 2004). It is plausible that antibodies to CPLs may inhibit production of microfilariae as in Experiment 2.

A reduction in the granulomatous response, numbers of ILT, was seen in ES immunized gerbils in Experiment 2. This may be due to the lower numbers of microfilariae in the SPCD lymphatics in these treatment groups. It is known that
microfilariae contribute to ILT formation. This is evident from previous experiments where microfilariae were found in the nidus of ILT in microfilaremic gerbils (Vincent et. al., 1980; Jeffers et al., 1987). It is logical to assume that if there are more microfilariae in the SPCD, then more ILT will be formed, while fewer microfilariae will result in fewer ILT. It is also known that presensitizing naive gerbils with somatic microfilarial antigen will induce an immune response to subsequent challenge, resulting in a decreased microfilaremia (Klei et. al., 1982). This decreased microfilaremia could be due to the response induced by presensitization either decreasing the fecundity of adults or increasing the killing of microfilariae. Both of these scenarios would result in a decrease in the numbers of circulating microfilariae. However, ILT formation was actually increased in presensitized gerbils, suggesting the decreased microfilaremia was probably due to the increased killing of circulating microfilariae rather than reducing fecundity. This increase in ILT indicates that at one time, there were higher numbers of microfilariae that were subsequently killed by the immune response induced by the presensitization, thereby creating more ILT in which microfilariae consisted of the nidus.

In the present study, ES immunization decreased the numbers of total circulating microfilariae, as well as the numbers of microfilariae produced per female. Also, ILT formation was decreased, unlike the presensitization study. As a result, the reduction in ILT formation may be due to the decreased levels of circulating microfilariae observed as a result of the decreased fecundity of adult worms. Therefore, it is likely that proteins in L3 ES are involved in microfilarial production and subsequent ILT formation. If this is the case, then the immune response to ES is able to inhibit these mechanisms.
In conclusion, parasite secretions have become some of the most intensively studied molecules, showing a strong association with larval invasion and host parasitism. Although it appears that antibodies to L3 ES are not sufficient to induce protection in gerbils infected ID, they are capable of recognition by the immune response. However the immune response to ES has been shown to reduce early migration rates, parasite fecundity, and lymphatic lesion formation. It is now important to analyze the biological functions of these ES products in the host-parasite interaction in order to develop a clearer picture of how these products function in a host.
Chapter 3: Conclusions

In order to study the significance of *Brugia pahangi* L3 ES on parasite migration and establishment, two *in vivo* investigations using Mongolian gerbils were conducted. These were carried out using an intradermal infection model previously established (Porthouse et al., 2006; Chirgwin et al., 2006). There were some differences detected between the two preparations of ES used. This is most evident in silver stained SDS-PAGE gels and in the Western blots using acute and chronic sera hybridized to ES used in both experiments. In spite of these minor differences, antibodies produced to both preparations did not induce significant protection in either experiment. These results in some ways contradict previous studies which used recombinant *Brugia malayi* ES proteins (Gregory et al., 2000; Ramachandran et al., 2004). Our studies suggest that the gerbil immune responses to L3 ES are not sufficient to induce a host protection. Nonetheless, L3 ES is still able to induce a potent Th1/Th2 immune response to multiple ES proteins.

However, ES immunization does reduce early migration rates like that seen in other systems such as *A. suum* in pigs (Tsuji et al., 2004) and mice (Tsuji et al., 2003) and *A. caninum* in mice (Sen et al., 2000; Bethony et al., 2005). Furthermore, the final location of adult worms in ES immunized animals was significantly different from AJ immunized animals, supporting the effect of anti-ES immunity on migration after 3 DPI. A decrease in microfilarial production was also seen in ES immunized animals and has been previously shown using *A. caninum* (Bethony et al., 2005) and *A. ceylanicum* (Mendez et al., 2005). There are perhaps proteins in L3 ES also involved in microfilariae production.
In addition, lymphatic lesion (intralymphatic thrombi) development was also decreased probably as a result of the decrease in SPCD microfilariae.

The resulting study contributes to the growing field of research on nematode secretions and their effects on the host during infection. Further molecular studies of *Brugia* spp. L3 ES are needed to determine the function(s) of these secreted proteins. By accomplishing this, a wide variety of vaccine candidates may emerge.
References


Appendix:
Supplemental Data
Table A. Experiment 1 Total Cells and Cell Types in Complete Blood Counts (CBC) (X 10^3/µl). Values are expressed as mean values ± S.D. according to type of leukocyte from different immunizations and infections.

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<tr>
<td>ES + BP + ID</td>
<td>16.25 ± 3.74</td>
<td>12.22 ± 3.55</td>
<td>3.82 ± 1.84</td>
<td>0.13 ± 0.14</td>
<td>0.03 ± 0.06</td>
<td>0.02 ± 0.06</td>
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<td>AJ + BP + ID</td>
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<td>AJ + MC + ID</td>
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<td>ES + BP + IP</td>
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<td>0.05 ± 0.07</td>
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<td>AJ + BP + IP</td>
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<td>0.11 ± 0.15</td>
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Animals were immunized with: ES and challenged ID with *B. pahangi* L3s (ES+BP+ID), RIBI adjuvant and challenged ID with *B. pahangi* L3s (AJ+BP+ID), ES and challenged ID with RPMI (ES+MC+ID), RIBI adjuvant and challenged ID with RPMI (AJ+MC+ID), ES and challenged IP with *B. pahangi* L3s (ES+BP+IP), RIBI adjuvant and challenged IP with *B. pahangi* L3s (AJ+BP+IP), ES and challenged IP with RPMI (ES+MC+IP), or RIBI adjuvant and challenged IP with RPMI (AJ+MC+IP).

a, b Letters indicate significance at $p < 0.05$ within cell type between experimental and control groups.
Table B. Experiment 2 Total Cells and Cell Types in Complete Blood Counts (CBC) (X 10^3/µl). Values are expressed as mean values ± S.D. according to type of leukocyte from different immunizations and infections.

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<td>ES + BP + ID</td>
<td>14.24 ± 1.26</td>
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<td>3.33 ± 2.17</td>
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<td>0.258 ± 0.17</td>
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<tr>
<td>AJ + MC + IP</td>
<td>14.06 ± 1.49</td>
<td>10.54 ± 1.96</td>
<td>3.13 ± 1.91</td>
<td>0.237 ± 0.18</td>
<td>0.081 ± 0.13</td>
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Animals were immunized with: ES and challenged ID with *B. pahangi* L3s (ES+BP+ID), RIBI adjuvant and challenged ID with *B. pahangi* L3s (AJ+BP+ID), ES and challenged ID with RPMI (ES+MC+ID), RIBI adjuvant and challenged ID with RPMI (AJ+MC+ID), ES and challenged IP with *B. pahangi* L3s (ES+BP+IP), RIBI adjuvant and challenged IP with *B. pahangi* L3s (AJ+BP+IP), ES and challenged IP with RPMI (ES+MC+IP), or RIBI adjuvant and challenged IP with RPMI (AJ+MC+IP).

^a,b^ Letters indicate significance at *p* < 0.05 within cell type between experimental and control groups.
Fig. A. Mean larval recoveries in the peritoneal cavity (peritoneal cavity wash, viscera, and body soak) and in other sites (all other tissues) in Experiment 1. Animals were immunized with either ES in RIBI adjuvant and challenged IP (ES + BP + IP) or with RIBI adjuvant alone and challenged similarly (AJ + BP + IP). Three DPI, larvae were recovered and reported as mean percent recoveries. Letters indicate significance ($p<0.05$) between experimental and control groups within and between each necropsy site.
Fig B. Mean adult recovery percentages according to proximity to injection site in ES + BP + ID and AJ + BP + ID animals in Experiment 2. The only proximal site examined was the LPOP. Distal sites are RPOP, RRLN, LRLN, RSUB, LSUB, RSPCD, LSPCD, RTEST, and LTEST. Letters indicate significance ($p<0.05$) between experimental and control groups within each site grouping as well as between groupings.
Fig C. Mean adult recoveries in the peritoneal cavity (peritoneal cavity wash, viscera, and body soak), other sites (all other tissues except heart and lungs), and heart and lungs in Experiment 2. Animals were immunized with either ES in RIBI adjuvant and challenged IP (ES + BP + IP) or with RIBI adjuvant alone and challenged similarly (AJ + BP + IP). At 106 DPI, adults were recovered and reported as mean percent recoveries. Letters indicate significance ($p<0.05$) between experimental and control groups within and between each necropsy site.
Fig D. Mean numbers of microfilariae in ES immunized or AJ immunized animals challenged ID or IP at 101 DPI in Experiment 2. A Knotts test was performed using 500µl blood. Error bars represent SEM.
Vita

Ginger Ann Robertson was born in Baton Rouge, Louisiana, in 1981. She graduated with a Bachelor of Science degree in biological sciences from Louisiana State University in Baton Rouge, Louisiana. In May 2006, she completed a Master of Science degree in the Department of Pathobiological Sciences, Louisiana State University School of Veterinary Medicine.