DETECTION OF ANTIBODIES AGAINST PARASCARIS EQUORUM EXCRETORY-SECRETORY ANTIGENS

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DETECTION OF ANTIBODIES AGAINST *PARASCARIS EQUORUM* EXCRETORY-SECRETORY ANTIGENS

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DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Agriculture at the University of Kentucky

By

Steffanie Valentine Burk

Lexington, Kentucky

Co-Directors: Dr. Mary G. Rossano, Associate Professor of Animal Science and Dr. Eric Vanzant, Associate Professor of Animal Science

Lexington, Kentucky

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DETECTION OF ANTIBODIES AGAINST *PARASCARIS EQUORUM* EXCRETORY-SECRETORY ANTIGENS

*Parascaris equorum* is a nematode parasite that infects young horses, sometimes causing unthriftiness, respiratory signs, or intestinal impaction in severe cases. Infection can be diagnosed by detection of eggs in feces, but this is only possible after the worms are fully mature. The goal of this study was to develop an antibody-based test for prepatent diagnosis of *P. equorum* infection. To produce western blot (WB) antigen, *P. equorum* larvae were cultured for collection of excretory-secretory antigens (ESA). Sera from 18 pregnant broodmares, their subsequent foals, and a group of 12 older mares and geldings were analyzed. In order to check for cross-reactivity between *P. equorum* and other ascarid species and equine parasites, additional sera were analyzed. Sera from a horse with monospecific *P. equorum* infection was compared to horses with monospecific *Strongyloides westeri* or cyathostome infections, rabbits inoculated with *Baylisascaris procyonis* or *Toxocara canis* eggs, dogs naturally infected with *T. canis*, and rabbits immunized with *B. procyonis* or *P. equorum* ESA. Molecular weights of silver-stained *P. equorum* larval ESA ranged between 12 to 94 kDa. In WB analysis, sera from 94% of broodmares contained IgG(T) antibody that recognized multiple *P. equorum* larval ESA. Foals showed no IgG(T) antibodies pre-suckle, but antibodies similar to their dams were observed post-suckle and thereafter. Of the older mares and geldings, 58% had IgG(T) antibodies recognizing larval ESA. Serum IgG(T) antibodies against *P. equorum* larval ESA were also found in parasite-free and monospecific infection equine sera. Ascarid positive foals did not produce detectable amounts of IgE or IgM antibodies against larval ESA. When *P. equorum*, *T. canis*, and *B. procyonis* antibody reactivity was compared, antigens at 19 kDa and 34 kDa had the highest potential for identification of larval *P. equorum* infections. When immature adult *P. equorum* ESA was examined, IgG(T) antibody recognition was demonstrated in 50% of broodmares and 17% of the older horses, and appeared several weeks prior to patency in foal serum. Results indicate that IgG(T) antibodies against *P. equorum* ESA are common in mature horses, and are transferred from mare to foal, limiting the diagnostic potential of an antibody-based test.
KEYWORDS: *Parascaris equorum*, horse, western blot, diagnosis, antibody
DETECTION OF ANTIBODIES AGAINST *PARASCARIS EQUORUM* EXCRETORY-SECRETORY ANTIGENS

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August 2013
This dissertation is dedicated to the memory of my grandparents:

Dorothy and Sam Burk

Florence and Paul Dresser
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ABBREVIATIONS

EPG- Eggs per gram

ESA- Excretory-secretory antigens

ELISA- Enzyme-linked immunosorbent assay

FEC- Fecal egg count

FECRT- Fecal egg count reduction test

(H+L)- Heavy and light immunoglobulin chains

HRP- Horseradish peroxidase

IgE- Immunoglobulin E

IgG- Immunoglobulin G

IgG(T)- Combination of equine IgG isotypes IgG3 and IgG5

IgM- Immunoglobulin M

L1- First stage, larva

L2- Second stage, larva

L3- Third stage, larva

L4- Fourth stage, larva

L5- Fifth stage, immature adult
MCF- Maine Chance Farm

MW- Molecular weight

MWCO- Molecular weight cut off

PIC- Protease inhibitor cocktail

PVDF- Polyvinylidene difluoride

SD- Standard deviation

TBST- Tris buffered saline with Tween 20

VSF- Veterinary Science farm

WB- Western blot

Parasite Scientific Names

*Ascaris lumbricoides*- Human ascarid

*Ascaris suum*- Swine ascarid

*Baylisascaris procyonis*- Raccoon ascarid

*Parascaris equorum*- Equine ascarid

*Strongyloides westeri*- Equine threadworm

*Toxocara canis*- Dog ascarid
*Toxocara cati*- Cat ascarid

*Toxascaris leonina*- Ascarid found in cats, dogs, and foxes

*Toxocara vitulorum*- Cattle and buffalo ascarid
CHAPTER 1: INTRODUCTION

*Parascaris equorum* is a parasitic roundworm commonly found in young horses. This parasite was formerly known as *Ascaris megaloecephala*, and was studied as early as 1883, when Edouard Van Beneden used eggs of the species to first describe the heredity of chromosomes through meiosis [1]. *Parascaris equorum* is a member of the phylum Nematoda, a diverse group of terrestrial and marine roundworms. One review estimated the number of nematode species to range between 100,000 to 100 million [2]. Along with *P. equorum*, many other parasites in the order Ascaridida are host-specific, especially those that parasitize mammals.

*Parascaris equorum* has a wide geographical range, and has been found in North America [3, 4], South America [5], Europe [6, 7], Africa [8], Asia [9, 10], and Australia [11]. In addition to horses, *P. equorum* infects other equids, including zebras [12], Przewalski’s horses [12], donkeys [8, 10], and mules [10]. The parasite typically infects younger equids, with foals and yearlings more likely to have patent infections than animals two years of age or older [7, 11]. Mature horses appear to develop immunity to *P. equorum*, and efforts to induce patent egg shedding in adult horses have been unsuccessful [13, 14].

The life cycle of *P. equorum* begins when an infected equid sheds eggs in the feces. A single foal can contaminate the environment with a large number of eggs. One foal with an artificial infection was found to shed up to 50 million eggs per day [15]. After an equid ingests infective eggs, larvae hatch and migrate from the small intestine
to the liver, then to the lungs, and finally back to the small intestine via the tracheoesophageal route [15]. The worms mature and reproduce in the small intestine, thus beginning the life cycle again. Pathogenicity may occur at several points during the life cycle. Larval migration can cause lesions in the liver, lungs, and bronchial and hepatic lymph nodes [16]. During larval migration, equids may display clinical signs such as coughing and nasal discharge [17]. As infection progresses, other clinical signs include depression, anorexia, weight loss [17]. Large ascarid burdens in the small intestine can lead to intestinal impaction and colic, or even intestinal rupture [18-20]. Because of the damage caused by heavy ascarid infections, prevention and control are of paramount importance, particularly in young horses that have not yet developed an adequate immune response.

The eggs of P. equorum are very durable, and can remain viable for years [21, 22]. The eggs have a proteinaceous outer coating [23], allowing them to affix to objects and spread passively. However, there are several methods to control the eggs environmentally. Eggs can become unviable at very high temperatures [24, 25], as is found when composting manure [26]. Removal of manure from stalls and pasture also helps to decrease prevalence of ascarid eggs [7]. More often, owners focus on the use of anthelmintics to treat ascarid infections [7].

There are several anthelmintics currently on the market that are used to treat P. equorum infections. With the rise of cyathostome resistance to several anthelmintic classes, parasite resistance is becoming a major concern. Benzimidazole compounds,
including fenbendazole and oxibendazole, are effective in treating ascarid infections at a dosage of 10 mg/kg body weight [27]. One case study reported possible resistance to fenbendazole in a miniature horse [28], and veterinarians have suggested that resistance may exist in Kentucky [29], but there have been no published reports of resistance to this class of drug. Tetrahydropyrimidines, including pyrantel tartrate and pyrantel pamoate, are also used to treat *P. equorum* infections. There are multiple reports of *P. equorum* resistance to tetrahydropyrimidines in North America [30-32]. Resistance of *P. equorum* to the macrocyclic lactones (ivermectin and moxidectin) is well documented in North America and Europe [30, 32-36]. Concern over drug-resistant ascarid populations increases the importance of prevention and timely diagnosis.

Methods for *P. equorum* diagnosis include microscopic examination for helminth eggs in feces (copromicroscopic techniques) and necropsy. Occasionally infection can be diagnosed by the presence of an adult worm in the feces, typically after treatment with anthelmintics, or as the horse develops immunity. For horse owners, copromicroscopic examination is currently best method for diagnosis. There are several methods that can be used to check for infection, including the fecal egg count (FEC) or the qualitative fecal flotation. The FEC provides a quantitative measure of infection by calculation of the eggs per gram (EPG) in feces, and performs well at identifying positive horses [37]. However, there are several issues with copromicroscopic methods that could be mitigated by the development of new diagnostic tools.
CHAPTER 2: STUDY RATIONALE, HYPOTHESIS, AND OBJECTIVES

The development of additional diagnostic tools could allow for detection of parasites earlier in the infection process. Infection cannot be diagnosed using copromicroscopic methods until the life cycle of the ascarid is completed and female worms begin reproducing. As clinical respiratory signs typically occur during the migratory stage, diagnosis occurs after clinical symptoms are observed. Additionally, copromicroscopic methods rely on the presence of reproductively active adult female worms, and do not detect the presence of migratory larvae, immature adults, or mature adult male parasites. The EPG is not correlated with actual worm burden in the small intestine [37]. Although the FEC has high specificity and positive predictive value, a negative result on the test does not conclusively indicate that the horse is uninfected [37]. The development of new diagnostic tools could target diagnosis at earlier stages of ascarid infection, and attempt to increase sensitivity and negative predictive value. This could be useful for research and clinical purposes.

Furthermore, the immune response of equines to ascarids has not been studied in great detail. The timing of the appearance of *P. equorum* antibodies that recognize *P. equorum* larval ESA is currently unknown. This project will study the value of a serological test for *P. equorum*, test adult horses for the presence of antibody, and identify when antibodies to *P. equorum* larval ESA first appear in foal sera.
Hypothesis

Hₐ: Excretory-secretory proteins produced by *P. equorum* contain at least one antigen that can be used for serological diagnosis of infection in horses.

Goal and Objectives

Goal: Develop a western blot test specific for *P. equorum* larval ES antigens.

Chapter 4 Objectives

1. Hatch and maintain *P. equorum* and *Baylisascaris procyonis* in culture for collection of ESA.
2. Compare ESA produced by *B. procyonis* and *P. equorum* using SDS-PAGE and silver stain.
3. Identify IgG or IgG(T) recognition of *P. equorum* ESA through western blot analyses using sera from animals with *P. equorum*, *B. procyonis*, or *Toxocara canis* inoculations, immunizations, or natural infections.
Chapter 5 Objectives

1. Compare IgG(T), IgE, and IgM antibody recognition of larval *P. equorum* ESA using sera from infected horses.

2. Test the sera from a cohort of broodmares and their foals from birth until a period of no egg shedding.

3. Examine equine controls with monospecific parasite infections to identify possible cross-reactivity.

Chapter 6 Objectives

1. Compare IgG(T) recognition of larval *P. equorum* ESA by a group of low-exposure horses with previous results from a group of high *P. equorum* exposure horses.

2. Compare IgG(T) recognition of adult *P. equorum* ESA by sera from broodmares, older horses, and foals shedding ascarid eggs.
CHAPTER 3: REVIEW OF THE LITERATURE

Life cycle

Infection with *P. equorum* begins when a foal ingests larvated eggs from the environment [38]. Infection with some ascarid species, such as *Toxocara canis* (the canine ascarid) and *Toxocara cati* (the feline ascarid), begins when larvae are transmitted to the offspring through the mother’s milk [39, 40]. *Toxocara canis* can also be transmitted through the placenta [41]. Additionally *Strongyloides westeri*, the equine threadworm, arrests in the mare’s tissue and then passes to the foal through the milk [42]. However, it does not appear that *P. equorum* is transmitted from mare to foal through the milk, as attempts to recover larvae from colostrum or milk have been unsuccessful [38].

The life cycle of *P. equorum* begins when fertilized eggs are released by adult female worms in the horse’s small intestine (Figure 3.1). The eggs then pass through the rest of the gastrointestinal tract and are released to the environment in the horse’s feces. Within the egg, embryos become vermiform and molt once, going from a first stage larva (L1) to a second stage larva (L2) [23]. At optimum temperatures of 25-37 °C, eggs passed in feces require 10 days to become infective L2 [23, 38]. At 15 °C, embryonation occurs more slowly, and at 0 °C, eggs will not embryonate but will remain viable indefinitely [23]. Eggs have been shown to embryonate in 10-19 days in laboratory conditions when obtained directly from female worms [17, 25]. The period during which eggs are in the environment is the only free-living phase of the life cycle.
Once eggs are ingested by an appropriate host, the parasitic phase of the life cycle begins. Although *P. equorum* larvae have been shown to hatch and migrate through the liver and lungs in guinea pigs and mice, the larvae are not able to return to the small intestine and reach maturity [43]. Thus, it appears that the complete *P. equorum* life cycle is only possible in the equine.

In the horse, larvated eggs appear to hatch in the stomach or small intestine and penetrate the wall of the jejunum, as larvae have been found in the villi of the jejunum 24 hours post-infection [16]. The larvae then migrate to the liver through the lymph and blood vessels [16]. Larvae have been found in the mesenteric lymph nodes 24 hours post-infection [16]. After 7 days post-infection, the majority of larvae can be found in the liver [15]. In *P. equorum*, it appears that the molt from L2 to third stage larvae (L3) may occur before larvae reach the liver, although this has not been confirmed with multiple larvae [15]. Research with *Ascaris suum* (swine ascarid) indicates that the second molt is initiated within the egg, prior to hatching [44, 45]. The time of completion of the second molt varied from within the egg to the time at which larvae reached the liver [45]. By day 14, most *P. equorum* larvae have migrated to the pulmonary parenchyma and airways of the lungs [15, 46]. On day 16, some larvae have traveled to the lymph follicles of the lungs [16]. Between 17 and 23 days after infection, some larvae remain in the pulmonary parenchyma of the lungs, but the majority of larvae had migrated up the trachea, where they have been coughed up, swallowed, and migrate back to the small intestine [15, 16, 46]. Shortly after returning to the small intestine, larvae undergo another molt, from L3 to fourth stage (L4) larvae. [15]. The
final molt from L4 to immature adult (L5) must then occur in the small intestine. The worms mature in the duodenum and proximal jejunum, and complete the life cycle by reproducing 10-15 weeks post-infection [15, 17, 46, 47]. Earlier appearance of eggs in foal feces may be due to coprophagy [48].

Infected animals can shed large quantities of eggs that could infect future generations of foals. An individual female *Ascaris lumbricoides* (the human ascarid) can shed up to 200,000 eggs per day [49]. Egg-shedding estimates for one foal with an artificial *P. equorum* infection reached a maximum of 50 million eggs per day [15]. Once the eggs become infective and are ingested by another animal, the life cycle begins again.

**Biology**

**Chromosomal Makeup**

Genetic research has led to some debate over the number of ascarid species infecting equines. Chromosomal investigations suggest that there are two distinct species: *P. equorum* and *Parascaris univalens*. The diploid genome of *P. equorum* has 4 chromosomes, while *P. univalens* has 2 chromosomes (and therefore only one pair of chromosomes) [50]. It has been proposed that *P. equorum* and *P. univalens* can interbreed, with sterility in the resulting offspring [50]. Evidence of hybridization has been noted in other parasite species, including *A. suum* and *A. lumbricoides* [51], leading to some debate over whether they are even two separate species [52].
Additional study is needed to determine the geographic range and prevalence of *P. univalens*. One group of researchers using DNA fingerprinting found low genetic diversity among ascarids in equids from the US (Kentucky), Sweden, Norway, Iceland, Germany, and Brazil; although genetic differences in one Norwegian sample were explained by the possibility of *P. univalens* infection [53]. The prevalence of *P. univalens* has not been well characterized and the majority of published research refers to *P. equorum* without regard to chromosomal examination. Thus, the nomenclature *P. equorum* will be used here to refer to ascarids found in equids.

**Reproduction**

With a mean body size of 370 mm$^2$, *P. equorum* is one of the largest parasitic nematodes [54]. Like most nematodes, *P. equorum* is dioecious and sexually dimorphic. Mature females are typically larger than males, and can grow to over 25 cm in length. Males can also be distinguished from females by their curved tail [55]. Internally, male *P. equorum* have a long, coiled reproductive system, consisting of the testis, seminal vesicle, and vas deferens [56]. The copulatory spicules are located within the cloaca of the male worm, and are used to fertilize female oocytes through the discharge of round, amoeboid-type sperm [56]. The female worm has two long ovaries that extend to oviducts and then connect to uteri. The uteri meet at the vagina, which leads to the vulva, or genital pore, about a third of the way from the anterior end of the worm [56]. Immediately after fertilization occurs in the uteri, the innermost layers of the egg shell begin to form [56]. As fertilized eggs progress down the uterus, they gain a sticky, proteinaceous outer coat from the uterine secretions [23]. The eggs are then passed
through the vulva into the digesta of the small intestine of the equid. Eggs are clear in color within the uteri, but once passed in the feces, they gain a brown coloration.

*Parascaris equorum* eggs are spherical and approximately 90-100 µm in diameter [57]. In general, ascarid eggs have four layers; (1) an inner lipid layer, (2) a tough chitinous layer 3 to 4 microns thick [58], (3) a vitelline layer, and (4) a mammilated mucopolysaccharide/protein uterine layer [56, 59]. The chitinous layer is the thickest portion of the shell, and functions to provide strength [59]. The protein coat presents a sticky surface that allows the eggs to attach to objects and enhance spread and transmission. The shell has been shown to provide protection against harmful solutions, including formalin, sulfuric acid, and hydrochloric acid [23]. However, there is some permeability of the shell. Ascarid eggs exposed to low humidity can slowly lose water [59], and embryonation of eggs is an aerobic process, requiring permeability of the shell to oxygen and carbon dioxide [23]. Permeability of the shell increases at higher temperatures [59].

The resistant shell allows *P. equorum* eggs to remain viable for long periods of time in the environment. In order to maximize chances of successfully infecting a host, ascarid eggs do not hatch in the environment. Instead, they are prompted to hatch by conditions of the gastrointestinal tract. These conditions experimentally determined with *A. lumbricoides* include temperature of 37 °C, neutral pH, and the presence of carbon dioxide [60]. Upon hatching of *A. lumbricoides* or *A. suum*, there is an increase in the permeability of the lipid layer of the shell [61]. Trehalose from the perivitelline fluid within the egg begins to leak out, reducing osmotic pressure and promoting increased
movement of the larvae [60, 61]. It is also thought that enzymes may play a role in the process, as proteases [62] and chitinases [63] have been identified in hatching fluid. It has also been hypothesized that the operculum-like region of A. suum eggs [64] may be more vulnerable to enzymes [61], although P. equorum and B. procyonis eggs do not appear to have this region [64]. Hatching of P. equorum does not appear to have been studied or performed in-vitro, but it has been studied in other species. The final stage described in the hatching of nematodes is eclosion, where a larva explores the egg, presses against the shell with the cephalic region, and some non-ascarid species use stylet thrusts to penetrate the shell [61].

**Body Structure and Locomotion**

The exterior of the ascarid is made of a durable 3-4 layered cuticle secreted by the epidermis [65]. Ascarid cuticles have large transverse grooves called annulae, which assist the body in flexing for locomotion [65]. The surface of the cuticle is made up mostly of glycoproteins (proteins containing sugar chains) [65]. These surface glycoproteins appear to be shed readily by T. canis larvae, possibly to assist in evasion of the host’s adaptive immune response [65]. Labeled lectins (proteins that bind to carbohydrate groups) have been used to identify carbohydrates on the cuticle of larval A. suum [66]. Larval stages (L2, L3, L4) differed in cuticular carbohydrates present [66]. These carbohydrates appeared to be a structural component of the surface of the cuticle, as A. suum larvae did not appear to shed the carbohydrates at any time other than molting [66]. In the deeper layers of the cuticle, the main components are collagen-like proteins [65].
The epidermis and the somatic muscle cells lie below the nematode cuticle. The somatic muscles run longitudinally, and have dorsal and ventral halves that are controlled by two separate nerve cords [67]. This allows for nematodes to swim by undulating in sinusoid-type waves [68]. Nematodes are also pseudocoelomates, and thus have a fluid-filled central cavity that supports the body by acting as a hydrostatic skeleton [56], while also circulating fluid throughout the body [69].

Feeding

The anterior of *P. equorum* has a mouth with three prominent labia [55]. Each labium is lined with tiny tooth-like projections called denticles [55]. The exact function of the denticles is unknown, although in *A. suum*, they have been shown to wear over time as the parasites age [70]. The mouth opens into the buccal cavity, leading to the pharynx, intestine, rectum, and then the anus in the female or cloaca in the male [71]. Adult *P. equorum* are anaerobic and feed by swimming freely in the small intestine, using pharyngeal muscles to pump host digesta through the mouth [23, 72]. The worms do not appear to feed by absorption of nutrients through the cuticle, as efforts to view cuticle absorption with labeled isotope or glucose have been unsuccessful [23]. The ascarid intestine also appears capable of digestion without necessity for cuticular absorption. Multiple digestive enzymes have been identified in the intestine of *A. lumbricoides* [23], and the luminal surface of the intestine is lined in microvilli [72].

*Parascaris equorum* larvae do not begin feeding until they hatch in the small intestine of the equid. The exact feeding mechanism of larvae during migration is unknown [72]; however, larvae of other ascarid species, such as *A. suum* [73-75], *A.
*lumbricoides* [75], *T. canis* [76], *B. procyonis* [77], and *Toxocara vitulorum* (the cattle or buffalo ascarid) [78], have been maintained in cell culture media for multiple weeks. In *A. suum*, larvae were grown to maturity *in vitro* [79]. Thus, it appears that some, if not all, nutritional requirements can be met using cell culture media.

**Excretion/Secretion**

The intestine of mature *A. lumbricoides* is emptied every 3 to 4 minutes, although some digesta remains, probably for absorption through the microvilli [72]. Excretion in nematodes occurs through contraction and relaxation of body wall muscle and intestinal muscle [72]. Ascarids also produce secretions, but difficulties in separating and characterizing excretions and secretions has led to common use of the term “excretory-secretory products” [69]. In other nematodes, enzyme secretions have been shown to assist worms in migration by cleaving molecules in different cell types. For example, hookworms have been shown to secrete hyaluronidase, which can cleave hyaluronic acid, a major component of the epidermis [80]. Similarly, soybean cyst nematodes have been shown to secrete enzymes that hydrolyse cellulose [81]. Secretions have also been shown to have a variety of immunomodulatory effects. First-instar cattle grub larvae produce a serine protease that can cleave bovine IgG [82]. A cysteine protease inhibitor produced by filarial nematodes was shown to decrease T-cell proliferation and upregulate production of interleukin-10 (IL-10), an anti-inflammatory cytokine [83]. Many animal-parasitic nematodes secrete acetylcholinesterase [84], which has been hypothesized to prevent worm expulsion by blocking processes of innate immunity in the host’s intestine [85]. Other functions of ESA include modification
of host cells and gene expression to improve feeding sites or provide a protective environment [69].

Secreted factors of larval *P. equorum* have not been studied, but ESA have been identified for larval *A. suum*. This parasite secretes serine proteases (chemotrypsin, trypsin, and elastase) during L2, L3, and L4 stages [86], which could assist larvae during migration through host tissue. *Ascaris suum* larvae appear to produce aminopeptidase and cysteine proteases while molting from the L3 to L4 stage [87]. Excretory-secretory antigens of larval *T. canis* have been well-characterized because of the zoonotic potential of this species, and secreted lectins, mucins, and enzymes have been identified [88]. A major glycoprotein ESA of larval *T. canis*, TES-120, is also the main component of the surface of the cuticle [89, 90]. Shedding of this glycoprotein may play a role in evasion of host immune response. Antibodies against *T. canis* larval ESA have been shown to bind to the intact cuticle of larvae [91]. Lectins (C-type) have been identified in larval *T. canis* ESA [92], including TES-70, which was shown to bind selectively to canine cells [93]. Protease inhibitors from homogenized adult *P. equorum* have shown activity against bovine trypsin and chymotrypsin, and one protease inhibitor demonstrated activity against porcine elastase [94]. Extracts from mature *A. suum* and *A. lumbricoides* were found to contain trypsin inhibitors, which may help them to survive in the host’s gastrointestinal tract [95].

**Behavior**

Behavior of nematodes is controlled by the cranial ganglia (feeding) and perianal ganglia (mating) [67]. Nematodes also have sense organs for detection of stimuli. Sense
organs include chemoreceptors, mechanoreceptors, thermoreceptors, and even photoreceptors in some cases (mostly in marine nematodes) [96]. Nematodes can communicate with one-another through secretion of pheromones. Most pheromone work has been done using *C. elegans*, and these nematodes have been found to communicate information about population density and mate-finding [69].

**Pathology and Clinical Signs**

Pathologies due to *P. equorum* can occur during different stages of the parasite’s life cycle. A full day after artificial infection, many eosinophils and agranulocytes are present in the jejunum of the small intestine, along with ascarid larvae [16]. *Parascaris equorum* larvae do not seem to cause noticeable damage to the wall of the small intestine; lesions in the small intestine have been described as rare [97].

While migrating through the liver, larvae are estimated to range in size from 0.580 to 0.963 mm long [15]. Migration of these larvae causes liver pathologies around 3-14 days after artificial infection [98, 99]. Petechial hemorrhages (small dark spots caused by broken capillaries), edema, and scattered necrotic foci containing mononuclear cells, neutrophils, and eosinophils are found at this time [16]. In one study, necrotic foci were only found in older foals (6-12 months) inoculated with one large (8,000 eggs) dose [99]. Necrotic foci have been proposed to be areas formed around dead ascarid larvae [99], and dead larvae have been found in these areas [16].

Hematology results showed eosinophilia and leukopenia in foals 9 days post-inoculation with 100,000 eggs [16], but eosinophilia appeared to be limited to foals receiving large single doses or repeated low doses (50 eggs twice weekly) of eggs [100]. White lesions
1-3 mm in diameter also appeared 7-30 days after inoculation [99]. These were more common in animals inoculated with large (8,000 eggs) doses rather than small trickle inoculations [99]. The hepatic lymph nodes were also enlarged [16] and there were narrow tracts in the liver containing neutrophils, eosinophils, and macrophages [101]. By 16-23 days after infection, chronic necrotic lesions could still be found in the liver [16], and by day 30, there were still fibrotic lesions present [99].

The migration of *P. equorum* through the lungs produces pathologies and clinical signs of respiratory disease. During the lung migratory phase, young foals (those less than 6 months of age) display respiratory signs such as coughing, frothy white mucus in the trachea, and thick nasal discharge [38]. Yearlings naïve to *P. equorum* develop more severe respiratory symptoms upon first infection, such as frequent coughing, depression, and increased density of the lobes of the lungs as seen via radiographs [38]. Yearlings also exhibited anorexia, depression, and weight loss at this time, unlike the younger foals [17]. The severe respiratory illness displayed by these animals between days 16-23 indicates that they were more intensely affected during larval migration through the lungs. While migrating through the lungs, larvae range in size from 0.867 mm to 2.436 mm [15]. Damage to the lungs immediately following migration includes small lesions showing edema, hemorrhage, and necrosis, along with the presence of numerous eosinophils [16]. In artificially infected foals, green or grey subpleural nodules around 5 mm in diameter were present on all lobes, and over time, some nodules even became calcified [100]. Hyalinization of the alveoli also occurs, and on day 23 after infection, some alveoli displayed hemorrhaging and contained larvae [16]. There were
also granulomas containing eosinophils, neutrophils, and mononuclear cells in other areas of the lungs [16]. The lungs of infected foals displayed pneumonic areas that appeared red and collapsed [100].

On day 42 after infection, larvae were again found in the villi of the small intestine [16]. The mucosa and submucosa of the small intestine displayed edema, and contained amplified numbers of eosinophils, mononuclear cells, and plasma cells [16]. The return of larvae to the small intestine concludes the migratory phase of the parasite. However, in foals with natural infections, it is likely that ascarids in multiple stages are all present within the host at the same time.

Once ascarids reach the small intestine and mature, there are new concerns and clinical signs. After 50 days post-inoculation, foals exhibited weight loss and depression [17]. Eventually, these foals became emaciated and appeared to have stunted growth [97]. As these foals had been dosed initially with 8,000 eggs, clinical signs observed during most natural infections should be less exaggerated. Infected yearlings did not exhibit any clinical signs at this time [17]. Serum and total body albumin were numerically lower in *P. equorum* infected foals than in controls, although only four animals were compared [102]. As there was no increase in albumin catabolism in the infected animals [102], it appears that the lower albumin levels were due to decreased synthesis. Albumin synthesis is dependent on nutritional intake; starvation leads to low albumin production, and a lack of protein in the diet leads to an even greater reduction [103]. It seems likely that parasites were consuming some of the foals’ nutrients, although the small number of animals in this study prohibited statistical comparisons,
and the intake for infected foals was numerically lower than the intake for controls [102].

Although vital signs are often used to identify equine health problems, temperature of foals infected with ascarids is expected to be within the normal range [17]. Temperature does not seem to be affected by ascarid infection, as both foals and yearlings maintained normal temperatures throughout migratory and patent infection stages [17]. Other vital signs were not examined in these studies.

A major concern with infections of ascarids in the small intestine is colic or intestinal rupture. Large ascarid infections can cause acute non-strangulating intestinal obstruction [20]. Although these consequences are severe, ascarid impaction accounts for only 0.4% of surgical colic cases involving horses less than one year of age [104]. In two studies, the median age of equines with ascarid impaction was 5 months, and the majority of cases occurred in the fall (in the northern hemisphere) [104, 105]. This type of colic may be suspected when there are abnormalities in abdominal fluid, abdominal distention, and persistent pain [20], and irregularities may be detected by ultrasound [104]. During surgery, ascarids can be visualized and felt through the intestinal wall, and can removed by small intestine enterotomy [20], milking of the small intestine into the cecum using lubricant, or resection of the impacted segment [106]. The prognosis for surgical treatment of foals or young horses with ascarid impaction can be poor, especially when certain procedures are used. In one study, all foals (7/7) treated with enterotomy died or were euthanized [105], and another study found that horses that underwent enterotomy were less likely to survive to discharge [106]. Survival of greater
than one year post-surgery has been calculated at 10% [105], 24% [104], and 60% [106]. The difference in percentages may be because the study which calculated long-term survival at 60% used a larger variety of surgical methods, as well as more recent records [106]. This study found better prognosis for foals treated by milking of the small intestine, as all of these horses survived [106]. The change in techniques may improve the prognosis of horses with ascarid impaction, but it is still important to take preventative measures.

The occurrence of intestinal impaction with roundworms has been associated with recent anthelmintic use. This association between has been documented in children with *A. lumbricoides* infections [107]. In horses, studies have reported that 72% [104] to 80% [106] of horses admitted for ascarid-related colic had received anthelmintic treatment less than 24 hours before the appearance of clinical signs. Another study reported that 58% of horses had been treated within 6 days of ascarid-related colic [105]. The most common anthelmintics used were ivermectin and pyrantel pamoate [104, 105]. Dead or paralyzed worms may form a bolus and block the intestine [20], so some researchers believe that it may be prudent to use anthelmintics with a slower effect, such as fenbendazole, when foals are thought to have heavy ascarid burdens [108]. These risks of intestinal obstruction in foals increase the importance of accurate and timely diagnosis.

**Diagnosis**

Diagnosis of *P. equorum* is primarily conducted using copromicroscopic methods, including qualitative fecal flotations or the quantitative FEC. There are several different
methods used to conduct fecal flotations or FECs. All of these methods use some type of flotation solution that is dense enough to separate parasite eggs from debris in the feces, and float eggs to the top of solution for collection and examination.

The specific gravity of *P. equorum* eggs has been reported at 1.0969 [109]. In order to recover both strongyle-type and *P. equorum* eggs from horse feces, the specific gravity of the flotation solution should be between 1.25-1.38 [109, 110]. At lower specific gravities, strongyle-type eggs may still be recovered, but the solution will not be dense enough to float the heavier *P. equorum* eggs [110]. Flotation solutions used include sodium nitrate, sucrose, sodium sulfate, zinc sulfate, magnesium sulfate, or sodium chloride [110, 111]. In some procedures, fecal material is poured through a strainer to remove large pieces of fecal debris. At 500, 350, and 250 µm mesh sizes, this has been shown to have no effect on the number of *P. equorum* eggs recovered [110]. A variety of techniques and flotation solutions can be used, but fecal tests can be broken down into two major categories: qualitative or quantitative.

The qualitative fecal flotation generally entails modifications of the Willis method, first described in 1921 [112]. The initial steps of the fecal flotation involve mixing of fecal material and flotation solution and pouring the mixture into a tube. In some cases, the feces may be mixed with water instead of flotation solution, passed through a strainer, and then centrifuged, decanted, mixed with flotation solution, and centrifuged once more to float the eggs [113, 114]. The final steps involve placing a coverslip on top of the tube to capture the eggs. The coverslip is then placed on a
microscope slide and examined under the microscope for the presence of absence of parasite eggs. Although the test is qualitative, some researchers have been known to assign a score or provide a rating to describe the number of eggs present on the slide [111]. However, this test cannot be used a quantitative measure for treatment decisions or to evaluate the efficacy of dewormers [111]. Despite the fact that an exact count is not performed, the fecal flotation does have some advantages. Only one gram of feces is required [111], which is advantageous when the quantity of fecal matter is limited (such as with young foals). The fecal flotation is also purported to be more sensitive than fecal egg count methods [111, 114, 115].

There are three major quantitative FEC categories for diagnosis of equine parasites: the modified McMaster, the modified Stoll, and the Cornell-Wisconsin method. All three stem from initial work performed by Stoll in 1923 [116]. There are multiple variations found within the modified McMaster and modified Stoll methods. Additionally, new methods with altered McMaster-type chambers have recently been developed [117, 118].

Depending on the particular method used, the amount of feces needed for a modified McMaster test generally ranges from 1-4 g [119]. Feces may be mixed with water and centrifuged prior to decanting and mixing with flotation solution, or they may be mixed directly with flotation solution [119]. With any of these techniques, a subsample of the flotation/fecal mixture is pipetted into two chambers of a McMaster slide, and the number of eggs present are counted under a microscope, and multiplied
by a coefficient specific to the modification used [119]. In the United States, the most common procedure for testing horses is to use 4 g feces and 26 ml flotation solution, count the eggs in both chambers of the slide (0.3 ml total), and multiply that number by 25 to get the EPG [57]. One advantage of the modified McMaster is that, depending on the method used, a centrifuge may not be required. This makes FEC testing faster and more available to individuals who do not have expensive laboratory equipment.

The modified Stoll technique differs primarily from the modified McMaster in that a centrifuge is always required, and traditional microscope slides and coverslips are used in place of the McMaster slides. The modified Stoll involves combining 5-10 g of fecal matter flotation with flotation solution (or water, then combining a subsample with flotation solution), and centrifuging once with a coverslip [46, 57, 120, 121]. The number of eggs on the cover slip are counted and multiplied by a coefficient particular to the method used. For example, if 5 g of feces are mixed with 45 ml water (or 10 g feces mixed with 90 ml water), and 1 ml of this solution (0.1 g feces) is mixed with flotation solution in a test tube, the number of eggs counted on the slide will be multiplied by 10 to get the EPG [46, 121]. The Cornell-Wisconsin method is another variation of the modified Stoll technique, and differs in that it requires centrifuging each entire sample twice (once after mixing 1-5 g feces in water and straining, and again after decanting and mixing feces in flotation solution) [57, 121, 122].

As it is typically used, the modified McMaster test has a detection limit of 25-50 EPG, with better detection limits obtained with larger fecal samples [57]. New variations
of the modified McMaster, such as FECPAK or FLOTAC use altered counting chambers and feature improved sensitivity [117, 118]. Traditionally, for FEC tests where test sensitivity is important, the modified Stoll or Cornell-Wisconsin methods have been used. The modified Stoll has a detection limit of 5 EPG, and the Cornell-Wisconsin method is even more sensitive, at a detection limit of as low as 1 EPG [57]. These detection limits were developed using eggs from parasites other than *P. equorum*.

Although the FEC offers a non-invasive and relatively cost-effective method of parasite diagnosis, the test has several limitations. The performance of the modified Stoll technique in diagnosis of *P. equorum* infections in the small intestine has been examined and found to have an estimated specificity of 0.94 [37]. This indicates that within the population examined, the test performed well at correctly diagnosing uninfected horses. Sensitivity was estimated at 0.72, so the test did not perform as well at correctly identifying infected horses [37]. One explanation is that the FEC only accounts for mature egg-laying female parasites. An equid with migrating or immature adult ascarids would yield a false negative result through the FEC during the 3-month period in the life cycle before patent shedding occurs [38]. Likelihood ratios calculated in this study indicated that a horse with a positive result on the test is likely to have a *P. equorum* infection in the small intestine (12.11), but a horse with a negative result on the test is not as likely to be uninfected (0.3) [37]. As this study examined *P. equorum* infections in the small intestine, the likelihood ratio for a negative result could be expected to be closer to 1 if migratory larvae were accounted for.
These data were also tested for a linear correlation of the modified Stoll FEC results and *P. equorum* intestinal worm burden. However, the correlation was found to be poor (r<30) [37]. It is possible that other non-linear correlations between *P. equorum* FEC results and worm burden exist, but these have yet to be described. A linear correlation between worm burden and EPG in children with *A. lumbricoides* infection also could not be found [49]. Interestingly, the average egg output per female worm was found to be lower in children with high numbers of female worms [49]. An equine study found that when larger *P. equorum* egg inoculations are given to foals, there are more worms in the small intestine, yet the worms are smaller in size [100]. Smaller worms may have lower fecundity than larger worms. This has been shown to be the case with other ascarid species: mean weight of female *A. lumbricoides* is positively associated with egg output [123]. This may make it impossible to estimate worm burden solely through the FEC.

In children with *A. lumbricoides* infections, EPG were found to fluctuate by day, and were thought to be affected by the volume of feces produced [49]. Water content may also affect EPG, as drier samples are expected to contain more fecal material per gram [111]. Variation of EPG within each fecal sample has been found to occur in children with *A. lumbricoides* infections [49], and horses with strongyle-type eggs [124]. Variation within each equine sample was more marked in samples with low (<50) EPG values, as there may be fewer worms in these horses and more clumping of eggs [124]. Although there are issues with variation in FEC results and identification of early stages of disease, the FEC remains the primary tool for diagnosis of *P. equorum* infections.
Epidemiology

As mentioned previously, *P. equorum* is ubiquitous throughout the world, and has been found on all continents inhabited by equids [4, 5, 8, 10, 11]. There is some evidence that parasite body size correlates with prevalence, and may be partially due to the greater fecundity of larger nematodes [54]. In any case, *P. equorum* is a large parasite, with high prevalence of infection in young animals. In addition to horses, *P. equorum* infects other equids, including zebras [12], Przewalski’s horses [12], donkeys [8, 10], and mules [10]. The mode of transmission and prevalence of this parasite make it difficult to control, although it appears that there are ways to minimize risk of infection.

Transmission of *P. equorum* begins when a horse with a mature infection passes eggs in fecal matter, and the eggs have sufficient time and environmental conditions to embryonate (typically 10 days at temperatures of 25-27 °C, or longer at lower temperatures) [23, 38]. *Parascaris equorum* eggs are different from those of some other parasites, such as strongyles, in that they do not hatch in the environment. While strongyle larvae hatch in the environment and are able to migrate short distances [125, 126], ascarid eggs naturally hatch only within the host [23, 38]. This increases the importance of the structure of the egg for protection and transmission. The multi-layered shell provides protection [23], allowing *P. equorum* eggs to remain viable for at least 18 months to 3 years [21, 22], although the eggs can probably survive even longer. Eggs have been shown to survive temperatures ranging from -20 °C to 60 °C [25], allowing ascarid eggs to be transmitted across generations of foals. Within the egg,
Trehalose content may help to protect the egg from dessication or freezing [61].

Because *P. equorum* are unable to actively migrate in the environment, they rely on other methods for dispersal. The sticky outer proteinaceous coat of the eggs allows them to cling to surfaces and be transported from one area to another [23].

There are several risk factors that have been linked to *P. equorum* infection. Young age is probably the most commonly described risk factor. One study of 2000 German horses of varying ages found that only foals and weanlings were positive for ascarids by FEC [7]. An Australian study of 150 necropsied horses, and a Kentucky study of 350 necropsied horses had comparable results, with only horses 2 years of age or younger positive for ascarids in the small intestine [11, 127]. A Spanish coprological study found the highest prevalence of ascarid infection (22%) to occur in horses younger than 3 years of age [128]. In another German coprological study, 33% of foals and 6% of yearlings were positively diagnosed for ascarids [129]. Higher prevalence of eggs in feces was found in French 3-4 month old foals when compared with 8-9 month old foals [130]. In central Kentucky, 22% of foals aged 10-223 days were recorded as having ascarids following a series of fecal egg counts [4], while another study in the same area found 43% of foals 2-7 months old were positive for *P. equorum* in the small intestine by necropsy [131]. In Kentucky, positive foals were found on 86% of Thoroughbred farms surveyed [4]. Conversely, one study on horses in Lesotho, a small country bordering South Africa, found no association between age and infection, and reported an overall coprological prevalence of 21.6% [132]. The authors proposed that the usual pattern of development of immunity to *P. equorum* may diverge in developing countries [132].
Although young horses are the typical host for mature *P. equorum*, patent infections can still be found in mature animals and other types of equids. In Greece and Macedonia, 2% of “riding” horses and 1.3% of “working” horses were coprologically positive for *P. equorum* [6]. In some areas of the world, prevalence reports in mature horses appear to be much higher. One study found *P. equorum* eggs in feces of 16.3% of Ethiopian horses older than 8 years of age [133], while another study positively diagnosed 22% of Pakistani horses over the age of 2 [10]. It is difficult to discern why prevalence is higher in these areas, but it is possible that management factors may play a role. In Ethiopia, *P. equorum* infections in mature animals appeared to be more common in donkeys than in horses, as 50.1% of donkeys over the age of 8 had patent infections [133]. In contrast to these results, a study of equids in Pakistan found similar prevalence in horses (22%), mules (16%), and donkeys (19%) over the age of 2 [10]. In animals younger than 2 years, prevalence was as high as 64% for horses, 56% for mules, and 52% for donkeys [10]. It is possible that the high prevalence of ascarids in donkeys and mules may be due more to management than to genetics. In Greece, out of 37 donkeys and 37 mules, none were found to be shedding ascarid eggs (ages not described) [6]. Ascarid prevalence also appears high in other types of equids. A study monitoring parasites in two wildlife parks in Germany found *P. equorum* eggs in 39.2% of Chapman zebra fecal samples, and in 11.2% of samples from a breeding herd of Przewalski’s horses, but the exact ages of the animals were not specified [12]. These animals had also not been dewormed except in the case of “massive egg excretion” or clinical disease [12].
It is anticipated that some variation in prevalence reports may occur not just by geographic location, but also by the method used. As previously noted, there is variation in the sensitivity of the FEC test depending upon the particular technique used [57]. Furthermore, FEC or necropsy did not account for migrating larvae in these studies, so it is expected that some infections were missed, and actual prevalence is higher than described. For example, one German study found 3% prevalence of *P. equorum* by FEC, and 8.8% prevalence of *P. equorum* in the small intestine by necropsy on the same horses at the same time [134]. Of horses with small worm burdens (1-10 worms), only 16.7% were diagnosed as positive through modified McMaster FEC (detection limit 10 EPG), while horses with larger worm burdens were more likely to be correctly diagnosed by the FEC [134].

In addition to age, other factors have been evaluated for association with *P. equorum* prevalence in equines. Sex is one factor that has been assessed in multiple studies, with mixed results. One study found that males were more likely to be diagnosed as positive through FEC or necropsy [134], while another stated that females were more likely to shed eggs [128], and yet another found no effect [135]. However, the study that found females more likely to shed eggs also stated that males were more likely to have higher EPG counts [128]. One study found that geldings were less likely to be infected than intact male or female animals [11], but this was likely confounded by an association with age.
Breed is another factor that has been examined. In one Spanish study, horses designated as English Pure Breed were more likely to be shedding *P. equorum* eggs when compared to horses designated as Spanish Sport Horse, Spanish Pure Breed, or Pura Galega Raza [128]. An Australian study found no difference in *P. equorum* prevalence at necropsy between Thoroughbreds and “hacks” [11]. A Polish study found no difference in prevalence between 9 breeds [135], and a French study found no difference between Thoroughbreds and French Trotters [130]. It is possible that some of the differences in prevalence found by breed may occur because of management practices on farms catering to various breeds.

As one might expect, manure management and hygiene practices are related to the prevalence of *P. equorum* infections. There appear to be differences in the prevalence of *P. equorum* by farm. A Kentucky study found prevalence by farm to range from 0% to 64% [114], and a French study found prevalence by farm to range from 6.9% to 76.2% [130]. Some management practices could actually increase the spread and transmission of ascarid eggs. For example, foals on stud farms where manure was used to fertilize pastures had 44 times the odds of shedding *P. equorum* eggs when compared to stud farms where other types of fertilizer were used [7]. Similarly, regular removal of all fecal material from stalls is important in reducing transmission of *P. equorum*. Horses on deep litter bedding, where only surface waste is removed, were found to have 3.35 times the odds of shedding ascarid eggs, when compared to horses in stalls that were routinely cleaned [7]. One study found that housing (stall, pasture, or several paddock types) was not associated with *P. equorum* egg shedding, nor was farm type (stable,
stud farm, riding club, or ordinary farm) [135]. The authors of this study attributed the lack of association to low statistical power due to the smaller number of horses with *P. equorum* infection compared to strongyle infection [135].

Prevalence of *P. equorum* in regards to temperature, rainfall, and season has also been studied. Rainfall and temperature in Poland were found to be positively correlated with *P. equorum* infection [135]. This meshes well with the life cycle of *P. equorum*, as warmer temperatures would help with larvation of the eggs. Rainfall could possibly help with dispersal of eggs, or this correlation may have resulted because of the association between rainfall and temperature in Poland [135]. Additionally, eggs have been shown to larvate only at relative humidity close to 100% [23]. Conversely, a USDA study found that the number of horses shedding ascarid eggs more than doubled in the winter when compared to the summer [136], although an Australian study found no effect [11].

Another factor associated with *P. equorum* infection was type of deworming drug used. Deworming with ivermectin was positively associated with mean EPG, although ivermectin was much more commonly used than the other deworming drugs [135]. This agrees with research indicating parasite resistance to macrocyclic lactones [34], and emphasizes the importance of minimizing potential for infection.

**Prevention**

Prevention of ascarid infection should be of concern to those who own young equids. Because of the durability of the egg and its ability to remain viable for years [21,
23], it may be difficult, if not possible, to completely remove the threat of *P. equorum* infection. Regardless, certain measures can be taken to reduce the viability, spread, and transmission of the parasite eggs and decrease the risk of heavy infection.

As noted previously, manure management practices, such as spreading fresh manure back on pasture and using deep litter bedding have been linked to *P. equorum* infection [7]. Another study found contradictory results: removal of feces from pasture and “resting” of pasture were associated with positive FEC [137]. No explanation was proposed, but it is possible that there were other, unmeasured confounding factors leading to these results; for example, pasture size. Another study comparing management systems for donkeys in South Africa found the highest EPG results for donkeys confined to small areas around the owner’s house, when compared with those allowed to roam their village or graze on pasture [138]. Grazing donkeys had the lowest mean EPG (0) [138]. It is possible that donkeys allowed to roam or graze larger areas may have been choosier in their grazing, and less likely to contact eggs in fecal material. However, these data were not adjusted for age or other factors, and grazing donkeys were from a different geographical area than donkeys under some of the other management systems. Although there are some conflicting results about practices related to housing and grazing systems and more data is needed, it seems prudent to employ hygienic practices for cleaning and disposal/treatment of manure.

One way to treat manure prior to use as fertilizer is by composting. *Parascaris equorum* eggs become unviable at temperatures of 45 °C for one day [24], or at temperatures of 60 °C or higher for one minute [25]. One study found that within a
windrow compost pile, mean minimum and maximum temperatures ranged from 37.9 °C to 58.3 °C, respectively [26]. Furthermore, location in the manure pile may impede embroyonation of ascarid eggs, as they require aerobic conditions to larvate [23]. After only 8 days within the pile, mean percent \( P. \textit{equorum} \) egg viability was 0 for eggs permanently located within the pile, and for those alternating by day either inside or on top of the pile [26]. The effect of temperature on eggs that have already larvated does not appear to have been examined, but it is probable that high temperatures would destroy larvae within the eggs. These studies indicate that composting is an effective way to render freshly passed eggs unviable [26].

Another possible method of environmental management is paddock soil type. One study examined sacrifice-lot type paddocks used for multiple years for the presence of ascarid eggs in soil. The upper portion of sand/gravel paddock surfaces was found to have fewer eggs per 100 cm\(^3\) when compared to clayey or morainic soil, although the actual number of eggs shed initially in each paddock could not be estimated [22]. Sandy/gravel soil or larger particle size may help rainwater to wash eggs from the surface of the soil, where horses are likely to ingest the eggs [22]. Egg contamination appears to be high in areas frequently used by young horses. Another study found a mean of 30 EPG in soil of a drylot used to house weanlings, but did not describe the soil type [108]. For eggs that have already been spread from manure piles to the soil or to objects or stall areas, there may be some options for chemical treatment.

Early work with disinfectants found \( P. \textit{equorum} \) eggs to be fairly resistant to chemical treatment [23]. The use of Lysol or phenolic compounds has been suggested
for disinfecting stalls, but was not tested by these authors [97]. One study found Amphyl aerosol spray (active ingredients alkyl dimethyl benzyl ammonium saccharinate and ethanol) to effectively prevent embryonation, while treatment with Lysol did not show practical significance [139]. Four days after treatment with 2 ml of disinfectant, none of the eggs treated with Amphyl aerosol spray were embryonating [139]. Amphyl aerosol spray could be recommended for disinfecting barn tools and small objects, but may not be cost-effective or practical for treatment of larger areas. Similarly, *A. lumbricoides* eggs were tested with 16 different disinfectant products, and only ortho-benzyl-para-chlorophenol was effective at preventing embryonation under all dilutions and times tested [98]. Another study using *T. canis* eggs found that when eggs were incubated in 70% ethanol for 24 days, 0% of eggs developed to the infective L2 stage [140]. Sodium hypochlorite (2%) did not prevent infectivity, but showed some efficacy, as only 8% of eggs developed to the L2 stage [140]. However, the extent of treatment in this study does not simulate practical application of the products. Additional work with disinfectants is needed to identify practical methods for disinfecting large areas used by young horses, and help reduce the need for treatment.

**Treatment**

Horse owners in North America have some options for commercially available anthelmintics for treatment of *P. equorum* infections. The drug classes typically used today include the benzimidazoles (fenbendazole and oxibendazole), the tetrahydropyrimidines (pyrantel pamoate and pyrantel tartrate), and the macrocyclic lactones (ivermectin and moxidectin). All of these classes were originally marketed as
effective against adult *P. equorum* and L4 stages, although macrocyclic lactones are
advertised as effective for L3 stages as well [141].

The first of these drug classes to be introduced on the market were the benzimidazoles, which became widely used beginning in the 1960’s [142]. The benzimidazoles have a wide margin of safety [143], and work by disrupting the energy metabolism of the parasites. This happens when the drug binds to the β-tubulin subunit of the ascarid, preventing microtubule assembly [143]. When nematodes are unable to assemble microtubules, they are unable to undergo cellular division or transport nutrients [143]. This effectively starves the nematodes, and most die and are expelled within 3 days of treatment [143]. This mode of action provides the benzimidazoles with ovicidal action [144], which the other dewormer classes do not have.

Benzimidazole compounds, including fenbendazole and oxibendazole, are effective in treating ascarid infections at a dosage of 10 mg/kg body weight [27]. For other parasites, such as susceptible cyathostomes, large strongyles, and pinworms, the dosage needed for treatment is 5 mg/kg body weight [145]. The higher dosage required to kill *P. equorum* makes it the dose-limiting parasite for most anthelmintics [145]. Dose-limiting parasites are thought to be more prone to developing resistance to deworming drugs [145]; however, *P. equorum* resistance to benzimidazole compounds has not yet been confirmed through published studies. One case study documented intestinal rupture caused by *P. equorum* one month after deworming with fenbendazole, although the horse was dosed at 7.5 mg/kg [28]. Additionally, resistance to fenbendazole has
been suspected by veterinarians in Kentucky [29], but further evidence is needed before resistance to this drug is confirmed.

Of the tetrahydropyrimidines, pyrantel pamoate was introduced in the 1970’s, and pyrantel tartrate was first marketed in 1990 as a daily anthelmintic treatment [142]. These drugs also have a wide margin of safety, as long as they are not administered at the same time as other drugs with a similar a mechanism of action [143]. The mechanism of action for pyrantel is as a depolarizing neuromuscular blocker [143]. This occurs because the drug mimics the action of acetylcholine [143], a neurotransmitter that causes muscle contraction. The muscle contractions induced by these drugs are sustained, so the worms become paralyzed [143] and can no longer swim or feed. The efficacy of these drugs is now in question, as multiple studies report *P. equorum* resistance to pyrantel [30-32].

The macrocyclic lactones are the newest of the drug classes marketed for *P. equorum* treatment in North America. Ivermectin was first introduced in the early 1980’s [142], and moxidectin in the late 1990’s [142]. Macro cyclic lactones work by binding to or inducing binding at gamma-aminobutyric (GABA-A) mediated and glutamate-gated types of ligand-gated chloride channels [143, 146]. Opening of these channels leads to an influx of chloride ions, causing inhibition of nerves and flaccid paralysis [143, 146]. This leads to starvation and death of the parasites, similar to the tetrahydropyrimidine mechanism. Although the macrocyclic lactones are still considered safe, the use of moxidectin in foals less than 6 months of age is contraindicated because
of the possibility of moxidectin toxicity [147]. Mammals do not have glutamate-gated chloride channels, but ivermectin can open GABA chloride channels at overdose levels [143]. Numerous studies have documented resistance of *P. equorum* to the macrocyclic lactones [30, 32-36]. Treatment with ivermectin has even been associated with having higher mean *P. equorum* EPG values, although the values were not of practical significance. Because of the impaired efficacy of two of the three major classes of deworming drugs, new strategies to monitor and prevent the development of resistance have been developed.

There are several explanations as to why anthelmintic resistance is becoming so prevalent in *P. equorum*. Anthelmintic resistant genes could be novel or pre-date the development of anthelmintics [148]. Mutations for anthelmintic resistance in *P. equorum* have the potential to spread between populations. A study on the population genetics of *P. equorum* found low genetic diversity among worms from Sweden, Norway, Iceland, Germany, Brazil, and the US (Kentucky) [53]. As the global population appears homogenous, the authors suspect that the level of gene flow caused by shipment of horses is high enough to rapidly spread new mutations [53]. The issue with new alleles for drug resistance comes about when these alleles are selected for [149]. When anthelmintic treatment is administered repeatedly, this is thought to put selection pressure on the nematode populations, and hasten the development of resistant populations [149]. There are also other factors, such as stocking density or timing of treatment, which could increase the frequency of resistant alleles [149].
The use of selective deworming, or the treatment of only individuals with higher levels of egg shedding, has been advocated to slow the development of resistance by providing refugia [142, 150-152]. The idea behind this type of deworming program is that by leaving some animals untreated, anthelmintic-susceptible alleles will be maintained in the population [152]. Although selective deworming has been advocated for cyathostomes, it is recommended to treat all foals on a scheduled treatment plan because of the higher parasite loads experienced at this age [150]. If foals must be treated regularly, the selective deworming technique will not be of use in preserving the effectiveness of anthelmintics against ascarids. This increases the importance of tracking the spread of resistance to deworming drugs.

The presence of anthelmintic resistant nematodes in equine populations is monitored through the fecal egg count reduction test (FECRT). Although the FECRT is more commonly used to test for anthelmintic resistance of cyathostomes, it is also advocated for monitoring anthelmintic resistance of \textit{P. equorum} [150]. The steps of the FECRT as it was originally developed for sheep involve (1) a washout period for the effects of previous anthelmintic treatment, (2) classifying a control group and experimental group, (3) conducting pre-treatment FEC and (4) 7-10 day post-treatment FEC tests, then (5) calculating results [153]. Sheep group mean FECs of at least 150 EPG were required for proceeding with the FECRT. The calculations involved the mean EPG, the percent reduction of eggs, and the 95% confidence interval [153]. The original cutoff level for anthelmintic efficacy was a 95% reduction in the mean number of eggs, when compared with the control group, combined with a 95% confidence interval that did not
extend below 90% [153]. Others have used the FECRT at different cutoff levels; generally between 80%-95%, with differences by anthelmintic [142, 150, 151]. As farms with equines may not have large numbers of animals with high egg counts, the use of each animal as its own control is accepted [154]. For documenting *P. equorum* resistance, it has been suggested to wait 14-21 days before taking the second fecal sample after anthelmintic treatment [145]. Although the FECRT is relatively cheap and easy to perform, there are some issues with the test. The FECRT was originally designed using the modified McMaster technique [153], but the test could have greater or lower sensitivity depending on the FEC technique used. Tracking the spread of resistance may be hampered by the variability of methodology and inconsistencies in interpretation. Furthermore, the FECRT test measures a reduction in eggs laid by female worms, and so may not be an accurate depiction of the reduction of the total number of adult worms. As this is the only method used to classify anthelmintic resistance of *P. equorum*, there also is no way to examine efficacy of anthelmintics against larvae (except through necropsy). Surveillance of anthelmintic resistance could be improved by development of new diagnostic tools with the capacity to quantify current *P. equorum* infection. However, it would first be necessary to obtain additional knowledge of the equine immunoglobulin response to *P. equorum*.

**Immunoglobulin Types and Levels in the Young Horse**

In order to understand the humoral equine immune response to parasites, some background information on immunoglobulin types and levels in the young horse is necessary. Immunoglobulins are antibodies produced by B-cells in order to bind
antigens and protect against infection [155]. Depending on the structure of the heavy chains in the immunoglobulin molecule, immunoglobulins can be broken down into several classes, each with a different function. Immunoglobulin classes produced by horses include immunoglobulin A (IgA), immunoglobulin D (IgD), immunoglobulin E (IgE), immunoglobulin G (IgG), and immunoglobulin M (IgM) [156]. Several equine studies have serologically examined IgG, IgE, and IgM isotypes in relation to foal immunity and parasite infection or Th2 type response. These immunoglobulins will be the focus here [157-161].

Horses have multiple types of IgG; seven heavy chain genes have been discovered, which is more than has been found in any other mammalian species [156]. The seven isotypes include IgG1, IgG2, IgG3, IgG4, IgG5, IgG6, and IgG7 [156]. Formerly, IgG3 and IgG5 were classified together as one isotype called IgG(T), until affinity chromatography [162] and gene sequencing [163] showed IgG(T) to be two separate isotypes. Additionally, when goat anti-horse IgG(T) polyclonal antibodies were tested against each of the seven isotypes, they were found to react most strongly to IgG5, but also reacted to IgG2 and IgG3. However, many commercial producers of anti-equine immunoglobulins continue to produce “IgG(T).”

The newborn foal does not receive IgG or other immunoglobulins from the mare in utero. Equines have a diffuse epitheliochorial placenta [164]. Immunoglobulin molecules cannot pass from mare to foal through this type of placenta [164]. Rather, immunoglobulins (IgG, IgG(T), IgE, IgM, and IgA) are first passed from mare to foal via
colostrum [165-167]. Colostrum is routinely tested for IgG using a colostrometer or sugar refractometer prior to allowing the newborn to nurse, with 5000 mg/dl being considered “adequate”[168]. To ensure a healthy immune system, it is recommended that foal sera contain at least 800 mg IgG/dl by 6-9 hours after intake of colostrum [169]. Failure of passive transfer of IgG is associated with increased risk of severe bacterial disease [170], so it is recommended to treat foals experiencing failure of passive transfer through plasma transfusion [170]. The most common cause of failure of passive transfer is loss of colostrum due to premature lactation [171].

In the young horse, levels of immunoglobulins seem to vary by age, and endogenous production of immunoglobulins appears to be limited for very young foals. One day after birth, levels of serum IgG and IgG(T) are equal to adult horses [172]. From the day after birth until one month of age, levels of IgG and IgG(T) drop sharply [172, 173]. This is proposed to be due to catabolism of maternal antibody and possibly because of dilution of antibody due to increasing plasma volume from foal growth [173]. The half-life of foal serum IgG has been estimated at 18-23 days, with shorter half-life being associated with higher serum antibody concentrations [173]. Another study estimated the half-life of IgG(T) at 35 days [174]. In foals born to dams hyperimmunized with *Clostridium welchii* antitoxin, maternal IgG antibody persisted in the foals until around 4 months, when levels fell to negligible amounts [173].

Maternal antibody transfer is thought to protect foals during a period of time when endogenous antibody production is just beginning or still very low. The expression
of RAG-1 and RAG-2, which are genes necessary to produce mature B-cells and T-cells, are limited in foals younger than 3 months of age [175]. Because mature B-cells are necessary for the production of immunoglobulins, low RAG-1 and RAG-2 expression may be a factor leading to the low serum immunoglobulin levels in the 1-month-old foals. However, it appears as though endogenous antibody production can still occur in very young foals. Between 1 and 3 months of age, serum IgG and IgG(T) levels in foals begin to rise [172, 173]. Another study found a more delayed but similar pattern, with the lowest serum IgG and IgG(T) concentrations at 1-2 months of age, with an increase around 3-4 months of age [176]. Furthermore, foals deprived of colostrum and milk and instead fed a milk substitute were shown to begin serum IgG production around 2 weeks [173]. By 1 month of age, the IgG levels of these foals had surpassed the IgG levels of foals with adequate passive transfer that received colostrum and were kept with their dams [173]. Around 8 months of age serum IgG(T) levels have been shown to increase rapidly in Fjords, Dole horses, and Shetland ponies, while there was more of a plateau for Thoroughbreds [172, 176]. Serum IgG(T) was higher in 2-year-old horses than in adults, and so it appears to be important in young horses [172]. Serum IgG(T) levels were also higher in 2-4 year old Shetland ponies than in 2-3 year old Thoroughbred racehorses [176]. This could have been due to genetic differences or to management practices, as the ponies were on pasture and the Thoroughbreds would have been stalled, had higher levels of exercise, and were fed a high concentrate diet [176].
Functions of the different IgG isotypes and their significance in young horses are still being determined. There are some indications that the IgG1, IgG3, IgG4, and IgG7 isotypes appear to be important for effector function, and thus, for elimination of intracellular pathogens [177]. Some studies have indicated that certain immunoglobulin classes or isotypes may be associated with Th2 responses, which are commonly associated with parasitic infections as well as allergic responses [178]. Evidence points to a Th2 response for IgG(T), as it has been associated with response to allergens and parasites. Equine serum IgG1 and IgG(T) have been shown to bind to allergens from biting midges [157]. In weanlings infected with trickle doses of *Strongylus vulgaris*, serum levels of IgG(T) were found to increase, while levels of other IgG isotypes remained constant [161]. Correspondingly, IgG(T) performed better than other IgG subtypes in serological diagnosis of *S. vulgaris* infection [179]. Another study found an enzyme-linked immunosorbent assay (ELISA) for detection of IgG(T) better able to diagnose high levels of equine *Anoplocephala perfoliata* infections when compared with IgG [158]. Additionally, in adult ponies, IgG was found in serum at a mean concentration of 1334 mg/100 ml, and IgG(T) at 821 mg/100ml, which were much higher than any other immunoglobulin types [180]. The high levels of IgG(T) in the serum and its association with Th2 type responses make IgG(T) a good candidate for serological testing.

Immunoglobulin E is another immunoglobulin class associated with parasite infection and type I allergic diseases [178]. This immunoglobulin is less commonly found in circulation, as it is produced in smaller quantities and quickly binds to receptors on
mast cells, basophils, and eosinophils [155]. Although IgE is associated with parasite infection, detection of this isotype may be limited during the age when foals are most likely to be infected with ascarids. One study found that serum IgE peaked 2-5 days after foals received colostrum, and then disappeared by 3-4 months of age, indicating that IgE was transferred from mare to foal via colostrum [166]. Passively transferred maternal IgE also can bind to foal peripheral blood leukocytes, and bound IgE was found at high levels at days 2-5, and decreased to undetectable levels by 2 months of age [166]. This immunoglobulin isotype has the shortest half life; it has been estimated at 2 days in other species [181]. Serum levels of IgE in foals were not detectable again until 9-11 months of age, and these horses did not have adult levels until 18 months of age [166]. Serum IgE levels in adult horses have been found to be up to 1000 times higher than IgE levels found in human serum [182], which has been proposed to be caused by the lack of parasitism in humans living in westernized countries. The relatively high IgE levels in equine serum and the association of IgE with parasitism may also make IgE worth examining using a western blot test for *P. equorum*, although levels in the young foal may be too low for detection.

Another immunoglobulin type that could be worth examining for use in serological diagnosis is IgM. This is the first kind of antibody to be produced in any immune response, and it provides protection before the period when somatic hypermutation and isotype switching for production of other antibody types are underway [155]. Serum IgM concentration patterns in foals appear similar to those found for IgG, with some differences. Levels of IgM in day old foals were similar to those
found in adults [176]. Levels declined quickly and were at their lowest at one month of age [176]. However, endogenous production of IgM appeared to reach adult levels faster than the other immunoglobulin isotypes. Levels higher than those present at one day of age were produced by around 2 months for Shetland ponies, and 5 months for Thoroughbreds [176]. In serum, IgM is found in higher concentrations than IgE, but lower than IgG [166, 183]. For example, in day old foals, the concentration of IgM in plasma was about 1/13th of the IgG concentration present. Immunoglobulin M is known to bind to antigens from parasites, such as *Toxoplasma gondii* [184]. The diagnostic test for this parasite also uses antibody tests for IgM in conjunction with tests for IgG so that new infections can be distinguished from previous infections [184, 185]. Nonetheless, this technique is not thought to be effective for *T. canis*, as IgM is produced for a longer duration after initial infection with this species [185].

**The Immune Response to *P. equorum* and other Parasites**

The development of immunity in the horse differs depending on the type of parasite. Strongyle infections appear to lead to incomplete immunity, with the majority of older horses still susceptible to infection, although with fewer parasites when compared with younger horses [7]. In contrast, the development of immunity to *P. equorum* occurs rapidly at a young age, and adult parasites are typically not found in older horses [7, 42, 186]. *Parascaris equorum* egg shedding typically begins when the foal is around 3 months of age, and maximum egg shedding occurs around 5 months of age, after which time egg shedding decreases [186]. Another study measuring worm burden found the highest number of both immature and mature *P. equorum* in foals 3
months of age [3]. After 3-5 months of age, worm burdens decreased monthly to nearly zero by 10 months of age. Epidemiological studies have found foals and yearlings to be more likely to shed ascarid eggs when compared with older individuals [7, 136].

Interestingly, immunity appears to develop by around 6 months of age, even for *P. equorum* naïve foals [38, 186]. When 6-12 month old ponies (reared parasite-free) were experimentally infected with larvated *P. equorum* eggs, those ponies had lower numbers of parasites in the small intestine when compared with parasite-free 2-4 week-old pony foals [186]. Additionally, other researchers, as cited by Clayton and Duncan (1979) tried to induce a patent *P. equorum* infection in adult horses, with unsuccessful results [13, 14]

Another phenomenon has been observed in horses naïve to ascarids. As might be expected, older foals (8-10 months) naïve to *P. equorum* that become infected show more severe respiratory and systemic signs when compared to younger infected foals [186]. These foals show increased density on thoracic radiographs, indicating the intensity of the immune response to migrating larvae [38]. However, larvae do not seem to be able to reach maturity in naïve older horses. In a study using naïve 6-12 month-old horses, when compared with younger foals, few larvae were able to complete the cycle back to the small intestine [186]. This signifies potential for a strong primary immunologic response in the liver and lungs, despite not having encountered the parasite previously.
The secondary immune response may be important to developing immunity to ascarids and other equine parasites. One study found that administration of attenuated (irradiated) *P. equorum* eggs to foals prior to artificial infection with viable eggs decreased worm burden in those foals when compared with controls [47]. Similarly, when weanlings were administered a challenge dose of *S. vulgaris* larvae, those who had previously been given trickle doses of *S. vulgaris* survived, while control ponies naïve to *S. vulgaris* did not [161]. This signifies the importance of multiple small exposures for production of immunity. Another study compared foals that had been raised parasite-free and those raised naturally [186]. After the naturally raised foals were dewormed, both groups were inoculated with 8,000 *P. equorum* eggs [186]. No differences were found in the number of parasites in the small intestine upon necropsy, so age may be more of a factor than previous exposure for the development of immunity [186].

Ascarid infection in horses has also been associated with a change in cell-mediated immunity. From 10 to 40 days following ascarid infection, the number of eosinophils in the foal’s blood rises in proportion to the intensity of the infection [38]. This corresponds with pulmonary eosinophilia during the migration of the ascarid [38]. Eosinophilia has also been implicated with infection of other equine parasites. Pony foals infected with *S. vulgaris* were found to have higher eosinophil levels compared with uninfected ponies, and eosinophil levels decreased following treatment with ivermectin [187]. Eosinophils appear to assist in host defense through the expulsion of cytotoxic granules, presentation of parasite antigen to T-cells, and production of
interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-5 (IL-5), cytokines that are associated with parasite infection and Th2 immune response [188].

The humoral immune response of horses to ascarids has been the topic of limited study. Early immunological testing found a continual increase in the level of serum precipitin titers (indicating increased antibodies) against whole worm antigen during the development of immunity to *P. equorum* in foals and yearlings [47]. Interestingly, the dams were also tested and had high serum precipitin titers throughout the year, even though they were negative for ascarid eggs in the feces [47]. This was thought to have occurred because of the daily challenge of grazing on pastures populated by foals [47]. However, the exact mechanism leading to the development of immunity in mature horses is currently unknown.

Basic immunologic mechanisms of defense have been described for other parasite species. The primary mechanism of defense against parasites is a Th2 CD4 T-cell response, whereas an ineffective Th1 CD4 T-cell response leads to inflammation, tissue damage, and worm survival [155, 189]. The chain of events leading to a Th2-type response involves the presentation of parasite products by dendritic cells (although dendritic cells are not always required for this type of response), which prompt naïve T-cells to become Th2-type T cells [155, 190]. These cells produce cytokines such as IL-4, IL-5, IL-10, and IL-13 that help to upregulate of IgE production and recruit mast cells and eosinophils [88, 155].
The initial development of resistance may be marked by expulsion of parasites. When large numbers of *P. equorum* are present in the small intestine, an immunological reaction may occur to expel the worms [97]. In *A. suum*, larval expulsion has been shown to occur after different dosage levels when larvae complete their migration and return to the small intestine 21 days post-inoculation [191]. This process occurs in conjunction with increases in mucosal jejunum *A. suum*-specific IgA, and serum IgM, IgG1, and IgA specific to L3 ESA [191]. Maximal levels for serum IgM were found on day 14 post-inoculation, whereas maximal levels for serum IgG1 were found on day 21 [191]. An increase in eosinophils was found after the expulsion process had occurred, and there was no change in IgG2 or mucosal mast cell numbers [191]. In uninfected and infected children, there did not appear to be a difference in response of IgG, IgG subtype, IgM, or IgA to three different *A. lumbricoides* antigens [192]. With one of the antigens, infected children had significantly higher IgE response, so IgE may be important for immunity to *A. lumbricoides* [192]. In other parasite infections, IgE has been shown to bind to mast cells and become activated by parasite antigen, causing release of histamine, and subsequent expulsion of worms through contraction of the small intestine [155, 189]. In human *T. canis* infections, parasite ESA appear to prompt a Th2 response [193]. The primary antibody generated against these ESA seems to be IgG1, with substantial levels of IgM and IgE also produced [88]. Host production of these parasite-specific antibodies forms the basis for development of diagnostic tools.
Immunological Diagnostics for Ascarid Infections

Immunological diagnostic tools are presently available for several ascarid species. Whole worm antigen was once the focus of diagnostic test development until the late 1970’s when De Savigny described procedures for the culture of \( T. \) canis larvae, ESA collection, and use of ESA in diagnostic testing [76, 194]. Diagnosis of \( T. \) canis larval migrans in humans is now conducted using a commercial ELISA kit, or through western blotting for identification of low molecular weight proteins [88]. \( Toxocara \) cati infections can be diagnosed through the same tests, as \( T. \) canis and \( T. \) cati ESA are cross-reactive [195]. Recombinant proteins have also been created to try to improve the performance of these tests and reduce cross-reactivity with other species [88]. Most tests for \( T. \) canis larval migrans assess total IgG, although one test saw increased specificity and decreased sensitivity by measuring IgG4 [196]. Serological diagnosis of \( T. \) canis is also possible in dogs. A test for \( T. \) canis infection in dogs and puppies was developed to measure circulating ESA [197]. This sandwich ELISA had a positive correlation between circulating \( T. \) canis antigens and worm burden, and found the highest ESA levels in one-month-old puppies [197].

Excretory-secretory antigens, whole worm antigens, and adult worm body fluid of \( A. \) suum have been examined through SDS-PAGE and western blotting [75, 198]. \( Ascaris \) suum larval ESA exhibit a great deal of homology with \( A. \) lumbricoides and \( T. \) canis ESA, but the antigens can still be distinguished by SDS-PAGE or western blot profile [75, 199]. An ELISA for \( A. \) suum using a protein purified from adult worm body fluid has shown high sensitivity and specificity, and improved results over the FEC [200]. ELISAs
have been used to detect IgG4 responses to ESA or somatic proteins from adult *Ascaris lumbricoides* [201, 202]. Diagnostic tests for *B. procyonis* larval migrans in humans have also been developed. These include western blot assays and ELISAs to detect IgG using crude ESA [203] and recombinant antigen [204]. The recombinant antigen ELISA showed improved sensitivity and specificity over the crude ESA ELISA, along with reduced *Toxocara* cross-reactivity [204]. The number of diagnostic tests developed for other ascarid species indicates strong potential for serological diagnosis of *P. equorum* in horses.

The current methods of *P. equorum* diagnosis in horses are lacking in that they do not allow for detection of migratory larvae and do not correlate with worm burden [37]. Methods of prevention can be difficult for horse owners to implement, and the efficacy of treatment is now questionable. As heavy *P. equorum* burdens can lead to clinical signs and pathological effects, it is essential to learn more about equine immunity to this parasite and develop new tools for monitoring infection.
Figure 3.1. Life cycle of *P. equorum*.
CHAPTER 4: IN VITRO CULTURE OF PARASCARIS EQUORUM AND PRELIMINARY INVESTIGATION OF LARVAL EXCRETORY-SECRETORY ANTIGENS

Introduction

*Parascaris equorum* is a roundworm parasite found ubiquitously in young equids. Infection with this parasite begins when larvated eggs are ingested. The larvae hatch in the host’s small intestine, migrate through the liver and lungs, and return to the small intestine once more to mature and reproduce [15, 16]. It is at the reproductive stage of the life cycle, 10-15 weeks after initial infection [15, 17, 46, 47], when diagnosis can be made using coprological methods. However, this parasite is known to cause damage to the liver and lungs, along with respiratory symptoms during the migrational phase of the life cycle [16, 17, 99]. Because of the delay in diagnosis by coprological methods, early infections in foals may go undetected or clinical signs may be misdiagnosed. This emphasizes the importance of developing new diagnostic tools for *P. equorum* infection.

Diagnostic tests for other ascarid species have been investigated using larval excretory-secretory antigens (ESA). The ESA produced by ascarids are thought to serve a variety of purposes, including migration through host tissue [86, 87], molting [87], survival within the host’s digestive tract [95], and evasion of host immune response [93]. The ESA produced by other ascarid parasites, such as *Toxocara canis* [205, 206], *Toxocara cati* [195], *Ascaris suum* [75, 205, 207-209], *Ascaris lumbricoides* [75, 209], *Baylisascaris procyonis* [77, 203], and *Toxocara vitulorum* [210] have been characterized, but there is no known work describing the ESA of *P. equorum*. Two protease inhibitors have been identified from homogenized whole adult *P. equorum*
(after removal of body fluid). Both showed activity against bovine trypsin and bovine chymotrypsin, and one protease inhibitor showed activity against porcine elastase [94]. Although the proteases were not from the appropriate host species, it still appears that *P. equorum* may produce protease inhibitors to block the action of digestive enzymes and survive in the gastrointestinal tract of the horse. A description of the ESA produced by *P. equorum* larvae would be the first step in identifying potential antigens of importance for diagnostic testing. Comparison of ESA produced by *P. equorum* L2/L3 and L5 stages may also be of interest, as these stages could produce different antigens to assist with the functions of migration versus survival in the gastrointestinal tract. Separation and visualization of these proteins would also lead the way for future analyses to categorize these proteins and their functions.

In-vitro larval culture has been conducted for the collection of ESA produced by other ascarid species [75-77]. The literature does not indicate any hatching or culture attempts for *P. equorum* larvae. Thus, there are no published culture methods particular to this parasite, nor estimates of larval size upon hatching, larval mortality in culture, changes in larval stage in culture, or estimates for antigen production. Such information would be of use for those attempting to collect ESA for characterization or diagnostic purposes.

The specific objectives of this study were to (1) hatch and maintain *P. equorum* and *B. procyonis* larvae in culture for collection of ESA, (2) compare ESA produced by *B. procyonis* and *P. equorum* using sodium dodecyl sulfate polyacrylamide gel
electrophoresis (SDS-PAGE) and silver stain, and (3) identify antigens specific to *P. equorum* larvae through western blot analyses using sera from animals with *P. equorum*, *B. procyonis*, or *T. canis* inoculations, immunizations, and natural infections. Sera used for western blot analyses was obtained from *P. equorum* naturally infected horses, *T. canis* naturally infected dogs, rabbits inoculated with *B. procyonis* or *T. canis* eggs, and rabbits immunized with *P. equorum* or *B. procyonis* ESA collected from larval culture. *Baylisascaris procyonis* and *T. canis* were examined because of the close proximity in which horses, cats and dogs, and raccoons often live. One review cited the use of hay, straw, or animal feed as a major factor associated with *B. procyonis* infection [211]. Although not documented, it is possible that a horse could mount an antibody response against migratory *T. canis* or *B. procyonis* larvae after ingestion of eggs, causing a false positive result on the western blot test. Studies comparing other ascarid species have found similarities in ESA and cross-reactivity on serological analyses [75, 77, 199, 203, 205, 212]. *Toxocara cati* was not examined because of the degree of ESA similarity with *T. canis* [185]. As other ascarid species were expected to have a higher potential for cross-reactivity with *P. equorum* ESA when compared with other equine parasites, comparisons with *B. procyonis* and *T. canis* were emphasized here.

**Materials and Methods**

Antibody collection protocols for immunized rabbits were approved by the Institutional Animal Care and Use Committee at Bethyl Laboratories (20-2011-EM). All sampling procedures were approved (2012-0924) by the University of Kentucky Institutional Animal Care and Use Committee.
Parasite Egg Collection

Mature *P. equorum* specimens were collected from the small intestines of five necropsied foals from the University of Kentucky Veterinary Science Department farm, and from 15 foal necropsy submissions to the University of Kentucky Veterinary Diagnostic Laboratory. Mature *B. procyonis* specimens were collected from the small intestines of 11 raccoons trapped in Kentucky’s Whitley, Bell, or Knox counties between the months of November and February. Additional *B. procyonis* were collected from 16 raccoons collected by a wildlife control company based in Lexington, Kentucky between the months of February and June. Raccoons had been kept at -20 °C for approximately one month prior to worm collection.

Adult worms were maintained in 0.5% formalin, 0.85% saline at 4 °C until dissection. Prior to dissection, female worms were surface-decontaminated in 10% formalin, and rinsed in distilled water. Worms were dissected in distilled water, and the vagina and first 1-2 centimeters of the uterus were removed. Eggs were released from uteri in solution (0.1% formalin, 0.85% saline) using a Tenbroek homogenizer. Eggs were then embryonated in 0.1% formalin, 0.85% saline at room temperature and an egg density of ≤25 eggs per μl. Eggs were checked weekly for larvation, and after 3-5 weeks, eggs were either hatched immediately or stored at 4 °C until hatching. The thickness of the chitinous layer of the shell for *B. procyonis* and *P. equorum* was compared using the measurement tool on Adobe Photoshop 7.0 (Adobe Systems Incorporated, San Jose, CA).
Larval ESA Collection

Eggs that had been stored at 4 °C were maintained at room temperature for two days prior to hatching. Eggs were washed three times in 0.85% NaCl via centrifugation for one minute at 200 x g to remove formalin. Eggs were decorticated by adding 5.25% sodium hypochlorite in a 1:1 solution, and rocking on an orbital rocker until the majority of the chitinous layer of the eggs had been removed. For *P. equorum*, the eggs were washed 10 times in 0.85% NaCl, and stored at room temperature for one week. After one week, the washing steps were repeated, with a 1-2 minute bleaching step to eliminate any bacteria present. For *B. procyonis* eggs, which were less resilient, the washing and bleaching steps were only conducted once, proceeding to hatching on the same day. After washing in 0.85% NaCl, eggs were washed twice in warm (37 °C) Hank’s Balanced Salt Solution (HBSS) without phenol red (Thermo Scientific, Waltham, MA). On the last wash, 10 μl triple antibiotic solution (penicillin 10,000 IU/ml, streptomycin 10 mg/ml, amphotericin B 25 μg/ml; MP Biomedicals, Santa Ana, CA) was added per ml HBSS. Eggs in HBSS and antibiotic solution were hatched with mechanical disruption using a stir bar and glass beads (3 mm and 5 mm in diameter). Hatching solution was transferred to a Baermann apparatus according to the method of Urban et al. [213]. Larvae were collected from the Baermann apparatus four hours later. Larvae were then washed in warm (37 °C) Dulbecco’s Phosphate Buffered Saline (Thermo Scientific, Waltham, MA) by centrifuging 8 times at 200 x g for one minute each time, followed by two washes with warm (37 °C) RPMI-1640 (with L-glutamine and phenol red; Thermo Scientific, Waltham, MA). RPMI-1640 was added as necessary to make a larval
concentration of 10,000 larvae per ml solution. Protease inhibitor cocktail (described below) and triple antibiotic solution (penicillin 10,000 IU/ml, streptomycin 10 mg/ml, amphotericin B 25 μg/ml; MP Biomedicals, Santa Ana, CA) were added to RPMI-1640 to make a 1x concentration of each, and 2-4 ml of the resulting larval solution were transferred to individual 50 ml culture flasks (Research Products International Corporation, Mount Prospect, IL). The type of protease inhibitor cocktail (PIC) used was changed between *P. equorum* culture batches to attempt to decrease mortality, and included a protease inhibitor cocktail with EDTA (AEBSF, aprotinin, bestatin, EDTA, E-64, leupeptin; Sigma-Aldrich, St. Louis, MO), a protease/phosphatase inhibitor cocktail (sodium fluoride, sodium orthovanadate, b-glycerophosphate, sodium pyrophosphate, aprotinin, bestatin, leupeptin; Thermo Scientific, Waltham, MA), and a protease inhibitor cocktail without EDTA (AEBSF, aprotinin, bestatin, E-64, leupeptin and pepstatin A in DMSO; Thermo Scientific, Waltham, MA). Only one type of protease inhibitor (AEBSF, aprotinin, bestatin, E-64, leupeptin and pepstatin A in DMSO; Thermo Scientific, Waltham, MA) was used for the culture of *B. procyonis*.

Larvae were incubated in medium at 37 °C and 5% CO₂. Media were changed every 4 days and used media were filtered using 0.2 μm syringe filters (Corning, Tewksbury, MA). Filtered media were then dialyzed (SnakeSkin Dialysis Tubing, 3.5 MWCO, Thermo Scientific, Waltham, MA) at 4 °C against 0.1M ammonium bicarbonate. Dialyzed media were then frozen at -20 °C and subsequently lyophilized to concentrate protein. Lyophilized media were resuspended in 0.1M ammonium bicarbonate and pooled between culture batches and weeks of collection for the same species. Protein
concentrations were estimated using the Pierce BCA Protein Assay (Thermo Scientific, Waltham, MA) according to the manufacturer’s directions. Protein in solution was then aliquotted and stored at -20 °C.

Larval mortality was monitored weekly by gently shaking the culture flasks, removing a 5 μl subsample from each flask, and viewing larvae by microscopy. Day 0 samples were obtained before the larvae were separated into flasks; therefore, the number of samples taken on day 0 was less than on subsequent days. Cultures were terminated when larval mortality reached 60% or greater, or if bacterial contamination was noted. Weekly measurements of five randomly selected larvae from each flask were also taken using an eyepiece micrometer. Larval length and width at the widest part (approximately 1/3 of the way from the anterior end) were estimated. Sizes for a total of 10 B. procyonis larvae were obtained weekly, and all were from the same culture batch. Sizes for a total of 25 P. equorum larvae were obtained weekly, and were collected from multiple culture batches.

**Immature Adult P. equorum ESA Collection**

At necropsy, two live immature adult L5 P.equorum (approximately 5 centimeters in length) were collected from the small intestine of a yearling from the University of Kentucky Veterinary Science Department farm, and transported in phosphate buffered saline (PBS). The ascarids were washed in warm (37 °C) phosphate buffered saline (PBS) on an orbital rocker 8 times for 2 minutes each time for decontamination. The worms were washed twice in RPMI-1640 (Thermo Scientific,
Waltham, MA) medium with triple antibiotic solution (penicillin 10,000 IU/ml, streptomycin 10 mg/ml, amphotericin B 25 μg/ml; MP Biomedicals, Santa Ana, CA) and then maintained individually in 50 ml culture flasks (Research Products International Corporation, Mount Prospect, IL) in RPMI-1640, triple antibiotic solution, and protease inhibitor cocktail (AEBSF, aprotinin, bestatin, EDTA, E-64, leupeptin; Sigma-Aldrich, St. Louis, MO). Media was changed on day 2, day 3, and day 5, upon which cultures were terminated. Antigen processing procedures were identical to those for larval ESA.

**Sera Collection**

Sera were collected from animals with artificial or natural infection with *P. equorum*, *B. procyonis*, or *T. canis* for qualitative examination by western blot.

Polyclonal antibodies were obtained from two rabbits immunized with *P. equorum* larval ESA and two rabbits immunized with *B. procyonis* larval ESA (Bethyl Laboratories, Montgomery, TX). As the primary focus of this study was on the larval stages, no anti-sera were produced against immature adult *P. equorum*. On day 0 each rabbit was immunized with 100 μg antigen in complete Freund’s adjuvant. Each rabbit was subsequently immunized with 100 μg (*B. procyonis*) antigen or 50 μg (*P. equorum*) antigen and incomplete Freund’s adjuvant on days 14, 28, 42, and 56. Less *P. equorum* protein was used because of the limited availability of this protein. Sera were collected on day 0 prior to immunization, and on day 63. Sera were stored at 4 °C and were tested several days after receipt.
Sera from animals infected with *T. canis* were also collected. Sera remaining from routine veterinary procedures were obtained from two naturally infected adult dogs at the Lexington Humane Society and one naturally infected adult dog at a local veterinary clinic, and were stored at -20 °C. Two animals were found to be shedding eggs in the feces by fecal flotation using sodium nitrate flotation solution (specific gravity 1.25 to 1.30). One animal had expelled adult worms in feces.

Additional sera were obtained from New Zealand White rabbits (kindly provided by Drs. Kevin Kazacos and Sriveny Dangoudoubiyam, Purdue University). Sera were collected from two rabbits pre-inoculation, from two rabbits inoculated with embryonated *T. canis* eggs, and from five rabbits inoculated with embryonated *B. procyonis* eggs. These sera were used in prior studies [77, 203] and had been stored at -20 °C.

Sera were collected from two horses with natural *P. equorum* infections. Serum was obtained from an 8 –month-old colt immediately prior to necropsy for a different study (kindly provided by Drs. Eugene Lyons and Martin Nielsen, University of Kentucky). Upon necropsy, 54 immature and 18 mature *P. equorum* were found in the small intestine. This foal also had a fecal egg count of 40 eggs per gram (EPG), as determined by a previously described modified Stoll method with a detection limit of 10 EPG [46]. Serum was also obtained from a University of Kentucky (Maine Chance Farm) 3-month-old filly positive for *P. equorum* eggs in feces, as confirmed by a previously described fecal flotation method [113]. Blood was collected in a 10 ml serum tube (Vacutainer
Serum Plus Tubes; Becton, Dickinson, and Company, Franklin Lakes, NJ) and centrifuged for 10 minutes at 1000 x g prior to removing and storing serum. Sera were stored at -80 °C until used for western blotting.

**Gel Electrophoresis and Silver Staining**

SDS-PAGE was conducted using a 1.5 mm width 4-20% linear gradient gel with a 4% stacking gel. Broad Range Precision Plus Protein Unstained Standards (Bio-Rad, Hercules, CA) were used in the first lane of the gel (2 µl) to provide a molecular weight reference. Samples were heat denatured for 5 minutes 95 °C in 5x sample buffer (1.5 M Tris HCl, pH 6.8, with 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.01% bromophenol blue). Samples included 5 μg each of *P. equorum* larval ES protein, *P. equorum* immature adult ES protein, and *B. procyonis* larval ES protein. Following gel electrophoresis, the gel was silver-stained using the manufacturer’s instructions for the Silver Stain Plus Kit (Bio-Rad, Hercules, CA).

**Gel Electrophoresis and Western Blotting**

SDS-PAGE was conducted using 0.75 mm 2-D/prep 12% gels with a 4% stacking gel. Broad Range Precision Plus Protein Unstained Standards (Bio-Rad, Hercules, CA) were used in the first lane of the gel (2 µl) to provide a molecular weight reference. Samples were heat denatured for 5 minutes 95 °C in 5x sample buffer (1.5 M Tris HCl, pH 6.8, with 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.01% bromophenol blue), with an additional 100 µl of 1x sample buffer (62.5 mM Tris HCl, pH 6.8, with 2% SDS, 10% glycerol, 0.01% β-mercaptoethanol, 0.01% bromophenol blue) to help
distribute the solution in the large 2-D/prep well. Samples to be run in the large well of the gel included 4 µg of either *B. procyonis* larval ESA or *P. equorum* larval ESA for the serum from rabbit immunizations or 20 µg of either *B. procyonis* larval ESA or *P. equorum* larval ESA for the serum from rabbit inoculations. An increased amount of protein was used when blotting sera from rabbit inoculations because these sera had been stored for longer period of time. After electrophoresis, proteins were electrophoretically transferred to a 0.45 µm polyvinylidine fluoride (PVDF) membrane (Westran Clear Signal; GE Healthcare, Piscataway, NJ). Membranes were washed twice for 5 minutes on an orbital rocker in 1x Tris Buffered Saline with Tween 20 (TBST). Next, membranes were washed in a 0.1% India Ink solution for 15-20 minutes for visualization of proteins, and then washed 4 times for 5 minutes each in 1X TBST. Membranes were air-dried and stored at 4 °C for up to 2 weeks. For blotting, membranes were allowed to equilibrate to room temperature, and then were blocked on an orbital rocker for one hour in 5% fat-free milk solution prepared in 1x TBST. Membranes were washed an additional 3 times in 1x TBST prior to clamping into a multiscreen apparatus (Mini-PROTEAN II, Bio-Rad, Hercules, CA).

For primary antibody preparation, sera were mixed with blocking buffer in a 1/1250 dilution, except for sera from inoculated rabbits, which were mixed in a 1/500 dilution. Primary antibody incubation was conducted in the multiscreen for 2 hours at room temperature on a platform rocker at a very slow speed. Samples were vacuumed from the multiscreen and lanes were washed 6 times with 1x TBST on the platform rocker for 5 minutes each. Next, secondary antibody was prepared at different dilutions.
in blocking buffer depending on the type of primary antibody. Secondary antibodies included horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG with heavy and light chains (H+L) at a 1/50,000 dilution in blocking buffer (Thermo Scientific, Waltham, MA), goat anti-canine IgG (H+L) at a 1/50,000 dilution (Southern Biotechnology Associates, Birmingham, AL), and goat anti-horse IgG(T) at a 1/100,000 dilution (AbD Serotec, Raleigh, NC). Molecular weight standards were incubated with StrepTactin-HRP (Bio-Rad, Hercules, CA) at a 1/100,000 dilution in blocking buffer. Secondary antibody incubation was conducted for one hour at room temperature on a platform rocker. Lanes were then washed 6 times in 1X TBST before using a chemiluminescent kit according to the manufacturer’s instructions (Amersham ECL Prime; GE Healthcare, Piscataway, NJ). Antibody recognition was visualized using a FluorChemE imager (ProteinSimple, Santa Clara, CA). Molecular weights of proteins were estimated using the AlphaVIEW SA program (Version 3.2.4.0; Cell BioSciences Inc., Santa Clara, CA).

**Statistical Analysis**

Statistical analysis was conducted using SAS version 9.3 (SAS Institute Inc., Carey, NC). Differences for *B. procyonis* prevalence by geographical area were examined using PROC FREQ. Descriptive statistics for larval mortality and larval sizes were obtained using the PROC MEANS command, and mean larval sizes were compared by week between the two species using PROC TTEST (unpaired). In the case of unequal variances, the Satterthwaite method was used to calculate the p-value. Mean larval sizes for day 0 and day 21 were also compared within species using PROC TTEST (paired). Where statistical comparisons were made, a p-value of <0.05 was deemed significant.
Results

Specimen Collection

Of 55 raccoons captured in central Kentucky between February and June, 29.1% were positive for *B. procyonis*, with a mean of 3 worms (SD=2.3 worms) in the small intestine. Of 61 raccoons trapped in southeastern Kentucky between November and February, 18.0% were positive for *B. procyonis*, with a mean of 14 worms (SD=9.3) in the small intestine. There was no statistical difference for prevalence by geographical area using the chi-square test (p=0.159).

Larval Culture

Larval culture of *P. equorum* proved challenging, and required a modification in order to promote sufficient hatching and larval recovery. Without the modification (proceeding directly from bleaching and washing to hatching), the mean *P. equorum* larval recovery percentage was 3.2% (SD=4.3%) from seven attempts. With the modification (bleaching and washing, maintaining in saline solution for a week, and then briefly bleaching and washing again), the mean larval recovery percentage was 48.3% (SD=21.5%) from seven attempts. Differences in the thickness of the eggshell for *B. procyonis*, *T. canis*, and *P. equorum* were noted visually (Figure 4.1) and the chitinous layer shell of *P. equorum* was found to be 4.7 times thicker than the shell of *B. procyonis*.

Percent mortality of *P. equorum* larvae after hatching also differed by protease inhibitor (Table 4.1). Due to the high mortality of larvae in cultures containing the PIC with EDTA (AEBSF, aprotinin, bestatin, EDTA, E-64, leupeptin; Sigma-Aldrich, St. Louis,
MO), subsequent culture attempts used two other types of PIC. Cultures of *P. equorum* were sustainable for approximately 3 weeks, after which time mortality was too high to maintain cultures for production of ESA. *Baylisascaris procyonis* was also maintained for 3 weeks, at which time mean mortality within the six culture flasks reached 77.3% (SD=17.6%).

Morphological differences were noted for *P. equorum* larvae when compared with *B. procyonis*. At all time points, *B. procyonis* larvae were wider than *P. equorum* larvae (p<0.001) (Table 4.2). While *B. procyonis* larvae appeared to grow noticeably in culture, changes in size for *P. equorum* were not as apparent (Table 4.2). However, paired t-test results indicated an increase in length and width by both *P. equorum* and *B. procyonis* between day 0 and day 21 (p<0.001). Shedding of the cuticle was also observed for *P. equorum* (Figure 4.2) and *B. procyonis* (Figure 4.3) while in culture.

*Parascaris equorum* cultured in Thermo Scientific PIC (AEBSF, aprotinin, bestatin, E-64, leupeptin and pepstatin A in DMSO; Thermo Scientific, Waltham, MA) produced 38 µg protein/10,000 hatched larvae over the course of 21 days. In comparison, *B. procyonis* larvae cultured in the same PIC produced 64 µg protein/10,000 hatched larvae during a 21 day culture period. Immature adult *P. equorum* produced 93 µg of protein per worm over the 5 day culture period.

**SDS-PAGE**

Silver-stained SDS-PAGE results displayed multiple ESA produced by larval *P. equorum*, larval *B. procyonis*, and immature adult *P. equorum* (Figure 4.4). Proteins that
were visualized by silver stain ranged in size from an estimated 12-94 kDa for *P. equorum* larval ESA, 12-189 kDa for *P. equorum* immature adult ESA, and 13-244 kDa for *B. procyonis* larval ESA. Banding patterns indicate that many proteins of homologous molecular weights were excreted/secreted by the two species of larvae, but *P. equorum* larvae did not appear to produce some of the high molecular weight proteins produced by *B. procyonis* larvae and *P. equorum* immature adults.

**Western Blotting**

In western blot analysis, the two horses naturally infected with *P. equorum* had IgG(T) antibodies that recognized larval *P. equorum* ESA (Figures 4.5, 4.6, Table 4.3). Antibody recognition by both horses occurred at approximately 19 kDa, 22 kDa, 26 kDa, and 34 kDa. Pre-immunization with *P. equorum* or *B. procyonis* ESA, rabbits showed some antibody reactivity at high molecular weights (approximately 90 kDa and 120 kDa) (Figure 4.5). Pre-inoculation with *P. equorum* or *B. procyonis* eggs, rabbits did not appear to have any IgG antibodies to *P. equorum* larval ESA (Figure 4.6). Serum from one rabbit immunized with *P. equorum* ESA had IgG antibodies that recognized proteins at 19 kDa, 22 kDa, 26 kDa, and 34 kDa (Figure 4.5 and Table 4.3). Serum from rabbits immunized with *B. procyonis* ESA recognized only the 26 kDa *P. equorum* protein, along with high molecular weight proteins (Figure 4.5 and Table 4.3). Serum from rabbits inoculated with *B. procyonis* eggs also recognized the high molecular weight proteins (Figure 4.6). Similarily, rabbits inoculated with *T. canis* eggs showed reactivity at these high molecular weights, along with 22 kDa and 26 kDa, as did one of the dogs with natural *T. canis* infection (Figure 4.6 and Table 4.3). One dog did not show any antibody
reactivity to *P. equorum* antigens (Figure 4.6), and another recognized only high molecular weights.

Sera blotted on *B. procyonis* larval ESA recognized antigens of different molecular weights. Serum from the *P. equorum* positive necropsied colt had very faint reactivity at approximately 52 kDa and 250 kDa (Figures 4.7 and 4.8). Serum from the filly positive for *P. equorum* eggs in feces faintly recognized ESA at approximately 30 kDa and 34 kDa (Figure 4.7). Rabbits immunized with *P. equorum* ESA showed antibody reactivity when the imager exposure time was increased, and recognized antigen at approximately 13 kDa, 40 kDa, 68 kDa and around 250 kDa (Figure 4.7). Serum from one rabbit showed additional antibody recognition at 33 kDa and 35 kDa. As might be expected, a greater number of *B. procyonis* than *P. equorum* antigens were detected by animals naturally infected with *T. canis*, or inoculated with *B. procyonis* or *T. canis* eggs (Figures 4.7 and 4.8).

**Discussion**

**Larval Culture**

The hatching method originally attempted with *P. equorum* has been reported to yield recovery of 80-90% of *A. suum* larvae [213, 214]. The *P. equorum* yield obtained by following this protocol was extremely low. This could be related to the morphology of the egg shell. In other species, such as *A. lumbricoides* and *A. suum*, the chitinous layer has been reported to be 2 µm [215] or 3-4 µm [58] in thickness. Although measurements of the egg shells were not taken in this study, the shell of *P. equorum*
was comparatively thicker than that of *B. procyonis*. *Parascaris equorum* eggs are also more spherical when compared to *T. canis* and *B. procyonis* eggs, which could potentially contribute to the strength of the shell. The modification of bleaching and washing, and then allowing eggs to remain in physiological saline solution may have increased permeability of the shells. The bleaching time for the second hatching attempt was very short, and is not expected to have had much effect on further decreasing the chitinous layer of the shell. For future *P. equorum* culture attempts, it may also be worthwhile to examine extensive bleaching times and concentrations above what are reported for other species. Although the modification of bleaching and washing twice led to improved yields, these were still lower than what was obtained for *A. suum*. Additional investigation is needed to improve hatching yields for *P. equorum*.

Larval mortality by week for *P. equorum* and *B. procyonis* in unsupplemented media for ESA collection was similar to what has been described in some previous work. Dangoudoubiyam and Kazacos (personal communication) maintained *B. procyonis* in Dulbecco’s modified Eagle’s medium for 3 weeks until mortality reached 50%, while another researcher was able to maintain *B. procyonis* larvae in the same medium for 6 weeks [77]. *Toxocara vitulorum* was reported to survive in RPMI-1640 media in culture for up to 3 months, but mortality data was not provided [78]. *Toxocara canis* can be cultured in Eagle’s minimal essential medium with Hank’s salts for up to 18 months without significant mortality [76]: this is thought to be because of the ability of *T. canis* larvae to remain dormant and encysted in adult female dogs. In the same type of medium, *A. suum* has been shown to survive for only 3 weeks without substantial
mortality [76]. Larval survival for *P. equorum* was shorter when the protease inhibitor cocktail containing EDTA was used. Although the same protease inhibitor containing EDTA did not appear to harm cultures of *Strongylus vulgaris* (the equine large strongyle) [179], it appears that it is not a good choice for *P. equorum* larval culture.

Size differences in culture were noted for *B. procyonis* and *P. equorum*. Although only rough estimates of larval length can be provided due to the methods used in this study, the estimates for *B. procyonis* (Table 4.2) were extremely similar to what has been previously described. Mean lengths of fixed *B. procyonis* after hatching have been reported at 278 µm on day 0, 333 µm on day 7, 409 µm on day 14, and 451 µm on day 21 [77]. For *P. equorum*, lengths of larvae recovered at necropsy have been reported. On days 1, 3, and 5 after infection, larvae in the stomach, small intestine, and liver were reported to be 200 µm in length [16]. By day 7, larvae in the liver had increased to 600 µm in length [16]. As our larvae did not reach this size, it appears that development occurred more slowly in culture than it would have within the host. However, cuticle shedding was noted for both *B. procyonis* and *P. equorum*. Other studies have identified cuticle shedding and larval growth during culture of *A. suum* [216] and *B. procyonis* [77], while *T. canis* remained in the L2 stage [76]. If *P. equorum* cuticle shedding occurs in culture, it appears that the infective unhatched larva is in the L2 stage. The molt to L3 stage appears to be completed after hatching, and probably prior to or shortly after reaching the liver.

The amount of protein produced by each species may not be completely accurate, as the silver-stained gel indicated that the 5 µg sample of *B. procyonis* ESA
contains more protein than the *P. equorum* lanes (Figure 4.4). The BCA kit may have underestimated production by *B. procyonis*, or overestimated production by *P. equorum* larvae and immature adults. Regardless, it appears that *B. procyonis* larvae produce a greater amount of protein in culture than *P. equorum*. While Boyce and colleagues estimated that each *B. procyonis* larva produced 1-3 ng of protein per day for the first 3 weeks in culture [77], we estimate that each *P. equorum* larva produced 0.18 ng protein/day, and each *B. procyonis* larva produced 0.30 ng protein/day. It is possible that differences in egg storage or culture methods could have affected larval *B. procyonis* ability to produce ESA.

**SDS-PAGE**

SDS-PAGE results indicated that *P. equorum* larvae and immature adults excrete/secrete a range of molecules (Figure 4.4). Silver-stain results indicate that *P. equorum* larvae produced dominant proteins that were smaller than 100 kDa, with approximately 13 bands that could be visualized. There were some common bands among the three parasite types, along with several differences. Differences observed between *P. equorum* larvae and immature adults could have been attributed to variation in individual populations, as other researchers have noted changes in banding by culture batch [217], or they could have been due to changes in proteins produced by life stage. Other researchers who examined homogenized whole adult *P. equorum* worm identified two broad and unresolved low molecular weight bands (7 and 9 kDa) as protease inhibitors [94]. *Parascaris equorum* larvae and immature adults produced diffuse areas of banding below 10 kDa, but we cannot speculate on the identity or
function of these proteins. Another study examined somatic antigens of adult *P. equorum*, and found a large number of bands ranging in size from below 15 kDa up to 200 kDa [218]. We observed fewer proteins, but it is expected that a smaller number of proteins would be excreted/secreted than would be contained within the body structures.

**Western Blotting**

Silver-stained *P. equorum* larval proteins at approximately 22 kDa, 26 kDa, and 34 kDa were also recognized by IgG(T) antibodies of both *P. equorum* infected horses (Figures 4.4, 4.5, and 4.6). The protein at 19 kDa was not clearly visualized by silver-staining. This indicates that the amount of that protein in the gel may have been too low to be detected by silver stain, but that protein may elicit a relatively strong immune response in the host. Of the *P. equorum* antigens of importance recognized by *P. equorum* infected horses, only the 26 kDa band was recognized by the rabbits immunized with *B. procyonis* ESA. Of the rabbits immunized with *P. equorum* ESA, one rabbit responded similarly to the horses, while the other rabbit responded more like the rabbits immunized with *B. procyonis* ESA. This could have been due to the lower amount of protein used for *P. equorum* immunizations, or differences in immune response of the two rabbits. As many of the same antigens were recognized by IgG(T) antibodies of horses and the IgG antibodies of the immunized rabbit, it appears that the rabbit IgG isotypes corresponding to equine IgG(T) are the antibodies of major importance. Of the rabbits inoculated with *B. procyonis* or *T. canis* eggs, cross-reactivity with antibodies to *P. equorum* was only noted at the 22 kDa and 26 kDa antigens. These results indicate
that the antigens with the greatest potential for distinguishing the two types of infection are located at 19 kDa and 34 kDa. Antibody recognition of the higher molecular weight molecules appears common to all of the sera and *P. equorum* larval ESA combinations examined.

Western blots using *B. procyonis* larval ESA and sera from *B. procyonis* and *T. canis* infected animals indicated that sera were acceptable for use as controls (Figures 4.7 and 4.8). Although our main goal was to examine the diagnostic potential of a western blot test for *P. equorum*, these western blots provided new information on the potential for cross-reactivity when testing for *B. procyonis* infection. Antibody recognition of *B. procyonis* larval ESA was very weak for *P. equorum* infected horses. Antibody binding to *B. procyonis* ESA was also weaker for rabbits immunized with *P. equorum* ESA when compared with rabbits immunized with *B. procyonis* ESA, as might be expected. Recognition of antigen by *P. equorum* immunized rabbit sera could only be visualized by increasing the imager exposure time above what was needed for the *B. procyonis* immunized rabbits. Dangoudoubiyam and Kazacos used antibody binding between 30-45 kDa as criteria to identify infections with *B. procyonis* [203], and although weaker, we did identify antibody binding in this region by rabbits immunized with *P. equorum* ESA. This suggests that there is potential for cross-reactivity when using the western blot test for diagnosis of *B. procyonis* infection. It also appears that there were differences in ESA produced or collected during our *B. procyonis* culture when compared with prior studies, as antigen recognition patterns by rabbits inoculated
with *B. procyonis* and *T. canis* eggs did not provide an exact match for what has been previously described using the same serum [203].

**Specimen Collection**

It is worthwhile to report the prevalence of *B. procyonis*, as ingestion of infective eggs can lead to larval migrans and health concerns in humans. In the eastern and midwestern United States, the prevalence of *B. procyonis* in raccoons has been found to decrease from north to south, with prevalence as high as 86% in Illinois, decreasing to 0% in Florida and Alabama [211]. The prevalence of *B. procyonis* in raccoons has been previously described for western Kentucky, but reports for other areas of Kentucky could not be found in the literature. One study found a very low prevalence of 3% out of 145 raccoons collected throughout the year in western Kentucky and northern Tennessee [219]. Another study in western Kentucky found 30% prevalence in 70 raccoons collected between December and May [220], which is similar to the findings for central Kentucky (29%) in our study. The majority of the positive raccoons from western Kentucky were from an agricultural region [220]. In midwestern states, *B. procyonis* prevalence was found to be higher in rural areas than urban areas [221], and *B. procyonis* is also thought to be more common in mountainous areas [211]. Conversely, a study in Georgia found that prevalence was higher in urban/suburban raccoons, when compared to rural raccoons [222]. The majority of raccoons collected from central Kentucky were from urban or suburban locations, while the area in southeastern Kentucky is more rural and mountainous. However, we did not find a
statistical difference in prevalence between the two locations, and the prevalence in the urban area was numerically higher. If the parasite is more sparsely distributed in southern states, *P. equorum* could be more prevalent in urban areas because raccoon population densities are also higher in those areas [223], helping to maintain transmission of the parasite. Also, because *B. procyonis* is more prevalent in juveniles than adults, results could differ because of the ages of raccoons sampled, as well as the time of year of collection [211]. The highest prevalence of *B. procyonis* appears to occur in the fall, followed by a rapid decline in the winter months [211]. The raccoons from southeastern Kentucky were all obtained during trapping season in the winter months; thus, the prevalence in this region may be higher at other time points throughout the year.

**Conclusion**

The results of this study provided new information on the culture and ESA production of *P. equorum* larvae, and the potential for IgG/IgG(T) antibody recognition of *P. equorum* ESA. Larval culture results indicated that a modified hatching technique may be required to obtain sufficient numbers of *P. equorum* for culture, but that hatching and *in-vitro* maintenance of this parasite are possible. Excretory-secretory antigen production of this parasite was low, so larger cultures of this parasite may be required to obtain adequate antigen for diagnostic testing. *Parascaris equorum* larvae appear to molt and grow in culture, indicating that they hatch as the L2 stage. For
western blotting, antigens with the potential to diagnose *P. equorum* infection were identified at 19 kDa and 34 kDa. Some cross-reactivity between species was noted at 22 kDa, 26 kDa, and at high molecular weights. It appears that there may be potential for diagnosis of *P. equorum* infection using larval *P. equorum* ESA.
Table 4.1. Mean and standard deviation of *P. equorum* larval mortality percentage by type of protease inhibitor cocktail.*

<table>
<thead>
<tr>
<th>Days Post-Hatching</th>
<th>Sigma-Aldrich PIC (with EDTA)</th>
<th>Thermo Scientific Phosphatase PIC (without EDTA)</th>
<th>Thermo Scientific PIC (without EDTA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>16.1 (12.6) n=2</td>
<td>8.3 (2.5) n=4</td>
<td>5.5 (2.5) n=3</td>
</tr>
<tr>
<td>7</td>
<td>63.7 (16.9) n=3</td>
<td>34.6 (27.9) n=14</td>
<td>24.2 (9.0) n=12</td>
</tr>
<tr>
<td>14</td>
<td>71.4 n=1</td>
<td>47.9 (14.7) n=11</td>
<td>41.9 (13.4) n=12</td>
</tr>
<tr>
<td>21</td>
<td>-</td>
<td>76.0 (14.0) n=10</td>
<td>75.3 (7.1) n=12</td>
</tr>
</tbody>
</table>

*The number of culture flasks is represented by “n.” On day 0, samples were taken prior to aliquoting into flasks.
Table 4.2. Mean and standard deviation of lengths and widths of *P. equorum* and *B. procyonis* larvae (µm).

<table>
<thead>
<tr>
<th>Days Post-Hatching</th>
<th><em>P. equorum</em> Length</th>
<th><em>B. procyonis</em> Length</th>
<th><em>P. equorum</em> Width</th>
<th><em>B. procyonis</em> Width</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>263 (19)</td>
<td>275 (26)</td>
<td>12* (2)</td>
<td>25* (3)</td>
</tr>
<tr>
<td>7</td>
<td>277* (17)</td>
<td>328* (26)</td>
<td>14* (2)</td>
<td>30* (5)</td>
</tr>
<tr>
<td>14</td>
<td>274* (20)</td>
<td>367* (73)</td>
<td>15* (4)</td>
<td>37* (11)</td>
</tr>
<tr>
<td>21</td>
<td>291* (25)</td>
<td>498* (50)</td>
<td>17* (5)</td>
<td>40* (8)</td>
</tr>
</tbody>
</table>

*Length or width means within a row differ between species, *P < 0.05.*
Table 4.3. Number of animals exhibiting antibodies to *P. equorum* ESA.

<table>
<thead>
<tr>
<th>Host Species</th>
<th>Infection/Injection Type</th>
<th>Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse</td>
<td><em>P. equorum</em> natural infection</td>
<td>2/2 2/2 2/2 2/2</td>
</tr>
<tr>
<td>Rabbit</td>
<td><em>P. equorum</em> ESA</td>
<td>1/2 1/2 2/2 1/2</td>
</tr>
<tr>
<td>Rabbit</td>
<td><em>B. procyonis</em> ESA</td>
<td>0/2 0/2 2/2 0/2</td>
</tr>
<tr>
<td>Rabbit</td>
<td><em>B. procyonis</em> eggs</td>
<td>0/5 0/2 0/2 0/2</td>
</tr>
<tr>
<td>Rabbit</td>
<td><em>T. canis</em> eggs</td>
<td>0/2 2/2 2/2 0/2</td>
</tr>
<tr>
<td>Dog</td>
<td><em>T. canis</em> natural infection</td>
<td>0/3 1/3 1/3 0/3</td>
</tr>
</tbody>
</table>
Figure 4.1. Eggs of *B. procyonis*, *T. canis*, and *P. equorum* (not to scale). A: *B. procyonis* egg undergoing division. B: *T. canis* unicellular egg. C: *P. equorum* unicellular egg. D: *P. equorum* egg with outer proteinaceous layer removed and mechanical disruption of chitinous layer of shell.
Figure 4.2. Cuticle shedding by *P. equorum* larvae, day 21 in culture (A and B). Arrows indicate the shed cuticle. The larva in photo B shed a thicker cuticle than typically noted in culture.
Figure 4.3. Cuticle shedding by *B. procyonis* larva, day 14 in culture. The arrow indicates the shed cuticle.
Figure 4.4. Silver-stained *P. equorum* and *B. procyonis* ESA on a 4-20% gradient gel. Lane 1, molecular weight standards; Lane 2, *P. equorum* larval ESA; Lane 3, *P. equorum* immature adult ESA; Lane 3, *B. procyonis* larval ESA.
Figure 4.5. Western blot of larval *P. equorum* ESA recognized by *P. equorum* naturally infected equine IgG(T) and *P. equorum* ESA immunized rabbit IgG. Lane 1, molecular weight standards; Lane 2, colt positive for *P. equorum* at necropsy, anti-horse IgG(T) secondary antibody; Lanes 3-6, pre-immunization rabbit sera; Lanes 7-8, rabbits immunized with *B. procyonis* ESA (day 63); Lanes 9-10, rabbits immunized with *P. equorum* ESA (day 63).
Figure 4.6. Western blot of larval *P. equorum* ESA recognized by *P. equorum* naturally infected equine IgG(T), *B. procyonis* or *T. canis* inoculated rabbit IgG, and *T. canis* naturally infected canine IgG. Lane 1, molecular weight standards; Lane 2, colt positive for *P. equorum* at necropsy; Lane 3, filly positive for *P. equorum* eggs in feces; Lanes 4-6, pre-immunization/inoculation rabbits; Lanes 7-11, rabbits inoculated with *B. procyonis* eggs; Lanes 12-13, rabbits inoculated with *T. canis* eggs; Lanes 14-16, dogs with natural *T. canis* infections.
Figure 4.7. Western blot of larval *B. procyonis* ESA recognized by *P. equorum* naturally infected equine IgG(T) and *P. equorum* or *B. procyonis* immunized rabbit IgG. A: Lane 1, molecular weight standards; Lane 2, colt positive for *P. equorum* at necropsy; Lane 3, filly positive for *P. equorum* eggs in feces; Lanes 4-7, pre-immunization rabbits; Lanes 8-9, rabbits immunized with *P. equorum* ESA (day 63). B: Lanes 10-11, rabbits immunized with *B. procyonis* ESA (day 63).
Figure 4.8. Western blot of larval *B. procyonis* ESA recognized by *P. equorum* naturally infected equine IgG(T), *B. procyonis* or *T. canis* inoculated rabbit IgG, and *T. canis* naturally infected canine IgG. Lane 1, molecular weight standards; Lane 2, colt positive for *P. equorum* at necropsy; Lane 3, filly positive for *P. equorum* eggs in feces; Lanes 4-6, pre-inoculation/immunization rabbits, Lanes 7-11 rabbits inoculated with *B. procyonis* eggs; Lanes 12-13, rabbits inoculated with *T. canis* eggs; Lanes 14-16, dogs with natural *T. canis* infections.

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CHAPTER 5: ANTIBODY RESPONSE OF HORSES TO *PARASCARIS EQUORUM* EXCRETORY-SECRETORY ANTIGENS

**Introduction**

*Parascaris equorum* is an ascarid parasite that infects foals and young horses. This parasite has been found on every continent except Antarctica [5, 6, 8, 10, 11, 131], and in Kentucky, prevalence in foals by farm has been reported to range from 0% to 64% [114]. The successful survival of this parasite is at least partially due to the life cycle and the structure of the egg. Transmission occurs when eggs are shed in the feces of an infected equid, the eggs larvate, and an equid ingests the infective eggs [38]. The microscopic eggs of *P. equorum* have been reported to remain viable for at least 18 months to 3 years [21, 22], making this a difficult parasite to manage. Once infective eggs are ingested, larvae hatch and migrate from the small intestine through the liver and lungs, and then return to the small intestine for maturation and reproduction [15, 16]. During the migrational phase of the life cycle, larvae can cause pathologies of the liver and lungs, along with respiratory symptoms [16, 17, 99]. Diagnosis can be made using microscopy to check for parasite eggs in feces. However, the appearance of eggs does not occur until parasites reach the reproductive stage of the life cycle, 10-15 weeks after initial infection [15, 17, 46, 47]. Thus, prepatent infections cannot currently be diagnosed. Once larvae return to the small intestine to mature, new issues arise. Large worm burdens in the small intestine can lead to weight loss, depression, lowered serum and total body albumin, and even intestinal blockage or rupture [17, 20, 97, 102, 104]. Copromicroscopic methods for detection of the parasite eggs do not provide an
accurate depiction of the total worm burden in the small intestine [37]. Therefore, it becomes problematic to use copromicroscopic methods, such as the fecal egg count (FEC), for making treatment recommendations or evaluating the efficacy of anthelmintics.

As copromicroscopic methods are currently the only methods available for diagnosis of *P. equorum*, the development of new diagnostic tools would be beneficial to equine health, and could help to improve our knowledge of the host immune response to this parasite. Serological tests have been developed for diagnosis of other ascarids or equine parasites, and often utilize excretory-secretory antigens (ESA) collected from parasites in-vitro [77, 160, 179, 194, 195, 203, 224]. Excretory-secretory antigens have been used in the development of serological tests for infection or larval migrans of *Toxocara canis* [194, 205, 206, 210], *Baylisascaris procyonis* [77, 203], *Ascaris suum* [75, 205, 225, 226], and *Ascaris lumbricoides* [201, 205].

Tools for serological diagnosis of other equine parasites have been developed using ESA. For example, a western blot test, and subsequently, an ELISA test have been developed using ESA from the equine tapeworm, *Anoplocephala perfoliata* [158, 160]. Diagnostic potential of this test was improved by monitoring for IgG(T) instead of IgG, and using a recombinant protein based on the 12/13 kDa ESA [158]. In horses, IgG(T) actually consists of two isotypes (IgG3 and IgG5) that were formerly classified together as one [162, 163]. These isotypes, together as IgG(T), have also been used for diagnosis of other equine parasites. Recently, ESA from a large equine strongyle species, *Strongylus vulgaris*, were evaluated using a western blot test, and a recombinant
protein was developed for ELISA use [179]. This study also found that IgG(T) had better diagnostic accuracy than IgGa or total IgG [179]. These studies indicate the potential for a new serological tool for diagnosis of *P. equorum* utilizing ESA.

Sera from two foals with *P. equorum* infection had been previously examined and found to contain IgG(T) antibodies that recognized antigens that migrated at approximately 19 kDa, 22 kDa, 26 kDa, and 34 kDa, indicating the potential for diagnostic use (Figures 4.5 and 4.6). Other secondary antibody types have not yet been examined for diagnosis of *P. equorum*. Although not currently used in other diagnostic tests for equine parasites, investigation of other immunoglobulin isotypes is warranted. For example, Immunoglobulin M (IgM) is the first immunoglobulin type produced in response to infection [155], and antigen binding by IgM is used for diagnosis of *Toxoplasma gondii* infection [184]. Immunoglobulin E may also be useful for diagnosis, as IgE is commonly associated with parasitic disease [178], and serum levels of IgE in horses are much higher than levels found in human serum [182].

The objectives of this study were to (1) compare IgE, IgM, and IgG(T) antibody recognition of larval *P. equorum* ESA using sera from infected horses, (2) test the sera from a cohort of broodmares and their foals from birth until a period of no egg shedding, and (3) examine equine controls with monospecific parasite infections to identify possible cross-reactivity.

**Materials and Methods**

The University of Kentucky Institutional Animal Care and Use Committee (2012-0924) approved all procedures used in this study.
Antigen Collection

*Parascaris equorum* egg collection, embryonation, and hatching were conducted as previously described (Chapter 4). Larvae (L2/L3 stages) were maintained *in-vitro* for collection of ESA, as described elsewhere (Chapter 4).

Animals and Housing

A total of 18 Thoroughbred broodmares at University of Kentucky’s Maine Chance Farm (MCF) were used for this study. The mean age of the mares was 8.8 years (SD=4.4 yr), and the range was 4-20 years. All mares were in foal to Thoroughbred stallions. Broodmares were kept on pasture for the majority of the year, and were moved to smaller 5 acre pastures in groups of 4-7 near the foaling barn at least one month prior to the expected foaling date. Mares were housed in foaling stalls within several days prior to giving birth. Actual foaling dates were between 2/29/12 and 4/7/12. Mares are designated Mare1-18, and foals are correspondingly designated Foal1-18, based on the order in which foals were born.

Immediately after giving birth, mare colostrum was checked using a Brix equine colostrum refractometer (Animal Reproduction Systems, Chino, CA). For four mares (Mare6, Mare7, Mare10, and Mare11), the colostrum score was at or below 20% (corresponding to a concentration of approximately 50 g IgG/L or lower), and the foal was supplemented with banked colostrum from a MCF mare with a good colostrum score. Serum was collected the day after foaling to check serum IgG levels. Two foals (Foal5 and Foal16) with failure of passive transfer (IgG <800 mg/dl) were given plasma provided by a plasma bank (Equine Medical Associates, Lexington, KY). Overall, 12 foals
received colostrum only from their dam and had adequate passive transfer, while four foals required supplemental colostrum, and two foals required plasma. Results from all foals were analyzed.

For the first 1-2 weeks after birth, foals were kept in stalls with their dams at night and were turned out in small paddocks in individual mare-foal pairs during the day. Mare and foal pairs were later grouped together in larger pastures and were stalled only for health care or training. The locations of the fields and groupings changed several times during the study. Foals were weaned in August or September by gradually removing mares from each field.

**Sera and Fecal Collection Procedures**

Blood from each horse was collected (schedule below) in a 10 ml serum tube (Vacutainer Serum Plus Tubes; Becton, Dickinson, and Company, Franklin Lakes, NJ) and centrifuged for 10 minutes at 1000 x g prior to removing and storing serum. Sera were stored at -80 °C until used for western blotting.

For MCF broodmares, ≥10 g of feces were collected from a freshly deposited sample (schedule below). Samples were stored at 4 °C and processed within one week after collection using a modified Stoll FEC method. For this method, 10 g feces were mixed with 90 ml double distilled water, and a 1 ml subsample was added to a 14 ml conical tube. Sucrose solution (1.275 specific gravity) was added to the tube. A cover slip was added and the tube was centrifuged at 200 x g for 10 minutes. Parasite eggs counted by microscopy were multiplied by 10 to calculate the eggs per gram (EPG).
For MCF foals, ≥1 g of feces was collected either rectally or from a freshly deposited sample (schedule below). Samples were stored at 4 °C and processed within one week after collection. Parasite eggs were detected using a previously described qualitative fecal flotation test [113, 115], using sucrose solution (1.275 specific gravity) and 1 g of feces. This methodology was chosen because of the small amount of feces required and the purported high sensitivity of the test [111, 115].

**Sera and Fecal Collection Schedule**

Sera and manure were collected from MCF mares within one month of expected foaling date. Mares had not been treated with an anthelmintic for at least 10 weeks prior to sampling, and were not treated with an anthelmintic for *S. westeri* after giving birth, as is the typical procedure at this farm.

Serum was collected from each MCF foal immediately after birth, prior to suckling. Serum was collected one day after suckling, and then weekly thereafter. As *P. equorum* eggs from patent infections were not expected until the foals were at least 10-15 weeks of age [15, 17, 46, 47], fecal samples were taken weekly starting on week 8. Foals were not treated with anthelmintics until the week following the first *P. equorum* positive fecal. Once foals were confirmed to be positive for *P. equorum* through fecal flotation, they were switched to a monthly fecal and serum sampling schedule. A regular anthelmintic treatment schedule was then followed in order to maintain the farm’s foal health care practices and provide realistic results. The schedule was as follows: fenbendazole (Panacur; Merck Animal Health, Summit, NJ) double dose one week after
first *P. equorum* positive fecal, pyrantel pamoate (Durvet, Blue Springs, MO)
approximately one month later, fenbendazole double dose approximately one month
after pyrantel, and ivermectin with praziquantel (Zimectrin Gold; Merial, Duluth, GA) in
November. Foals were sampled every 4-6 weeks, depending on anthelmintic use.
Sampling for each foal ceased when two consecutively negative fecal samples were
obtained. If anthelmintic use was suspected to have affected fecal results, a third fecal
sample was examined 3-6 weeks later. Yearlings remaining on the study were
dewormed with pyrantel in January and ivermectin in March. Two foals did not
complete the study: Foal15 was still shedding ascarid eggs when sold in February 2013,
and Foal13 was still shedding eggs in May 2013.

Additional sera were obtained from another study using mixed-breed foals from
the University of Kentucky Veterinary Science Farm (VSF) for use as positive controls
(courtesy of Drs. Eugene Lyons and Martin Nielsen, University of Kentucky). Sera and
manure were collected from five 3-5 month old VSF foals in July of 2011. *Parascaris
equorum* results for these foals ranged from 30-820 EPG. Serum from one of these foals
(VSF1) was collected again immediately prior to necropsy, when the horse was 8 months
of age. At necropsy, this horse had 54 immature adult *P. equorum* and 18 mature *P.
equorum* in the small intestine, and a FEC of 40 EPG (by modified Stoll method). Sera
from two other foals were collected immediately prior to necropsy. An 11-week-old foal
(VSF2) had 3 migrating *P. equorum* L4 in the lungs, and 159 L4 and 118 L5 *P. equorum* in
the small intestine. One 8-week-old foal (VSF3) had 4 migrating *P. equorum* L4 larvae in
the lungs, and 115 L4 and 31 L5 *P. equorum* in the small intestine. These foals were from
a previously characterized herd of macrocyclic lactone naïve horses [227], and the foals had never been treated with anthelmintics. It is unknown if these foals had adequate passive transfer of antibodies.

Sera from foals with various parasite infections were obtained for this study (kindly provided by Dr. Jacqui Matthews, Moredun Research Institute). One specimen was from pooled sera from a group of ponies that were raised parasite-free. Briefly, foals were removed from their dams 12 to 18 hours after birth, raised in cages, and fed formula and pelleted horse feed. These animals were described in more detail in a previous study [21]. Sera were also acquired from two ponies (numbers 101 and 105) that were reared indoors with their dams as helminth-naïve. Sera were obtained from these foals before (0-1 weeks pre-infection) and 13-15 weeks after artificial infection with cyathostome larvae. These animals were also described in a previous study [228]. Finally, samples were obtained from two ponies that had been raised parasite-free but were found to be infected with either *Strongyloides westeri* or *P. equorum* at necropsy (M. Chapman, unpublished), as were referenced in another study [218]. These sera were stored at -20 °C prior to use for western blotting.

**Western Blotting**

Identical western blotting procedures were used to test for IgM, IgE, and IgG(T) antibodies, with modifications only in primary and secondary antibody dilutions. First, Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate antigen. SDS-PAGE was conducted using 0.75 mm 2-D/prep gels (12% running and 4% stacking). *Parascaris equorum* ESA (4 μg) were thawed and combined with 5x
sample buffer (1.5 M Tris HCl, pH 6.8, with 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.01% bromophenol blue), and an additional 100 µl of 1x sample buffer (62.5 mM Tris HCl, pH 6.8, with 2% SDS, 10% glycerol, 0.01% β-mercaptoethanol, 0.01% bromophenol blue). Samples were then heat denatured for 5 minutes 95 °C. A molecular weight standard (Broad Range Precision Plus Protein Unstained Standards; Bio-Rad, Hercules, CA) was used in the first lane of each gel (2 µl), and P. equorum larval ESA were run in the large lane of each gel.

After electrophoresis, proteins were transferred electrophoretically to a 0.45 µm polyvinylidene difluoride (PVDF) membrane (Westran Clear Signal; GE Healthcare, Piscataway, NJ). Membranes were washed in 1x Tris Buffered Saline with Tween 20 (TBST) twice for 5 minutes each time on an orbital rocker. Membranes were washed in a 0.1% India Ink solution for 15-20 minutes, washed in 1X TBST 4 times for 5 minutes, and then were air-dried and stored at 4 °C for up to 2 weeks. Membranes were allowed to equilibrate to room temperature prior to western blotting, and then were blocked on an orbital rocker for one hour in 5% fat-free milk solution prepared in 1x TBST. Next, membranes were washed an additional 3 times in 1x TBST prior to securing in a multiscreen apparatus (Mini-PROTEAN II, Bio-Rad, Hercules, CA).

Primary antibody was prepared at dilutions dependent upon the type of secondary antibody to be used. For use with anti-equine IgG(T), equine sera were mixed with blocking buffer in a 1/1250 dilution, or a 1/500 dilution for the banked sera from the parasite-free and monospecifically infected foals. For use with anti-equine IgM,
samples were mixed with blocking buffer at a 1/100 dilution. For use with anti-equine IgE, serum dilutions included 1/1000, 1/500, 1/100, and 1/25. Primary antibody incubation was conducted in the multiscreen for 2 hours at room temperature on a platform rocker at low speed. Samples were vacuumed from the multiscreen and lanes were washed 6 times with 1x TBST on the platform rocker for 5 minutes each. Next, secondary antibody was prepared in blocking buffer. Secondary antibodies included horseradish peroxidase (HRP) labeled goat anti-horse IgE at a 1/1000 dilution (Alpha Diagnostic International, San Antonio, TX), HRP-labeled goat anti-horse IgM at a 1/2000 dilution (Bethyl Laboratories, Montgomery, TX), HRP-labeled goat anti-horse IgG(T) at a 1/50,000 dilution for banked monospecific infection or parasite-free control sera, and at a 1/100,000 dilution for all other samples (AbD Serotec, Raleigh, NC). Molecular weight standards were incubated with StrepTactin-HRP (Bio-Rad, Hercules, CA) at a 1/100,000 dilution in blocking buffer. Secondary antibody incubation was conducted for one hour at room temperature on a platform rocker. Lanes were then washed 6 times in 1X TBST for 5 minutes each time before using a chemiluminescent kit according to the manufacturer’s instructions (Amersham ECL Prime; GE Healthcare, Piscataway, NJ). Antibody recognition was visualized using a FluorChemE imager (ProteinSimple, Santa Clara, CA). Molecular weights of proteins were estimated using the AlphaVIEW SA program (Version 3.2.4.0; Cell BioSciences Inc., Santa Clara, CA).
Results

IgE, IgM, and IgG(T) Antibodies

When sera from *P. equorum* positive horses VSF1 and VSF2 were tested using anti-equine IgE at the manufacturer’s recommended dilutions, no bands were present on the western blots. Sera from Mare4 and Foal4 samples were also tested, with the same results. Use of anti-equine IgE was discontinued following these results, although a true control was not available to test efficacy of the secondary antibody.

A doublet at approximately 53 and 58 kDa was recognized by IgM from Mare4 and Foal4 post-suckle, on weeks 1 and 2, and faintly on week 3 (Figure 5.1). Sera from ascarid positive foals VSF2 and Foal4 did not contain measurable IgM antibodies that recognized *P. equorum* ESA. Thus, anti-equine IgM was not pursued further for diagnostic use.

All 8 VSF ascarid positive foal serum samples contained IgG(T) antibodies that recognized proteins at approximately 19 kDa, 22 kDa, 24kDa, 26 kDa, 34 kDa, and 250 kDa or larger.

MCF Mare and Foal Fecal Flotation Tests

None of the 18 mares were found to be shedding *P. equorum* eggs in the feces by FEC. The only parasite eggs identified were strongyle-type eggs, which were found in the feces of 67% of the mares. Of those shedding strongyle eggs, the mean EPG was 105 (SD= 91; range 10-290).
All foals became naturally infected with *P. equorum*. Foals first began shedding *P. equorum* eggs in feces 11-18 weeks after birth (Figure 5.2). The mean age at first shedding was 13.5 weeks (SD=1.6 weeks). Foals also became infected with other types of parasites (Table 5.1). When fecal results from the complete study were examined, all foals shed strongyle-type eggs and *S. westeri* eggs, and most foals also shed *Eimeria leukarti* oocysts at some point during the study. Prevalence of parasite eggs in feces was also examined (Table 5.1), although these results are weighted towards the first portion of the study when foals were sampled weekly. The most commonly noted parasite ova in the feces were strongyle-type eggs and *S. westeri* eggs (Table 5.1). The second negative *P. equorum* fecal flotation result (Figure 5.3) was obtained between 21 to over 54 weeks (for Foal13 that did not complete the study). Excretion of adult ascarids by two foals was observed during the study. Foal2 excreted an adult ascarid one month after his second negative fecal flotation test (without recent dewormer use), and Foal16 excreted an adult female ascarid two days after deworming with fenbendazole.

**MCF Mare Sera and Foal Sera over Time**

Mare and foal sera recognized antigen at 19 kDa, 22 kDa, 26 kDa, and 34 kDa molecular weights (Table 5.2). All but one mare sera exhibited IgG(T) antibodies against these immunodominant antigens. All post-suckle foal sera and foal sera drawn at the time of first ascarid positive fecal had antibodies that recognized *P. equorum* ESA (Table 5.2). No foal sera contained IgG(T) antibodies against ESA prior to drinking colostrum. Foal antibody recognition of ESA appeared to decrease over time (Figures 5.4-5.6). By the time two successive negative fecal flotation examinations were conducted, antibody
recognition of most immunodominant antigens were still present, although antibody recognition of the 22 kDa and 26 kDa antigens became less apparent for some foals (Table 5.2, Figure 5.6). Fifteen of the foals showed a decrease in IgG(T) recognition of immunodominant antigens over the first 13 weeks of life. For three foals (Foal9, Foal11, and Foal13), IgG(T) recognition of ESA appeared to increase several months after initially shedding *P. equorum* eggs in feces (Figure 5.7-5.8). Sera from one mare and foal pair (Mare1 and Foal1) showed little initial IgG(T) antibody recognition of ESA, but Foal1 sera displayed increased antibody recognition about 6 weeks prior to first shedding *P. equorum* eggs (Figure 5.9). A reduction in band intensity was noted for this foal during the last few months on the study (Figure 5.10).

**Parasite-Free and Monospecific Infection Sera**

Although banding intensity was low, the pooled parasite-free pony sera recognized antigen at 19 kDa and 26 kDa (Figure 5.11). Serum from the horse monospecifically infected with *P. equorum* recognized antigens at 19 kDa, 26 kDa, 34 kDa, 66 kDa, and approximately 110 kDa. Serum from the foal monospecifically infected with *S. westeri* recognized only the 19 kDa antigen. With the exception of weak IgG(T) recognition of ESA at 66 kDa and 110 kDa by pony 105, antibody recognition of ESA was not apparent by pre-cyathostome infection pony foals 101 and 105. Post-cyathostome infection, antibodies from pony 105 recognized antigen at 19 kDa, 22 kDa, 34 kDa, 66 kDa, and approximately 110 kDa. Only the 66 kDa and 110 kDa antigens were recognized by antibodies from pony 101 after cyathostome infection.
Discussion

IgE, IgM, and IgG(T) Antibodies

Western blot analyses for IgE antibodies against *P. equorum* ESA did not appear to have diagnostic potential. As controls for the anti-equine IgE secondary antibody were not available, we cannot rule out the possibility that the secondary antibody was not effective, or that IgE antibodies within the sera had deteriorated prior to testing due to the short half-life. Alternatively, the horses may not have produced measurable quantities of IgE antibodies against *P. equorum* larval ESA. Very low levels of unbound IgE have been detected in young foals, but these antibodies were of maternal origin [166]. We did not detect any IgE antibody recognition of ESA or evidence of passive transfer when using serum from Mare4 and Foal4. Other researchers have proposed that endogenous IgE production in the foal does not begin until 6 months [229] or 9-11 months [166] of age. Similarly, none of our ascarid positive foal sera exhibited IgE antibody recognition of the *P. equorum* antigen. Another study using western blot analysis of sera from humans with toxocariasis found that although the test had high specificity, only 35% of symptomatic patients and 24% of asymptomatic patients displayed serum IgE reactivity to *T. canis* larval ESA [230]. Levels of free circulating *P. equorum*-specific IgE may be too low to measure, even in positive horses.

Serum IgM antibodies did show some reactivity against *P. equorum* larval ESA. Passive transfer of IgM was observed from Mare4 to her foal. Other researchers have also found passive transfer of IgM, with levels in foals dropping rapidly within the first month after birth [176]. Our results were similar, as IgM recognition of ESA by Foal4 was
apparent for only the first 3 weeks after birth. It is possible that IgM could be associated with immunity against patent infections and could provide some protection against ascarid infection for the first few weeks in life, but this would need to be studied further. Immunoglobulin M against *P. equorum* ESA may have been present in mare sera as a result of recent infection with *P. equorum* larvae, or due to cross-reactivity with similar antigens. Transfer of maternal IgM, IgA, and IgG antibodies directly into the small intestine of newborn offspring can provide protection against parasites [231]. Interestingly, although IgM has been observed as the first antibody produced in young foals [176], endogenous IgM antibody production against *P. equorum* larval ESA was not noted for this foal during the first 14 weeks of age. Monitoring for *P. equorum* specific IgM antibodies in serum for diagnostic purposes does not appear promising, as the two infected foal samples displayed no IgM recognition of antigen on the western blot. This test would also not allow for diagnosis of migrating larvae, as there was no indication of antibody binding to ESA by Foal4 during the migratory period prior to patent infection.

Recognition of a greater number of *P. equorum* larval antigens was observed with IgG(T). As all of the *P. equorum* positive VSF foals had IgG(T) antibodies that recognized *P. equorum* immunodominant larval ESA, IgG(T) appears more promising than IgM or IgE for diagnostic test development. Serum IgG(T) has been linked to a Th2 immune response [157], and increases in IgG(T) have been associated with artificial *S. vulgaris* infection in horses [161]. Serum IgG(T) has also been used for diagnosis of infection with *S. vulgaris*, *A. perfoliata*, and cyathostominae in horses [158, 179, 232]. It appears that horses produce IgG(T) antibodies against *P. equorum* larval ESA as well.
MCF Mare and Foal Fecal Flotation Tests

Fecal flotation test results for the MCF broodmares were not surprising, as horses older than 2 years of age typically do not have patent *P. equorum* infections [7, 11, 127]. The identification of low-level strongyle-type egg shedding by the majority of mares was also expected.

The time at which MCF foals began shedding *P. equorum* eggs matched what has previously been described in the literature. A central Kentucky study reported the mean age at first appearance of *P. equorum* eggs in the feces to be 11.3 weeks, with a maximum of 15 weeks [233]. Our mean was slightly higher, at 13.5 weeks. The foal that did not become positive until week 18 (Foal 7) had been stalled for several weeks: first due to a broken rib at birth, and second due to *Rhodococcus equi* infection, so this foal may have had less exposure to *P. equorum* eggs when compared to the other foals that lived on pasture. All of the MCF foals became naturally infected with *P. equorum*, as had been reported previously when weekly examinations were conducted on Thoroughbred foals in Kentucky [233]. Incidence of *P. equorum* infection in foals may be considerably higher than prevalence, as studies have reported copromicroscopic prevalence in the central Kentucky area to be at 22% in 10-223 day old foals [4] and 39% in 28-330 day old foals [114]. We found only 29% of MCF foal fecal flotation tests to be positive for *P. equorum* eggs, so there may be potential to miss infections if samples are not taken as frequently. Similarly, as a diagnostic tool, the FEC (modified Stoll) has been shown to have lower sensitivity (0.72) than specificity (0.94), indicating the potential for missed infections of adult *P. equorum* [37].
Although we cannot prove that two consecutive negative fecal examinations signifies immunity to *P. equorum*, the ages at which this occurred was similar to what has been reported for the development of immunity. Epidemiological studies have concluded that foals and yearlings are more likely to shed ascarid eggs than older equids [7, 136]. One study found that after 3-5 months of age, worm burdens decreased monthly to nearly zero by 10 months of age (as determined by necropsy) [3]. Other research indicates that immunity may begin to develop around 6 months of age, even for *P. equorum* naïve foals [38, 186].

Along with *P. equorum*, MCF foals developed other natural infections. It is not uncommon to find strongyle-type eggs, *S. westeri* eggs, and *E. leukarti* oocysts in foal fecal samples. Our percentage of fecal samples containing *S. westeri* may have been higher than other recent reports [4, 114] because the mares were not treated with ivermectin prior to or following giving birth. Also, we sampled more frequently when the young foals had not yet begun shedding *P. equorum* eggs, so this is expected to bias the percentages. Concurrently, the percentage of fecal samples with strongyle-type eggs also appeared high when compared with other reports [4, 114]. This could have been because foals were not treated with an anthelmintic until one week after shedding ascarid eggs, and so the treatment protocols of the farm were delayed by approximately 1-2 months. Strongyle resistance to fenbendazole, one of the primary anthelmintics used, has also been documented on this farm [234].
MCF Mare Sera and Foal Sera over Time

Although none of the mares had patent *P. equorum* infections, all of the mares except one had IgG(T) antibodies against larval *P. equorum* ESA. In another study, Quarter Horse broodmares were found to have high serum precipitin titers to *P. equorum* antigen from homogenized whole adult worms [235]. The mares maintained these titers throughout the 52 week study. Broodmares live in an environment with high exposure to *P. equorum* eggs, and are expected to have constant challenge from ingestion of eggs. It appears that the larvae hatch out and migrate in the adult horse, causing an immune response, but then are unable to complete the life cycle. Clayton and Duncan found that in previously helminth naïve 6-12 month old horses, fewer *P. equorum* returned to the small intestine following artificial infection, when compared with younger foals [186]. This indicated that the larvae may be destroyed during migration, possibly in the liver or lungs in the older horse. Alternatively, larvae could return to the small intestine and be expelled immediately afterwards, but this does not seem likely as larvae would be noticed during fecal examinations. The exact mechanisms and the role of IgG(T) regarding immunity to *P. equorum* in the older horse have yet to be described.

The pre-suckle foal sera displayed no antibody recognition of *P. equorum* ESA, as would be predicted. Because of the diffuse epitheliochorial placenta of the horse [164], the foal is first exposed to the dam’s antibodies through the colostrum [164]. The post-suckle sera for all foals contained IgG(T) antibodies that recognized *P. equorum* larval ESA similarly to the dams or colostrum donors, indicating passive transfer of those
antibodies from mare to foal. This would not be so problematic for diagnostic analyses if the maternal antibody waned quickly, but foal sera samples over time show that maternal antibody is still present during the points in time when foals become infected with *P. equorum*. This clearly limits the usefulness of a western blot test for IgG(T) antibodies in *P. equorum* infected equids.

Nevertheless, this provides structure for new questions regarding immunity to *P. equorum*. The transfer of IgG(T) from mare to foal does not appear to prevent infection, so the function of these immunoglobulin isotypes is not yet obvious. Even though maternal antibodies did not prevent infection, they could still have a protective effect. In rats experimentally infected with *Taenia taeniaeformis*, those receiving maternal antibodies against *T. taeniaeformis* through colostrum had smaller tapeworm burdens following experimental infection when compared with controls [236]. In foals, maternal antibodies appear to have a protective effect against some pathogens. Successful passive transfer of IgG in foals is imperative, as failure of passive transfer has been associated with contracting infectious diseases and consequently, foal mortality [170, 180, 237].

Equine maternal transfer of antibodies specific to other infectious diseases, such as West Nile Virus [238], equine protozoal myeloencephalitis [239], strangles [167], piroplasmosis [240], tetanus [174], and influenza [174] has been documented previously. In some cases, maternal antibody transfer can actually be harmful to foal health. Maternal antibody transfer of anti-tetanus IgGa, IgGb, and IgG(T) antibodies were found to inhibit foal endogenous production of antibodies following vaccination
Studies of other parasite and host species have found that passive transfer of antibody can lead to negative effects, such as suppression of priming of immune cells and inhibition of endogenous antibody production [231]. Further research is needed to determine whether maternal antibodies have a protective effect, harmful effect, or no effect at all on foal immunity to parasites.

Although maternal antibodies masked the endogenous antibody production by foals, Mare1 sera did not exhibit strong antibody recognition on the western blot test. Accordingly, Foal1 sera displayed weak antibody recognition post-suckle and thereafter. This foal appeared to produce detectable quantities of endogenous antibody by around 8 weeks of age, particularly to the 19 kDa antigen. Other studies have estimated IgG(T) endogenous production to occur between 1 and 3 months of age [172, 173], closer to 3-4 months of age [176], or as early as around 2 weeks for foals deprived of maternal antibody [173]. Foal1 did not begin shedding *P. equorum* eggs in feces until 14 weeks of age, so endogenous antibody production was detectable 6 weeks prior to egg shedding. By the time Foal1 began IgG(T) antibody production, the first larvae would already have reached the small intestine. IgG(T) antibody production began to wane around 4 months after Foal1 began shedding ascarid eggs, and one month before his last *P. equorum* positive fecal.

The majority of the foal sera displayed a reduction in band intensity over the first 13 weeks of age, probably due to the decrease in maternal antibody. Because all of the foals became infected with ascarids, we cannot determine the timeline for persistence of these maternal antibodies in foal sera. Other studies have found a sharp decline in
foal IgG(T) occurring by 1 month of age [172, 173]. Maternal IgG(T) antibodies specific to influenza have been found to decrease to undetectable levels in foals by 12 weeks, while maternal IgG(T) antibodies against tetanus persisted for over 26 weeks [174]. There was also individual variation among foals [174].

A few of the foals that were followed for longer time periods displayed more intense banding several months post-shedding. These foals may have been infected enough times to build up antibody levels more similar to the dams. One study using a mouse model found fewer *Toxocara vitulorum* migratory larvae in mice that had been immunized with *T. vitulorum* larval ESA when compared to controls, and the degree of protection was associated with the number of immunizations given [241]. However, Foal13 was still shedding eggs several months after the increase in band intensity. The majority of foals appeared to cease egg shedding (at least temporarily) without apparent changes in band intensity, so this may not be associated with the development of immunity.

**Parasite-Free and Monospecific Infection Sera**

The parasite-free pooled pony sera most likely recognized *P. equorum* larval ESA because upon necropsy, one control pony foal was found to have eighteen *P. equorum* in the small intestine, and several ponies in the experimental groups also became infected [21]. Also, these ponies may have had residual maternal antibodies against *P. equorum* from colostrum. These results point out the difficulties in keeping foals parasite free, as many precautions were taken during this study.
Similarly, the foals with monospecific *P. equorum* or *S. westeri* infections were originally intended to be parasite free (M. Chapman, unpublished, as referenced in [218]). The foal with the monospecific *P. equorum* infection had antibodies that recognized antigen similarly to infected MCF and VSF foals. The foal with the *S. westeri* infection may have recognized the 19 kDa molecule because of cross-reactivity, or due to passive antibody transfer, but the suckling status and age of this foal at necropsy are not known.

The helminth-naïve Pony 101 pre-inoculation serum [228] could be considered a parasite-free negative control, as no antibody recognition was noted. These foals had been raised with their dams, but by the time the pre-infection serum was collected (age 6-12 months) maternal antibody could have declined, or the dams may not have had antibodies to *P. equorum*. However, the post-cyathostome inoculation sera did exhibit antibody binding of ESA at the immunodominant molecular weights characteristic of a *P. equorum* infection. Either cyathostome infection is highly cross-reactive with *P. equorum* infection on the western blot, or the ponies may have contracted *P. equorum* infections. Pony 103, a control pony, was reported to sporadically excrete *P. equorum* eggs [228]. Although Pony 101 and Pony 105 were not reported to have *P. equorum* infections, it is possible that eggs from Pony 101 were transmitted to the other ponies.

**Conclusion**

IgE immunoglobulins with reactivity against *P. equorum* ESA were not recognized using the western blot test. Mare IgM antibodies were found to bind to *P. equorum* larval ESA at approximately 53 and 58 kDa, and antibodies were passively transferred to
the foal. Foals positive for *P. equorum* did not exhibit antibody recognition of *P. equorum* antigen; thus, anti-equine IgM is not useful for diagnostic purposes. Anti-equine IgG(T) showed the most potential of the secondary antibody types, with antibody binding of ESA at approximately 19 kDa, 22 kDa, 26 kDa, and 34 kDa in most infected VSF and MCF foals. However, passive transfer of antibody distorts interpretation of results on the western blot test. Control sera from parasite-free foals and foals with monospecific parasite infection also recognized some of the same antigens as the *P. equorum* positive sera, emphasizing the difficulty in preventing *P. equorum* infection, and obtaining true controls for equine parasitology studies. Future work should examine the prevalence of antibodies to *P. equorum* in other equine populations, identify the function of IgG(T) antibodies that recognize *P. equorum* ESA, and explore other avenues for diagnostic testing in foals. These data indicate that serological testing for antibodies in foals may be contraindicated, especially in situations where maternal antibodies against the infective organism are prevalent.
Table 5.1. Percentage of foals and percentage of fecal flotation tests positive for different parasite types over the course of the study. No other parasite types were identified.

<table>
<thead>
<tr>
<th>Parasite Type</th>
<th>Percentage of foals positive (n=18)</th>
<th>Percentage of fecal tests positive (n=197)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. equorum</em></td>
<td>100%</td>
<td>29%</td>
</tr>
<tr>
<td>Strongyle-type</td>
<td>100%</td>
<td>89%</td>
</tr>
<tr>
<td><em>S. westeri</em></td>
<td>100%</td>
<td>78%</td>
</tr>
<tr>
<td><em>Eimeria leukarti</em></td>
<td>89%</td>
<td>18%</td>
</tr>
</tbody>
</table>
Table 5.2. Percentage of mares and foals with IgG(T) antibodies recognizing immunodominant *P. equorum* larval antigens.

<table>
<thead>
<tr>
<th>MW (kDa)</th>
<th>Mares n=18</th>
<th>Pre-Suckle Foals n=18</th>
<th>Post-Suckle Foals n=18</th>
<th>First Positive Fecal Foals n=18</th>
<th>Second Negative Fecal Foals n=16</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>94%</td>
<td>0%</td>
<td>100%</td>
<td>100%</td>
<td>88%</td>
</tr>
<tr>
<td>22</td>
<td>83%</td>
<td>0%</td>
<td>89%</td>
<td>89%</td>
<td>56%</td>
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<tr>
<td>26</td>
<td>89%</td>
<td>0%</td>
<td>94%</td>
<td>100%</td>
<td>63%</td>
</tr>
<tr>
<td>34</td>
<td>94%</td>
<td>0%</td>
<td>100%</td>
<td>100%</td>
<td>88%</td>
</tr>
</tbody>
</table>
Figure 5.1. Western blot of VSF2, Mare4, and earliest Foal4 samples with IgM recognition of larval *P. equorum* ESA. Lane 1, molecular weight standards; Lane 2, ascarid positive foal VSF2; Lane 3, Mare4; Lane 4, Foal4 pre-suckle; Lane 5, Foal4 one day post-suckle; Lanes 6-18, Foal4 weeks 1-13; Lane 19, Foal4 week 14 at first *P. equorum* positive fecal.
Figure 5.2. Number of MCF foals that have begun shedding *P. equorum* eggs by age in weeks.
Figure 5.3. The number of MCF foals remaining on the trial by age. Foals were removed from the trial after two consecutive fecal flotation tests were negative for *P. equorum* eggs. One foal had a second consecutive negative fecal on week 44, and the remaining two foals were censored while still shedding eggs.
Figure 5.4. Western blot of earliest Foal3 samples with IgG(T) recognition of larval *P. equorum* ESA. Lane 1, molecular weight standards; Lane 2, ascarid positive foal VSF1; Lane 3, Mare3; Lane 4, Foal3 pre-suckle; Lane 5, Foal3 one day post-sucke; Lanes 6-17, Foal3 weeks 1-12; Lane 18, Foal3 first *P. equorum* positive fecal week 13.
Figure 5.5. Western blot of earliest Foal4 samples with IgG(T) recognition of larval *P. equorum* ESA. Lane 1, molecular weight standards; Lane 2, ascarid positive foal VSF1; Lane 3, Mare4; Lane 4, Foal4 pre-suckle; Lane 5, Foal4 one day post-suckle; Lanes 6-18, Foal4 weeks 1-13.
Figure 5.6. Western blot of latest Foal3 and Foal4 samples with IgG(T) recognition of larval *P. equorum* ESA. Lane 1, molecular weight standards; Lane 2, ascarid positive foal VSF1; Lane 3, Foal3 pre-suckle; Lane 4, Foal3 week 13 first *P. equorum* positive fecal; Lanes 5-7, Foal3 months 1-3; Lane 8, Foal4 week 13; Lane 9, Foal4 week 14 first *P. equorum* positive fecal; Lanes 10-12, Foal4 months 1-3.
Figure 5.7. Western blot of earliest Foal13 samples with IgG(T) recognition of larval *P. equorum* ESA. Lane 1, molecular weight standards; Lane 2, VSF1 ascarid positive foal; Lane 3, Mare13 mare; Lane 4, Foal13 pre-suckle; Lane 5, Foal13 post-suckle; Lanes 6-18, Foal13 weeks 1-13.
Figure 5.8. Western blot of last Foal13 samples with IgG(T) recognition of larval *P. equorum* ESA. Lane 1, molecular weight standards; Lane 2, VSF1 ascarid positive foal; Lane 3, Foal13 pre-suckle; Lanes 4-6, Foal13 weeks 13-15; Lane 7, Foal13 week 16 first *P. equorum* fecal; Lanes 8-15, Foal13 months 1-8.
Figure 5.9. Western blot of earliest Foal1 samples with IgG(T) recognition of larval *P. equorum* ESA. Lane 1, molecular weight standards; Lane 2, VSF1 ascarid positive foal; Lane 3, Mare1; Lane 4, Foal1 pre-suckle; Lane 5, Foal1 post-suckle; Lanes 6-18, Foal1 weeks 1-13.
Figure 5.10. Western blot of last Foal1 samples with IgG(T) recognition of larval *P. equorum* ESA. Lane 1, molecular weight standards; Lane 2, VSF1 ascarid positive foal; Lane 3, Foal1 pre-suckle; Lane 4, Foal1 week 13; Lane 5, Foal1 week 14 first ascarid positive fecal; Lanes 6-12, Foal1 months 1-7.
Figure 5.11. Western blot of monospecific or parasite-free control IgG(T) antibody recognition of larval *P. equorum* ESA. Lane 1, molecular weight standards; Lane 2, Foal3 pre-suckle; Lane 3, parasite-free pooled pony sera; Lane 4, *P. equorum* monospecifically infected foal; Lane 5, *S. westeri* monospecifically infected foal; Lane 6, pony 105 pre-cyathostome infection; Lane 7, pony 105 post-cyathostome infection; Lane 8, pony 101 pre-cyathostome infection; Lane 9, pony 101 pre-cyathostome infection; Lane 10, Foal18 at first *P. equorum* positive fecal.
CHAPTER 6: SERUM IgG(T) RESPONSES TO L2/L3 AND L5 PARASCARIS EQUORUM ANTIGENS BY HORSES IN LOW AND HIGH RISK ENVIRONMENTS

Introduction

Parascaris equorum is a roundworm parasite that is often found in the small intestine of foals and yearlings. The first stage of the life cycle begins when an infected horse sheds eggs in the feces. This stage is important to the epidemiology of the parasite, as the eggs have a thick, multilayered shell [23] and can remain viable for at least 18 months to 3 years [21, 22]. One horse can shed millions of eggs per day [15]. For the life cycle to continue, a horse must ingest the larvated eggs. Larvae then hatch and migrate from the small intestine to the liver, then to the lungs, and back to the small intestine [15]. The worms mature and reproduce in the small intestine. This parasite is of concern to horse owners because of the clinical signs and pathogenicity that can occur. Larval migration can lead to lesions in the liver, lungs, and bronchial and hepatic lymph nodes [16], and horses may display respiratory symptoms [17]. Large burdens of P. equorum in the small intestine may lead to depression, anorexia, weight loss [17], or even intestinal impaction or intestinal rupture in severe cases [18-20].

The durability of the egg in the environment makes transmission difficult to prevent, and the potential for pathogenicity increases the importance of correct and timely diagnosis. Currently, copromicroscopic methods are the only means for diagnosis. However, the use of serological testing for antibodies has recently been explored (Chapters 4 and 5). Although testing for IgE, IgM, or IgG(T) antibodies against P. equorum larval excretory-secretory antigens (ESA) does not appear valid for diagnosis
(Chapter 5), the results lead to more questions about the immune response of the horse to this parasite, particularly in the adult horse.

In a recent study using western blot analyses, 18 Thoroughbred broodmares were tested for IgG(T) response to *P. equorum* larval ESA (Chapter 5). None of these mares were coprologically positive for *P. equorum*, yet all had IgG(T) antibodies against at least some of the proteins at 19 kDa, 22 kDa, 26 kDa, and 34 kDa molecular weights. Earlier work found similar results, as coprologically negative Quarter Horse broodmares had high serum precipitin titers against *P. equorum* whole worm antigen throughout the year [47]. The same study demonstrated a continual increase in the level of serum precipitin titers of foals and yearlings against whole *P. equorum* worm antigen as the horses developed immunity [47]. The function of these antibodies in the adult horse is not yet known, but maternal transfer of the antibodies did not prevent *P. equorum* infection in foals (Chapter 5). It is also unknown if all horses maintain a high titer of antibodies to *P. equorum* larval ESA throughout life, or if these results occurred because broodmares live in a high-exposure environment, contaminated annually by each foal crop. Broodmares maintained antibodies against *P. equorum* larvae, yet did not have patent infections. This could be explained by the ingestion of infective eggs that hatch, migrate briefly, but then are stopped by the immune system prior to returning to the small intestine to mature. Examination of IgG(T) response to adult *P. equorum* ESA could be of value in describing the antibody response of the mature, immune horse. The objectives for this study were to (1) compare IgG(T) antibody recognition of larval *P. equorum* ESA by a group of low-exposure horses with previous results from a group of
high *P. equorum* exposure horses (2) compare IgG(T) antibody recognition of adult *P. equorum* ESA by sera from broodmares, older horses, and foals shedding ascarid eggs.

**Materials and Methods**

**Antigen Collection**

Collection, embryonation, and hatching of *P. equorum* eggs was conducted as previously described (Chapter 4). Larvae were maintained in culture for collection of ESA, and antigen was processed and stored as previously described (Chapter 4). Two live immature adult (L5) *P. equorum* (approximately 5 centimeters in length) were collected from the small intestine of a foal at necropsy. These ascarids were also cultured for ESA collection, and antigen was collected and processed as previously described (Chapter 4).

**Animals and Housing**

Eighteen Thoroughbred broodmares from University of Kentucky’s Maine Chance Farm were used for this study. All broodmares were in-foal to Thoroughbred stallions, and had a mean age of 8.8 years (SD=4.4 yr, range=4-20 yrs). Broodmares were maintained on pasture most of the year. At least one month prior to expected foaling date, broodmares were moved to smaller 5 acre pastures in groups of 4-7 near the foaling barn. Mares were moved to foaling stalls prior to giving birth. Foaling dates were between 2/29/12 and 4/7/12. Mares were designated Mare1-18, and foals were correspondingly designated Foal1-18, based on the order in which foals were born.

During the first 1-2 weeks after birth, foals were stalled with dams at night and turned out in small paddocks during the day. Afterwards, mare and foal pairs were
grouped with others in larger pastures and only stalled for brief periods of time for management procedures.

Sera from a group of older horses were obtained from a veterinarian after use for routine health testing in March 2012. This group included twelve horses of varying breeds (Quarter Horse, Haflinger, Thoroughbred, Appaloosa, Paso Fino, and mixed-breed), with a mean age of 21.8 years (SD=4.0 yr, range=17-29 yr). The mean length of time at the facility was 4.5 years (SD=2.2 yr, range=0.3-7 yr). Six of the horses were mares and six were geldings. This group of horses was selected to compare with the broodmares because of differences in expected exposure to *P. equorum* eggs. Although the facility was not examined for the presence of eggs, several factors indicate that risk of environmental contamination of eggs should be low. The facility was built in 2005 on land that had not previously housed equids, and topsoil was stripped prior to construction of the facility. Once horses were accepted into the facility, they were not permitted to leave the grounds. All horses accepted into the facility were mature or aging (as described below). Horses were turned out in groups on pasture for approximately 12 hours each day, and were stalled for the remaining 12 hours. Several mature outside horses were brought in for one day each year for an intercollegiate horse show. Fecal examinations had been performed once every two months since 2011 using a modified McMaster’s method (egg detection limit 25 EPG) [57], and no *P. equorum* eggs had ever been identified.
**Sera and Fecal Collection**

The University of Kentucky Institutional Animal Care and Use Committee approved (2012-0924) all procedures performed on University of Kentucky horses in this study. Sera were collected by jugular venipuncture and processed as previously described (Chapter 5).

From older horses in the low-exposure environment, freshly deposited fecal samples were collected on the same day and processed using a modified Stoll method described elsewhere (Chapter 5). These horses had not been dewormed for 2-5 months prior to sample collection, depending on strongyle egg shedding levels.

Sera and feces were collected from broodmares in the spring of 2012, one month prior to expected foaling date, and from foals weekly beginning at 8 weeks of age until the first ascarid positive fecal (Chapter 5). Feces from broodmares were collected from freshly deposited samples and were examined using a previously described modified Stoll method (Chapter 5). Mares had not been dewormed for at least 10 weeks prior to sampling, and were not treated with anthelmintics for *S. westeri* after giving birth, as is the typical procedure at this farm. Feces from foals were collected either rectally or from freshly deposited samples, and examined using a qualitative fecal flotation test [113].

Serum was also collected from an 8-month-old colt positive for *P. equorum* in the small intestine, as confirmed by necropsy (kindly provided by Drs. Eugene Lyons and Martin Nielsen) for use as a positive control. For use as an antibody-negative control, a
pre-suckle sample was collected from a Maine Chance Farm foal immediately after birth, and prior to ingesting colostrum.

**Western Blot Analyses**

Samples (4 µg of either *P. equorum* larval ESA or immature adult ESA) were heat denatured for 5 minutes 95 °C in 5x sample buffer (1.5 M Tris HCl, pH 6.8, with 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.01% bromophenol blue), with an extra 100 µl of 1x sample buffer (62.5 mM Tris HCl, pH 6.8, with 2% SDS, 10% glycerol, 0.01% β-mercaptoethanol, 0.01% bromophenol blue). Larval and immature adult *P. equorum* ESA were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 0.75 mm 2-D/prep 12% gels with a 4% stacking gel. Broad Range Precision Plus Protein Unstained Standards (Bio-Rad, Hercules, CA) were used as a molecular weight reference. Western blotting was conducted as previously described (Chapter 4), using horseradish peroxidase (HRP) conjugated goat anti-horse IgG(T) at 1/100,000 dilution (AbD Serotec, Raleigh, NC) for chemiluminescent detection. Molecular weights of proteins were estimated using the AlphaVIEW SA program (Version 3.2.4.0; Cell BioSciences Inc., Santa Clara, CA).

**Statistical Analyses**

Statistical analyses were conducted using SAS version 9.3 (SAS Institute Inc., Carey, NC). Western blot banding patterns were compared using PROC FREQ and the Fisher’s exact test function. Where statistical comparisons were made, a p-value of <0.05 was deemed significant.
Results

Copromicroscopic Results

None of the older horses were found to be shedding *P. equorum* eggs at the time serum was collected. In 42% of the horses, strongyle-type eggs were identified in the feces (Table 6.1). Of those shedding strongyle-type eggs, the mean EPG was 180 (SD=249 EPG, range=10-620 EPG). No other types of parasite eggs were identified. Fecal results for the broodmares and foals were described in detail in Chapter 5. Briefly, none of the broodmares were shedding ascarid eggs at the time of sampling, and all of the foals began shedding *P. equorum* eggs by age 11-18 weeks.

Older Horse IgG(T) Recognition of L2/L3 ESA

Sera from older horses recognized larval *P. equorum* antigen at some of the same molecular weights as has been previously described for immunized rabbits and naturally infected horses (Chapters 4 and 5). Sera from five of the twelve horses (42%) did not have any IgG(T) antibody recognition of the antigen, while 58% had antibodies that recognized proteins at 26 and 34 kDa, and 50% at 19 and 22kDa (Figure 6.1). When compared with the broodmare results reported in Chapter 5, the older horse sera were less likely to recognize the 19 kDa antigen (p=0.009) and 34 kDa antigen (p=0.026). There was no significant difference between the groups with regards to the 22 kDa antigen (p=0.102) but there was a trend towards higher recognition of the 26 kDa antigen (p=0.084) by broodmare sera.
Foal, Broodmare, and Older Horse Recognition of L5 ESA

Positive control necropsied foal serum recognized *P. equorum* immature adult ESA at approximately 12 kDa, 70 kDa, and higher molecular weights at and above 120 kDa (Figure 6.2). Pre-suckle foal serum did not recognize any antigens.

Sera from 39% of broodmares recognized *P. equorum* immature adult ESA at 12 kDa, 22% at 70 kDa, and 39% at molecular weights of 120 kDa or higher. Sera from 17% of older horses recognized the 12 kDa molecule, and 8% recognized the 70 kDa molecule or molecular weights of 120 kDa or higher. There were no significant differences between the broodmares and older horses for recognition of the molecules. All broodmare sera that recognized *P. equorum* immature adult ESA also recognized larval ESA, except one. Of the two older horse sera that recognized *P. equorum* adult ESA, one strongly recognized both adult and larval ESA, while the other did not recognize larval ESA, and had only weak recognition of the 12 kDa L5 protein.

Sera from 17% of the MCF ascarid positive foals recognized the 12 kDa antigen, and 61% recognized the 70 kDa molecule and molecular weights of 120 kDa or higher (Figure 6.2). There was no significant difference between sera from mares and foals with patent infections for recognition of the 12 kDa molecule or at molecular weights of 120 kDa or above, but foal sera were more likely to recognize the 70 kDa molecule (p=0.041). Overall, antibody recognition of adult ESA was lower than antibody recognition of larval ESA by sera from broodmares and older horses. Of broodmares, 6% of sera did not exhibit any antibody recognition of larval ESA, while 50% did not
recognize adult ESA. Of older horses, 42% did not recognize larval ESA, while 83% did not recognize adult ESA.

When sera from one seronegative mare and her foal were examined through western blot, faint antibody recognition of two high molecular weight proteins and the 12 kDa antigen were apparent by week 11 after birth (Figure 6.3), which was three weeks before the foal began shedding ascarid eggs (Figure 6.2).

Discussion

Older Horse IgG(T) Recognition of L2/L3 ESA

The older horses were not expected to show much antigen recognition because of their ages, and amount of time spent in an environment with presumably little to no exposure to *P. equorum* eggs. However, over half of the horse sera recognized *P. equorum* larval ESA. It is possible that because of the durability of the chitinous layer and the sticky nature of the proteinaceous layer of the shell [23], eggs could have been transported into the facility by staff, students, or a health care professional, and remained infective until ingested. Alternatively, a visiting horse could have been shedding *P. equorum* eggs during the annual intercollegiate horse show, although these horses were mature and not likely to have patent infections. Cross-reactivity cannot be ruled out, as it has not been fully examined for other equine parasites (Chapter 5). However, this is not the most likely explanation because of the similarities to responses by *P. equorum* immunized or infected animals (Chapters 4 and 5). It is also possible that some of the horses maintained antibodies against *P. equorum* over a long period of time. Lengthy persistence of serum IgG antibodies against other infectious diseases has
been reported. For example, by western blot, IgG antibodies remained detectable for at least a year against *Campylobacter jejuni* [242] and at least three years against *Toxocara canis* [243] and *Borrelia burgdorferi* [244]. It appears that the degree of egg contamination in the environment and/or age may have some effect on IgG(T) antibody production against *P. equorum* larval ESA, as fewer old horse sera recognized two of the antigens. This effect could be amplified with larger sample sizes. Additional samples could be examined to report the seroprevalence of antibodies to *P. equorum* larval ESA.

Of the five older horses that were positive for strongyle-type eggs, three displayed no antibody recognition of the *P. equorum* larval ESA (Table 6.1). One of these mares had a strongyle EPG of 620. Of the seven horses with a negative strongyle EPG, five were displayed antibody recognition of *P. equorum* larval ESA. These data suggest that there may not be issues with cross-reactivity with strongyles on western blot analyses, but testing of additional samples and determination of actual strongyle species may be necessary to conclusively declare this.

**Foal, Broodmare, and Older Horse Recognition of L5 ESA**

Western blot banding patterns for *P. equorum* positive horses indicate that immature adult *P. equorum* produce different ESA molecules that stimulate an antibody response when compared with newly-hatched larvae. Alternatively, the protease inhibitors used were changed between the larval and L5 cultures, which could have preserved different molecules. The main dissimilarity was that aspartic proteases could be present in the larval antigen, but not in the L5 antigen. More likely, there are some differences in proteins produced by stage, with L5 producing mainly immunogenic ESA.
of higher molecular weight. Other researchers have found differences in antigens produced by ascarid stage. One researcher found small differences in *B. procyonis* larval protein production during the first few weeks in culture [77]. Other research has identified stage-specific ESAs produced by L2 and L3/L4 *Ascaris suum* larvae [208].

Production of varied proteins by life stage could aid in survival by performing different functions, as L2/L3 larvae are migratory and L5 remain in the small intestine. Excretory-secretory proteins produced by other nematode species have been shown to assist in migration by cleaving cellular molecules [80, 81, 86], aid in modulation or evasion of the host’s immune response [82, 83, 88, 89], improve feeding sites and environment [69], and possibly prevent worm expulsion from the host’s intestine [85]. Protease inhibitors from homogenized adult *P. equorum* have shown activity against bovine trypsin and chymotrypsin (activity against equine digestive proteases was not tested) [94]. Further study is needed to characterize the excreted/secreted proteins of larval and adult *P. equorum*.

Examination of a mare that was seronegative to *P. equorum* immature adult ESA provided information about the timeline for antibody development in a naturally infected foal. *Parascaris equorum* larvae complete their migration back to the small intestine by between 17-23 days after ingestion of embryonated eggs [15, 16, 46] and reproduce 10-15 weeks post-infection [15, 17, 46, 47]. This suggests that Foal4 became infected sometime between weeks 0-4, and that *P. equorum* were present in the small intestine for 3-8 weeks prior to production of detectable levels of IgG(T) antibodies against adult ESA at week 11. When compared to the response of a previously examined
foal (Foal1) to *P. equorum* larval ESA (Chapter 5, Figure 5.9), antibodies to immature adult ESA were detected three weeks later. Both foals began shedding *P. equorum* eggs on week 14, but antibody to larval ESA was detected by week 8, while antibody to L5 ESA was detected by week 11. This could have been due to individual differences between foals, but it is likely that antibody production against larval ESA occurs prior to antibody produced against immature adult ESA.

It is also of interest to compare the IgG(T) antibody recognition of immature adult ESA by the Mare4/Foal4 pair with previous findings. Although Mare4 did not have detectable IgG(T) antibodies against L5 ESA, the same mare did passively transfer IgG(T) and IgM antibodies against larval ESA to her foal (Chapter 5). Similarly, fewer broodmares and older horses recognized adult ESA when compared with larval ESA. It is possible that in some horses, the immune response destroys migratory parasites before they are able to begin excretion/secretion of adult-stage proteins.

**Conclusion**

Sera from older horses in a low-egg exposure environment were less likely to recognize the *P. equorum* larval 19 kDa molecule (p=0.009) and 34 kDa molecule (p=0.026) when compared with broodmare sera. However, more of the old horse sera recognized antibody than expected, given their environment. This suggests that either (1) horses maintain high titers of antibodies to *P. equorum* larvae over very long periods of time or (2) adult horses are likely to encounter and ingest ascarid eggs regardless of whether or not foals are housed at the facility.
Sera from horses confirmed as positive for *P. equorum* recognized antigens of different molecular weights when *P. equorum* larval and immature adult ESA were compared. This indicates that the parasite produces some stage-specific proteins, thus inducing stage-specific antibody production in the host. Additionally, fewer adult horse sera recognized the immature adult ESA when compared with larval ESA. This could occur if the horses destroy larvae before they reach the L4 or L5 stage in the small intestine. However, this could mean that in some adult horses without patent infections, larvae survive long enough to produce adult antigens. Although only one foal was examined for endogenous antibody production, his results indicate that larvae are present in the small intestine for at least 3 weeks before detectable quantities of antibody against immature adult ESA are endogenously produced. The exact identities and functions of the larval and immature adult ESA should be the subject of further study.
Table 6.1. Older horse data.

<table>
<thead>
<tr>
<th>Horse Number</th>
<th>Horse Age</th>
<th>Years at Facility</th>
<th>Strongyle EPG</th>
<th>IgG(T) Response to larval ESA</th>
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<tr>
<td>1</td>
<td>28</td>
<td>7</td>
<td>100</td>
<td>Yes</td>
</tr>
<tr>
<td>2</td>
<td>26</td>
<td>7</td>
<td>0</td>
<td>Yes</td>
</tr>
<tr>
<td>3</td>
<td>23</td>
<td>7</td>
<td>0</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>6</td>
<td>620</td>
<td>No</td>
</tr>
<tr>
<td>5</td>
<td>29</td>
<td>5</td>
<td>0</td>
<td>Yes</td>
</tr>
<tr>
<td>6</td>
<td>21</td>
<td>5</td>
<td>60</td>
<td>No</td>
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<td>20</td>
<td>5</td>
<td>0</td>
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<td>22</td>
<td>4</td>
<td>0</td>
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<td>17</td>
<td>3</td>
<td>0</td>
<td>No</td>
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<tr>
<td>12</td>
<td>21</td>
<td>0.3</td>
<td>110</td>
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Figure 6.1. Western blot of older horse IgG(T) antibody recognition of *P. equorum* larval ESA. Lane 1, molecular weight standards; Lane 2, foal positive for *P. equorum* at necropsy; Lane 3, pre-suckle foal; Lanes 4-15, older horses negative for *P. equorum* eggs in feces in order from greatest number of years at facility to fewest.
Figure 6.2. Western blot of IgG(T) antibody recognition of *P. equorum* immature adult
ESA by foals with patent infections. Lane 1, molecular weight standards; Lane 2, foal positive for *P. equorum* at necropsy; Lane 3, Foal1 pre-suckle; Lanes 4-18, Foals1-12 and 14-16 at first week of patent shedding.
Figure 6.3. Western blot of Foal4 IgG(T) antibody recognition of *P. equorum* immature adult ESA over time. Lane 1, molecular weight standards; Lane 2, foal positive for *P. equorum* at necropsy; Lane 3, Pregnant Mare4; Lane 4, Foal4 pre-suckle; Lane 5, Foal4 post-suckle; Lanes 6-18, Foal4 weeks 1-13. Week 14 was the first week of patent infection.
Prior to this research, the larval culture of *P. equorum* for collection of ESA and the antibody response of the horse to these proteins had not been described in the literature. Results indicate that *P. equorum* larvae can be hatched using a modified technique and maintained in culture for up to 3 weeks with adequate survival for ESA collection. Larvae molted while in culture, indicating that the molt from L2 to L3 stages occurs within the first week or two after hatching. SDS-PAGE of *P. equorum* larval ESA revealed heterogenous proteins ranging from 12-94 kDa, and immature adult (L5) proteins ranged between 12-189 kDa. Western blot results suggest that IgG(T) or IgG antibody binding to proteins at 19 kDa and 34 kDa may be important for distinguishing *P. equorum* infections from *B. procyonis* or *T. canis* infections. Cross-reactivity between species was noted at 22 kDa, 26 kDa, and at high molecular weights.

Western blot analyses did not detect IgE recognition of larval *P. equorum* ESA. Mare IgM antibodies recognized antigen at approximately 53 and 58 kDa, and antibodies were passively transferred to the foal. However, serological testing for IgM is not useful for diagnostic purposes, as foals with patent infections did have IgM antibodies that recognized antigen. Serological examination for IgG(T) had the most potential, with antibody binding at approximately 19 kDa, 22 kDa, 26kDa, and 34 kDa in most foals with patent infections. Nevertheless, the majority of broodmares passively transferred IgG(T) antibody against *P. equorum* larval ESA to their foals. This illustrates the complications of antibody testing in foals for diagnostic purposes, especially in situations where the infectious pathogen is common and induces an antibody response.
in mares. The purpose of the IgG(T) antibodies against larval ESA is not yet known, but the antibodies did not prevent infection, as all of the foals became naturally infected with *P. equorum*.

To further explore the antibody response of the immune horse, older horses in a low-egg exposure environment were examined. These horses were less likely to recognize two of the larval molecules when compared with broodmares, but more than half of the older horses displayed an antibody response. This points to the ease in which eggs can be transmitted between facilities, and indicates that many adult horses probably do maintain IgG(T) antibodies to *P. equorum* larvae.

When *P. equorum* larval and immature adult ESA were compared, differential banding patterns by positive foals were noted on western blot analyses. It appears that *P. equorum* produces stage-specific proteins and prompts stage-specific antibody production in the host. When adult horses without patent infections were compared, fewer recognized the immature adult ESA when compared with larval ESA. It is possible that in some horses, larvae are destroyed before producing late stage proteins, while in others horses, larvae survive long enough to produce adult antigens. The exact identities and functions of *P. equorum* ESA and the antibody response of the host require further study before the development of immunity to this parasite can be fully understood.

Future attempts to develop new tools for diagnosis of *P. equorum* infection should not focus on circulating antibody detection. As *P. equorum* is primarily a disease of foals, maternal antibody will be too much of a confounding factor if serological antibody detection is used. For the detection of migratory larvae, testing for antigen
instead of antibody could be examined. Other researchers have utilized tests for circulating ESA in diagnosis of *T. canis* infection [197, 245]. In dogs, antigen has been detected by sandwich ELISA assay [197]. Higher levels of antigen were found early during infection, with lower but still detectable levels identified during chronic infection [197]. Another study found that levels of antigen detected by sandwich ELISA decreased at the time when antibody levels increased [245]. In experimentally infected mice, antigen was detected early during infection, but then was complexed with antibody [245]. It is possible that maternal antibody could distort the results of an antigen-based test by binding to antigen.

Similarities in ascarid migration during the early phase of infection in immune adult horses and foals may make it difficult to distinguish between the two types of infection. Other approaches may need to be examined for diagnosis. In sheep, a diagnostic test has been developed for the presence of IgA in saliva against a particular trichostrongylid surface antigen [246]. One advantage of this test is the non-invasive sampling procedure, which would be practical for routine diagnosis. This test was also found to be correlated with worm burden [246]. Migratory *P. equorum* larvae induce coughing while in the lungs, and then migrate up the trachea and be swallowed; therefore, it is possible that ESA or anti-*P. equorum* IgA could be detected in saliva. As this stage of the life cycle is towards the end of the migratory phase, a saliva test could help to distinguish between responses of immune and susceptible horses. Another possible route for diagnosis might include testing for the presence of antigen in bronchoalveolar lavage fluid, although this would be more invasive than using saliva. In
summary, there are several alternative approaches for diagnosis that could be explored. Although we did not find testing for antibodies to *P. equorum* ESA to be useful for diagnosis, our research still provides new information about *P. equorum* and the immune response of the horse.
## APPENDIX A:

Appendix 1: Mare ages and foaling data.

<table>
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<tr>
<th>Mare/Foal Code</th>
<th>Mare Name</th>
<th>Mare Age (2012)</th>
<th>Expected Foaling Date</th>
<th>Actual Foaling Date</th>
<th>Acceptable Colostrum?</th>
</tr>
</thead>
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<tr>
<td>Mare 1/ Foal 1</td>
<td>Brave Boco</td>
<td>11</td>
<td>2/22/12</td>
<td>2/29/12</td>
<td>Yes</td>
</tr>
<tr>
<td>Mare 2/ Foal 2</td>
<td>So Beautiful</td>
<td>6</td>
<td>2/26/12</td>
<td>3/6/12</td>
<td>Yes</td>
</tr>
<tr>
<td>Mare 3/ Foal 3</td>
<td>Italian Opera</td>
<td>5</td>
<td>3/8/12</td>
<td>3/8/12</td>
<td>Yes</td>
</tr>
<tr>
<td>Mare 4/ Foal 4</td>
<td>Tabadabado</td>
<td>15</td>
<td>3/7/12</td>
<td>3/13/12</td>
<td>Yes</td>
</tr>
<tr>
<td>Mare 5/ Foal 5</td>
<td>Marinade</td>
<td>13</td>
<td>2/26/12</td>
<td>3/13/12</td>
<td>Yes, but plasma a few days later</td>
</tr>
<tr>
<td>Mare 6/ Foal 6</td>
<td>Classy Ensign</td>
<td>20</td>
<td>3/21/12</td>
<td>3/22/12</td>
<td>No, Italian Opera and Emerald Buddha</td>
</tr>
<tr>
<td>Mare 7/ Foal 7</td>
<td>Whom Shall I Fear</td>
<td>6</td>
<td>3/15/12</td>
<td>3/22/12</td>
<td>No, used Tabadabado</td>
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<td>Vinlear</td>
<td>9</td>
<td>3/29/12</td>
<td>3/23/12</td>
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<td>Mare 9/ Foal 9</td>
<td>Engaging Gigi</td>
<td>4</td>
<td>3/3/12</td>
<td>3/29/12</td>
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<tr>
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<td>To the Right</td>
<td>11</td>
<td>3/22/12</td>
<td>3/31/12</td>
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<tr>
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<td>Run Nola Run</td>
<td>5</td>
<td>3/27/12</td>
<td>4/5/12</td>
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<td>4</td>
<td>3/29/12</td>
<td>4/6/12</td>
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<tr>
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<td>Distinctive View</td>
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<td>4/9/12</td>
<td>4/9/12</td>
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<tr>
<td>Mare 14/ Foal 14</td>
<td>Blue Stream</td>
<td>12</td>
<td>4/3/12</td>
<td>4/10/12</td>
<td>Yes</td>
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<td>Mare 15/ Foal 15</td>
<td>Ziffy</td>
<td>6</td>
<td>4/6/12</td>
<td>4/11/12</td>
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<td>Mare 16/ Foal 16</td>
<td>Possesting</td>
<td>5</td>
<td>4/9/12</td>
<td>4/18/12</td>
<td>Yes, but plasma a few days later</td>
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<tr>
<td>Mare 17/ Foal 17</td>
<td>Sweet Champagne</td>
<td>9</td>
<td>4/21/12</td>
<td>4/21/12</td>
<td>Yes</td>
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<td>Mare 18/ Foal 18</td>
<td>Chatelian</td>
<td>11</td>
<td>5/5/12</td>
<td>5/7/12</td>
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APPENDIX B: LABORATORY PROTOCOLS

Ascarid Dissection Protocol

Materials Needed:
- Metal Tray
- Aluminum Foil
- 15 ml Tenbroek Homogenizer
- Microscope and...
  - Slides
  - Cover slips
- Scalpel(s)
- Probe (optional)
- Blunt tip tweezers
- Sharp tip tweezers
- Iris Forceps (Sharp)
- Thumbs Dressing Forceps (have teeth)
- Blunt Forceps
- 50 ml Beaker(s)
- Large Petri Dish
- 3 Autoclavable plastic dishes
- 50 ml Conical Tube
- Centrifuge
- Container of Ascarids

Safety Equipment Needed:
- Class II Biological Safety Cabinet
- Lab Coat, gloves
- Goggles/Surgical Mask (Baylisascaris/Toxocara)
- Autoclave and...
  - Autoclave Tray
  - Small Autoclave Bag(s)

*Make sure equipment has been sterilized (autoclaved) prior to use to reduce risk of contamination by other species’ eggs.

Chemicals Needed:
- Double Distilled H₂O
- 0.1% Formalin, 0.85% Saline
- 2% Formalin
- 10% Formalin

Ascarid Collection Procedure:

1. Put *Parascaris equorum* (equine ascarid), *Baylisascaris procyonis* (raccoon ascarid), and *Toxocara canis* (dog ascarid) or *Toxocara cati* (cat ascarid) in plastic specimen container with 0.5% formalin, 0.85% saline.
2. Transport to Garrigus in a lined cooler.
3. Record relevant information about collection and label container.
4. Refrigerate at 4°C until dissection.
Dissection Procedure:

1. Put a layer of aluminum foil on biological safety cabinet working surface.
2. Sex parasites. Keep females in container and autoclave any males.
   a. Males have a hooked tail and seem to not have internal white area as far up as females (white coloration seems to begin about 1/3 from the anterior end in females, and slightly further down for males).
3. Surface decontaminate females by putting several at a time in an autoclavable bowl of 10% formalin for 5 minutes.
4. Move females to another autoclavable bowl of 2% formalin.
5. Rinse females in a large petri dish containing distilled water for one minute.
6. Fill large petri dish with distilled water and place on metal tray.
7. Place worm in petri dish. Make sure it is submerged. Be careful not to splash water out of dish because it will be contaminated with eggs during following procedure!
8. Identify anterior end (will have bulbous lips) and posterior end.
9. Hold anterior end by mouth with blunt forceps. Cut cuticle down towards the tail with a scalpel or gently insert sharp Iris forcep tips into anterior end about a centimeter from the mouth and cut the worm lengthwise with forceps.
10. Take thumbs forceps and grab cuticle. Peel cuticle down like a banana. Peel segments until vagina and uterus come off with cuticle.
   a. Vagina is very thin and will be attached to cuticle. Uterus is Y shaped and light in color.
11. Move unwanted materials to the side in the dish.
   a. Ovaries are very thin and do not contain viable eggs. Pharynx will be flat and darker yellow or green color.
12. Remove vagina and anterior 1-2 centimeter(s) of uterus.
13. Place vagina and uterus piece in 50 ml beaker containing 0.1% formalin, 0.85% saline.
14. Add portion of uteri and solution to the Tenbroek homogenizer.
15. Gently insert pestle into homogenizer. Make sure pestle doesn’t reach to bottom or eggs will be damaged. Gently grind the uteri until large pieces are gone.
16. Add egg solution to beaker.
17. Cover beaker with aluminum foil with holes poked in top.
18. Check (see below) subsample of two 4 µl of egg solution for egg density and larvation.
19. Add enough 0.1% formalin, 0.85% saline solution to put eggs at density of (or below) 25 eggs/µl (Eriksen, 1990).
20. Autoclave dissected female parasites, male parasites, and uterine tissue.
21. Autoclave sharps, dishes, homogenizer, or any other equipment in the BSC.

Embryonation Procedure:

1. Maintain beaker at room temperature for embryonation. Alternatively, store at
   4°C in conical tube with screw cap loosened until ready for use.
2. Sit beaker on lab bench. Agitate gently 5x per week with applicator stick once
   per day.
3. Maintain in solution until second stage larval development (3-5 weeks).
4. Check 4µl subsamples of eggs for larvation weekly.
   a. Stir sample a few times with sterile applicator stick.
   b. Pipet out one 4 µl drop from center of beaker (before eggs have time to
      settle to bottom).
   c. Put on microscope slide with cover slip.
   d. Count number of eggs, recording number of eggs in each stage of
      development. Do this quickly before eggs have time to dry out (they look
      like little spores and you can’t see inside of them).
5. If solution in beaker evaporates, add double distilled water as needed in case
   saline has remained in beaker.
Ascarid Hatching Protocol

Materials Needed:
- 50 ml screw-top conical tubes
- 15 ml screw-top conical tubes
- 50 ml beaker
- Waste beaker
- 125 ml flask containing...
  - Glass beads (3 mm and 5 mm diameter)
    - Fill flask to about 1 cm in height
  - Stir bar
- Baermann apparatus
  - Funnel attached to clamped tubing
  - Stand
  - Beaker to hold conical tube
  - Metal sieve fit in funnel (large holes)
  - Two fluffed cotton balls
  - 2 g layered cotton gauze

*Make sure equipment has been sterilized (autoclaved) prior to use to reduce risk of contamination by other species’ eggs.

Chemicals Needed:
- Bleach - Great Value unscented
- 0.85% NaCl solution (sterile)
- Hank’s Balanced Salt Solution (sterile) without phenol red
- Dulbecco’s Phosphate Buffered Saline
- RPMI-1640 with phenol red, 25 mM HEPES, and L-Glutamine
- 70% Ethyl alcohol
- Protease Inhibitor Cocktail (Thermo Scientific 87785)
- Antibiotics: 100x concentrated solution (MP Biomedicals 1674049)
  - Penicillin-10,000 IU/mL
  - Streptomycin-10mg/mL
  - Amphotericin B-25µg/mL

Safety Equipment Needed:
- Class II Biological Safety Cabinet
- Lab coat
- Gloves
- Goggles/surgical mask (Baylisascaris/Toxocara)
All procedures must be performed aseptically (especially important after bleaching step). Perform all pipetting or any procedures where eggs are exposed to air in BSC. Use 70% ethanol to clean gloves and all items placed in cabinet. When removing tubes from BSC, cap tightly.

Formalin Removal Procedure:

1. If eggs had been stored at 4 °C, remove from refrigerator at least two days prior to hatching so that larvae can acclimate to room temperature and become more active.
2. Pipet eggs into sterile 50 mL conical tube. Cap tightly.
3. Gently rotate tube to mix solution and eggs.
4. Estimate number of active larvated eggs by counting viable larvae in at least two 5 ul subsamples. Record amount of solution and calculate an estimation for total viable larvae.
5. Wash eggs three times in 0.85% NaCl.
   a. Centrifuge at 200 x g for 1 minute with no braking of centrifuge. Acceleration: 8, deceleration: 4. Do not use bucket covers- they can turn sideways and jam.
   b. Carefully remove tube and put in BSC without shaking it!
   c. Use bulb pipet filler to carefully remove supernatant without disturbing eggs. Eggs should be visible at the bottom of the tube.
   d. Use electronic pipet to insert fresh 0.85% NaCl solution. Do not let pipet touch sides of tube.
   e. Repeat for two more washes.
   f. After last wash, remove supernatant to just above the eggs.

Egg Decoating Procedure:

22. Spray off rocker platform and place in BSC/hood.
23. Turn on Isotemp incubator (37 °C) and CO2 incubator (5% CO2, 37 °C – better to turn on a few days beforehand).
24. Turn on water bath and set at 37 °C.
25. Pipet straight bleach (5.25% sodium hypochlorite) into conical tube with egg solution at ratio of 1 ml bleach: 1 ml egg soln. Make sure to use Great Value bleach (Chlorox Lemon Fresh will decoat too quickly).
26. Rotate tube several times to coat eggs in bleach and place on shaker platform for 3 minutes. Do not let tube sit without rocking or bleach could harm eggs.
27. At minute 3, begin taking subsamples to examine the eggs once per minute.
a. Pipet 4 µl subsample onto microscope slide. Do not add cover slip or eggs may appear to burst prematurely.

b. Check eggs on 10x, then 40x for decoating of layers of egg shell. Shells of larvae may decoat faster than unlarvated eggs. Decoating should remove the outermost acid mucopolysaccharide/protein uterine layer, lipoprotein vitelline layer, and part of the thicker chitin/protein layer. This will leave the part of the chitin layer and the inner lipoprotein layer (ascaroside layer). When bleached enough, eggs may not be perfectly round anymore. Bleach until the chitinous layer of the shell is very thin.

c. Length of time for bleaching should be at least 8 minutes but will vary by batch of eggs. The egg pictured above should be bleached for a couple more minutes to thin the chitinous layer a little more.

28. Remove tube from rocker and pipet in sterile 0.85% saline solution to 50 ml mark and centrifuge for 1 minute at 200 xg (acc. 7, dec. 4). This will help eggs pellet to bottom and decrease bleaching action. Pipet out supernatant after wash.

29. Wash out bleach solution with 8 more washes of sterile 0.85% saline.

a. Centrifuge for 1 minute at 200 xg (acc. 8, dec. 4).

b. Most bleach will be washed out by wash #5.

c. For *P. equorum*, wash 2 more times in 0.85% saline, then put cap on loosely and let sit for one week at room temperature in 0.85% saline for a week. Then repeat decoating steps 1-8 again, bleaching for 1-2 minutes to remove any bacteria. When decoating the second time, switch to Hank’s Balanced Salt Solution (HBSS) for the last two washes, and continue to hatching.

d. For other species, Use sterile 1x Hank’s Balanced Salt Solution (HBSS), pH 6.8-7.0 without phenol red starting on wash #8. Then continue straight from step 8 to hatching.
Hatching Procedure:

1. Set up sterile Baermann apparatus in CO2 incubator. Funnel should contain metal sieve with layered gauze and finally fluffed cotton balls on top. Tubing should be attached to funnel and clamped with metal clamp. Funnel should sit in stand and end of tubing should go into sterile conical tube.

2. Pipet out most of HBSS from the conical tube containing washed eggs, taking care not to disturb eggs.

3. Pipet in 10 µl of 100x concentrated antibiotic solution per ml of HBSS/egg solution.
   a. Gives 100 IU penicillin/ml, 100 µg streptomycin/ml, 0.25 µg/ml amphotericin.
   b. Cap tube and gently mix antibiotic and HBSS.

4. Pipet egg solution into flask containing stir bar and glass beads. Only pipet in enough solution so that beads are coated with solution (probably around 7 ml total). Do not pipet in so much solution that it sits above the beads (or the eggs won’t receive enough mechanical stimulation)!

5. Rinse and pipet any remaining eggs from conical tube into flask using HBSS/antibiotic solution, monitoring level of solution in flask.

6. Cover top of flask with sterile aluminum foil or parafilm so that no air can get in and cause bacterial contamination.

7. Place flask on stir plate (one that can rotate at slow speeds, 1-2x per second) in the incubator at 37 °C (CO2 is not necessary). Monitor to make sure stir bar doesn’t get stuck.
   a. For larval homogenization, let stir faster in cold room overnight.
   b. Pipet some HBSS into conical tube and put in water bath. Make sure lid area does not touch water (bacteria can get in)!
   c. Check hatching by aseptically removing a 5 µl subsample every 10 minutes and putting on microscope slide without cover slip. Cover slipping can cause premature explosion of the eggs once the shells are thinner. Count unhatched larvated eggs, healthy hatched larvae, and damaged larvae in each subsample. When 80-90% of eggs have hatched, remove from stir plate. Should take 20-40 minutes. Debris will be apparent from hatched eggs.

Larval Washing Procedure:

a. Remove larvae from beads by pipetting solution out and into 50 ml conical tube. Rinse flask several times with HBSS and pipet rest of solution into conical tube. Fill conical tube up to 50 ml line with warm HBSS.

b. Centrifuge at 100 x g (acc. 7 dec. 4) for 5 minutes to clarify suspension. This will help to remove egg debris.
c. While larvae is washing, wash cotton twice in incubator with HBSS to remove fine particles.
d. Repeat washing of larvae 2 more times, pipetting out supernatant and adding warm HBSS.
e. Pipet out majority of HBSS without disturbing eggs.

Baermannizing Procedure:
1. Add 10 µl antibiotic per ml larvae/HBSS solution. Cap and gently rotate to mix.
2. Make sure tubing in Baermann is clamped and pour larval pellet into funnel.
3. Rinse conical tube with warm HBSS to get more larvae out and to ensure cotton is saturated and solution sits above cotton. Add 10 µl antibiotic per ml larvae/HBSS solution, cap, and gently rotate to mix.
4. Put sterile aluminum foil on top of funnel to keep larvae wet.
5. Let stand in incubator for 4 hours.
   a. Healthy larvae should migrate down the flask while debris, dead larvae, and egg shells remain on cotton.
6. Warm a conical tube of HBSS in water bath to 37 °C.
7. Remove tube from water bath. Add 10 µl antibiotic per ml HBSS to tube and vortex to mix.
8. Remove larvae by unclamping tubing and put sterile conical tube below the tubing to collect larvae.

Culture Prep Procedure:
1. Turn on water bath and set to 37 °C.
2. Pipet Dulbecco’s Phosphate Buffered Saline (DPBS) into a couple 50 ml conical tubes.
3. Tightly screw on lids and put tubes in water bath to warm them. Do not let lids touch water (avoid contamination)!
4. Pipet warm DPBS into conical tube containing larvae.
5. Wash by centrifuging at 200 x g for 1 minute (8 acc., 4 dec.).
6. Pipet out supernatant without disturbing larvae. Pipet in fresh DPBS.
7. Wash in DPBS 7 more times (8 DPBS washes total).
   a. In between washes, put RPMI-1640 in a conical tube and put in water bath to warm up.
8. After 8th wash, pipet out supernatant and add warm RPMI-1640 to 50 ml line.
9. Pipet out 25 µl of solution and count number of active larvae. Repeat for a total of 50 µl.
   a. Determine # of larvae.
i. (X larvae/25 µl) (1000 µl/1 ml) (50 ml/tube) = larvae in tube
b. Determine # of culture flasks to make.
i. Larvae in tube/10,000 = # ml RPMI-1640 needed.
   1. Larval concentration should be 10,000 larvae/ml for ES collection.
c. Measure sizes of newly hatched larvae.
i. Lengths and widths (1/3 of way from anterior end) of 5 larvae.
10. Centrifuge larvae in RPMI-1640.
11. Pipet out supernatant and do one more wash in RPMI-1640 (10 post-collection washes total: 8 washes in DPBS and 2 washes in RPMI-1640).
12. Remove supernatant.
13. Add fresh warm RPMI-1640.
   a. Determine how many ml RPMI-1640 remain in the tube, and how many need to be added.
   b. Choose appropriately-sized conical tube and pipet in...
      i. RPMI-1640 (amount to be added to the larvae).
      ii. 10 µl of antibiotic solution per ml total RPMI 1640.
      iii. 10 µl 100x concentrated protease inhibitor cocktail (Thermo Scientific 87785) per ml RPMI-1640. (40 µl 25x concentrated Sigma P2714 protease inhibitor cocktail was used during early cultures but led to higher larval mortality).
      iv. Vortex tube to mix RPMI-1640, antibiotics, and protease inhibitor.
   c. Pipet mixed solution into conical tube containing larval pellet.
14. Gently tap or shake tube to homogenize larvae, then pipet aliquots of 2-5 ml into T25 culture flasks.
   a. Incubate flasks at 37 °C and 5% CO2.
   b. Sit flasks upright.
Excretory-Secretory Protein Collection Protocol

Materials Needed:
- 50 ml, 15 ml screw-top conical tube
- Pipettors (1000 µl, 200 µl, 50 µl)
- 1000 µl pipet tips
- 200 µl electrophoresis pipet tips
- 50 µl normal pipet tips
- 1 L beaker
- Scintillation vial
- CO2 Incubator
- Water bath
- 5-10 ml syringe
- 18-20 gauge needle
- 0.2 µm syringe filter
- Vortex
- Snakeskin dialysis tubing (3.5 MWCO)
- Two dialysis tubing clips
- Stir bar
- Freeze dryer (lyophilizer)

*Make sure equipment in contact with media/larvae has been sterilized prior to use to reduce risk of bacterial contamination.

Chemicals Needed:
- RPMI-1640 with phenol red, 25 mM HEPES, and L-Glutamine
- 70% Ethyl alcohol
- Antibiotics: 100x concentrated solution
  - MP Biomedicals 1674049
  - Penicillin-10,000 IU/mL
  - Streptomycin-10mg/mL
  - Amphotericin B-25µg/mL
- 100 x concentrated protease inhibitor cocktail (PIC) Thermo Scientific HALT 87785
- 0.1 M ammonium bicarbonate

Safety Equipment Needed:
- Class II Biological Safety Cabinet
- Lab coat
- Gloves
- Goggles/surgical mask (Baylisascaris/Toxocara)
- Sharps container

All procedures involving flasks must be performed aseptically. Perform all pipetting or any procedures where larvae are exposed to air in BSC. Use 70% ethanol to clean gloves and all items placed in hood. When removing tubes from hood, cap tightly.

1. Turn on water bath to 37 °C.
2. Put 0.1 M ammonium bicarbonate in refrigerator (4 °C) to chill.
3. Sterilize hood with 70% ethanol.
4. Make sure T25 flasks (in incubator) containing larvae are in the upright position.
   - If not already, flasks should sit in this position for at least 10 minutes to allow larvae to sink to bottom of flask.
5. Calculate amount of RPMI-1640, PIC, and antibiotic needed.
a. RPMI-1640 needed will be half the amount that is in the flask (eg. 2 ml needed for a flask currently containing 4 ml)

b. PIC needed is 10 µl per ml RPMI-1640.

c. Antibiotic needed is 10 µl per ml RPMI-1640.

6. Remove RPMI-1640 from fridge along with PIC and antibiotic. Pipet calculated amounts of each into a conical tube, vortex, and place in water bath. Do NOT allow water in bath to reach cap of conical tube or contamination may result.

7. Carefully remove flask containing larvae from incubator and gently sit it in the hood. Do not shake it or larvae will be disturbed!

8. Set pipet to 200 µl and attach electrophoresis tip.

9. Remove flask lid and very carefully pipet from TOP of media in flask. Continue to pipet until half the liquid in the flask is removed. Put removed supernatant media into the conical tube (or microcentrifuge tube if only a small amount of media).

10. Remove conical tube from water bath and vortex.

11. Use 1000 µl pipet to put appropriate amount of fresh media solution into flask.

12. Return flask to incubator.

13. Repeat steps for other flasks in incubator, combining removed media.

14. Cut several inches of 3.5 MWCO snakeskin dialysis tubing. See directions for how much to cut, but probably 3 inches minimum.

15. Fold one end of dialysis tubing over several times and clip with tubing clip.

16. Attach needle to appropriately sized syringe and draw up the removed media. A 10 ml syringe won’t fit in a 15 ml conical tube, but a 5 ml one will.

17. Recap needle and remove from syringe.

18. Screw syringe filter (0.2 µm pore size) on to syringe.

19. Push plunger down to push media through syringe filter, into the dialysis tubing.

20. Roll the top of the tubing over several times and clip with dialysis tubing clip. Check for leaks.

21. Pour chilled 0.1 M ammonium bicarbonate buffer into large beaker.

   a. Over the course of dialysis, the buffer will need to equal at least 300x the amount of the sample, and should be changed at least 3x.

22. Put dialysis tubing containing sample into buffer and add stir bar.

23. Put on stir plate at low speed in refrigerator (4 °C).

24. Leave for a few hours, then change buffer.

25. Change buffer again before leaving the dialysis to go overnight.

26. Change buffer again as necessary until red/pink color of phenol red leaves the baggie.

27. Remove baggie from buffer, carefully unclip one end, and pipet out sample.
28. Pipet sample into a labeled scintillation vial. Fill the vial to a maximum of halfway.
29. Turn the vial so that it is at a 45° angle, increasing surface area of liquid, and store in freezer (-20 °C) at this angle.
30. After freezing, sample can be freeze dried in scintillation vial. Freeze dry until nearly all of the liquid has been removed.
Protein Concentration Estimation

Materials needed:
- Freeze-dried ES proteins in scintillation vials
- Pipets (1000 µl, 200 µl, 50 µl, 10 µl) and tips
- Multichannel pipet, 200 µl (optional)
- Pierce BCA Assay kit
- Microcentrifuge tubes (2 ml)
- 0.1 M ammonium bicarbonate
- Conical tube
- Plate shaker/ rocker

Non-CO2 incubator
96-Well microplate
Microplate reader
Flash drive

Safety equipment:
- Gloves
- Lab coat

Thawing Procedure:

1. Turn on non-CO2 incubator to 37 °C.
2. Remove previously freeze-dried samples from -20 °C.
3. Thaw rapidly by putting bottoms of scintillation vials under running lukewarm water.
4. If proteins do not appear to resuspend, add as small amounts of 0.1 M ammonium bicarbonate as possible until proteins resuspend.
5. Combine batches of proteins for the same species in a microcentrifuge tube.

BCA Kit Procedure:

6. Perform dilutions to make BCA kit microplate standards as specified by kit instructions, copied below. For diluent, use 0.1 M ammonium bicarbonate.

<table>
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<th>Tube</th>
<th>Volume of Diluent (µl)</th>
<th>Volume and Source of BSA (µl)</th>
<th>Final BSA Concentration (µg/ml)</th>
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<tbody>
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<td>A</td>
<td>0</td>
<td>300 of Stock</td>
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</tr>
<tr>
<td>B</td>
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<td>375 of Stock</td>
<td>1,500</td>
</tr>
<tr>
<td>C</td>
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<td>325 of Stock</td>
<td>1,000</td>
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<tr>
<td>D</td>
<td>175</td>
<td>175 of tube B dilution</td>
<td>750</td>
</tr>
<tr>
<td>E</td>
<td>325</td>
<td>325 of tube C dilution</td>
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<td>F</td>
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<td>125</td>
</tr>
<tr>
<td>H</td>
<td>400</td>
<td>100 of tube G dilution</td>
<td>25</td>
</tr>
<tr>
<td>I</td>
<td>400</td>
<td>0</td>
<td>0 (Blank)</td>
</tr>
</tbody>
</table>
7. Calculate the amount of working reagent (WR) needed.
   Formula:
   \[(\text{# standards} + \text{# unknowns}) \times (\text{# replicates}) \times (\text{volume of WR per sample}) = \text{total volume WR required}\]
   Formula for microplate procedure:
   \[(9 + \text{# unknowns}) \times 3 \times 200 \mu\text{l} = \text{total volume WR required}\]
   Round total required WR volume up so that you don’t run out of WR due to pipetting error.

8. Prepare WR by mixing 50 parts BCA reagent A with 1 part BCA reagent B (50:1, Reagent A:B).
   Formula:
   Calculated total volume WR required (this will be your reagent A): X = 50:1
   Solve for x to get volume B needed.

9. Vortex conical tube containing A and B. Turbidity should disappear to yield a clear, green WR.
   a. WR is stable for several days at RT in a closed container.

10. Draw out standards and unknowns on a blank microplate reader plan, so you know what will be in each well.

11. Pipette 25 µl of each standard or unknown replicate into a microplate well.
   a. This will require 75 µl total for each standard or unknown.
      i. If sample size is limited, or if samples appear to contain traces of RPMI-1640, pipet 40 µl of sample and 40 µl 0.1 M ammonium bicarbonate into a microcentrifuge tube and vortex. This would give a dilution of 1:2, and will need to be accounted for when calculating protein concentration.

12. Add 200 µl of WR to each well. If a lot of samples on plate, may want to use a multichannel pipet to speed up the process.

13. Mix plate thoroughly on a shaker plate for 30 seconds.

14. Cover plate and incubate at 37 °C in non-CO2 incubator for 30 minutes.

15. Cool plate to RT on benchtop.

16. While plate cools, turn on computer next to microplate reader.

Using Microplate Reader:

17. Open Softmax Pro.

18. Click assays → protein assays → BCA
19. Click Template.
   a. Assign correct protein values to each standard.
   b. Assign sample names and dilutions, if applicable.
   c. Click OK.
20. Click Fit under Standard Curve. Change to 4-Parameter.
21. Make sure wavelength is at 562 nm.
22. Once plate is cool, turn microplate reader on using button in back. Turn on immediately before use so bulb doesn’t get burnt out.
23. Make sure plate cover is off and place plate in drawer, matching A1 on drawer to A1 on plate.
24. Click “Read” on computer screen.
25. Click the arrow by Standard Curve. R2 should be above 0.9.
26. Click Standards. Check CVs to make sure they are below 10.
   a. If 10 or above, identify the well of the outlier.
   b. If appropriate, remove the outlier by selecting the well on the template and pressing backspace.
27. Repeat for Samples.
28. Copy and paste template, standards, standard curve, and samples to an excel file. Save on flash drive.
29. Remove plate from machine and shut drawer.
30. Push power button on machine and replace plastic cover.
**Fecal Flotation Protocol**

Materials Needed:

- Microscope
- Microscope slide
- Cover slip
- Fecal sample in plastic bag
- Scale
- Timer
- Mortar and pestle
- Sieve with fairly large holes
- Double distilled water
- Centrifuge
- Beaker (50 ml)
- 15 ml test tube(s)
- Sheather’s sucrose solution (1.27 g/mL)
- Applicator sticks

This procedure is ideal for small volume samples, such as foal grab samples. It provides a more sensitive measure of whether the foal is positive or negative for parasites, but does not provide an EPG. This procedure was courtesy of Dr. Eugene Lyons.

Procedure:

1. Label tube and microscope slide.
2. Squish sample around in bag for 30 seconds to 2 minutes to make sample more uniform.
3. Weigh out 1 g of feces into mortar.
4. Return sample to refrigerator.
5. Pour a small amount of ddH₂O into plastic cup or mortar to moisten the sample.
6. Use pestle to make sample uniform in water.
7. Put sieve on top of beaker and pour solution from mortar or cup into sieve.
8. Rinse any remaining debris from mortar or cup into sieve using more ddH₂O.
9. Pour solution into a labeled test tube.
10. Centrifuge at 200 x g for 5 minutes.
11. Use flinging motion to decant supernatant into sink, keeping pellet in bottom of tube.
12. Fill tube halfway with sucrose solution.
13. Use applicator stick to stir pellet and sucrose, completely breaking up the pellet.
14. Put tube in centrifuge and fill tube to the top with sucrose without overflowing.
15. Sit cover slip on top (gently tap cover slip or add more liquid if it does not contact the sucrose).
16. Spin at 200 x g for 10 minutes.
17. Remove cover slip from tube and put on microscope slide.
18. Put a second cover slip on the tube (the one that you just took the other cover slip from). This will allow you to capture any remaining eggs that float to the top.
19. Check first slide for presence/absence of parasite eggs. Do not count them! This is only a qualitative (+/-) test.
20. Check for eggs on the second slide.
21. Record which parasites the foal or horse is positive or negative for.
Modified Stoll Fecal Egg Count Protocol

Materials Needed:
- Microscope
- Microscope slide
- Cover slip
- Fecal sample in plastic bag
- Scale
- Timer
- Plastic cup or beaker
- Sucrose solution (1.275 g/mL)
- 90 mL double distilled water
- Disposable 10 mL pipet
- Centrifuge
- Centrifuge bucket cover (optional)
- 14 mL tube

Procedure:
1. Label plastic cup, conical tube, and microscope slide.
2. Squish sample around in bag for 30 seconds to 2 minutes to make sample more uniform.
3. Weigh out 10 g of feces into tared plastic cup on scale.
4. Return sample to refrigerator.
5. Measure 90 mL ddH2O in a graduated cylinder, and pour into plastic cup.
6. Stir sample with disposable pipet or stir bar for 1 minute, trying to make the solution as consistent as possible.
7. Take 1 mL from middle of sample in cup using pipet and put into conical tube.
8. Refrigerate sample in plastic cup.
9. Sit tube in centrifuge.
10. Pour sucrose to top of tube, sit cover slip on top (gently tap cover slip or add more liquid if it does not contact the sucrose).
11. Spin at 200 x g for 10 minutes.
12. Put cover slip on slide and count number of ascarid eggs and strongyle eggs (added separately) using microscope.
13. Multiply the number of ascarid eggs on slide by 10 to get the ascarid EPG (Eggs Per Gram).
14. Multiply the number of strongyle eggs on slide by 10 to get the strongyle EPG.
Polyacrylamide Gel Casting Protocol

Materials Needed:
- Mini-gel glass plates
  - One spacer plate per gel
  - One short plate per gel
- Casting stand (clear plastic)
- Casting frame (green plastic clamp)
- Gel comb

Chemicals Needed:
- 70-100% ethanol
- ddH2O
- Ammonium persulfate
- TEMED
- Acrylamide for running gel (stored at 4°C)
  - For 8% gels: 29% acrylamide, 1% bis-acrylamide
  - For 12% gels: 19% acrylamide, 1% bis-acrylamide
- Acrylamide for stacking gel
  - 37.5% acrylamide, 1% bis-acrylamide
- 4X running buffer (1.5 M, stored at 4°C)
- 4X stacking buffer (0.5M, stored at 4°C)
- 10% SDS
- Water saturated butanol (80% butanol)
- 1x TBST (if storing gels)

Safety Equipment Needed:
- Lab coat
- Gloves
- Safety glasses

Acrylamide is a carcinogen! Wear PPE when working with it. Acrylamide or any items that contact acrylamide (gloves, pipets, tubes, etc.) should be disposed of in hazardous waste container.

Equipment Preparation:
1. Determine which combs you will need for your gels (10 well, 15 well, 2D prep).
2. Select appropriately sized glass spacer plate to correspond with the size of the comb.
3. Clean spacer and short plates by scrubbing with soap, being careful not to scratch them, until the water runs off cleanly as a sheet (no water droplets left on the plate).
4. Wipe both plates with 70-100% ethanol and a kimwipe. Let dry.
5. Wipe the gasket (grey foam rubber rectangle) down with 70-100% ethanol on a kimwipe.
6. Place a clean gasket down on the bottom of the casting stand within the grooves.
7. Place the short plate on top of the spacer plate.
8. Slide the two plates into the casting frame, keeping the short plate facing to the front (where the clamp flaps are).
9. Lock the clamp to secure the plates together. Run your finger along the bottom of the two plates to ensure that their bases are even.
10. Place the clamped plates on the gasket and clamp them into place on the casting stand.

Making the Running Gel:

1. Make 10% ammonium persulfate fresh in a microcentrifuge tube.
   a. For 2 gels, weigh 30 mg (0.03 g) APS into tared microcentrifuge tube. Add 300 µl ddH2O.
2. Make 10% TEMED in hood in a microcentrifuge tube.
   a. For 2 gels, pipet 30 µl TEMED into tube. Add 270 µl ddH2O.
3. Determine what % gel you want to make for your running gel.
4. Label a conical tube for the running gel and combine chemicals in the order as follows:

8% Running Gel

<table>
<thead>
<tr>
<th># of 1.5 mm Mini-Gels</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td># of 0.75 mm Mini-Gels</td>
<td>2</td>
<td>4</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>ddH2O (ml)</td>
<td>5.2</td>
<td>10.4</td>
<td>15.6</td>
<td>20.8</td>
</tr>
<tr>
<td>1.5 M Running Buffer (ml)</td>
<td>2.5</td>
<td>5</td>
<td>7.5</td>
<td>10</td>
</tr>
<tr>
<td>Acrylamide (29% acryl:1%bis-acryl) (ml)</td>
<td>2</td>
<td>4</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>10% SDS (ml)</td>
<td>0.1 (100 µl)</td>
<td>0.2 (200 µl)</td>
<td>0.3 (300 µl)</td>
<td>0.4 (400 µl)</td>
</tr>
</tbody>
</table>

APS and TEMED start the solidification process...add them last and be ready!

10% APS

<table>
<thead>
<tr>
<th>10% APS</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 (100 µl)</td>
</tr>
</tbody>
</table>

10% TEMED

<table>
<thead>
<tr>
<th>10% TEMED</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 (100 µl)</td>
</tr>
</tbody>
</table>
## 12% Running Gel

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td># of 1.5 mm Mini-Gels</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td># of 0.75 mm Mini-Gels</td>
<td>2</td>
<td>4</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>ddH2O (ml)</td>
<td>4.2</td>
<td>8.4</td>
<td>12.6</td>
<td>16.8</td>
</tr>
<tr>
<td>1.5 M Running Buffer (ml)</td>
<td>2.5</td>
<td>5</td>
<td>7.5</td>
<td>10</td>
</tr>
<tr>
<td>Acrylamide (19% acryl:1%bis-acryl) (ml)</td>
<td>3</td>
<td>6</td>
<td>9</td>
<td>12</td>
</tr>
<tr>
<td>10% SDS (ml)</td>
<td>0.1 (100 µl)</td>
<td>0.2 (200 µl)</td>
<td>0.3 (300 µl)</td>
<td>0.4 (400 µl)</td>
</tr>
</tbody>
</table>

APS and TEMED start the solidification process...add them last and be ready!

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% APS</td>
<td>0.1 (100 µl)</td>
<td>0.2 (200 µl)</td>
<td>0.3 (300 µl)</td>
<td>0.4 (400 µl)</td>
</tr>
<tr>
<td>10% TEMED</td>
<td>0.1 (100 µl)</td>
<td>0.2 (200 µl)</td>
<td>0.3 (300 µl)</td>
<td>0.4 (400 µl)</td>
</tr>
</tbody>
</table>

Once all ingredients have been added, vortex very briefly.

5. Save the rest of the APS and TEMED for the stacking gel.
6. Use a 1000 µl pipet to insert the gel solution into the slot between the spacer and gel plate.
   Some tips for pipetting:
   a. Pipet in a corner against the spacer plate so that the fluid runs down the side and spreads evenly.
   b. Use a kimwipe to prevent bubbles from getting into the assembly.
   c. Avoid bubbles by being careful and not fully emptying pipet tip.
   d. For 0.75 mm gels, you will need to pipet quickly and carefully so that the gel does not solidify in an uneven manner. May not want to attempt more than 2 of these gels at once.
7. Pipet carefully, but quickly until the solution reaches the top of the green clamp flaps (1 cm from the top of the short plate).
8. Keep the remainder of the gel and the pipet tip in the conical tube.
9. Shake water saturated butanol to mix (80% butanol).
10. Once the solution has reached the line, CAREFULLY and slowly pipet 80% butanol to the top of the gel. Butanol can distort the gel if squirted with too much force. Add 1 ml of the 80% butanol for 1.5 mm gels, or 500 µl for 0.75 mm gels. The butanol prevents air from entering and drying out the gel.
11. Leave the gel to harden for 30 minutes. Check the conical tube containing the gel and pipet tip to see if it has hardened. Also, moving the casting frame should move the butanol but not the gel.
12. Invert the gel over the sink to dump off water and butanol layers. Rinse with ddH2O wash bottle until butanol smell is gone (4+ times).
13. Wipe off excess H2O and turn clamping frame on its side. Use a kimwipe to wick the last of the water from the gel.

Making the Stacking Gel:

1. Label a conical tube for the stacking gel and combine chemicals in the order as follows:

<table>
<thead>
<tr>
<th># of 1.5 mm Mini-Gels</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td># of 0.75 mm Mini-Gels</td>
<td>2</td>
<td>4</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>ddH2O (ml)</td>
<td>1.86</td>
<td>3.72</td>
<td>5.58</td>
<td>7.44</td>
</tr>
<tr>
<td>0.5 M Stacking Gel (ml)</td>
<td>0.75 (750 µl)</td>
<td>1.5</td>
<td>2.25</td>
<td>3</td>
</tr>
<tr>
<td>Acrylamide (37.5% acryl:1%bis-acryl) (ml)</td>
<td>0.3 (300 µl)</td>
<td>0.6 (600 µl)</td>
<td>0.9 (900 µl)</td>
<td>1.2</td>
</tr>
<tr>
<td>10% SDS (ml)</td>
<td>0.03 (30 µl)</td>
<td>0.06 (60 µl)</td>
<td>0.09 (90 µl)</td>
<td>1.2</td>
</tr>
</tbody>
</table>

APS and TEMED start the solidification process...add them last and be ready!

| 10% APS | 0.03 (30 µl) | 0.06 (60 µl) | 0.09 (90 µl) | 0.12 (120 µl) |
| 10% TEMED | 0.03 (30 µl) | 0.06 (60 µl) | 0.09 (90 µl) | 0.12 (120 µl) |

2. Vortex the conical tube briefly.
3. Use a 1000 µl pipet to add the stacking gel on top of the running gel. Pipet carefully but quickly (especially for 0.75 mm gels). Try not to make any bubbles. If necessary, gel can be added from both sides for equal distribution.
4. Fill gel to the top of the short plate.
5. Leave the remainder of the gel and pipet tip in the conical tube.
6. Locate the gel comb of the appropriate size and well formation.
7. Wearing safety glasses, cover the front (side with the text) of the gel comb with a kimwipe to prevent splashing. Insert the comb straight down into the space between the glass plates.
8. Allow to harden for 60 minutes at room temperature.
9. After the gel has hardened, proceed to run the SDS-PAGE gel or...
Prepare the gel for storage:
   a. Lay a square of saran wrap on the benchtop.
   b. Lay a paper towel flat overtop the saran wrap, leaving a few inches of saran wrap around the edges.
   c. Squirt 1x TBST on the paper towel until it is saturated.
   d. Put the gel (still in plates with comb inserted) flat on the paper towel. Wrap it up in the paper towel and saran wrap.
   e. Store at 4°C.
   f. Gels can be stored for up to two weeks. Check occasionally to make sure the paper towel is still moist.

Composition of 4X stacking buffer (0.5 M Tris Base):

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount needed per liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris Base</td>
<td>60.5g</td>
</tr>
<tr>
<td>ddH2O</td>
<td>850 mL</td>
</tr>
<tr>
<td>6 or 10 N HCl</td>
<td>to adjust to pH = 6.8</td>
</tr>
<tr>
<td>ddH2O</td>
<td>to bring volume up to 1 L</td>
</tr>
</tbody>
</table>

Store at 4 °C.

Composition of 4X running buffer (1.5 M Tris Base):

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount needed per liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 M Tris base</td>
<td>181.65 g/L</td>
</tr>
<tr>
<td>ddH2O</td>
<td>750 mL</td>
</tr>
<tr>
<td>6 or 10 N HCl</td>
<td>to adjust to pH = 8.8</td>
</tr>
<tr>
<td>ddH2O</td>
<td>to bring volume up to 1 L</td>
</tr>
</tbody>
</table>

Store at 4 °C.
Polyacrylamide Gradient Gel Casting Protocol

Materials Needed:
- Mini-gel glass plates
  - One 1.5 mm spacer plate per gel
  - One short plate per gel
- Casting stand (clear plastic)
- Casting frame (green plastic clamp)
- Gel comb (1.5 mm)
- Stir plate
- Two small stir bars
- Empty syringe
- Microcentrifuge tubes
- 10-1000 µl Pipets
- 50 ml conical tubes
- Kimwipes
- Saran wrap and paper towels (if storing gels)
- 15 mL Gradient Mixer and tubing (clear plastic square with 2 chambers)

Chemicals Needed:
- 70-100% ethanol
- ddH20
- Ammonium persulfate
- TEMED
- 40% Acrylamide for (stored at 4˚C)
  - 37.5% acrylamide, 1% bis-acrylamide
- 4X running buffer (1.5 M, stored at 4˚C)
- 4X stacking buffer (0.5M, stored at 4˚C)
- 10% SDS
- Water saturated butanol (80% butanol)
- 1x TBST (if storing gels)

Safety Equipment Needed:
- Lab coat
- Gloves
- Safety glasses
  Acrylamide is a carcinogen! Wear PPE when working with it. Acrylamide or any items that contact acrylamide (gloves, pipets, tubes, etc.) should be disposed of in hazardous waste container.

Equipment Preparation
1. Clean spacer and short plates by scrubbing with soap, being careful not to scratch them, until the water runs off cleanly as a sheet (no water droplets left on the plate).
2. Wipe both plates with 70-100% ethanol and a kimwipe. Let dry.
3. Wipe the gasket (grey foam rubber rectangle) down with 70-100% ethanol on a kimwipe.
4. Place a clean gasket down on the bottom of the casting stand within the grooves.
5. Place the short plate on top of the spacer plate.
6. Slide the two plates into the casting frame, keeping the short plate facing to the front (where the clamp flaps are).
7. Lock the clamp to secure the plates together. Run your finger along the bottom of the two plates to ensure that their bases are even.
8. Place the clamped plates on the gasket and clamp them into place on the casting stand.
9. Sit stir plate on high area above benchtop.
10. Sit the casting stand on the benchtop.
11. Add a tiny stir bar to each chamber of the gradient mixer.
12. Make sure both valves on gradient mixer are in the closed position (upright)

Making the Running Gel:
1. Make 10% ammonium persulfate fresh in a microcentrifuge tube.
   a. For 2 gels, weigh 30 mg (0.03 g) APS into tared microcentrifuge tube. Add 300 µl ddH20. Vortex.
2. Determine what % light and heavy gels you want to combine.
3. Label a conical tube for the light gel and another for the heavy gel. Mix chemicals in the conical tubes using the amounts below:

<table>
<thead>
<tr>
<th>Gradient Running Gel Ingredients (for two 1.5mm gels)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH20 (ml)</td>
</tr>
<tr>
<td>6.3</td>
</tr>
<tr>
<td>40% Acrylamide (37.5% acryl:1%bis-acryl) (ml)</td>
</tr>
<tr>
<td>1.5 M Tris (pH 8.8) (ml)</td>
</tr>
<tr>
<td>10% SDS (ml)</td>
</tr>
<tr>
<td>Total Volume (ml)</td>
</tr>
</tbody>
</table>

4. Once all ingredients have been added, vortex very briefly.
5. Pipet 5 ml of the light solution into the back gradient mixer chamber (furthest from the tubing attachment).
6. Pipet 5 ml of the heavy solution into the front gradient mixer chamber (closest to the tubing attachment).
7. Add 50 ul of 10% APS to each chamber. The gel will begin to polymerize at this point.
8. Add 3 ul of straight TEMED into each chamber.
9. Sit gradient mixer on top of stir plate.
11. Insert pipet tip end into space between glass plates to fill the gel.
12. Open back chamber valve first, then open the front chamber valve.
13. Gravity should pull polyacrylamide solution down to the pipet tip to fill the gel.
14. Fill by positioning the pipet tip in the center of the plate, not on the sides.
15. Once the gel has all run out, disconnect the end of the tubing and flush excess solution out with an empty syringe (and into gel plates, if more solution is needed).
16. Gel should fill up to top of green clamp flaps (1 cm from top of short plate).
17. Flush tubing and gradient mixer with double distilled water immediately after use.
18. Add 1 ml of 80% butanol to top of gel.
19. Repeat steps 5-18 if a second gel is needed.
20. Leave the gel to harden for at least 60 minutes. Moving the casting frame should not move the gel.

Making the Stacking Gel:
Stacking Gel Ingredients (for two 1.5mm gels)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH2O (ml)</td>
<td>3.72</td>
</tr>
<tr>
<td>4x Stacking Buffer (0.5 M Tris) (ml)</td>
<td>1.5</td>
</tr>
<tr>
<td>40% Acrylamide (37.5% acryl:1%bis-acryl) (ml)</td>
<td>0.6 (600 µl)</td>
</tr>
<tr>
<td>10% SDS (ml)</td>
<td>0.06 (60 µl)</td>
</tr>
<tr>
<td>Total Volume (ml)</td>
<td>6</td>
</tr>
</tbody>
</table>

1. Vortex stacking gel ingredients well.
2. Pipet 3 ml of stacking solution into another tube.
3. Make 100 µl 10% TEMED by combining 10 µl TEMED with 90 µl ddH2O in a microcentrifuge tube.
4. Add 30 µl 10% APS and 30 µl 10% TEMED to one of the 3 ml tubes. Vortex very briefly.
5. Vortex the conical tube briefly.
6. Use a 1000 µl pipet to add the stacking gel on top of the running gel. Pipet carefully but quickly (especially for 0.75 mm gels). Try not to make any bubbles. If necessary, gel can be added from both sides for equal distribution.
7. Fill gel to the top of the short plate.
8. Leave the remainder of the gel and pipet tip in the conical tube.
9. Locate the gel comb of the appropriate size and well formation.
10. Wearing safety glasses, cover the front (side with the text) of the gel comb with a kimwipe to prevent splashing. Insert the comb straight down into the space between the glass plates.
11. Allow to polymerize for 60 minutes at room temperature.
12. After the gel has polymerized, proceed to run the SDS-PAGE gel or...

Prepare the gel for storage:
   a. Lay a square of saran wrap on the benchtop.
   b. Lay a paper towel flat overtop the saran wrap, leaving a few inches of
      saran wrap around the edges.
   c. Squirt 1x TBST on the paper towel until it is saturated.
   d. Put the gel (still in plates with comb inserted) flat on the paper towel.
      Wrap it up in the paper towel and saran wrap.
   e. Store at 4°C.
   f. Gels can be stored for up to two weeks. Check occasionally to make sure
      the paper towel is still moist.
Running an SDS-PAGE Gel Protocol

Materials Needed:
• Vortex
• Eppendorf tubes
• Heat block
• Electrophoresis Tank
• Tank Lid
• Green and white electrode clamp
• Previously made gel

Chemicals Needed:
• Samples and 5x Laemmli buffer
• Molecular weight standards
• Prestained molecular weight ladder (optional)
• 1x TBST (optional)
• Unused 1x electrophoresis buffer
• Used 1x electrophoresis buffer

Safety Equipment Needed:
• Lab coat
• Gloves

Acrylamide is a carcinogen! Wear PPE when working with it. Dispose of in hazardous waste container.

Sample Preparation:
1. Turn on the heat block to 95 °C. It will take about an hour to heat up. Put a microcentrifuge tube rack in the freezer to chill.
2. Calculate amount of samples and laemmli buffer to load using 5x Laemmli buffer calculator (excel file). Use concentrations determined previously by BCA kit when inputting concentrations into sheet. Type in desired µg protein per well, and final loading volumes should be calculated. 40 µl is the maximum loading volume for a 15-well 1.5 mm gel.
3. Fill out Gel Loading Plan sheet for appropriate well type (1, 10, 15 wells).
4. When heat block is almost ready, remove samples and 5x Laemmli buffer (if not already combined) from freezer and allow to thaw.
5. If sample is not already diluted in 5x Laemmli buffer:
   a. In eppendorf tubes, combine sample and 5x Laemmli buffer to make required loading volume. Use 4 parts sample, 1 part Laemmli buffer. Account for a few extra microliters to make pipetting easier.
   b. Aliquot and freeze (-20 °C) any excess Laemmli-diluted sample.
7. Vortex samples and standards.
8. Heat samples (but not standards if they have already been heated previously) in heat block for 5 minutes at 95 °C. This helps to unfold the protein.
9. Put samples and standards in chilled microcentrifuge rack while waiting to use.

Tank Preparation:
10. Remove gel from fridge and slowly remove comb. If afraid of tearing the wells, squirt 1x TBST in to lubricate.
11. Begin preparing the tank.
   a. Put gels in the green and white electrode clamp. Make sure spacer plates are facing the outside of the clamp. If only running one gel, use a buffer dam on the other side with the writing towards the inside of the clamp. Pull green clamps up to secure.
   b. Put electrode/gel assembly into tank, matching the red side of assembly to red side of tank.
   c. Check to make sure tank lid fits on electrodes correctly, matching red to red.
12. Centrifuge samples in gray buckets at 1000xg for 1 minute to make them easier to pipet.
13. Fill chamber of electrode/gel assembly with unused 1x electrophoresis buffer to the top. Check for leakage.
14. Fill outside of the tank with new or used 1x electrophoresis buffer (halfway for 2 gels, fully for 4 gels). If there was leakage from inner chamber, fill the tank up the entire way.
15. Find Gel Loading Plan Sheet that was previously filled out.
16. Using gel loading pipet tips, load amount of Laemmli-diluted standard/sample into each of the designated wells, without puncturing the gel with the tip or leaking the sample outside of the well.
   a. Hint: Wipe tip on kimwipe before loading to get excess sample from outside of tip.
   b. Hint: Pull tip up a little when pipetting in last little bit so that the bubble doesn’t force sample out of the well.

Running the Gel:
17. Plug PowerPac HC into electrical outlet.
18. Put lid on tank, matching red electrode to red plug.
19. Turn on PowerPac using black button on side.
20. Plug lid in to PowerPac, matching red plug to red socket.
21. Set voltage to 100V.
22. Press start (running man picture). Bubbles should be moving within the chamber.
23. Run for 15-30 minutes at 100V. The blue color should leave the wells. The stacking gel should help “stack” the proteins, and the dye should flatten in shape.
24. Increase voltage to 180V. Run at 180V until the blue color just comes off the bottom of the plate, then press stop. This should take around 30-40 minutes.
   a. The dye is smaller than the proteins, so they should remain on the gel.
   b. The length of the run will affect where particular proteins end up on the gel.
   c. Pre-stained standards can help you to time run appropriately for particular proteins if you know their size.
25. Push the black power button on the side of the PowerPac.
26. Unplug and remove lid.
27. In sink, pour used electrophoresis buffer into the used buffer container to save for future runs.
28. Unclamp gel and plates from electrode assembly.
29. Use green gel releaser or scalpel to carefully pry up short plate, leaving gel on spacer plate.
30. Cut off stacking gel (there is a line where the stacking and running gels meet) and put gel waste into hazardous acrylamide waste container.
31. Proceed to appropriate protocol (western blot, coomassie, silver stain).

Composition of 1X Electrophoresis buffer:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount needed per liter</th>
<th>4 L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>3.0285 g/L</td>
<td>12.114 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>14.4135 g/L</td>
<td>57.654 g</td>
</tr>
<tr>
<td>SDS</td>
<td>1 g/L</td>
<td>4 g</td>
</tr>
<tr>
<td>ddH2O</td>
<td>to bring volume up to 1 L</td>
<td>to bring volume up to 4 L</td>
</tr>
</tbody>
</table>

**pH should be between 8.3 and 8.5, but in the event that it is not, new buffer must be made (cannot “adjust” pH). Also a 10X running buffer can be made (multiply all values in the table above by 10) and then before using add 100 mL of 10X running buffer to 900 mL of ddH2O.

Composition of 5x Laemmli buffer:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount needed per 20 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 M Tris-HCl pH 6.8</td>
<td>4 ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>10 ml</td>
</tr>
<tr>
<td>SDS</td>
<td>2 g</td>
</tr>
<tr>
<td>B-mercaptoethanol</td>
<td>5 ml</td>
</tr>
<tr>
<td>1% bromophenol blue</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

*PREPARE SOLUTION IN HOOD*
Directions:

1) Add 1 ml of 1% bromophenol blue to 4 ml of 1.5 M Tris-Cl pH 6.8.
2) Add 10ml of glycerol and mix.
3) Add 2 g of SDS and mix (the SDS will take a few minutes to dissolve).
4) Add 5 ml of β-mercaptoethanol and mix.
5) Aliquot and store at -20°C.

Western Blotting Protocol

Materials Needed:
- Pre-run gel
- Polyvinylidene fluoride (PVDF) membrane
- 2 pieces Whatman blotting paper (per gel)
- Scissors
- Slide staining boxes
- Blunt end tweezers
- Orbital rocker
- Gel releaser (green wedge)
- 1000-2000mL volumetric flask
- Stir bar
- Conical Tubes
- Multiscreen apparatus (for 2-D/Prep gels)
- Vacuum flask (for 2-D/Prep gels)
- Microcentrifuge tube
- ½” binder
- Vari-mix platform rocker
- Rubbermaid transfer container
- Tupperware box for methanol
- Tupperware box for chilled 1x transfer buffer
- Tupperware box for used 1x transfer buffer
- Gel holder cassette (clear and black with holes)
- 2 Sponges (per gel)
- Electrode module (black and red with electrodes)
- Ice pack
- Buffer tank
- Buffer tank lid
- Flattener roller (roller with handle or pipet)
- Filter paper

Chemicals Needed:
- 100% methanol
- 1x transfer buffer (chilled and made the day of)
- 10x transfer buffer
- 0.1% India Ink

Safety Equipment Needed:
- Gloves
- Lab coat
- Hazardous acrylamide waste bin

Preparation:
1. Run proteins in gel as specified in the “Running an SDS-PAGE gel” protocol.
2. While running the gel, make fresh 1x transfer buffer and chill at 4°C
### 1x Transfer Buffer

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount needed for 1 liter (1-2 gels)</th>
<th>Amount needed for 2 liters (3-4 gels)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x Transfer Buffer (see end of protocol for instructions)</td>
<td>100 mL</td>
<td>200 mL</td>
</tr>
<tr>
<td>Methanol</td>
<td>200 mL</td>
<td>400 mL</td>
</tr>
<tr>
<td>ddH2O</td>
<td>~700 mL (bring to volume)</td>
<td>~1400 mL</td>
</tr>
</tbody>
</table>

3. Activate the membrane:
   a. Cut a piece of PVDF membrane with a notch in the top left corner.
   b. Fill a small tupperware box with 100% methanol. Use tweezers to grasp a corner of the membrane and submerge the membrane in the methanol. Make sure membrane is completely saturated and remains in methanol for no more than 15 seconds.
   c. Transfer the membrane to another Tupperware box with fresh chilled 1x transfer buffer. Put on the orbital rocker for 5 minutes.

4. Remove and equilibrate gel:
   a. Pour used 1x transfer buffer into another Tupperware box.
   b. Carefully and slowly use gel releaser to pry open glass plates surrounding gel. If the gel sticks to the short plate, submerge the gel and plates in the 1x transfer buffer box until the short plate can be removed.
   c. Cut off the stacking gel and put the stacking gel in the hazardous acrylamide waste bin.
   d. Cut a very small portion of the gel’s top right corner off (where the standard was).
   e. For 1.5 mm gels, carefully pick up gel with gloves and place in the Tupperware box with 1x used transfer buffer to equilibrate.
   f. For 0.75 mm gels, submerge the spacer plate and gel in the used 1x transfer buffer until the gel loosens from the plate. These gels rip very easily!
   g. Put Tupperware containing gel on orbital rocker on low for 5 minutes. This will remove salts that could generate heat during transfer.

5. Make the transfer sandwich:
   a. Fill the large Rubbermaid transfer container with enough used (or new) 1X transfer buffer to submerge the gel holder cassette.
   b. Place the gel holder cassette into the 1x transfer buffer in the open position.
   c. Get the sponges completely wet and push out all potential air bubbles by rolling with the flattener. Bubbles will appear as white spots on the sponge. Keep sponges immersed in the 1x transfer buffer.
d. Align one sponge on the black side of the gel cassette holder.

e. Put one piece of Whatman blotting paper on top of the sponge and apply gentle pressure to keep it wet and prevent any bubbles from forming between the sponge and the paper.

f. Transfer the gel to the top of the Whatman blotting paper by gently picking up the gel at the corners. Make sure there are no bubbles.

g. Place the activated PVDF membrane on top of the gel. Line up the notch on the PVDF membrane with the notch on the gel (top right).

h. Apply the second piece of Whatman blotting paper on top of the PVDF membrane, ensuring there are no air bubbles.

i. Place the second sponge on top of the Whatman blotting paper. Push out any more bubbles.

j. Clamp the gel cassette holder to secure.

6. Set up the buffer tank and run the transfer:

   a. Put the black and red electrode module in the buffer tank, matching red to red and black to black.

   b. Insert a plastic ice pack into the buffer tank.

   c. Put 500mL of new pre-chilled 1x transfer buffer in the buffer tank.

   d. Put the gel cassette in the electrode module, white clamp facing up. 2 gel cassettes can fit in one electrode module.

   e. Add more pre-chilled 1x transfer buffer if necessary, to bring solution in tank up to the “blotting” line.

   f. Put buffer tank lid on buffer tank (red-to-red, black-to-black).

   g. Plug buffer tank lid cord into Powerpac.

   h. Set Powerpac to run at 300 milliamps for 1 hour (may need to adjust run time for certain proteins). 300 milliamps = 0.3 amps.

       i. Should see small bubbles floating up from hole in electrode module.

   i. After one hour, pause, then stop the Powerpac. Remove lid.

7. Wash and stain the membrane:

   a. Remove the gel cassette sandwich from the buffer tank, squeezing out as much buffer as possible.
b. Place the gel cassette sandwich into the empty Rubbermaid transfer container. Put the black side on the bottom and open the clamp.

c. Carefully peel the top filter paper from the membrane. Note where the gel is underneath the membrane. If the membrane is larger than the gel and appears too large to fit in a glass slide staining box, carefully cut some of the excess membrane from the sides (do NOT cut off any membrane that was touching the gel).

d. Carefully peel the membrane off the gel using tweezers. Place the membrane protein side up (notch should now be in top left corner) in a glass slide staining box. Always keep membrane moist.

e. Wash in 1x TBST twice for 5 minutes each time on the rocker.

f. After pouring out second wash, keep the slide box rocking and pour 5-10 ml 0.1% India Ink into the box. Keep membrane rocking while pouring to avoid making swirl patterns on membrane.

   i. To make 50 ml 0.1% India Ink, add 50 µl India Ink to 50 mL 1x TBST.

   g. Stain on rocker in 0.1% India Ink for 15-20 minutes. Standards should start to appear after about 5 minutes.

   h. Pour out 0.1% India Ink solution.

   i. Wash membrane 4 times with 1x TBST on a rocker for 5 minutes each time.

   j. Determine whether you want to continue with the procedure or dry and store the blots. For overnight primary antibody incubations, continue with blocking. For short primary antibody incubations, dry and store the blot. Dried, refrigerated blots should last at least 2 weeks.

8. To dry:

   a. Remove membrane protein side up and lay on a piece of filter paper or paper towel.

   b. Let membrane air dry for at least 20 minutes, moving it to a dry spot several times.

   c. Place membrane within the folds of a kimwipe, then put in a labeled plastic baggie and seal.

   d. Store at 4 °C for up to 2 weeks. Make sure membrane doesn’t get bent or creased while storing.

9. To remove stored blots:

   a. Remove membrane from kimwipe and let sit on benchtop at room temperature for 10 minutes to equilibrate.

   b. Put 1x TBST in a glass slide box. Sit membrane on top of the liquid and let it slowly soak in.

   c. Rock in 1x TBST for 10 minutes to equilibrate. Proceed to blocking.

10. Block the membrane:

    a. Calculate amount of blocking buffer (5% w/v non-fat dry milk in 1x TBST) needed.
i. 10 mL solution (made with 0.5g milk powder, 1x TBST to volume) per membrane. Vortex well in conical tube.

b. Discard the 1x TBST from the slide box.

c. Add blocking buffer and block the membrane for 1 hour at room temperature with the slide box on the rocker.
   i. While blocking, do calculations and make samples as directed in Primary Antibody Incubation section.

d. Wash the membrane 3 times in 1x TBST on a rocker for 5 minutes each time.

e. Primary antibody incubation:

f. Follow the appropriate protocol for your gel/membrane type.
   i. Determine if you need to use the multiscreen apparatus (for 2-D/prep gels containing standards in one lane, and then the same protein across the gel in a second long lane).

For 10 or 15-well membranes:
   a. Make 5 mL of blocking solution.
      i. 0.25g non-fat dry milk
      ii. 1x TBST to volume
      iii. Serum for primary antibody.
         1. Dilution of 1/1250 for adult horse serum samples and post-suckle samples.
            a. 4 µl serum per 5 ml blocking solution.
         2. Dilution of 1/500 for older serum samples.
            a. 20 µl serum per 5 ml blocking solution.
      iv. Vortex and add solution to slide staining box. Rock overnight at 4°C.

For 2-D/prep membranes:
   a. Make sure the multiscreen is clean. It should be cleaned with ddH20 and Contrex and dried before use. Do not use >50% ethanol to clean it or it will degrade the plastic.
   b. Sample prep:
      i. Make 250 µl of each 1° antibody per lane.
      ii. Determine how many lanes you want to add 1° antibody to and multiply by 0.25 (representing the 250 µl per well) to get total ml blocking buffer needed. Round up.
         1. The MW standard will take up lanes 1 and 2 on the multiscreen, so you don’t need to add 1° antibody to these lanes. Leave lane 20 blank because it will probably not contain protein. This leaves 17 possible sample lanes.
      iii. Multiply total ml blocking buffer by 0.05 (representing 5% milk) to get grams of milk needed.
iv. Add milk to conical tube and vortex. Aliquot 250 µl per microcentrifuge tube for each sample.

v. Pipet 1° antibody (serum) into each microcentrifuge tube, expelling and drawing the buffer in to the pipet several times to get all serum out.
   1. Dilution of 1/1250 for recently collected serum samples.
      a. 0.2 µl per 250 µl lane.
   2. Dilution of 1/500 for old serum samples.
      a. 0.5 µl per 250 µl lane.

11. Assemble multiscreen apparatus:
   a. Remove blot from slide box using tweezers on one edge.
   b. Pick up the plastic piece containing the lanes and position the membrane face-down on the BACK side of this piece. You should see the protein side when looking from the front.
   c. You should still be able to see the standards at the left. Make sure they are in lanes 1 or 2. Check to make sure protein bands in the large well run across all of the lanes that you plan to use. Place this plastic piece on the gasket, lining up the pegs and screws.
   d. By hand, tighten the screws in the pattern below and tighten well:

   ![Multiscreen Apparatus Diagram]

   a. Sit the apparatus on a ½ inch binder so that the top of the apparatus is slightly higher than the bottom.
   b. Pipet 250 µl of each sample into the pre-designated lanes.
      i. Tips:
         1. Check tightness and keep membrane moist by pipetting 1x TBST in the standard lanes. Make sure there is no leakage before pipetting any primary antibodies.
         2. Pipet 1° antibody solution out from the tube slowly and smoothly to avoid bubbles.
         3. Place the pipet tip into the hole on the bottom of the lane and slowly eject the 1° antibody solution.
12. Wash the membrane after 1° antibody incubation:
   a. Remove 1° antibody solution.
      i. For 10 or 15 well membranes, pour it out.
      ii. For 2-d/Prep membranes, use a vacuum flask with tubing cut straight across on the vacuum end. Put vacuum flask tubing overtop of lane holes to remove the liquid.
   b. Wash membrane with 1x TBST for 5 minutes each time.
      i. For 10-15 well membranes
         1. Perform this step 6 times on the orbital rocker
      ii. For 2-D/Prep gels
         1. Pipet 250 µl 1x TBST into each lane in the manner described earlier.
         2. Rock on the Vari-Mix for 5 minutes each wash. Remove using vacuum suction after each wash. Wash in this manner at least 3 times if all lanes receive the same secondary antibody. If lanes receive different secondary antibody, you will need to wash at least 6 times in the multiscree
         3. If all lanes will receive the same secondary antibody, unscrew the top part of the apparatus, remove membrane, place it protein side up in a glass slide box, and wash at least 4 more times on the orbital rocker.
   c. This step is very important to remove the 1° antibody so that nothing other than antigen-bound antibody will react with the 2° antibody.

13. Incubate in 2° antibody solution:
   a. Prepare blocking buffer. For one membrane, use at least 10 ml blocking buffer solution.
   b. To make 20 ml blocking buffer, add the following to a conical tube and vortex well:
      i. 1.0 g non-fat dry milk
      ii. 1x TBST to 20 ml volume
      iii. 0.2 µl Strep-HRP (for binding to standards).
   c. Determine which secondary antibody you need to use, depending on the animal species that the primary antibody was produced in.
i. Anti-rabbit IgG
   1. 1/5,000 dilution (which is really 1/10,000 due to the mixing procedure when glycerol was added).
   2. Add 4 µl per 20,000 µl blocking buffer in the conical tube. Vortex.

ii. Anti-horse IgG(T)
   1. 1/100,000 dilution for fresh samples
      a. Add 0.2 µl per 20,000 µl blocking buffer in the conical tube. Vortex.
   2. 1/50,000 dilution for old control sera
      a. Add 0.4 µl per 20,000 µl blocking buffer in the conical tube. Vortex.

iii. Anti-horse IgE or IgM
   1. 1/1,000 dilution
      a. Add 20 µl per 20,000 µl blocking buffer in the conical tube. Vortex.

d. If doing same secondary antibodies for all samples:
   i. Pour into glass slide box after 1x TBST is poured out and cover.
      Rock on orbital rocker for 1 hour at room temperature.

e. If doing different secondary antibodies for all samples:
   i. Pipet 250 µl into each lane after removing 1x TBST. Rock on Vari-Mix rocker for 1 hour at room temperature.

f. While samples incubate, remove ECL prime from fridge. Per membrane, pipet 0.5 ml of solution A into one conical tube, and 0.5 ml of solution B into another. Let sit at room temperature, with tube A covered by aluminum foil or another object to protect from light exposure.

14. Wash and prepare for exposure:
   a. If doing same secondary antibodies for all samples:
      i. Pour out secondary antibody solution and rinse several times with 1x TBST.
      ii. Wash using 1x TBST on orbital rocker 6 times.
   b. If doing different secondary antibodies for all samples:
      1. Pipet 250 µl 1x TBST into each lane in the manner described earlier.
      2. Rock on the Vari-Mix for 5 minutes each wash. Remove using vacuum suction after each wash. Wash in this manner at least 3 times.
      3. Remove membrane, place it protein side up in a glass slide box, and wash at least 4 more times on the orbital rocker.
   c. Cut a non-glare sheet protector into 6 pieces.
   d. Put gloves, ECL tubes, an extra microcentrifuge tube, slide protectors, filter paper, and slide boxes containing membranes in a box for transport to Gluck.
15. Expose using FlourChemE in Gluck (Howe Lab 430):
   a. Add ECL.
      i. Remove membrane from 1xTBST and let corner drip onto kimwipe.
      ii. Lay membrane protein side up on a piece of filter paper. Using a cut piece of filter paper, gently dab the top of the membrane (don’t smear) to dry it.
      iii. Insert membrane into the sheet protector piece.
      iv. Mix 0.5 ml of ECL Prime A solution and 0.5 ml B solution in a microcentrifuge tube.
      v. Pipet 1 ml of solution on to the membrane. Cover with the top plastic piece and push out any bubbles.
      vi. Let sit for 5 minutes.
   b. Set up machine and expose.
      i. Open door and pull out sliding platform.
      ii. Remove clear plate and put black plate in for chemiluminescent blotting.
      iii. Center blot on black plate.
      iv. Use stylus to select “Chemiluminescence,” and adjust setting if necessary (or set on auto).
      v. Select “Exposure.”
      vi. After exposure, select the invert logo (black and white boxes) to change from white bands to black bands.
      vii. Select the logo with the four boxes. Select the best looking exposure.
      viii. Edit the blot image by entering username, editing the project title or description, and selecting “Print.”
      ix. Insert flash drive on top or side of machine and select “Copy.”
         1. To find photos again, select “Images” and search for your username.
      x. To restart with a new blot, select “Darkroom.”
      xi. When finished...
         1. Make an entry into the log stating how many prints you made.
         2. Replace clear plate on the platform and put black plate back into it’s bag.
Chemical solutions that can be made prior and stored:

### 10x Transfer Buffer

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount needed per liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris Base</td>
<td>30.3 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>144.13 g</td>
</tr>
<tr>
<td>ddH20</td>
<td>Bring to volume</td>
</tr>
</tbody>
</table>

### 10x TBS buffer

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount needed per liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris Base</td>
<td>24.2 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>80.0 g</td>
</tr>
<tr>
<td>HCl</td>
<td>To adjust pH to 7.6</td>
</tr>
<tr>
<td>ddH20</td>
<td>Bring to volume</td>
</tr>
</tbody>
</table>

### 1x TBST buffer

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount needed per liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x TBS Buffer</td>
<td>100 ml</td>
</tr>
<tr>
<td>Tween-20</td>
<td>1 ml</td>
</tr>
<tr>
<td>ddH20</td>
<td>Bring to volume</td>
</tr>
</tbody>
</table>

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LITERATURE CITED


37. Nielsen, M.K., et al., *Analysis of multiyear studies in horses in Kentucky to ascertain whether counts of eggs and larvae per gram of feces are reliable indicators of numbers of strongyles and ascarids present.* Veterinary Parasitology, 2010. **174**(1-2): p. 77-84.


233. Todd, A.C., et al., *Worm parasites in Thoroughbred sucklings and weanlings. A survey of incidence, development, and control*. 1949, Kentucky Agricultural Experiment Station, University of Kentucky: Lexington, KY.


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