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EQUINE IMMUNITY TO CYATHOSTOME INFECTIONS

A Dissertation

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

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

by
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To Mom and Dad

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Abstract

To study the protective responses of cyathostome-infected ponies, two challenges were performed employing animals with different histories of exposure to these parasites. The hypothesis developed and tested in these experiments was that ponies that had longer exposure to cyathostome contaminated pastures would express acquired resistance to infection. The assumption behind this hypothesis was that helminth-naïve ponies infected with cyathostomes would eliminate the infection using only innate immune responses. Whereas previously exposed ponies would eliminate the infection with acquired immune responses, and these would be more effective in ponies with longer exposure to cyathostomes. Thus, helminth-naïve animals would acquire the largest number of worms, followed in decreasing order by young and then adult ponies.

Two types of challenges were used: an controlled infection with 150,000 cyathostome infective third stage (L_3) given over a period of 5 days, and the natural acquisition of infection by grazing a cyathostome contaminated pasture for 7 weeks. The natural challenge was performed to confirm the data obtained with the experimental challenge, therefore to validate its use.

The parasitological data recovered showed that ponies with acquired resistance to cyathostome infections had reduced total number of worms, developing larvae, luminal fourth stage larvae, adult parasites and cyathostome species. The acquisition of resistance was also observed as elevations in the hypobiotic larvae numbers and of intestinal mast cells, intestinal and peripheral eosinophils, and antibody responses. These responses were consistent with increases in Th2 type cytokines, principally interleukin-4 (IL-4).

The data obtained suggest that the immune mechanisms of resistance developed in ponies with acquired protection to cyathostome are slow to develop and are targeted against each

parasite stage present in the host. These results warrant further research in the area, especially in the difference between immune mechanisms of helminth naïve ponies and animals with short exposures to cyathostome contaminated pastures.

Chapter 1: Literature Review

1.1 The Parasites

Within the large number of nematodes that infect horses, ponies, donkeys, zebras and their respective hybrids, the small strongyles, or cyathostomes, are the most abundant helminth (or nematode) parasites of these hosts. The lay term “small strongyle” includes all the genera of the subfamily *Cyathostominae* and the genera *Oesophagodontus*, and *Triodontophorus*, which are in the subfamily *Strongylinae*. For simplicity’s sake, and because 99.9% of the parasites recovered from the large intestine or cultured as L_3 from the feces belonged to the family *Cyathostominae*, the terms “cyathostomes” and “small strongyles” have been used interchangeably. More recently the term cyathostomins has been proposed and is becoming adopted in the literature (Lichtenfels et al., 2002). The more traditional term cyathostome will be used throughout this dissertation. A brief classification of these parasites follows (Lichtenfels et al., 1998).

Phylum *Nemathelminthes*
  Class *Nematoda*
    Order *Strongylida*
      Suborder *Strongylina*
        Family *Strongylidae*
          Subfamily *Strongylinae*.
            Genera *Oesophagodontus*
            Genera *Triodontophorus*
          Subfamily *Cyathostominae*
            Genera *Cyathostomum*
              Species *pateratum*
                catinatum
            Genera *Coronocyclus*
              Species *coronatus*
                labiatus
                labratus
            Genera *Cylicocyclus*
              Species *brevicapsulatus*
                elongatus
                insigne
                leptostomus

nassatus
radiatus
ashworthi
ultrajectinus
Genera *Cylicostephanus*
Species *asymmetricus*
bidentatus
calicatus
goldi
longibursatus
minutus
Genera *Cylicodontophorus*
Species *bicoronatus*
Genera *Petrovinema*
Species *poculatum*
Genera *Poteriostomum*
Species *imparidentatum*
ratzii
Genera *Parapoteriostomum*
Species *euproctus*
mettami
Genera *Gyalocephalus*
Species *capitatus*
Genera *Tridentoinfundibulum*
Species *gobi*

Adult cyathostomes can be differentiated by morphological features such as the shape buccal capsule, presence or absence of cutting plates or teeth in the buccal capsule, elements of the *corona radiata*, etc. Although, neither ova nor larvae can be differentiated into species. Large numbers of adult cyathostomes reside on the surface or within the luminal contents of the large intestine of equids, where the different species have preferential developing sites (Ogbourne, 1976). As an example, C*ylicocyclus nassatus, Cylicostephanus minutus, C. calicatus* and *Cyathostomum catinatum* predominate in the ventral colon whereas the predominant species in the dorsal colon are *Cylicostephanus longibursatus, C. goldi* and *C. insigne. Coronocyclus coronatum* and *Petrovinema poculatum* preferentially develop in the cecum, although members of several other genera colonize this region after emergence from the mucosa (Ogbourne, 1978).

The adult female worms lay their eggs, which are deposited in the external environment with the host's feces. The morulated eggs develop into first stage larvae within days of being deposited into a suitable external environment. These eggs are very resistant to environmental factors. Lucker (1941) studied the viability of morulated eggs, non-infective larvae and infective third stage larvae (L_3) in a range of temperatures under laboratory conditions and in the field. From his experiments it is known that the minimum temperature required for eggs to embryonate is approximately 10°C, that morulated eggs are more resistant to temperature changes than larvated ones, that cycles of freezing and thawing are more deleterious for eggs and larvae than continuous subfreezing temperatures, and that the infective L_3 is the most resilient of the stages that live in the environment.

The L_3 migrate to the grass surrounding the fecal pat, which maximizes their chances to be acquired by a suitable host. The pattern of development and survival of L_3 differs depending upon the local climatic conditions. In northern temperate climates, L_3 develop and accumulate in the pasture throughout the year (Ogbourne, 1973). The highest numbers are found during the late summer and early autumn, when the temperatures are more favorable for their development (Duncan, 1974; Ogbourne, 1973 and 1975). It has been suggested that although larvae are able to overwinter, especially under a permanent snow cover, they become more susceptible to climatic changes in the spring when their stored energy sources are exhausted (Baker et al, 1939, quoted in Ogbourne, 1973). Other authors indicate that even if the L_3 lifespan is shorter during the hottest months of the year in temperate climates, they effectively keep reinfesting pastures since fecal pats and grass mats act as refugia (Lucker, 1941; Ogbourne, 1973).

The survival of free-living larvae on herbage is maximal during the cooler months of the year in subtropical climates, such as northern Australia or the southern states of the U.S.

(English, 1979 a and b; Craig et al., 1983; Courtney and Asquith, 1985; Mfitilodze and Hutchinson, 1988; Hutchinson et al, 1989; Baudena et al, 2000). In laboratory experiments, L_3 have been shown to have very low survival rates at temperatures ranging from 33 to 37º C (Mfitilodze and Hutchinson, 1987). A probable explanation for this phenomenon is the rapid exhaustion of energy reserves due to heat related increases in activity. The combination of heat and humidity in the topsoil levels also impacts larval survival. Exposure to ultraviolet light also has deleterious effects on larval survival (Taylor, 1938). At high temperatures, a minimum amount of moisture is needed for the larvae to molt to the third stage, however, in temperatures above 33ºC, L_3 viability increases in dry conditions rather than in humid ones (Mfitilodze and Hutchinson, 1987).

1.2 Development of the Life Cycle in the Host

Upon ingestion, the L_3 exsheath on their migration through the gastrointestinal tract to invade the large intestine (Ogbourne, 1978; Reinmeyer et al, 1986). The exsheathed larvae burrow into the glands of Lieberkühn, where the majority of the species invade the mucosa, an example would be *Cylicostephanus longibursatus* (described by Tiunov, referenced in Ogbourne, 1978). Some species such as *Cylicocyclus* spp. and *Gyalocephalus capitatus* migrate deeper into the submucosa of the large intestine (described by Tiunov, referenced in Ogbourne, 1978). Once in the wall of the large intestine the larvae continue to develop to the L_4 stage or arrest their development as a hypobiotic L_3 (EL_3) (Eysker and Mirck, 1986). Poynter (1969) suggested that the EL_3 become surrounded by fibroblasts that form a capsule, the host's reaction to the invasion, thereby the inflammation seen around the larvae is minimal. However, Mathieson (1964) (cited by Ogbourne, 1978) described a marked goblet cell hypertrophy and hyperplasia around the encapsulated larvae. He also noted a marked eosinophilic infiltration in the submucosa directly

underneath of the encapsulated larvae. In few instances the eosinophils were in direct contact with the larvae. Extensive infiltration of eosinophils seemed to be directly associated with the emergence of the larva from the capsule (Mathieson, 1964, cited by Ogbourne, 1978). These observations suggest that larvae are quickly encapsulated upon invasion, and elicit an inflammatory response that is granulocytic in nature. When these larvae develop and are closer to emergence, they may become the target of the immune system. Few details of this host parasite relationship are known.

The larvae developing in the mucosa or submucosa of the large intestine begin to molt to the forth stage before or after entering the lumen of the large intestine (Ogbourne, 1978). Tiunov (cited by Ogbourne, 1978) states that *Cylicostephanus longibursatus* is one of the cyathostome species living in the mucosa that develops for 6 to 12 days before molting to the fourth stage in the intestinal lumen. Others species that seem to have a short development are *Cyathostomum catinatum*, *C. coronatum*, *Cilicostephanus minutus* and *C. calicatus*. However, *Cylicocyclus* spp. larvae develop in the submucosa in no less than eight weeks. Consequently, different cyathostome species develop at different rates implying that they have different life spans, thus, different prepatent periods. The shortest prepatent period is thought to be 35 days (Gibson, 1953; Tiunov, 1951, cited by Round, 1969; Poynter, 1969), and the longest 10-13 weeks, if arrested development is not taken into account.

As noted, not all the larvae readily develop to adult stage. In 1953 Gibson provided the first record of cyathostome hypobiosis, or arrested development, in the horse. He described small strongyles fecal egg counts of 4 stalled horses repeatedly treated with phenothiazine. After each treatment the fecal egg output would be reduced to zero. Five to 8 weeks post-treatment the fecal egg counts would start to rise again. Since the horses were fed strongyle free hay and grain,

Gibson concluded that the adult worms he found in the feces after each phenothiazine treatment had develop from larvae encysted in the mucosa of the intestine, and that removal of the adult worm population acted as a cue for the encysted larvae to emerge. Hypobiosis has been defined as the arrest, retardation, inhibition, or suppression of parasitic larvae that have not completed development in the host within the commonly accepted development interval for the species (Gibbs, 1986). Gibbs suggested that hypobiosis of nematodes in ruminants could be of 2 types: an immune mediated arrest, or a seasonally induced arrest. The factors that elicit the immune mediated arrest of L_3 are not known. Experimental evidence trying to differentiate immune arrested from seasonally arrested L_3 has been scant and non-conclusive; and immunosuppression of the host did not resulted in resumption of development of arrested L_3. The factors that are hypothesized to be involved in the seasonally induced arrest are: environmental stimuli such as temperature, humidity, and photoperiod. Host related factors are those alterations in the environment that alter the host physiological responses that in turn alter the parasite's behavior. Parasite related factors could be pheromones, which participate in worm interaction, genes that control obligatory arrest, or prior exposure of the worms to the environment, which promotes externally induced arrest. Since parasites evolve in specific relationships with their natural hosts, the resumption of development could be triggered by variable stimuli. This attempt to classify arrested development into two categories might not be accurate. As indicated by Gibbs (1986), most of the parasites that undergo hypobiosis present seasonally induced arrest with variable degrees of immune mediated arrest. Hypobiosis then would facilitate the survival or persistence of the parasites during periods of environmental adversity.

Only two factors are known to trigger resumption of L_3 development in cyathostome infections. One is the elimination of adult parasites from the intestinal lumen by using an

anthelmintic compound. The other is the natural synchronized senescence of adult cyathostomes during the late winter or early spring months. Both events trigger the development of EL_3 that replace the adult parasite population. The massive emergence of the developing larvae is termed larval cyathostomiasis. The signs and symptoms found in horses suffering from this condition vary with individual cases, however, most of them have a sudden onset of profuse diarrhea in the late winter or early spring months, showing varying degrees of emaciation and lower limb edema with production of soft feces (Chiejina and Mason, 1977; Ogbourne, 1978; Giles et al, 1985). These events could be associated with the incoming (Uhlinger, 1992) or the exiting larvae (Chiejina et al., 1977; Church et al., 1986) from the intestinal mucosa and submucosa.

In the case of a light cyathostome infections the damage to the intestinal wall is localized around the individual larvae. If massive infestations occur, a more generalized widespread catarrhal or hemorrhagic inflammation is seen. Animals with heavy infestations show edematous enteritis, with large areas of epithelial erosion and numerous ulcers that indicate the presence of emergent larvae. The most acute cases are seen in younger animals, usually two to six years of age (Gogolka, 1933 and Wagner, 1935 cited by Poynter, 1969; Ogbourne, 1978; Reinmeyer, 1986; Mair, 1994). Adult horses, animals older than 6 years of age, present two different patterns of infestation, which are likely related to their immune status. If the horses have a continuous exposure to cyathostome contaminated pastures, they develop resistance that is characterized by a lower number of adult parasites, lower egg counts (Klei and Chapman, 1999), and increased number of encysted L_3. Exceptions to this rule are individuals that idiosyncratically, or due to a breakdown of their resistance caused by age, or by exposure to cyathostome infections after being in a continuous anthelmintic treatment, acquire and maintain large numbers of cyathostomes which can cause severe cyathostomiasis.

1.3 Immunity to Gastrointestinal Nematodes

Studies of the immune responses against parasitic nematodes have primarily used laboratory rodents. Four murine gastrointestinal dwelling parasites (*Nippostrongylus brasiliensis*, *Trichinella spiralis*, *Heligmosomoides polygyrus*, and *Trichuris muris*) have been the focus of these investigations. Each of these parasites has a distinct and different life cycle: *T. muris* is a large intestine nematode, the other three are small intestine dwellers; *N. brasiliensis* and *T. spiralis* have tissue migratory larval stages, *H. polygyrus* and *T. muris* do not. *N. brasiliensis* and *H. polygyrus* adult stages live in the gastrointestinal lumen; *T. muris* adults induce syncitium formation and *T. spiralis* adults live in the intestinal epithelium for a short period and the first stage larvae induce the formation of nurse cells in the musculature where they reside (Else and Finkelman, 1998). It is not surprising that specific responses to these parasites differ. Nonetheless, the study of these infestations in mice has confirmed the role of Th2 type T cells in anti-nematode responses (Finkelman et al, 1997).

T. spiralis larvae penetrate the mucosa of the small intestine where they develop into adults. Mice generally expel them between 7 and 21 days post-infection (p.i.). The expulsion rate is mouse strain dependent (Else and Finkelman, 1998). The invasion of the intestine by this parasite is associated with a strong inflammatory response that includes eosinophils and mast cells. The most notable change in the intestine is mast cell hyperplasia. The role of mast cells in worm expulsion was studied using W/W^v mice, which lack functional mast cells, and a monoclonal antibody (mAb) against stem cell factor (SCF), that abrogates mast cell production (Finkelman et al, 1997). In both cases *T. spiralis* infected mice had delayed worm expulsions (Finkelman et al, 1997). In the reverse scenario, transgenic mice that overexpress interleukin (IL) 9, which increases mast cell proliferation and activity, showed elevated intestinal mastocytosis

and accelerated worm expulsion if infected with *T. spiralis*. Using IL-4 KO mice infected with *T. spiralis* it has been shown that IL-4 plays an important role in expulsion and is also responsible for intestinal enteropathy such as villus atrophy and crypt hyperplasia. In contrast, IFN-γ, a hallmark Th1 type cytokine, has been shown to have no role in either expulsion or host pathology (Finkelman et al, 1997).

T. muris is a trichuroid parasite that colonizes the murine colon and cecum. The anterior end of the parasite embeds and digests mucosal epithelium. While *T. muris* infections readily develop in a variety of mouse strains, expulsion is usually associated with the generation of a strong Th2 type cytokine response and chronic infections with IFN-γ and IL-12, Th1 type cytokines (Else and Finkelman, 1998). The adoptive transfer of $CD4^+$ T cells to severe combined immunodeficient (SCID) mice, that lack B and T cells, demonstrated antibodies are not needed for the expulsion of *T. muris* (Bancroft and Grencis, 1998). The importance of IL-4 in inducing a Th2 type response has been demonstrated by delivering IL-4 in the form of a complex with an antibody to susceptible strains of mice and by giving anti-IL-4 receptor mAb to resistant strains of mice. In both cases, the expulsion phenotype of the mice was completely altered (Bancroft and Grencis, 1998). Also, there is a strong inhibition of *T. muris* expulsion in mice deficient in IL-4 or IL-13. But IFN-γ deficient mice are protected if given either IL-4 or IL-13, which promotes expulsion of the parasite (Finkelman et al, 1997). In the same model, chronic infections were promoted by administering IL-12 to resistant mice. Increased proliferation of intestinal epithelial cells and crypt hyperplasia are characteristics of a chronic infection, reduction of these parameters are seen in resistant strains of mice that have strong Th2 type responses. In this model, IFN-γ has been shown to promote pathology. Chronic infections with this parasite are characterized by intestinal epithelial cell proliferation and crypt hyperplasia, which is a common

characteristic of *T. muris* susceptible mice. The intestinal epithelial cell proliferation is regulated by IFN-γ, as opposed to being a mechanical response caused by the presence of the worm. SCID mice, that have a peak production of IFN-γ after infection with *T. muris*, were depleted of IFN-γ with mAb. The role of IFN-γ in epithelial turnover was then confirmed, because the mice showed increased pathological changes in the absence of worm expulsion (Bancroft and Grencis, 1998).

The parasite *N. brasiliensis* uses the rat as the natural host, however mouse adapted strains have been developed. Expulsion of this parasite in mice has been found to depend on IL-4 and IL-13, since IL-4 KO mice do expel worms. Apparently IL-13 plays a major role in the expulsion of *N. brasiliensis*, since IL-13 KO mice showed a significantly delayed expulsion of the worms. This demonstrates the redundancy of the immune system to eliminate the infection, and how different worms trigger different effector mechanisms of the immune response (Else and Finkelman, 1998; Bancroft and Grencis, 1998).

Primary *H. polygyrus* infections in mice produce a chronic infection. However, challenge infections of mice that have had primary infections removed by anthelmintics are expelled more rapidly. This resistance has been correlated with increased numbers of mast cells, whose production is stimulated by IL-4. Mast cell numbers are reduced in chronic infections. Even though a primary infection with *H. polygyrus* produces a chronic infection, many Th2 type cytokines are induced. One of the most important in this system is IL-4. Administration of monoclonal anti–IL-4 or anti-IL-4 receptor antibodies effectively block protective immunity. However, studies on mice given anti-IL-5 mAb effectively reduce eosinophil levels without an effector worm expulsion. This observation demonstrated the absence of a role for eosinophils in protection.

Infection of lambs with the parasite *Haemonchus contortus* also induces a Th2 type response (Gill et al.; 2000). Cultures of spleen cells, abomasal or mesenteric lymph node cells from genetically resistant or randomly bred lambs had higher levels of IL-5 and lower of IFN-γ than uninfected controls. Furthermore, mitogen- and antigen–stimulated IL-5 responses were higher among resistant lambs when compared with random-bred lambs. These animals also presented higher parasite-specific IgG1 and IgE responses. Histological examination of abomasal tissue revealed higher densities of mast cells and eosinophils in the mucosa of resistant lambs (Gill et al., 2000).

The expulsion of a primary infection of the trichostrongylid parasite of cattle, *Cooperia oncophora*, has been associated with increased eosinophilia and mucosal IgA and IgG1 (Kanobana et al., 2002). The cytokine profile expressed by cattle infected with this parasite corresponded to the Th2 type. Based on correlations between the systemic immune response and parasitological data the authors proposed an effector role for the parasite-specific humoral responses. The authors found that the increase in the number of eosinophils was significantly negatively correlated with the expulsion rate of the parasite expressed by sex ratio (where male adult worms are expelled faster than female adult worms). Parasite specific IgA andIgG1 antibody levels were negatively correlated to the fecundity of the worms, expressed as number of eggs per female worm. These observations suggested that increases in eosinophils, concurrent with increases in $CD4^+$ T cells in mesenteric lymph nodes, and IgA were responsible for expulsion of *C. oncophora* adult worms. Mast cell counts showed high variability between challenged and control animals, therefore no relations could be established with the parasitological data. The presumption that eosinophils played a role in the elimination of primary infections of *C. oncophora* was solely based on the increased numbers of this cell type and on

increases of mucosal IgA and serum IgG1. Interestingly, this is the first experiment that assumed a direct relationship between eosinophils and adult worms. The authors (Kanobana et al., 2002) did not perform any *in vitro* studies to confirm degranulation of eosinophils in the presence of *C. oncophora* larvae or adult worms, or measured cytokine levels. Hence, further research would be needed in order to confirm the role of eosinophils and antibodies in the elimination of adult gastrointestinal parasites.

Ostertagia ostertagi is a widely studied parasite of cattle that inhabits the abomasum. Protective immune responses are slow to arise when compared to other gastrointestinal nematodes, such as *Cooperia* spp. and *Haemonchus* spp. (Gasbarre, 1997). However, reduction of parasite egg output in the feces and increase in the number of larvae undergoing inhibition usually is seen after brief exposure (3-4 months) to *O. ostertagi* contaminated pastures. Immune responses that reduce the number of parasites are not evident until the bovine's second year of exposure to the parasite. Lymph nodes draining the abomasum increase in size presenting increases in lymphocyte numbers, both parasite specific and not. Also, they present higher percentages of B cells and γδ T cells (Gasbarre, 1997). The interleukin profile of the *O. ostertagi* infection is dominated by IL-4, which is a hallmark for Th2 type responses such as those shown for murine gastrointestinal nematode infections. The development of resistance in cattle infected with this parasite is slow to develop and animals remain susceptible to infections for prolonged periods of time. However, continuous exposure to the parasite enhances the herd immunity. Manifestations of the immune response that increase herd immunity include a delay in the development time of the parasites, an increase in the number of larvae that undergo an inhibition in development, morphological changes in the worms, stunting of newly acquired worms, and reduction of the egg production in the adult female worms (Gasbarre et al., 2001). Although the

exact mechanisms of these immune responses are not known, the studies performed in cattle infected with *O. ostertagi* clearly indicated that infected animals responded to infections. Nevertheless, the manifestations of these immune responses are slow to develop due to adaptive strategies displayed by the nematode. It has been suggested that *O. ostertagi* could actively evade or suppress protective immune responses of the host (Gasbarre et al., 2001). Hence, experiments that study specific parasite-host interactions would be necessary.

1.4 Immunity to Equine Helminths

There is very little literature on equine immune responses to parasitic helminths. Most of the reports on the equine immune response to a helminth have been focus on using *Strongylus vulgaris* (Monahan et al, 1994; Klei and Chapman, 1999; Swiderski et al., 1999; Edmonds, 2001, PhD dissertation). Shortly after ingestion, the *S. vulgaris* L_3 penetrates the mucosa of the large intestine where it molts rapidly to L_4. These larvae penetrate the submucosal arterioles and migrate up the intestinal vasculature until reaching the cranial mesenteric artery where they molt to the fifth stage. The L_5 then travels the opposite route to reach the arterioles of the large intestine, from which they access the gastrointestinal lumen finally molting into the adult stage. Throughout the tissue migration, *S vulgaris* elicits a strong response from the host that includes fever, localized inflammation of the vasculature, eosinophilia, anorexia, depression, and abdominal pain. Horses produce specific and non-specific antibody responses to different *S. vulgaris* larval stages (Klei, 1992; Monahan et al., 1994), but antibodies are not sufficient to produce a protective immune response. The use of an irradiated *S. vulgaris* L_3 vaccine has been shown to be highly effective in reducing the immunopathological damages produced by a challenge infection (Monahan et al., 1994). Immunological parameters related to the protection of the irradiated *S. vulgaris* L_3 vaccine were studied by Edmonds (PhD dissertation, 2001). In

this experiment IL-4 mRNA levels were higher in all challenged ponies, and those who received the irradiated vaccine had increasing levels of IL-5 in the cecal lymph nodes. Also, all challenged ponies had higher levels of IL-13 than the non-challenged, but this increase was significantly higher in the groups vaccinated with the irradiated vaccine. Animals that showed more severe clinical responses to challenge also presented higher levels of IFN-γ response. Increases in IgG (T), IgG (a) and IgA responses to *S. vulgaris* L_4 and L_3 somatic antigens correlated with protection in the ponies receiving the irradiated larval vaccine.

Although *S. vulgaris* infections produce an excellent model to study equine immune responses, the regular use of the avermectins for parasite control has practically eliminated this parasite from equine populations. Thus cyathostomes have become the most prevalent parasites in these populations. Research on these parasites has been limited to the study of their life cycle (Gibson, 1953; Poynter, 1969; Eysker and Mirck, 1986; Lichtenfels et al., 1998), epidemiology (Ogbourne, 1971, 1973, 1975, 1976, 1978; Hutchinson et al., 1989; Baudena et al., 2000; Chapman et al., 2001) and description of clinical cases of cyathostomosis (Chiejina and Mason, 1977; Giles et al., 1985; Mair, 1994; Uhlinger, 1992). The acquisition of resistance in cyathostome infections is easily measured with parasitological parameters. For example, in horses maintained under well-managed conditions the fecal egg count (FEC), as measured by number of parasite eggs per gram (EPG), of yearling animals are much higher that those of their dams (Klei and Chapman, 1999). A survey carried out with control animals used for anthelmintic trials (Chapman et al., 2001) showed that ponies older than eight years of age have significantly lower number of parasites than yearlings. Acquisition of resistance to cyathostome challenge was demonstrated by Monahan et al. (1998) when parasite-free or previously exposed ponies were challenged with 410,000 cyathostome L_3. When the ponies of this experiment were divided by

age, the acquisition of resistance was more marked in the older parasite exposed ponies that had lower numbers of all parasite stages. In field studies, the exposure of tracer ponies to a short grazing season to cyathostome contaminated pastures increases the number of hypobiotic larvae but did not induce protection (Chapman et al., 2002). Antibodies of the IgG type against somatic extracts of *Cylicocyclus insigne*, a cyathostome species, were detected in serum samples from mares and their foals exposed to different levels of parasites but a direct correlation between antibody titer and resistance was not found (Kara, M.S. thesis, 1996).

In the absence of experimental studies, the general goal of this dissertation was to further characterize the acquired resistance to cyathostome infections. The working hypothesis was that the acquisition of resistance to cyathostome infections increases with exposure to contaminated environments over time; therefore adult animals would have a lower number of parasites. Correlations between parasites at specific stages of development of young and adult ponies previously exposed to cyathostomes, and young naive animals, with cellular and humoral measurements of the immune response to acquired resistance were made whenever possible. Chapter 2 describes the findings of a controlled challenge of 150,000 infective cyathostome L_3, administered over a period of five days. Complete parasitological data is reported, in conjunction with the measurement of some immunological parameters. Ponies with different histories of prior exposure to cyathostomes were used. The responses of parasite-free (PF) ponies, that had no prior exposure to helminths, were compared to those of ponies with little prior exposure to cyathostomes (Young ponies) or prolonged prior exposure (Adult ponies) to these parasites. It was hypothesized that Adult ponies would show acquired resistance to infection, therefore acquiring a minimum number of worms. Whereas Young ponies would show some protective responses, although the number of parasites that would be acquired would be higher than for

Adult ponies. In the case of the PF ponies, immune responses would correspond to a primary infection, therefore the acquisition of worms would be maximal. Chapter 3 reports the results obtained in a natural challenge, where ponies grazed a cyathostome contaminated pasture for seven weeks. This type of challenge corresponds with the natural acquisition of larvae by horses, and it was executed to confirm that the results obtained in a controlled challenge under experimental conditions mimic the results of a natural challenge. The hypothesis of worm acquisition established for the animals in the controlled challenge was applied also in this case. The results of the immunological parameters measured were compared to those reported in Chapter 2. Chapter 4 summarizes the findings of both experiments and compares the differences and similarities of both types of challenges.

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Chapter 2: Controlled Challenge

2.1 Introduction

The most common helminth parasites of horses maintained on well-managed farms are the small strongyles or cyathostomes (Kaplan and Little, 2000). Ten genera and 26 species have been identified in equids in the U.S. (Lichtenfelds et al., 1998). The adult stages colonize the mucosal surface of the equine large intestine in large numbers (Ogbourne, 1978; Reinemeyer, 1986). The parasites have a direct life cycle and the infective third stage larvae (L_3) survive for long periods of time in the environment given the right conditions of temperature, humidity and light exposure (Ogbourne, 1973 and 1975). Neither ova nor L_3 can be identified to species (Ogbourne, 1975), and monoespecific infections have not been produced (Klei, 2000). Therefore the species' mixtures have been studied as a group (Klei and Chapman, 1999). The L_3 exsheath and invade the mucosa and submucosa of the cecum, ventral and dorsal colons, where they go into hypobiosis, as early L_3 (EL_3) (Smith, 1976), or continue to develop to a pre-adult stage (Ogbourne, 1978). The ingestion of large numbers of L_3 is thought to produce generalized inflammation of the equine large intestine, i.e. typhlitis and colitis (Blackwell, 1973; Love et al., 1999) and it is likely the cause of mild colics (Uhlinger, 1990). The synchronized emergence of pre-adult larvae produces a clinical condition termed larval cyathostomosis. Gross pathological and histological examinations reveal focal necrosis around larvae that have emerged, eosinophilic infiltration surrounding encysted larvae, and generalized edema and inflammation (Ogbourne, 1978; Church et al., 1986). Horses with clinical larval cyathostomosis show rapid weight loss, diarrhea, subcutaneous edema of the ventral abdomen, limbs, muzzle, sheath or udder, pyrexia and variable degrees of colic (Giles et al., 1985; Church at al., 1986; Mair, 1994), i.e. from mild, non-life threatening colics (Uhlinger, 1990) to non-strangulating infarctions and

cecal tymphany (Murphy et al., 1997). The appetite usually remains good or may even be ravenous. The presentation of the signs previously described varies from case to case depending on risk factors, parasite load and environmental management. Risk factors such as early age (2 to 6 years of age), access to pasture, grazing with other horses, and anthelmintic treatment within 2 weeks prior to presentation of disease have been associated with the clinical disease (Reid et al., 1995).

The current knowledge of cyathostome infections is based on limited studies. Publications on cyathostomes describe experimental infections (Love et al., 1992 a; Murphy and Love, 1997; Chapman et al., 2002 a), epidemiological studies of larval migration on herbage (Duncan, 1974; Polley, 1986; Mfitilodze and Hutchinson, 1988; Hutchinson et al., 1989), seasonality of the infection (English, 1979 a and b; Bucknell et al., 1995), or are descriptions of clinical cases of natural infections (Giles et al., 1985; Mair, 1994). Experiments where helminth-naïve foals of similar age were given cyathostome L_3 in one dose or as a trickle infection, showed that cold conditioning of the larvae prior to challenge and trickle dosing decreased the establishment rates (Love and Duncan, 1992 a). Groups of helminth naïve, foals, yearlings and adult ponies (Love and Duncan, 1992 b) infected naturally showed that foals with prior exposure to cyathostomes had fewer numbers of developing larvae than helminth naïve foals of the same age. Arrested development was higher in foals and yearlings with a grazing history than in adult ponies (Love and Duncan, 1992 b). Furthermore, adult ponies had lower parasite egg counts. These differences in larval acquisition and adult worm development were also noted by Chapman et al. (2002 a; 2003) where ponies older than 8 years of age had lower total parasite numbers and decreased number of cyathostome species per animal than ponies less that 4 years of age. Recently published material showed that when young and adult ponies, helminth-naïve or

previously exposed, were given a challenge of 100,000 L_3, the animals with prior exposure had significantly higher numbers of EL_3 and lower number of adult parasites (Chapman et al., 2002 b). These findings suggest that the immune responses developed by the previously exposed animals drive the larvae into hypobiosis but that these responses might not be protective.

In animals infected with gastrointestinal parasites protection is mediated by CD4+ T helper cells (Garside et al., 2000). $CD4^+$ T helper cells are polarized into Th1 and Th2 producing cells. Production of Th2 cytokines, which include IL-3, IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13, have been associated with protective responses in gastrointestinal parasite infections. Whereas Th1 cytokines, which include IFN-γ, IL-2 and lymphotoxin, have been associated with protection against intracellular organisms. Primary infections of ruminants with the gastrointestinal nematode *Ostertagia ostertagi* induce very strong IL-4 responses, a hallmark Th2 type cytokine (Gasbarre, 1997). Immunological profiles of ponies infected with *S. vulgaris*, a close relative of cyathostomes, indicates that these worms may elicit a cytokine profile of a T helper cell, in a manner similar to that of rodents or cattle infected with gastrointestinal parasites. In ponies infected with *S. vulgaris*, anamnestic-like eosinophil response occurs in immune ponies when compared to non-immune controls following challenge infection (Monahan et al., 1994). Eosinophils taken from ponies infected with this parasite kill *S. vulgaris* L_3 in vitro in an antibody dependent manner (Klei et al., 1992) and were found to be activated *in vivo* (Dennis et al., 1988). Cytokine messenger RNA (mRNA) corresponding to a Th2 type profile was found to be present from lymphocytes of ponies immune to S. *vulgaris* (Swiderski et al., 1999). The same pattern of cytokine expression was found in a pony undergoing spontaneous expulsion of luminal cyathostomes when compared to a control animal (Horohov, et al., 1997). This pony had a

dramatic EPG drop over a 5 week period, and at necropsy it had an eight fold increase in IL-4 mRNA expressed in the cecal lymph nodes when compared to the control.

The current study is designed to further characterize acquired resistance of equids to cyathostomes and initially identify the immune components of the protective responses. Ponies with different grazing exposures to cyathostome contaminated pastures were compared to naïve animals in order to study differences in the acquisition of resistance following a controlled challenge infection. Measures of the immune response were compared with parasitological recovery data.

2.2 Methods and Materials

2.2.1 Animals and Experimental Design

Mix breed ponies with different levels of exposure to cyathostomes were identified from a group maintained on cyathostome-contaminated pastures. Levels of exposure were based on pony's age and subsequently on number of seasons of pasture exposure to cyathostomes. Previous studies indicate that resistance to cyathostome infections is attributable to age and previous exposure (Monahan et al., 1998). Therefore it was hypothesized that ponies with longer prior exposure to cyathostomes would show protective responses upon challenge. Whereas ponies with minimal prior exposure to these parasites or raised under helminth-free conditions would show responses but not of a protective nature. To further test these hypotheses young and adult ponies with different exposure, and young parasite-naïve animals were experimentally challenged with cyathostome L_3. Eight 1-3 year old ponies (Young), with little exposure to cyathostomes, and eight 10-19 year old (Adult) animals, with several seasons of exposure to cyathostomes, were chosen from this population. Eight animals 1-2 years of age, raised under

parasite free (PF) conditions since birth, were used as non-exposed controls (Monahan et al., 1997).

These twenty-four ponies were placed randomly in pairs within age groups into box stalls eight weeks prior to challenge. At day –56 all ponies were treated with 200µg/kg of ivermectin (Eqvalan®, Merial LLC, Iselin, NJ) plus 20mg/kg of oxibendazol (AnthelcideEQ, Pfizer Animal Health, Exton, PA) for five consecutive days to eliminate their adult and larval cyathostome infestations (Monahan et al, 1997; Chapman et al., 2001). Each group of eight animals was further subdivided into non-challenged (Non) animals and challenged (Ch) groups. The Ch groups consisted of six ponies each and the Non-challenged of two ponies. Beginning at day 0, all challenged animals received 30,000 infective cyathostome L_3 for five consecutive days, for a total challenge of 150,000 L_3. At day 49 post-infection (p.i.) all ponies were humanely sacrificed. Complete necropsies and parasite recoveries were performed at this time.

2.2.2 Challenge

Cyathostome infective third stage larvae (L_3) were cultured from feces recovered rectally from cyathostome-infected ponies. The fecal donor ponies had grazed pastures with those selected for this experiment. Thus, the population of L_3 used was similar to that infecting the experiment animals. The feces were mixed with tap water and vermiculite, divided in small portions in aluminum foil packets and held at 25ºC for 10-12 days. The larvae were recovered from the cultures using a Baermann apparatus. Larvae from different cultures were pooled and kept at 4ºC for a maximum of 5-7 days. For the challenge, aliquots containing 30,000 L_3 were placed in 6 ml syringes. The Ch ponies received one oral inoculation per day of L_3, for five consecutive days for a total of 150,000 L_3 per challenged animal. The inoculum was placed

behind the tongue; the syringe was then refilled with tap water, and the rinse was similarly inoculated. Non-challenged ponies did not receive an inoculation.

2.2.3 Samples Collected

Blood was collected by venipuncture on days –56, 0, 6, 14, 22, 28, 41 and 49 into Vacutainers ® containing EDTA for a complete blood count (CBC) or without additives to obtain sera.

On days –56, 0, 6, 14, 28 and 49 additional blood samples were obtained in Vacutainers® to which a sterile heparin sodium salt solution was added. Peripheral blood mononuclear cells (PBMCs) were isolated and used in lymphoproliferation assays and to isolate mRNA for cytokine measurement.

Rectal fecal samples were obtained on days –56, 0, 28, 42 and 49. The fecal samples were processed using a modified Stoll technique (Klei and Torbert, 1980). Results are presented as eggs per gram (EPG). The first fecal sample after challenge was obtained at day 28 because the commonly accepted prepatent period for cyathostomes is four to six weeks (Ogbourne, 1978).

Ponies were humanely sacrificed and necropsies were performed following a protocol previously described (Klei and Torbert, 1980; Monahan, et al., 1998). At necropsy, the large intestine was opened and the contents collected. A minimum of two 2x2 cm sections of tissue was taken from the cecum, dorsal colon and ventral colon for histological examination. The mucosal surfaces were then washed, and these washings were added to the contents. A ten percent aliquot of the mixed intestinal content was preserved with 10% formaldehyde solution. Isolation and identification of developing and adult parasites were performed on a 1% aliquot of the total contents per animal.

After washing the luminal surfaces of the cecum, ventral and dorsal colons, each organ was separated and individually weighed. A 1% by weight portion of each organ was cut from the areas between the teniae bands for mucosal digestion. Larvae were identified from an aliquot of digested mucosa with a stereomicroscope. Three 32-cm^2 samples were taken from the rest of the cecum, ventral colon and dorsal colon for transmural illumination (TMI).

2.2.4 Complete Blood Cell Counts

The parameters measured in the CBC were: white blood cell (WBC) count, white cell differential, hemoglobin, hematocrit, mean cell volume, mean cell hemoglobin concentration, packed cell volume, plasma protein, and platelet count. Samples were analyzed by the Louisiana Veterinary Medical Diagnostic Laboratory. Differential WBC counts were determined by counts of 100 cells examined at 1000x.

2.2.5 Intestinal Cell Enumerations

Small sections (2x2 cm) of the dorsal colon, ventral colon and cecum were preserved in 10% buffered formalin. These specimens were embedded in paraffin and 4μ cut sections were stained with Giemsa stain and examined microscopically. The mast cells had a pink cytoplasm with fine violet granules and a blue nucleus, which readily differentiated them from the eosinophils that had bright pink large cytoplasmic granules. Microscopic examination of the stained sections was performed at 400x. The microscope's ocular was fitted with a square grid to aide in the cell count. Mast cells and eosinophils were counted from 6 mucosal and 6 adjacent submucosal areas chosen at random within the section. Mean counts of the six mucosal areas and the mean counts of the six submucosal areas were averaged and presented as number of cells per high power field.

2.2.6 Parasites and Antigen Preparation

Adult cyathostomes were identified and recovered from the intestinal contents of an infected pony using a stereomicroscope. The larger worms were isolated and speciated without the use of a fixative solution. *Cylicocyclus insigne* was by far the most numerous cyathostome species harbored by this particular animal. *C. insigne* worms were isolated, pooled and washed three times in buffered saline solution, thoroughly dried by blotting and frozen fresh at –20ºC. When ready to use, the worms were thawed at room temperature, and ground with a mortar and pestle. Using a minimum amount of PBS the ground worms were transferred to a conical centrifuge tube. The resulting mixture was kept on ice and further processed with a Brinkmann homogenizer for 20 to 30 minutes. The crude antigen obtained was rocked overnight at 4ºC. The antigen was then spun for 30 minutes at 4 °C at 2500 rpm (~ 1400x G). The supernatant was homogenized using a glass tissue homogenizer for 10-20 minutes and clarified by centrifugation. The supernatant was then filtered using a 45µm filter, and a second time using a 22 µm filter. Aliquots of 0.5ml were placed in cryotubes and 5 µl of a mixture of protein inhibitors were added to every ml of parasite extract (Maizels et al., 1991). Aliquots were stored at –70 °C. The amount of protein present in the antigen aliquot was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA), based on the method of Bradford.

L_3 antigen was obtained as follows. Feces from cyathostome-infected ponies were collected and cultured for ten days. Larvae collected with a Baermann apparatus were then pooled and kept at 4ºC until further use. A small volume containing 1.5×10^6 viable larvae was homogenized at 30,000rpm with a BioSpec 0.7cm Tissue Tearor (Biospec, Bartlesville, OK). Homogenization of the larvae was monitored through microscopic examination of sub aliquots. When larvae were completely disrupted, the homogenate was then transferred to a sterile flask

and 2.5% of aprotnin was added. The mixture was stirred at 4ºC overnight, then clarified by centrifugation at 10,000g for 30 minutes at 4ºC. The supernatant was then filtered with a 45µm filter, and then with a 22µm filter. Sub-aliquots were placed in cryotubes and stored at –70ºC until use. The amount of protein present in the antigen aliquot was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories), based on the method of Bradford.

2.2.7 Enzyme-linked Immunosorbent Assay (ELISA)

Circulating titers of antibodies to soluble cyathostome L_3 and adult *C. insigne* soluble antigens were determined by ELISA. Soluble antigens were diluted in 0.01 M carbonate buffer (pH 9.6) to 10 µg / ml. These preparations, in 50 µl volumes, were added to wells of 96 well flat-bottom polystyrene microtiter plates (Immulon 1B, Dynex Technologies, Chantilly, VA) and incubated overnight at 4°C. Plates were washed with PBS-0.05% Tween 20 (PBST) and nonspecific binding sites were blocked using 1% casein-PBS, 100 µl/well, for 1 and half hours at room temperature. Serum samples were analyzed in triplicate. A high responder serum sample served as a standard positive control for each plate. Sera from a parasite free pony served as a negative control. Sera were diluted 1:100 in 1% casein-PBS. Serum dilutions were added to appropriate wells in volumes of 50 µl per well. Plates were incubated at 37°C in a humidified environment for 1 hour. Following incubation plates were washed 5x with PBST.

For total IgG, affinity purified antibody peroxidase labeled goat anti-horse IgG (H+L) (KPL, Inc. Gaithersburg, MD) in a 1:10,000 dilution was added at 50 µl/well or, for equine subisotypes IgG (a) and IgG (b) mouse anti-horse IgG (a kind gift from Dr. Paul Lunn) in a 1:2,500 dilution was added at 50 µl/well. Plates were then incubated at 37°C in a humidified environment for 1 hour, and washed 5x with PBST. In the case of the equine subisotypes an extra step was performed by adding affinity purified antibody peroxidase labeled goat anti-

mouse IgG (H+L)(KPL, Inc.). Plates were incubated at 37°C in a humidified environment for 1 hour, and washed 5x with PBST. Peroxidase substrate (1 component TMB Microwell Peroxidase Substrate, KPL, Inc. Gaithersburg, MD) was added at 75 µl/well for 15 minutes for IgG, or 8 minutes for IgG (T) and IgG (a). TMB Stop Solution (KPL, Inc. Gaithersburg, MD) was added at 75 µl/well. Optical densities (OD) were recorded using a Dynatech MR 700 automated microtiter plate reader (Dynatech Laboratories Inc., Chantilly, VA) with absorbance set at 450nm.

A standard curve was included in each plate. It consisted of seven two-fold dilutions of the sera of an animal known to be positive to a cyathostome infection. The most concentrated curve value (1/20 dilution of the positive serum) was given the value of 1,600 ELISA units (EU). The data obtained as OD were entered into an Excel worksheet (Microsoft®), and a mean was obtained from the triplicate samples. If two or more of the triplicate samples had an standard deviation of more than 30% (calculated by the ELISA reader), then the values were discarded and the sample was re-run at a later date. A graph was constructed using the log transformed EU of the standard curve on the X-axis, and its corresponding averaged OD on the Y-axis. A regression line was fitted through the data points. The linear equation and R-squared value for the regression line were obtained. The unknown EU values were calculated by solving the straight-line regression for x. The formula was $x=([\text{OD value}] - b)/m$, where $b = y$ – intercept of the standard curve and m = slope of standard curve line. Values were then antilogged.

2.2.8 Indirect Fluorescent Antibody Test (IFAT)

Antibody recognition of cyathostome larval surface antigens was evaluated using IFAT. Third stage larvae were cultured from feces of donor ponies' known to be free of large strongyles. These L_3 were exsheathed with 2% Clorox and used immediately or placed into *in vitro* cultures (Chapman et al., 1994) for 3 days. The freshly exsheathed larvae were considered

infective L_3 and the cultured larvae as developing L_3. Two fold serial dilutions of the sera were made in flat-bottomed polystyrene microtiter plates (Immulon 1B, Dynex Technologies, Chantilly, VA). Controls consisted in wells with no larvae but with test sera and anti-horse antibody, larvae and no test sera with FITC-conjugated anti-horse antibody, or larvae with test sera and no FITC-conjugated anti-horse antibody. Approximately 50 L_3 in 50μl 0.01M PBS ph 7.6 were added to each well. Plates were covered, gently agitated, and placed in a 37°C incubator for 30 minutes. Plates were then washed 3 times with PBS. Following the final rinse, 25 μl of fluorescein (FITC)-conjugated affinity purified rabbit anti-horse IgG (H+L) (Jackson Immuno Research Laboratories Inc., West Grove, PA) diluted 1/15 in PBS was added per well. Plates were covered, agitated, and incubated for 30 minutes at 37°C. Following the final incubation, plates were washed 4x in PBS and larvae observed on a Nikon Diaphot TMD inverted microscope equipped with epifluorescence (Nippon Kogatu, Tokyo, Japan).

For IgG subisotypes, 50μl/well of subisotype was added after the serum L_3 incubation and wash. Isotypes tested were mouse anti equine IgG (a) (Serotec, Inc., Raleigh, NC), and IgG (T) (kindly provided by Dr. Paul Lunn). An extra control of wells with larvae, test sera, no anti horse antibody and with FITC-conjugated anti-horse antibody was added in this assay. Subisotypes were diluted 1/30 (IgG (a) and IgG (T)) in PBS, added to the plates, and incubated as above. Following this step, plates were washed 3 times and 25 μl of FITC-conjugated affinity pure rabbit anti-horse IgG (H+L) (Jackson Immuno Research Laboratories Inc., West Grove, PA), added, diluted 1/25 in PBS for IgG (a) or 1/50 for IgG (T). Plates were again incubated for 30 minutes, washed 4 times, and viewed with epifluorescence as above.

The titer was determined to be the concentration of the serum where more than half of the larvae were positive (fluorescent).

2.2.9 Isolation of Equine Peripheral Blood Mononuclear Cells (PBMC)

Blood samples were drawn into 20 ml Vacutainer ® tubes containing 0.2 ml preservative free heparin sodium salts (Sigma-Aldrich Corp.). The tubes were spun at 800g for 10 minutes. The plasma was then removed with a sterile pipette to 1 cm above the buffy coat and discarded. The buffy coat cells were removed and transferred to a sterile conical polypropylene centrifuge tube containing 2 ml of Ca-Mg-free phosphate buffered saline (CMF-PBS). The suspension was mixed and diluted to 8ml with CMF-PBS, then under layered with 4 ml of Ficoll-Paque ® Plus (Pharmacia Biotech, Amersham Biosciences Corp., Piscataway, NJ). The samples were spun at 800g at 20-30 ºC, for 30 minutes without braking. The clear upper layer of diluted plasma was discarded, and the cells on the interface with the Ficoll-Paque ® were removed to another tube containing about 2ml of CMF-PBS. The suspension was then again mixed and diluted with 14ml with CMF-PBS, and spun at 800g for 10 minutes without braking. The supernatant fluid is removed by aspiration or decantation and the pelleted cells are resuspended by tapping or strumming the tube. Another 14 ml of CMF-PBS was added to the cell suspension, and the samples were spun at 150g for 10 minutes without braking, this lower speed facilitated the removal of platelets. The previous steps were repeated for a total of 3 washes. After the third wash, the cell pellet was again resuspended in 1 ml (per 20 ml of heparinized blood drawn) in RPMI-1640 (supplemented with Na_2HCO_3, 25nM HEPES, penicillin-streptomycin-glutamine and 5.5 x 10^{-5} M 2-mercaptoethanol, and 5% inactivated fetal bovine serum). Viable cells were counted using a 0.04% trypan blue solution. The total live cell yield was determined as the number of viable cells counted per ml. times the measured volume of the cell suspension.

2.2.10 Lymphoproliferation Assay

Viable cells (1 x 10^5 per well) were placed in warmed supplemented RPMI-1640, in sterile U bottom 96 well culture plates (Corning Glass Works, Corning, New York). Each plate contained one subject per row; each row had triplicate samples for a negative control (media), a positive control (pokeweed mitogen, PWM at 1µg/ml) (Sigma-Aldrich Corp., St. Louis, MO) and the two antigen concentrations under study (0.1 µg/ml or 0.3 µg/ml of *C. insigne* antigen). Two sets of plates were run per time point, one with no stimulation, the other set had equine IL-2 added (a kind gift from Dr. David W. Horohov), as a growth factor supplement. IL-2 supplementation was used to detect instances of IL-2 deficiency in these cultures. The plates were incubated during 5 days at 39 ºC, with 5% CO_2, in a humidified environment. At the end of this incubation the plates were pulsed with 1µci of tritiated thymidine per well, and incubated for another 4 hours at 39 ºC in a humidified environment. The plates were stored at –20 ºC until further processing.

To evaluate proliferation, the cells were harvested in a Harvester 96 Mach II (TOMTEC, Hamden, CT). Following the harvest, 10ml of Betaplate Scint® (Wallac Scintillation Products, Perkin Elmer Life Sciences Inc., Torrance, CA) were added to each plate, and plates were read in a LKB 1205 Betaplate® liquid scintillation counter (Perkin Elmer Life Sciences, Torrance, CA). Results are expressed as the corrected CPM (c-CPM) that corresponds to the average counts of the triplicate PWM or antigen stimulated samples minus the average c-CPM of the triplicate non-stimulated wells. The net CPMs are the averages of the c-CPM for the animals present in each treatment group. The stimulation index (SI) is the average of the triplicate PWM or antigen stimulated wells over (divided by) the average of the triplicate c-CPM of the non-stimulated wells, which is a fold increase over the non-stimulated c-CPMs.

2.2.11 RNA Isolation and Complementary DNA (cDNA) Synthesis

Two aliquots of 3 x 10^6 PBMC were counted and placed into two RNAse free tubes and kept on ice. The cells were pelleted for 30 seconds at 14,000 rpm in a microcentrifuge and the supernatant was discarded. Cell pellets were resuspended in 300 µl of RNA STAT® (Tel-Test, Friendswood, TX), vortexed thoroughly and stored at –70 ºC until use.

RNA extraction and reverse transcription to obtain cDNA was done following a published protocol (Swiderski et al., 1999). Additionally all samples were diluted with 80µl DEPC H_2O and stored at –20˚C until use.

2.2.12 Detection of Specific Equine Interleukin Messenger RNA

Levels of IL-4, IL-5, IL-13, γ-IFN and of the house keeping gene β-actin mRNA were determined using the ABI PRISM™ 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA). This detection system uses a fluorogenic 5' nuclease assay (6-FAM), or Taq Man® assay, which allows the real time detection of fluorescence in a polymerase chain reaction (PCR) based assay under universal PCR cycling conditions. Probes and primers (a kind gift from Dr. D.W. Horohov) were constructed using Primer Express® software (Applied Biosystems, Foster City, CA), this program is recommended to be used whenever using the Taq Man® assay in order to maximize utilization. Sequences of the primers and probes are shown in Table 1. Probes containing specific equine interleukin sequences labeled with 6-FAM were purchased from PE Biosystems. Primers were commercially prepared by GeneLab (School of Veterinary Medicine, Baton Rouge, LA). A PCR master mix containing 20µM of each primer and 10 pmoles Taq Man® probe, 1x Taq Man® PCR Reagent and double distilled water was prepared. Aliquots of 45µl of the mix were dispensed into MicroAmp® Optical Tubes placed in a 96-well reaction plate. Each tube was then loaded with 5µl of a known dilution of a standard curve,

double distilled water (as a negative control), or the unknown sample. All reactions were run in duplicates.

A standard curve was constructed for each equine interleukin and housekeeping gene. This curve consisted of eight 1-log increments to achieve final concentrations of 0.2×10^{-15} to 0.2×10^{-23} moles of plasmid/ml of the corresponding plasmid containing the cDNA sequence to each equine interleukin gene. The most diluted curve value (0.2×10^{-23} moles of plasmid/ml) is theoretically equivalent to Avogadro's number: 6.02×10^{-23} and was then given the value of 6.02 copy units (CU). A standard curve was run on each plate containing unknown samples. The ABI PRISM™ 7700 Sequence Detection System generated a C_T (cycle threshold), which is the cycle where amplification changes from geometric to exponential.

Table 2.1 Equine primers and probes used for the quantification of cytokines.
☝ labeled with TAMRA™ as the quencher dye, and FAM™ as the luminescent dye.

Equine primers and probes	Forward primer	Reverse primer	TaqMan® Probe☝
β actin	AGG GAA ATC GTG CGT GAC A	GCC ATC TCC TGC TCG AAG TC	CAA GGA GAA GCT CTG CTA TGT CGC CCT
Interferon γ	CGC AAA GCA ATA AGT GAA CTC ATC	CGA AAT GGA TTC TGA CTC CTC TTC	TCT GCT GCC CAA AGC TAA CCT GAG GAA
IL-4	TCG TGC ATG GAG CTG ACT GTA	GCC CTG CAG ATT TCC TTT CC	CCT TTG CTG GCC CGA AGA ACA CAG A
IL-5	AGC TCT TGG AGC TGC CTA CGT	CAG TGT CAA GGT CTC TGC CAC TAG	TGC CCT TGC TGT AGA AAG TCC CAT GAA
IL-13	TGT GGA GCG TCA ACC TGA CA	TCA GCA TCT TCC GCG TGT T	CAA CGT CTC CAC CTG CAG TGC CAT

Relative amounts of cDNA for each interleukin were evaluated following the User Bulletin #2 from ABI PRISM™ 7700 Sequence Detection System. Briefly, the data set produced by the ABI PRISM™ 7700 Sequence Detection System was imported into an Excel (Microsoft®) worksheet. The data set contained the C_T values of the curve and unknown the samples. The duplicates for each sample were averaged. If C_T values differed by more than 2 cycles the sample was re-run at a later date. A graph was constructed using the log transformed CU of the standard curve on the X-axis, and its corresponding averaged C_T on the Y-axis. A regression line was fitted through the data points. The linear equation and R-squared value for the regression line were obtained. The unknown CU values were calculated by solving the straight-line regression for X. The formula was X=([CT value] – b)/m, where b = y – intercept of the standard curve and m = slope of standard curve line. The anti logarithm values of X were then obtained.

To normalize the data, all the β-actin values (in CU) were logarithm transformed and a mean value was obtained, and antilogarithm transformed. This β-actin mean was then divided by 10^6, and the values for each interleukin were calculated by dividing the interleukin CU over the β-actin average.

2.3 Statistics

The software package used to analyze the data was Sigma Stat® 2.03. All data was analyzed by two-way repeated measures analysis of variance (RM ANOVA), followed by all pairwise multiple comparison procedures (Tukey test). With the exception of the parasite recoveries and tissue eosinophil and mast cell counts which were analyzed with one-way ANOVA, followed by the Tukey test or the Kruskal-Wallis One Way Analysis of Variance on Ranks if the data was not normally distributed. Significant differences were sought first in the normally distributed data. If no significant differences were seen the analysis of the logarithm

(x+1)-transformed data was performed. Significance, in all instances, was established with $P < 0.05$.

2.4 Results

2.4.1 Parasite Recoveries

Young Ch ponies had the highest worm counts.

It was expected from previous studies that the total number of worms recovered would correlate to the degree of acquired resistance, and numbers of parasites would decrease with increased exposure to cyathostome contaminated pastures. However, Young ponies had the largest numbers of total worms and of EL_3 (Table 2.2). This increase was almost completely due to the increased number of EL_3. Numbers of DL, luminal L_4 and adult worms were similar to those of PF Ch ponies, as were the DL counted by TMI (Table 2.2).

The Adult Ch ponies had approximately 3 times fewer worms than the Young Ch ponies (Table 2.2). Although reductions in numbers were seen at each life stage, the greatest percentage of the parasite population in the adult animals was in the EL_3 stage.

Adult Ch ponies had 77.5% fewer worms than the Young Ch (Table 2.3). This significant decrease was mainly due to reductions in DL, as measured by digestion and TMI, luminal L_4 and adult worms. In contrast, PF Ch and Young Ch animals had similar number of these parasite stages (Table 2.3).

There is an increase in EL_3 numbers in previously exposed ponies.

The percentages of DL and L_4 in the previously exposed ponies, Young Ch or Adult Ch, were significantly lower than in the PF Ch (Table 2.4). At the same time PF Ch animals had a much lower percentage of EL_3 than the other two Ch groups. Comparisons of the PF Ch and Young Ch groups show a noticeable increase of EL_3 in the latter group (Table 2.2). This is

Table 2.2 Enumeration of the different cyathostome life cycle stages in the mucosa and lumen of the large intestine based on transmural illumination, digestion and recovery of larvae and adults from the lumen.

	EL_3[¶]	DL[¢]	TMI[£]	Luminal L_4[¥]	Adults	Total counts[§]
PF Non	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
PF Ch	1908 ± 819[a] (0-5,448)	911 ± 176[a] (405-1,313)	1,156 ± 153[a] (724-1,665)	113 ± 60[a] (18-390)	526 ±100[a,b] (257-874)	3,457 ± 805[a] (1,725-7,163)
Young Non	0 ± 0	0 ± 0	0 ± 0	1 ± 1 (0-1)	35 ± 14 (21-48)	35 ± 13 (22-48)
Young Ch	13,174 ± 9,058[a] (0-57,521)	698 ± 258[a,b] (0-1,669)	1,730 ± 712[a] (558-5,178)	102 ± 83[a] (6-516)	734 ± 130[b] (158-982)	14,708 ± 9,270[a] (1,315-60,226)
Adult Non	212 ± 212 (0-424)	0 ± 0	54 ± 54 (0-108)	2 ± 0 (2-2)	61 ± 38 (23-98)	275 ± 250 (25-524)
Adult Ch	5,117 ± 2,375[a] (0-16,009)	201 ± 90[b] (0-417)	147 ± 97[b] (0-551)	29 ± 15[a] (0-86)	181 ± 65[a,c] (1-351)	5,527 ± 2,486[a] (1-16,841)

PF: parasite-free. Non: non-challenged (to animals per group). Ch: challenged (six animals per group). [¶]EL_3: hypobiotic or early L_3, based on counts from digestion. [¢]DL: developing larvae, including mid- to late L_3 and early L_4, based on counts from digestion. [£]TMI: developing larvae counted with the transmural illumination technique. [¥]L_4: fourth stage larvae recovered from intestinal contents. [§]Total counts: EL_3 + DL + luminal L_4 + adults.
This table shows the average number of the worms recovered for each pony group in each category, followed by the standard error of the mean and the range between parentheses. Different superscript letters indicate significant difference with $P < 0.05$, only Challenged groups were compared.

Table 2.3 Comparison between DL, luminal L_4 and adult worms in the Ch groups.

	DL[¢]	**Luminal L_4**[¥]	**Adults**	**% Reduction**
PF Ch	911	45	573	__
Young Ch	698	33	641	**10**
Adult Ch	201	28	106	**77.5**

PF: parasite-free. Ch: challenged (six animals per group). The results were obtained by subtracting the values obtained for the Non-challenged from the values of the Challenged animals. [¢]DL: developing larvae, including mid- to late L_3 and early L_4, based on counts from digestion. [¥]L_4: fourth stage larvae. The % reduction was obtained by adding the values of DL, luminal L_4 and adult worms, and comparing the results obtained for the Young Ch or the Adult Ch to those obtained for the PF Ch.

shown more clearly in Table 2.4 where the parasite life stages recovered at necropsy are presented as percentages of the population based on the total number of parasites of the group.

The percent of cyathostome establishment was similar for PF and Adult animals.

The percent of cyathostome L_3 establishment was calculated as the total number worms x100 / 150,000 L_3. Interestingly, a marked difference between the percentages of establishment of PF ponies (average: 2.3 %, range: 1.15 to 4.78 %, median: 2.06 %) and Adult ponies (average: 3.68 %, range: 0 to 11.23 %, median: 2.84 %), was not seen. However, Young ponies had an increase percent establishment of worms (average: 9.81%, range: 0.88 to 40.15 %, median: 3.94%).

The anthelmintic treatment was not 100% effective.

The Non-challenged animals had a few residual cyathostomes after the anthelmintic treatment. These accounted for 0.2% of the total number in the Young and 5% in the Adult. This data may be skewed due to the low number (two) of subjects in the Non-challenged groups. Nonetheless, most of the residual worms (98.6%) of the Young Non challenged ponies were

adults. In the Adult Non challenged ponies most were EL_3 (77.2%). The higher number of EL_3 and lower number of cyathostome species (10) than the Young (that had 13 species) or PF (that

Table 2.4 Percentages of cyathostome life cycle stages based on the total number of parasites obtained for each group.

	EL_3[¶]	DL[¢]	L_4[¥]	Adults
PF Non	0	0	0	0
PF Ch	55.2[a]	26.4[a]	3.3[a]	15.2[a]
Young Non	0	0	1.4	98.6
Young Ch	89.6[a]	4.7[a,b]	0.7[a,b]	5[a]
Adult Non	77.2	0	0.7	22
Adult Ch	92.6[a]	3.6[b]	0.5[b]	3.3[a]

PF: parasite-free. Non: non- challenged (two animals per group). Ch: challenged (six animals per group). [¶]EL_3: hypobiotic early L_3, based on counts from digestion. [¢]DL: developing larvae, including mid- to late L_3 and early L_4, based on counts from digestion. [¥]L_4: luminal fourth stage larvae. Different superscript letters indicate significant difference with $P < 0.05$, only Challenged groups were compared.

had 14 species) animals. Also, the order of the three most prevalent species (Table 2.5) changes from *Cylicocyclus nassatus*, *Cyathostomum catinatum* and *Cilicostephanus longibursatus* (in decreasing order) for the Young and PF to *Cilicostephanus longibursatus*, *Cylicocyclus nassatus*, and *Cyathostomum catinatum* in the Adult ponies, suggesting that the immune resistance developed by the latter group might target the least fittest species. All of these observations indicate that ponies with longer exposures to cyathostomes develop resistance to the different life stages.

Table 2.5 Cyathostome species identified from aliquots of the intestinal contents of the Challenged ponies.

Cyathostome species found in challenged ponies	Rank (Prevalence)			
	Total*	PF	Young	Adult
Cylicocyclus nassatus	1 (17/18)	1 (6/6)	1 (6/6)	2 (5/6)
Cylicostephanus longibursatus	2 (17/18)	3 (6/6)	3 (6/6)	1 (5/6)
Cyathostomum catinatum	3 (17/18)	2 (6/6)	2 (6/6)	3 (5/6)
Cylicostephanus goldi	4 (15/18)	4 (6/6)	4 (6/6)	4 (3/6)
Cylicostephanus minutus	5 (12/18)	7 (5/6)	5 (6/6)	5 (1/6)
Cylicostephanus calicatus	6 (14/18)	5 (6/6)	6 (6/6)	9 (2/6)
Coronocyclus coronatus	7 (12/18)	6 (6/6)	7 (6/6)	--
Cyathostomum pateratum	8 (9/18)	8 (4/6)	8 (3/6)	7 (2/6)
Cylicocyclus insigne	9 (5/18)	11 (1/6)	9 (1/6)	6 (3/6)
Poteriostomum ratzii	10 (7/18)	9 (2/6)	11 (4/6)	10 (1/6)
Coronocyclus labratus	11 (4/18)	10 (1/6)	13 (1/6)	8 (2/6)
Cylicocyclus ashworthi	12 (3/18)	--	10 (3/6)	--
Poteriostomum imparidentatum	13 (4/18)	13 (1/6)	12 (3/6)	--
Parapoteriostomum mettami	14 (1/18)	12 (1/6)	--	--
Cylicocyclus radiatus	15 (1/18)	14 (1/6)	--	--

PF: parasite-free. *Total = PF Ch + Young Ch + Adult Ch
--: No adult cyathostome were recovered for the mentioned species.
Rank = cyathostome species ranked by intensity. Intensity = number of adult cyathostomes of the mentioned species obtained per pony.
Prevalence = number of ponies infected with the mentioned species of cyathostomes / number of ponies examined. Data shown between brackets.

Parasites other than cyathostomes were recovered.

The tapeworm *Anoplocephala perfoliata* was found in 10 out of the 12 previously exposed animals (Table 2.6). The roundworm *Parascaris equorum* was found only in 3 of the

Young animals. Third instars of *Gasterophilus intestinalis* were found in the stomachs of 2 Young Ch and one Adult Ch ponies. And the large strongyle *Strongylus edentatus* was recovered from a Young Ch and an Adult Ch pony. It is believed that *G. intestinalis* and *S. edentatus* were leftover after treatment with IVM and OBZ. Neither of the anthelmintic drugs used are effective against *A. perfoliata*.

Table 2.6 Numbers of non-cyathostome parasites recovered from the intestinal contents at necropsy.

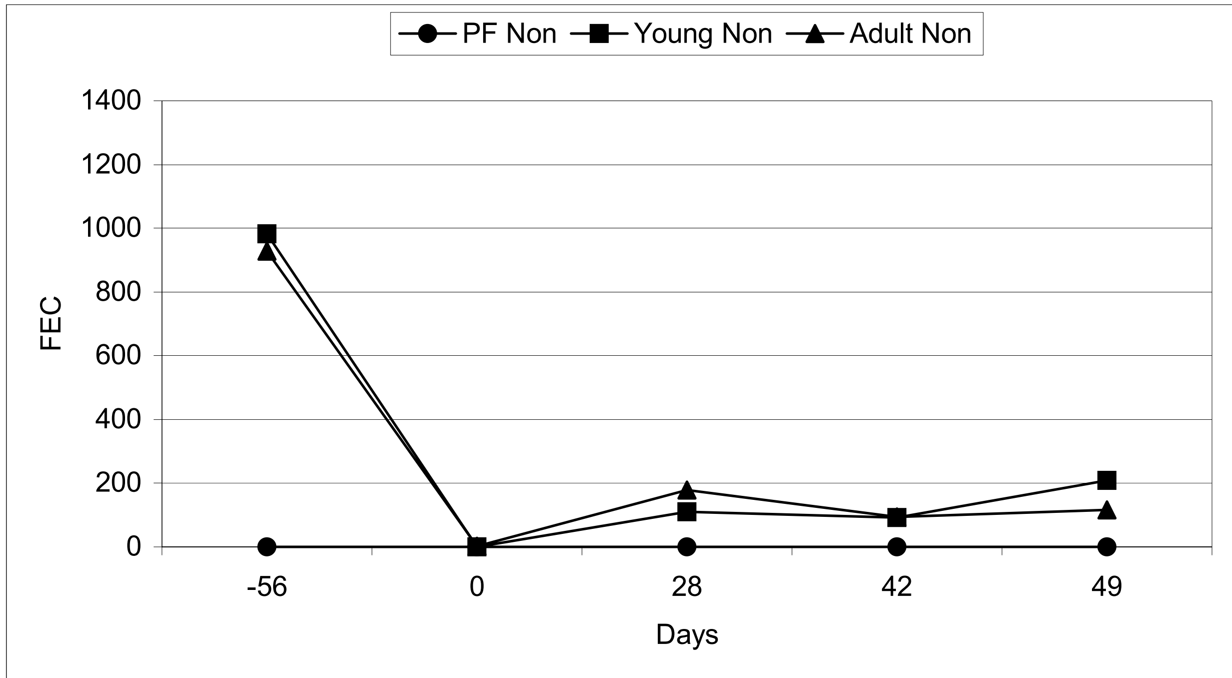
Group	Animal ID	Anoplocephala perfoliata	Parascaris equorum	Gasterophilus intestinalis	Strongylus edentatus
PF Non					
PF Ch					
Young Non	050	26	3		
	12 hip	3			
Young Ch	1 hip	47		1	
	13 hip		3		1
	003	4	7 (immature)		
	026	11		1	
	048	12			
Adult Non	039	3			
Adult Ch	035	1			
	036	1			
	041				1
	042			1	
	792	27			

PF: parasite-free. Non: non-challenged. Ch: challenged. Animal ID: pony identification number. *Anoplocephala perfoliata, Parascaris equorum, Strongylus edentatus*: adult or immature specimens. *Gasterophilus intestinalis*: third-stage instars.

2.4.2 Fecal Egg Counts

Adult Ch ponies had lower egg counts than Young Ch animals.

All previously exposed animals were shedding cyathostome eggs at day –56 (Fig.2.1). Egg production was reduced to near zero in all animals following the anthelmintic treatment used. The decision of not collecting the second data point until day 28 was based on the presumption that 100% of the worms present in the mucosa were eliminated, and that the egg counts present at day 28 in Young and Adult ponies indicates that FEC production present is



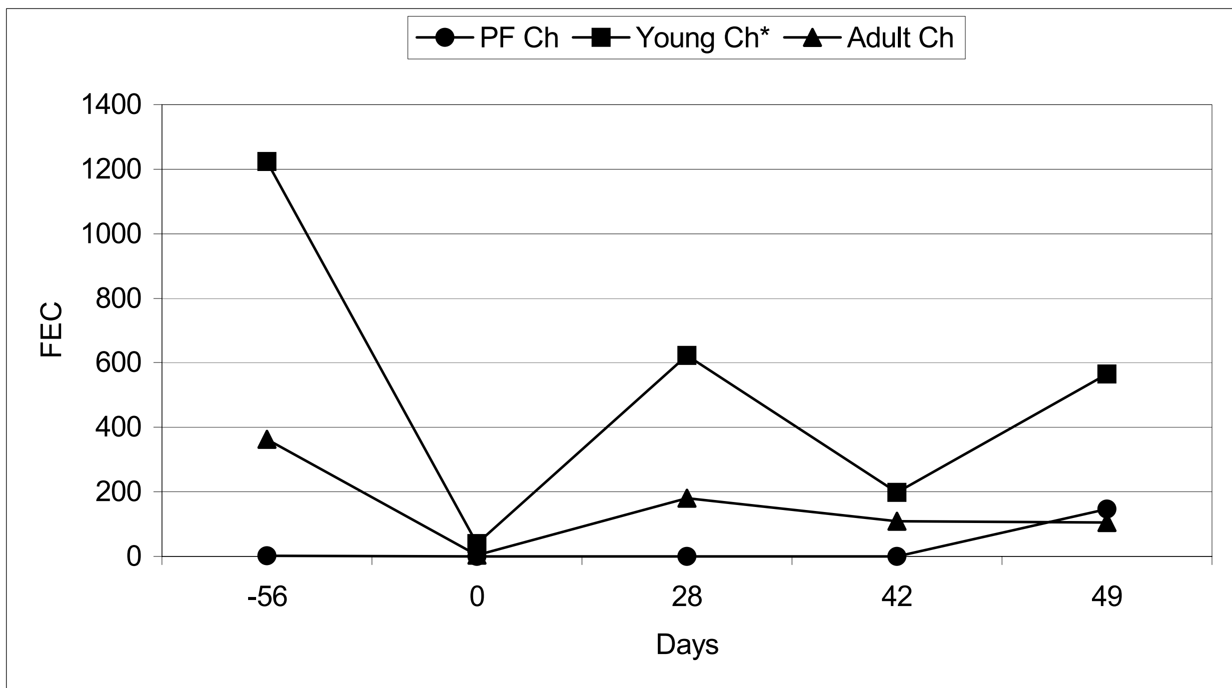


Fig. 2.1 Fecal egg counts presented as the average of eggs per gram per group. PF: parasite-free. Non: non-challenged (2 animals per group) (top chart). Ch: challenged (6 animals per group) (bottom chart). * Young Ch had significantly higher FEC throughout the experimental period than PF Ch or Adult Ch animals. No differences were found between PF Ch and Adult Ch groups. $P < 0.05$.

minimum prepatent period for cyathostome infections is 35 days (Poynter, 1970). Therefore, the egg counts present at day 28 in Young Ch and Adult Ch ponies indicated that egg production was the product of residual worms after anthelmintic treatment. At day 28 the Young Ch had an average of 623 eggs per gram (EPG) and Adult ponies had a mean of 180 EPG. The Young Ch group had significantly higher FEC than PF Ch or Adult Ch groups throughout the experimental period. No statistical differences were detected between Adult Ch and PF Ch groups. The difference of egg production between parasites of Young Ch versus Adult Ch ponies suggests a possible effect of the immune response directed to the adult parasites.

2.4.3 White Blood Cell Counts

Previously exposed ponies had anamnestic-like eosinophil responses, whereas PF Ch ponies had primary eosinophil responses.

The Young Ch and Adult Ch ponies showed a clear anamnestic-like eosinophil response that started on day 6 post challenge and peaked by day 22 (Fig. 2.2). The PF Ch showed a primary eosinophilic response that peaked on day 41-post challenge. Adult Ch animals had significantly higher numbers of circulating eosinophils on days 15 and 22 than the PF Ch or Young Ch groups. At day 41, PF Ch and Young Ch ponies had significantly higher levels of circulating eosinophils than the Adult Ch group, no statistical differences were seen between the first two mentioned groups. The rise in eosinophil counts was faster in the previously exposed ponies and slower to develop in the PF animals. Eosinophil levels in the Non-challenged ponies remained low throughout the experiment. PF Non animals had the lowest levels followed by the Young Non and Adult Non (Fig. 2.2).

No differences were found in other CBC parameters.

The neutrophil and total leukocyte counts did not show any trends or significant differences when analyzed statistically (data not shown). No differences were seen in any

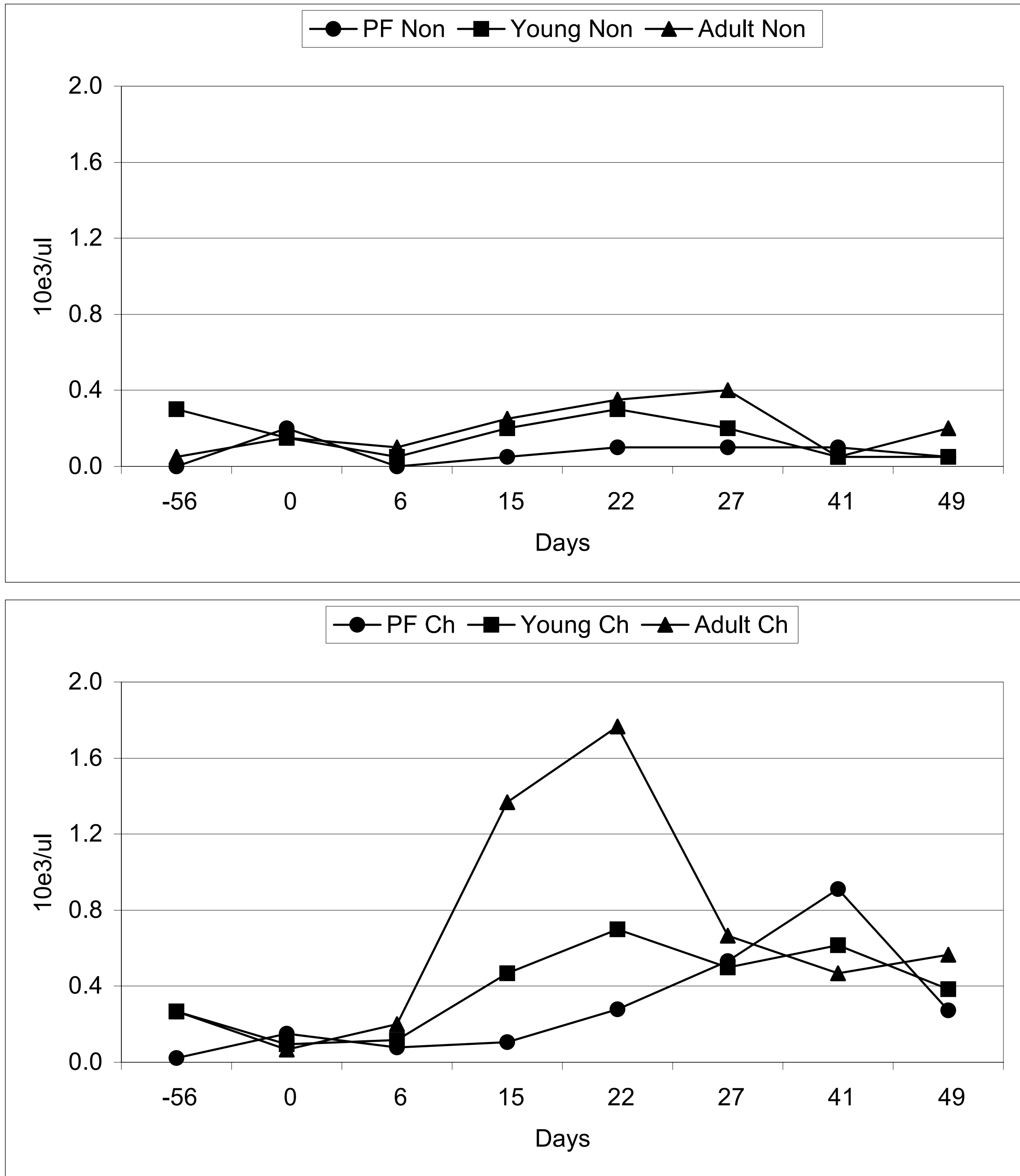


Fig. 2.2 Circulating eosinophil levels in the peripheral blood, presented as the average number of eosinophils counted per group. PF: parasite-free. Non: non-challenged (2 animals per group) (top chart). Ch: challenged (6 animals per group) (bottom chart). Adult Ch had significantly higher numbers of circulating eosinophils on days 15 and 22 when compared to PF Ch and Young Ch ponies. On day 41, PF Ch and Young Ch animals had significantly higher levels of circulating eosinophils than Adult Ch ponies. $P < 0.05$.

of the other CBC parameters evaluated (WBC, white cell differential, hemoglobin, hematocrit, MCV, MCHC, PCV, plasma protein, and platelet counts).

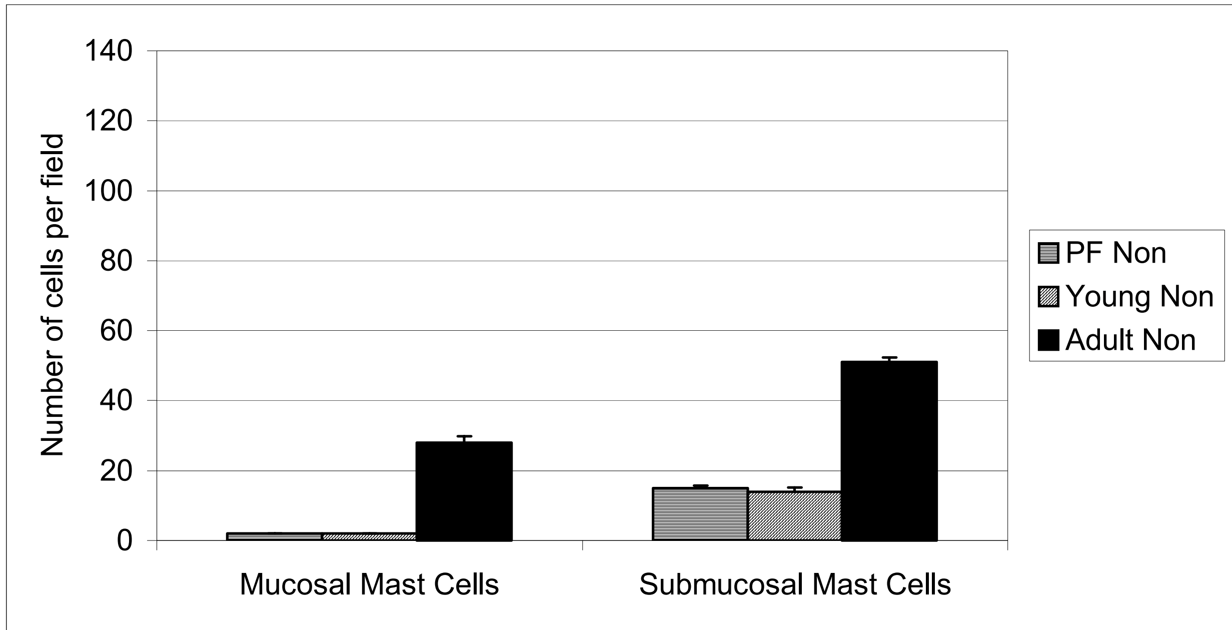
2.4.4 Intestinal Inflammatory Cell Responses

Adult ponies had increased numbers of mucosal and submucosal mast cells.

Adult Ch ponies had significantly higher numbers of mucosal mast cells than the PF Ch and the Young Ch groups (Fig. 2.3). Both Young Ch and Adult Ch ponies had significantly higher numbers of submucosal mast cells than PF Ch animals. Further, the ranking of groups by mucosal mast cell numbers corresponded to the level of pasture exposure to cyathostomes, with the Adults having the greatest numbers, followed by the Young and then the PF. The challenge greatly increased the presence of mast cells in the intestinal tissues, although statistical comparisons between non-challenged and challenged groups were not performed due to the low number of subjects present in the Non groups (two).

Increased numbers of mucosal and submucosal eosinophils were found in Young ponies.

Eosinophil counts were significantly higher in the Young Ch group. The increase was significant ($P < 0.05$) for both the mucosal and submucosal eosinophils when compared to PF Ch or Adult Ch ponies (Fig. 2.4). In general, eosinophils were present in much larger numbers in all groups when compared to the number of mast cells present in the same areas. These numbers could be related to marked increases of EL_3 seen in these animals but not to the level of exposure. Mucosal larvae were not observed histologically and thus the relationship between the eosinophils and the larvae were not noted.



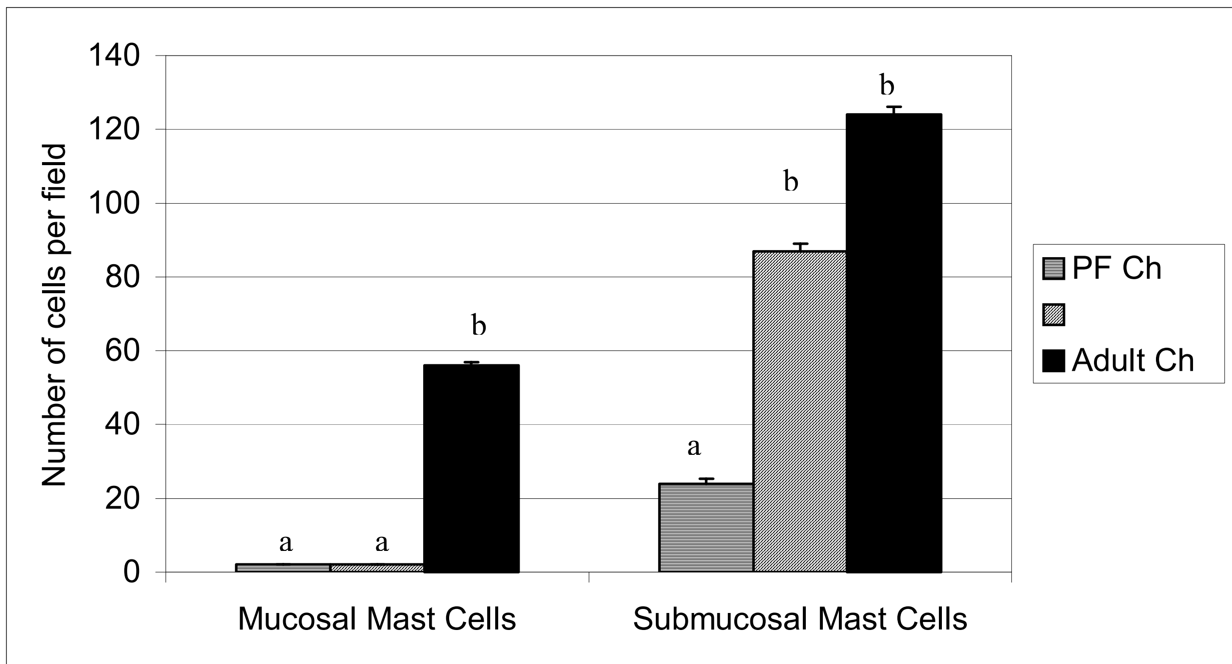
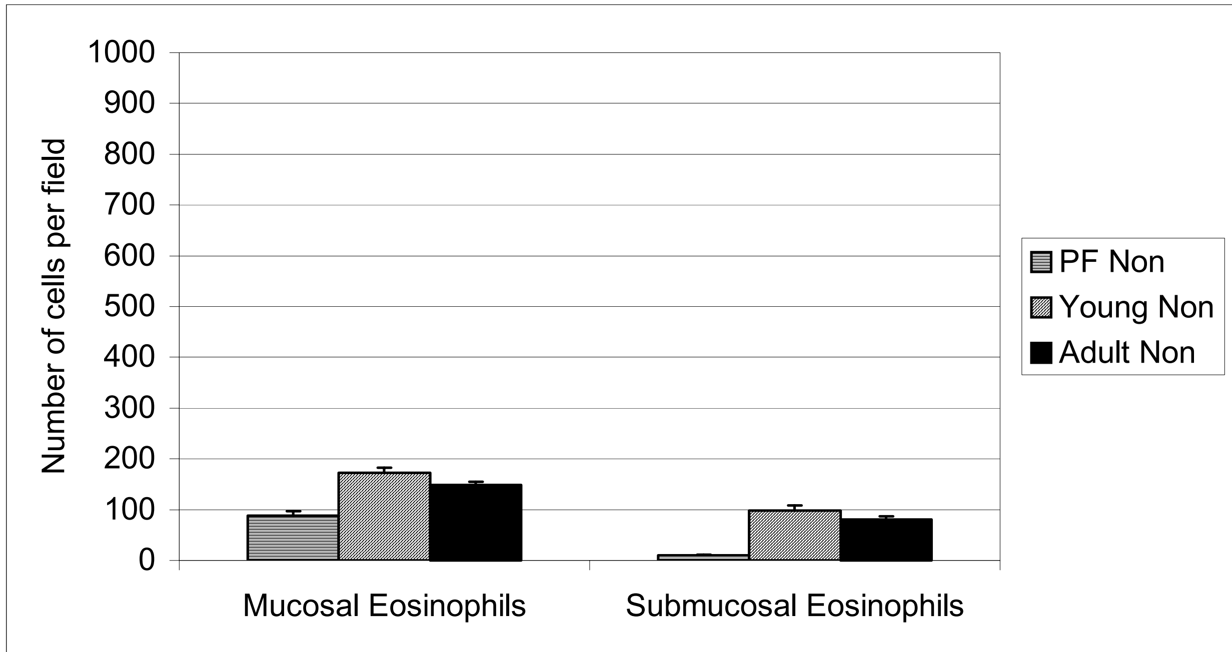


Fig. 2.3 Mucosal and submucosal mast cell counts from histological sections stained with Giemsa. PF: parasite-free. Non: non-challenged (two animals per group) (top chart). Ch: challenged (six animals per group) (bottom chart). The columns represent the sum of the averages of the number of cells counted per high power field obtained for the cecum, dorsal and ventral colons. The bars represent one standard error of the mean. Statistical analysis revealed differences per group. Different letters above the columns indicate significant differences. $P < 0.05$



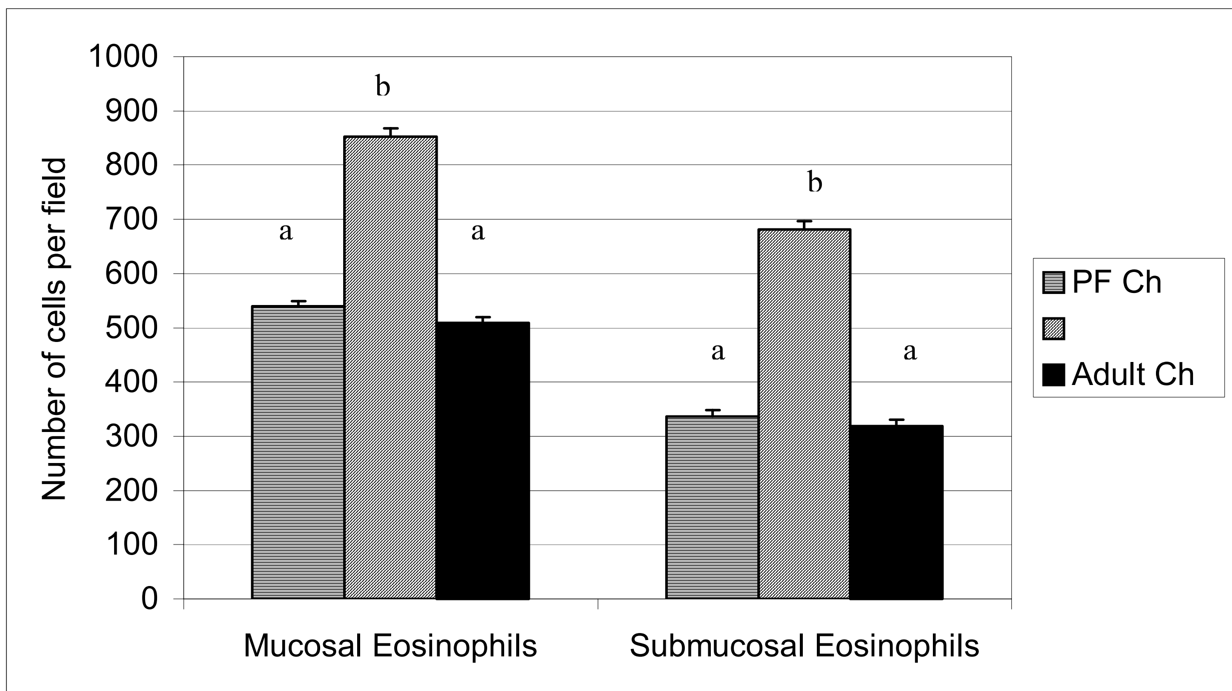


Fig. 2.4 Mucosal and submucosal eosinophil counts from histological sections, stained with Giemsa. The columns represent the sum of the averages of the number of cells counted per high power field obtained for the cecum, dorsal and ventral colons. The bars represent one standard error of the mean. Statistical analysis revealed differences per group and per treatment. Different letters above the columns indicate significant difference per group. $P < 0.05$.

2.4.5 Antibody Responses Measured by ELISA

PF ponies respond to challenge infection with an increased production of antibodies against somatic antigens.

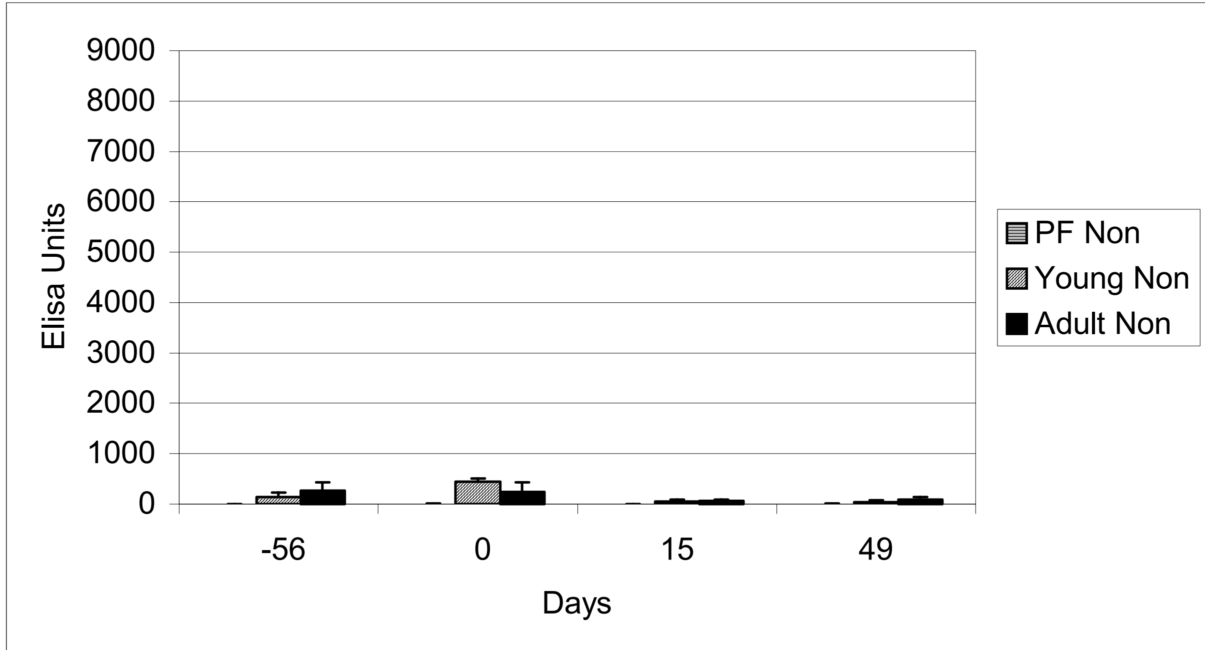
In all instances the naïve animals had very low levels of IgG, IgG (T) and IgG (a) on days –56 and 0, with titers sharply increasing after challenge. PF Ch ponies had increased levels of IgG and IgG (T) against adult somatic antigen and L_3 somatic antigens after challenge (Figs. 2.5 through 2.10). Significant increases were seen for IgG and IgG (a) against somatic extracts of adult antigens at days 15 and 49.

Adult ponies had significantly higher levels of IgG (T) to adult somatic antigen.

The statistical analysis of the logarithm transformed data showed that Adult Ch ponies had significantly higher IgG (T) titers against adult *C. insigne* antigens than any other group throughout the experiment (Fig. 2.6). Interestingly this appears to be due to the high circulating levels of IgG (T) on days –56 and 0, with titers falling after day 0. Levels of IgG (T) against somatic antigens of L_3 were significantly higher for the Adult Ch ponies than for PF Ch and Non-challenge, but were not different from Young Non, Young Ch or Adult Non challenged (Fig. 2.9).

Most isotypic antibody responses of previously exposed animals to somatic L_3 extracts remained elevated or increased following challenge.

Levels of IgG and IgG (T) against somatic extracts of cyathostome L_3 remained elevated for the Young and Adult ponies throughout the experiment, whereas they increased for the PF animals (Fig. 2.8 and 2.9). Interestingly, the levels of these two antibodies remained elevated for the Adult Non-challenged ponies throughout the experimental period but diminished for IgG for the Young Non-challenged animals by day 15 (Figs. 2.8 and 2.9). However, the titers of IgG (a) against cyathostome L_3 remained low until day 49 for the Ch groups (Fig. 2.10).



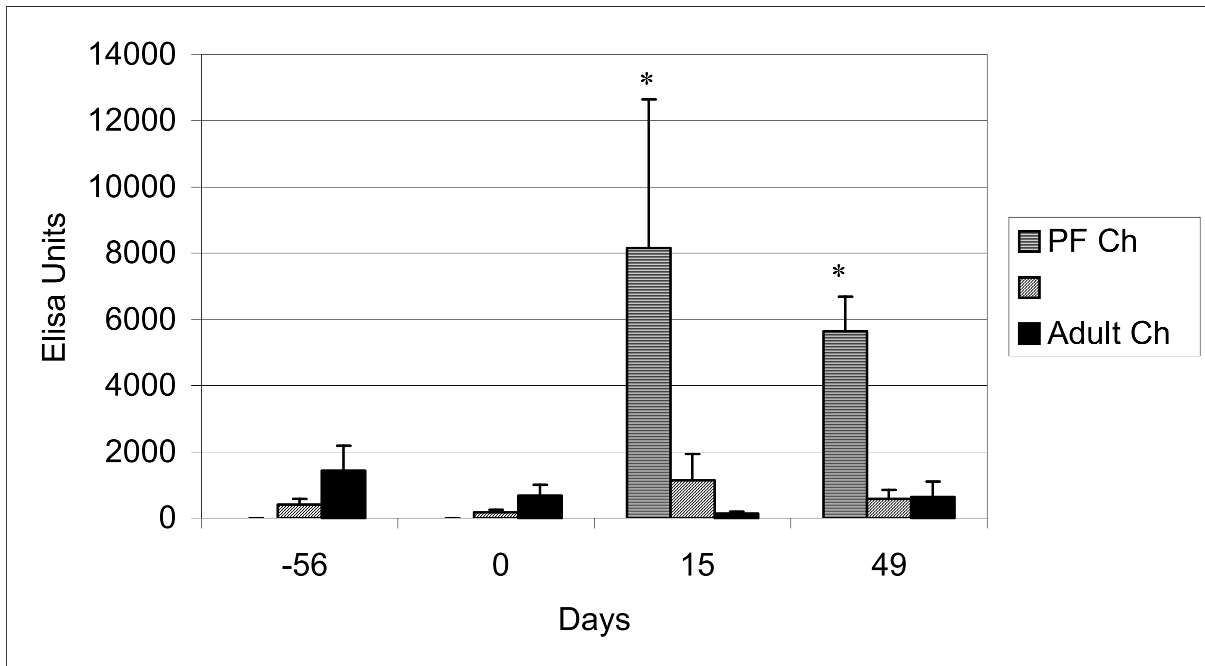
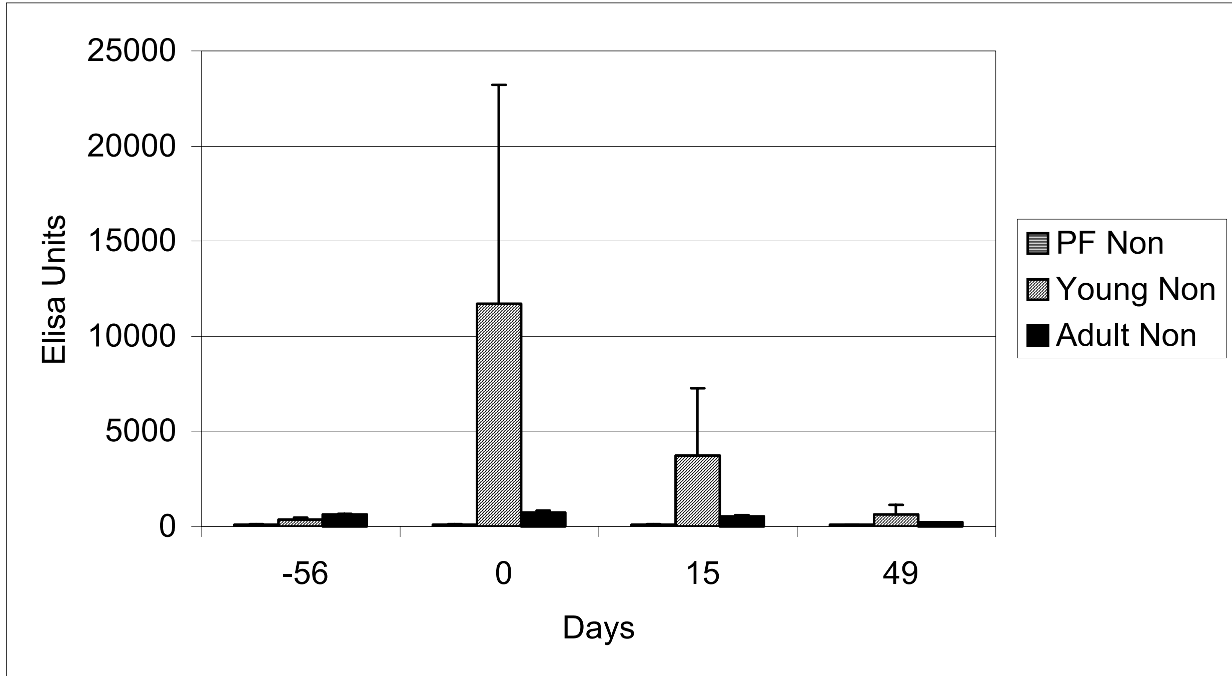


Fig. 2.5 Levels of IgG antibodies against the somatic extracts of adult *C. insigne*.
PF: parasite-free. Non: non-challenged (two animals per group) (top chart). Ch: challenged (six animals per group) (bottom chart). * denotes significant increase of IgG antibodies in the PF Ch group when compared to Young Ch and Adult Ch animals. $P < 0.05$.



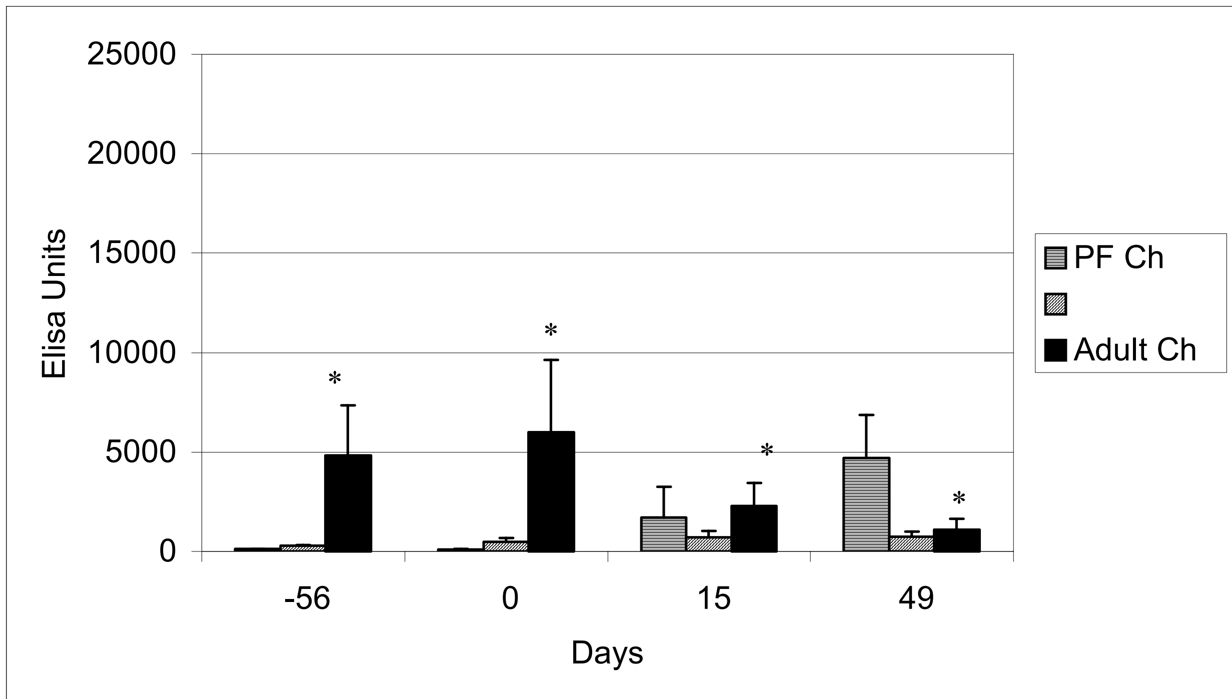
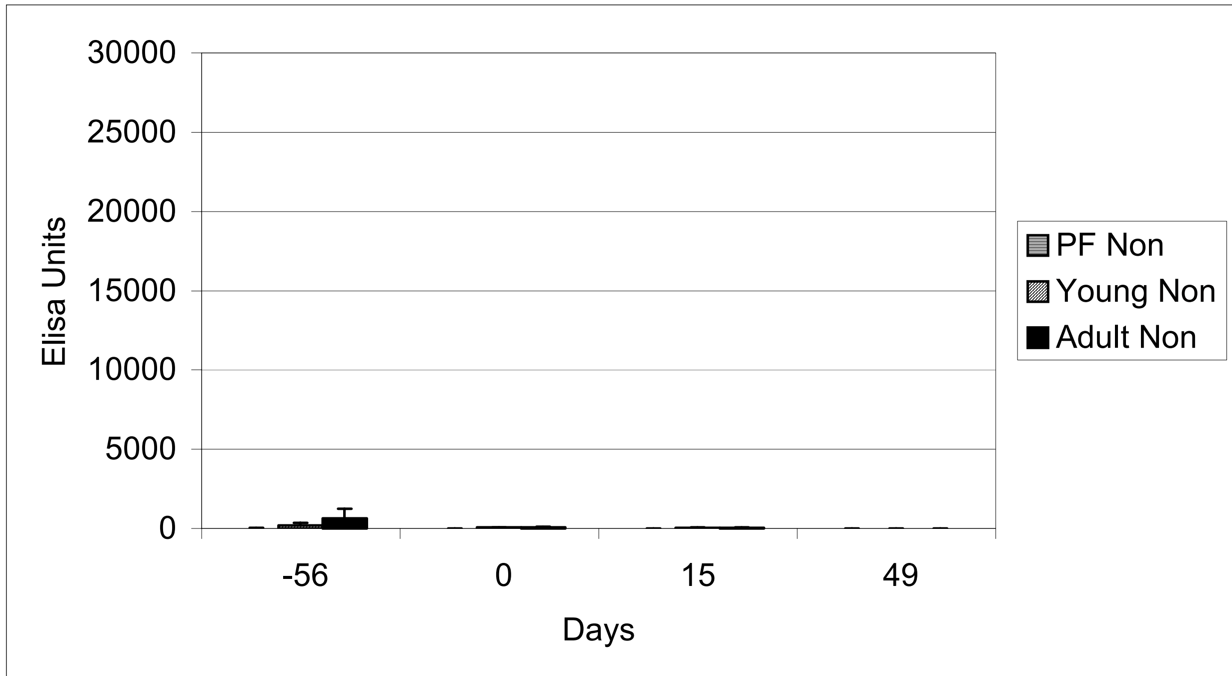


Fig. 2.6 Levels of IgG (T) antibodies against the somatic extracts of adult *C. insigne.*
PF: parasite-free. Non: non-challenged (two animals per group) (top chart). Ch: challenged (six animals per group) (bottom chart). * denotes significant higher levels of IgG (T) antibodies in the Adult Ch group when compared to PF Ch and Young Ch animals. $P < 0.05$.



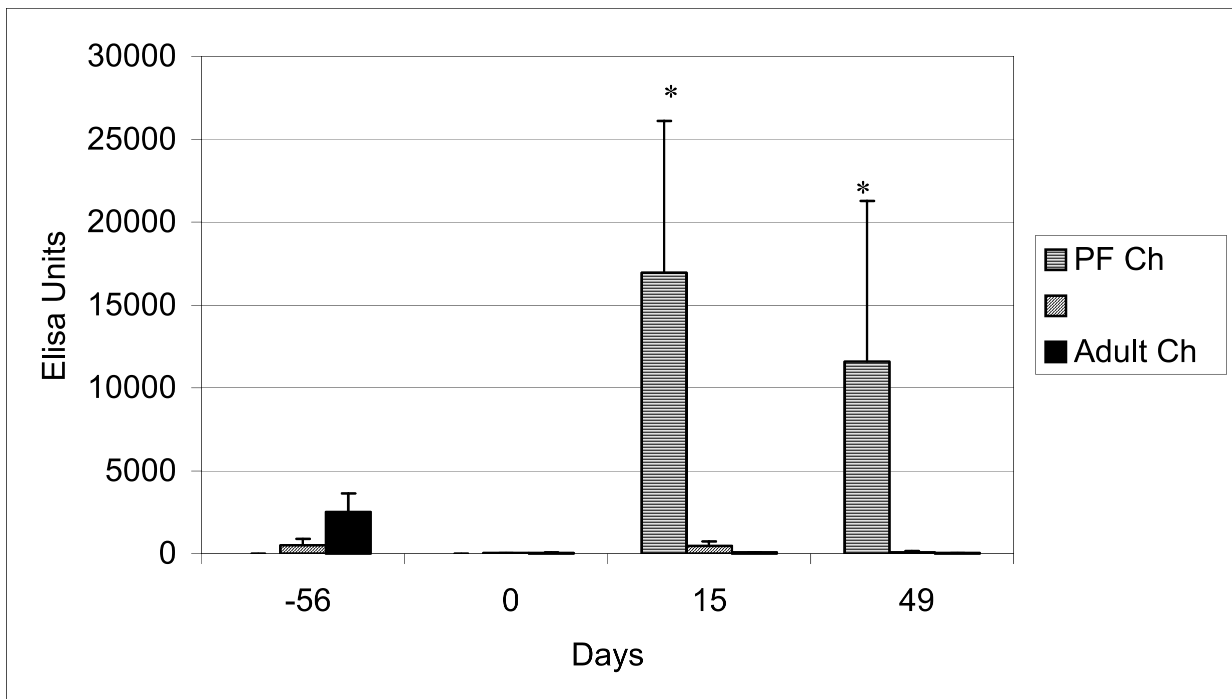
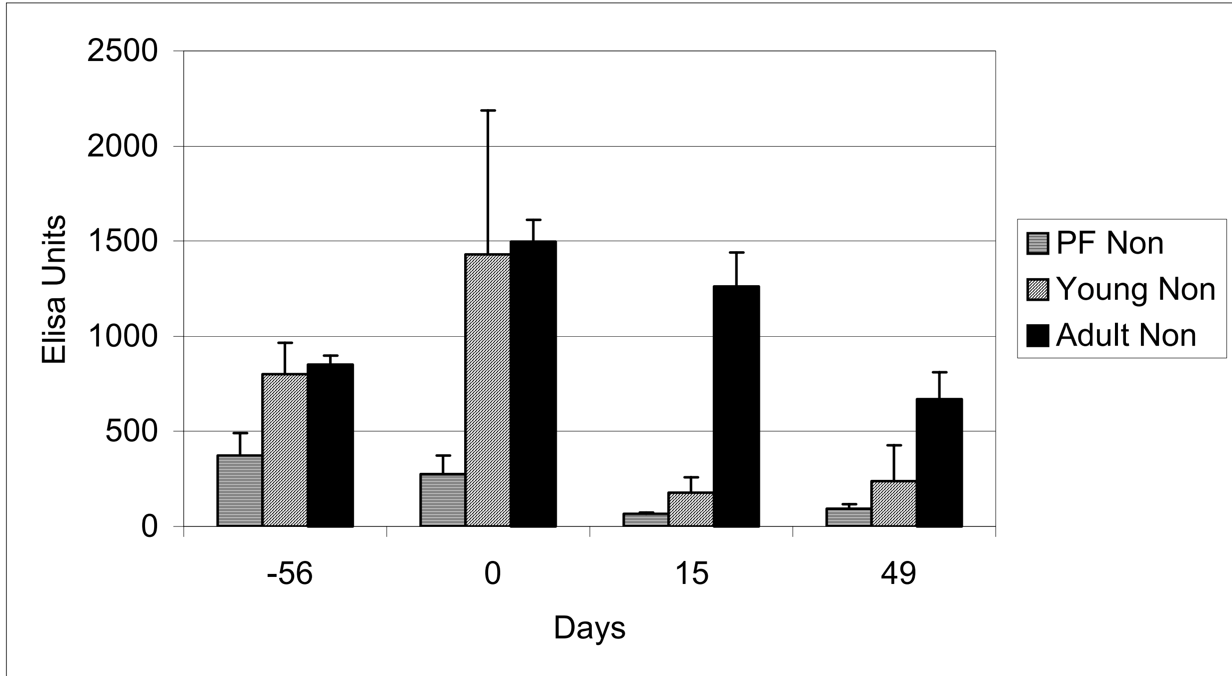


Fig. 2.7 Levels of IgG (a) antibodies against the somatic extracts of *adult* C. insigne.
PF: parasite-free. Non: non-challenged (two animals per group) (top chart). Ch: challenged (six animals per group) (bottom chart). * denotes significant increase of IgG(a) antibodies in the PF Ch group on days 15 and 49 when compared to Young Ch and Adult Ch animals. $P < 0.05$.



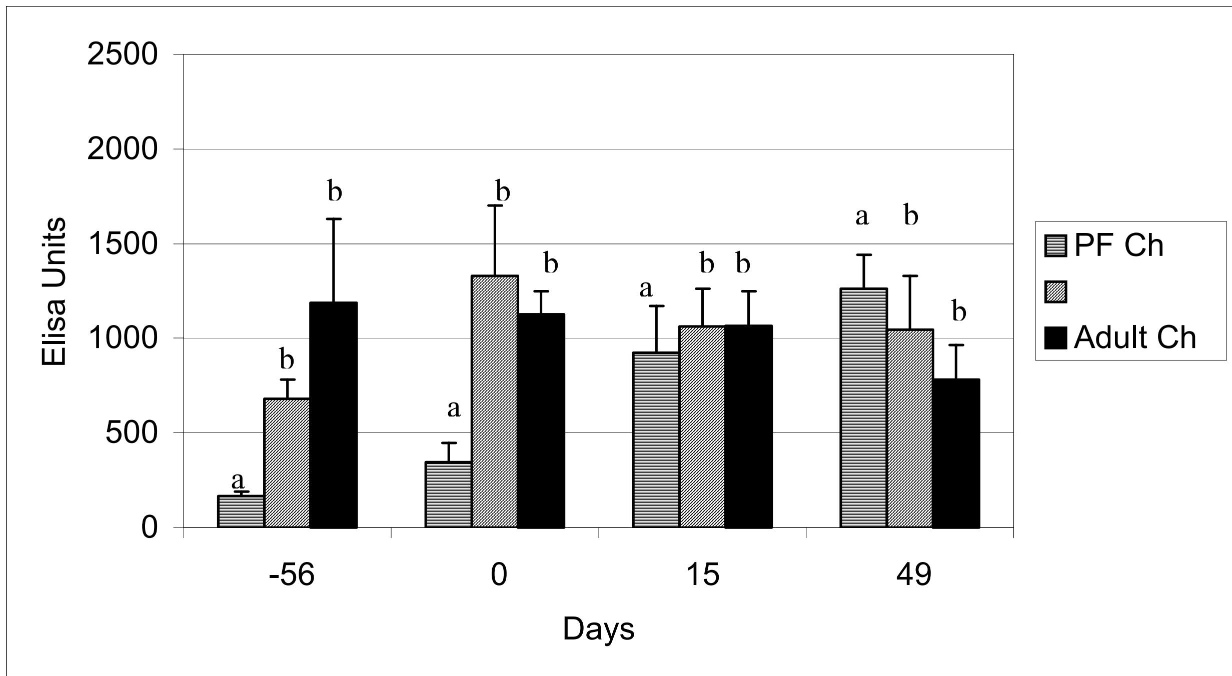
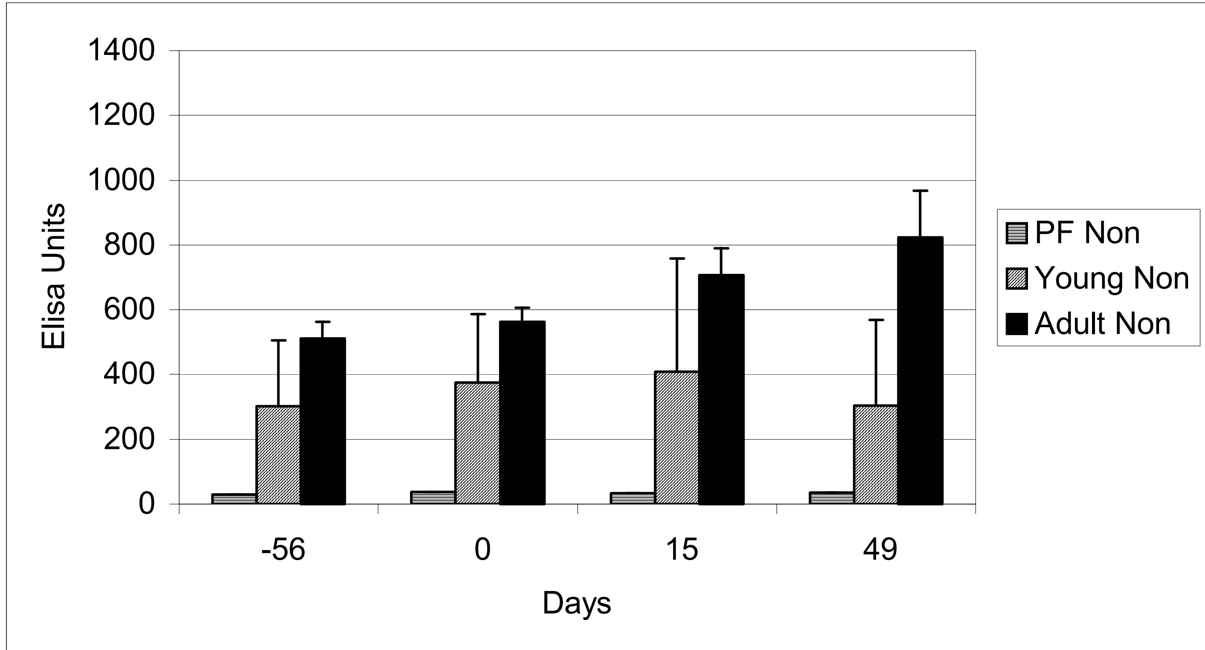


Fig. 2.8 Levels of IgG antibodies against the somatic extracts of cyathostome L_3.
PF: parasite-free. Non: non-challenged (two animals per group) (top chart). Ch: challenged (six animals per group) (bottom chart). Different superscript letters denote significant differences of IgG antibodies when Challenged groups were compared. $P < 0.05$.



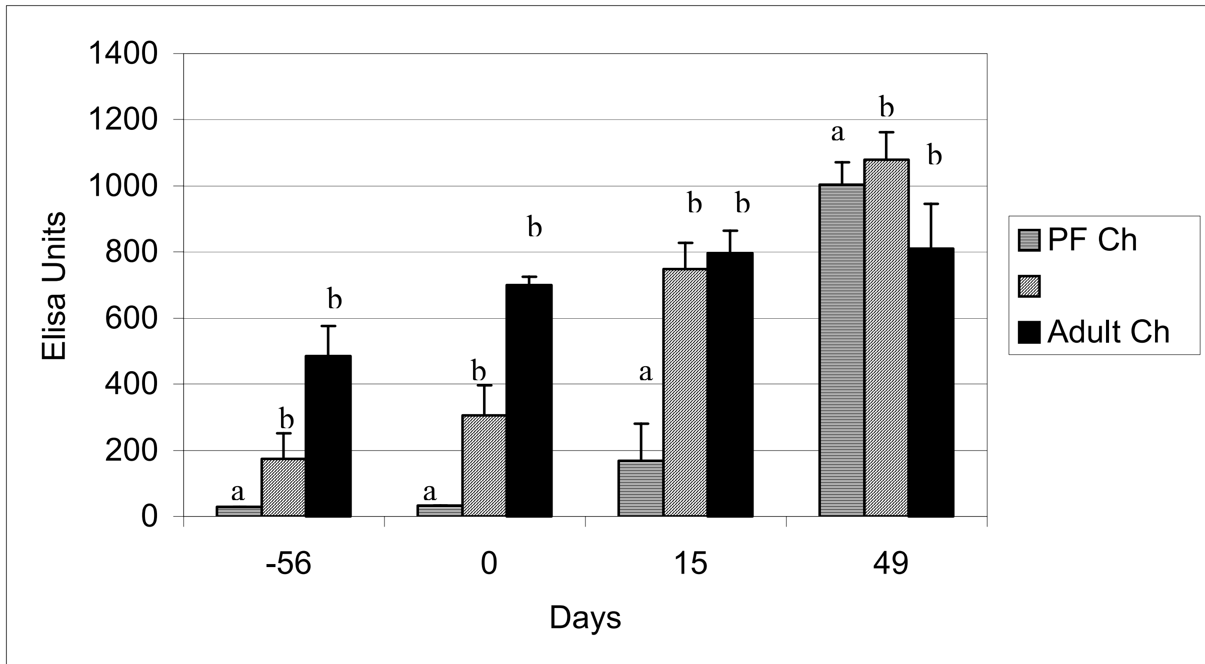
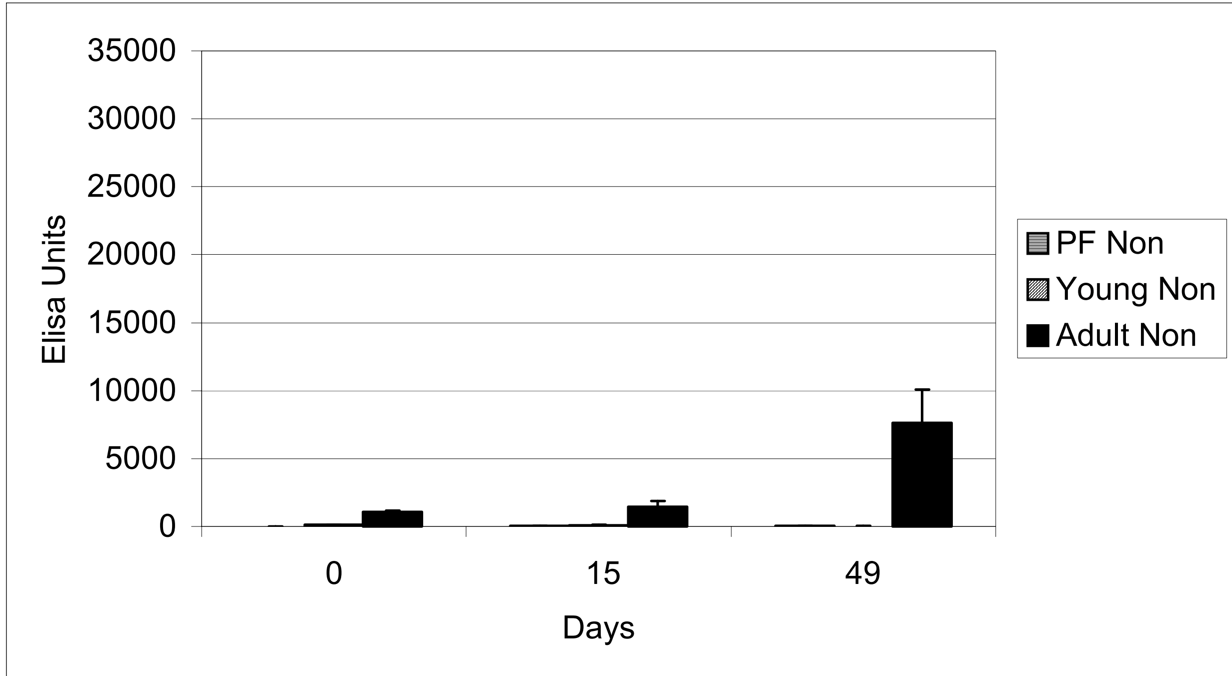


Fig. 2.9 Levels of IgG (T) antibodies against the somatic extracts of cyathostome L_3. PF: parasite-free. Non: non-challenged (two animals per group) (top chart). Ch: challenged (six animals per group) (bottom chart). Different letters above the columns indicate significant differences of IgG (T) antibodies when Challenged groups were compared. $P < 0.05$.



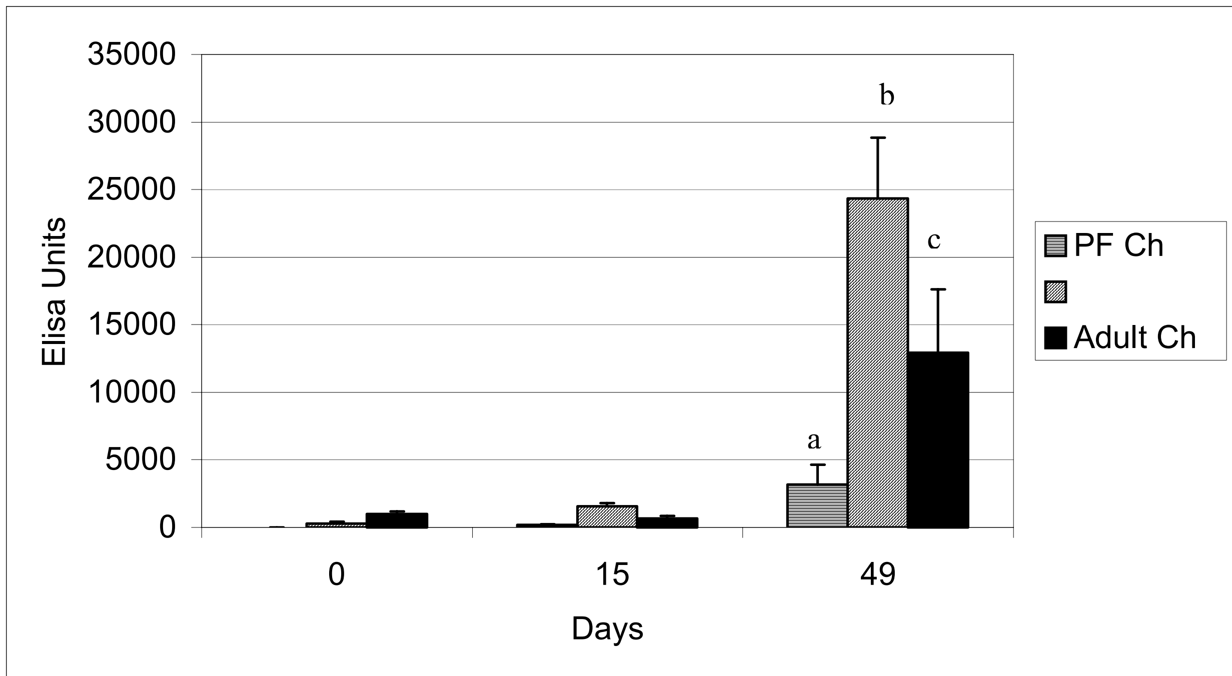


Fig. 2.10 Levels of IgG (a) antibodies against the somatic extracts of cyathostome L_3. PF: parasite-free. Non: non-challenged (two animals per group) (top chart). Ch: challenged (six animals per group) (bottom chart). Different letters above the columns indicate significant differences of IgG (a) antibodies at day 49 when Challenged groups were compared. $P < 0.05$.

2.4.6 Antibody Responses Measured by IFAT

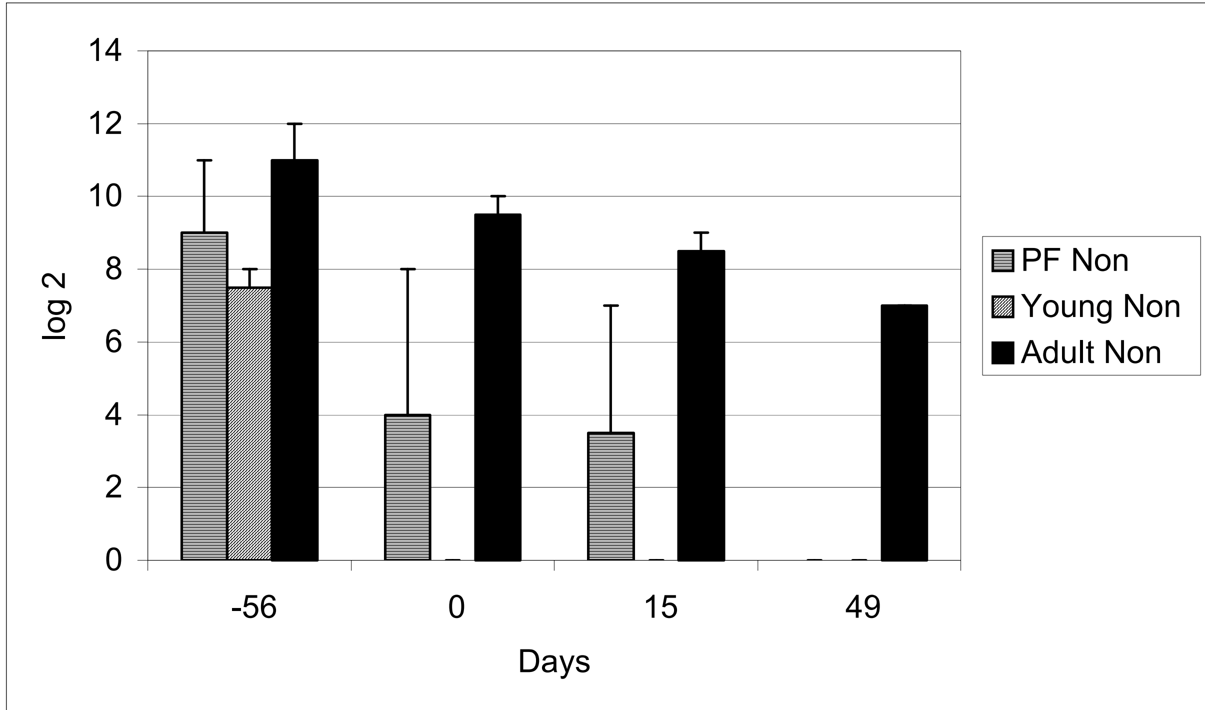
Antibodies to L_3 surface antigens increased in all ponies post challenge.

The results show that Young Ch and PF Ch ponies had the highest increases of both IgG and IgG (T) against surface antigens of freshly exsheathed L_3 (Figs. 2.11 and 2.12). Interestingly the IgG levels of PF Non and Young Non challenged ponies decreased to non detectable levels by day 49 (Fig. 2.11). The Adult ponies had high circulating levels of both immunoglobulin isotypes and these titers remained stable or slightly increased after challenge. When the data was analyzed statistically the levels of IgG and IgG (T) of the Adult Ch ponies (Fig. 2.11 and 2.12) were significantly higher than those of PF Non, Young Non and PF Ch ponies.

2.4.7 Lymphoproliferative Responses

The lymphoproliferative responses to *C. insigne* antigen of the Adult Ch group were significantly higher.

Lymphoproliferation assays were conducted using freshly isolated PBMC, unstimulated, or stimulated with 0.1µg/ml or 0.3µg/ml of adult *C. insigne* antigen or PWM, with or without the addition of equine IL-2. The kinetics of the proliferative responses with IL-2 were similar to those not supplemented with IL-2 at each time point, therefore the latter data is shown. The lymphoproliferative responses obtained with 0.1µg/ml of *C. insigne* antigen were less consistent than those using 0.3 µg/ml of antigen. Therefore, the latter results are reported. The responses obtained with PWM were proliferative at all time points, the data s not shown. The lack of difference between treatment groups could be due to mitogenic activity of the *C. insigne* antigen on the lymphocytes of the Non challenged animals. The data presented in the graph shows a reduction in proliferation immediately after challenge (day 6), the cause of this decrease is not known. The Adult Ch had significantly higher proliferative responses than the PF Ch or Young Ch ponies.



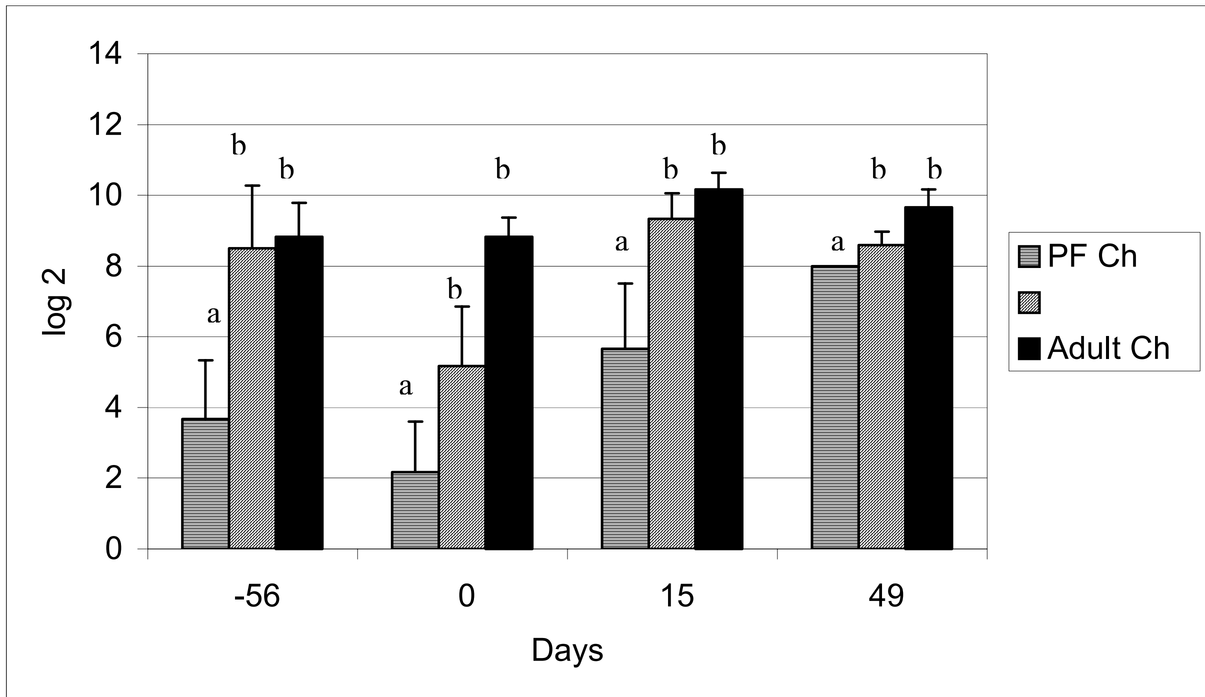
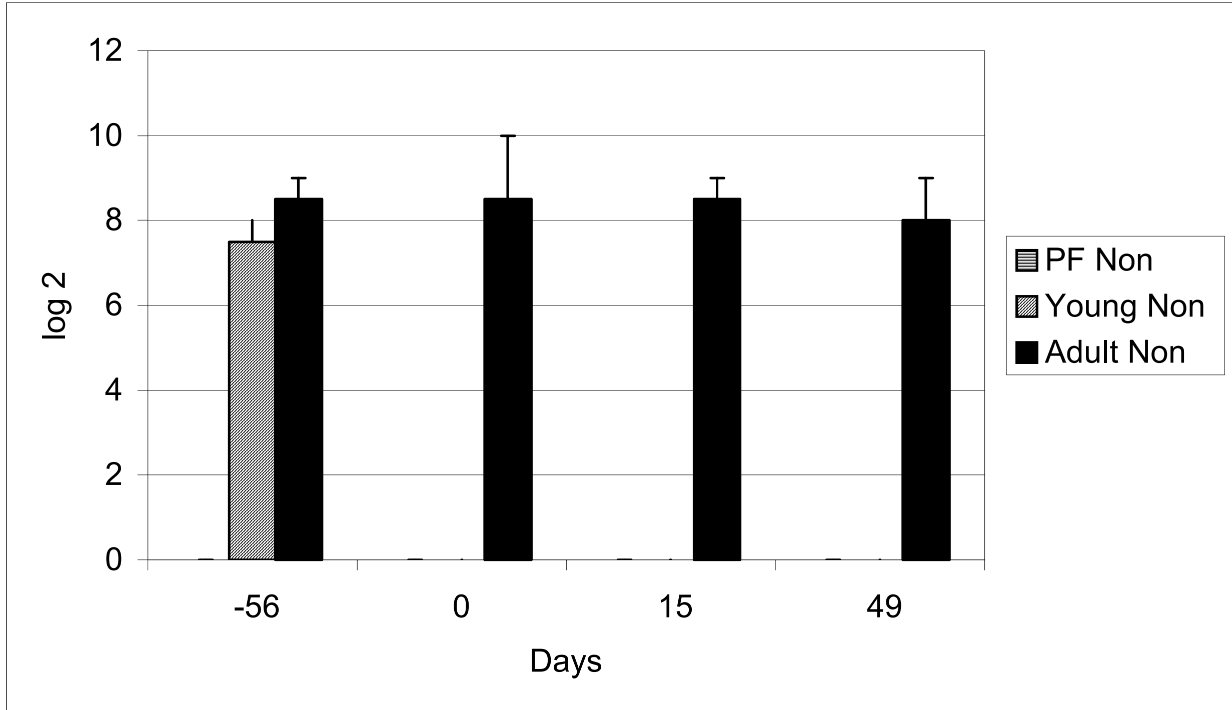


Fig. 2.11 Levels of circulating IgG antibodies against the surface of freshly exsheathed cyathostome L_3, as measured by IFAT. PF: parasite-free. Non: non-challenged (two animals per group) (top chart). Ch: challenged (six animals per group) (bottom chart). Different letters above the columns indicate significant differences of IgG antibodies when Challenged groups were compared. $P < 0.05$.



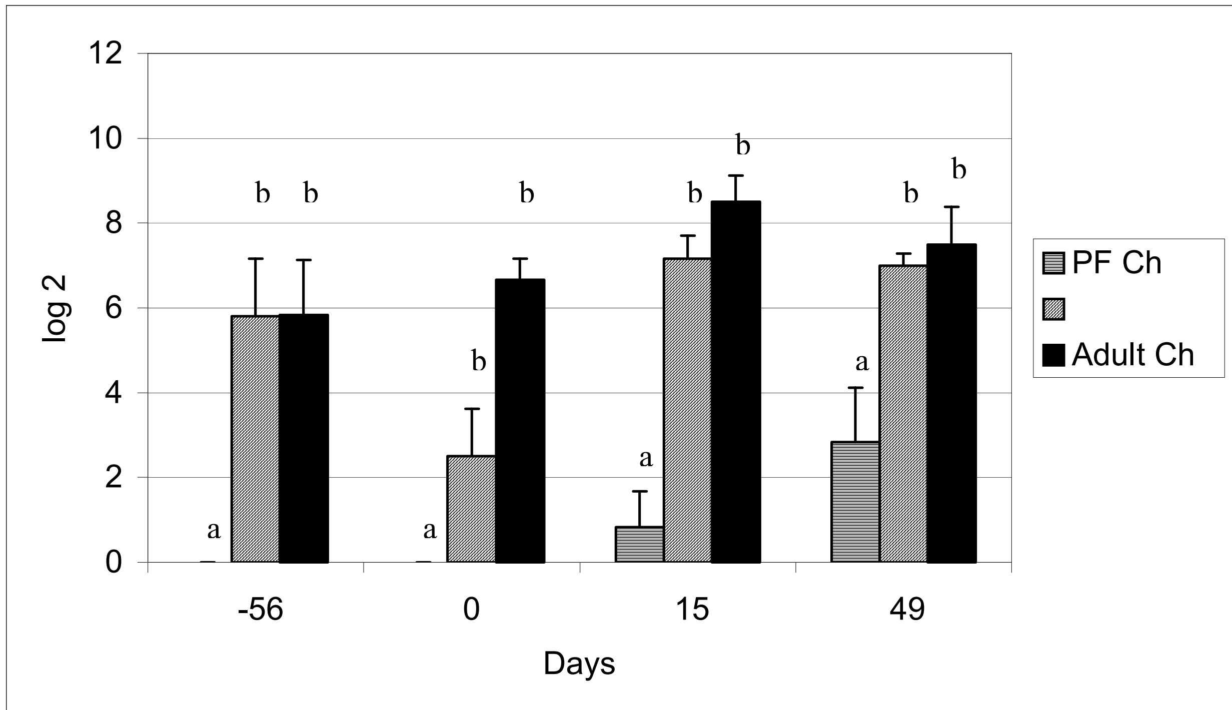
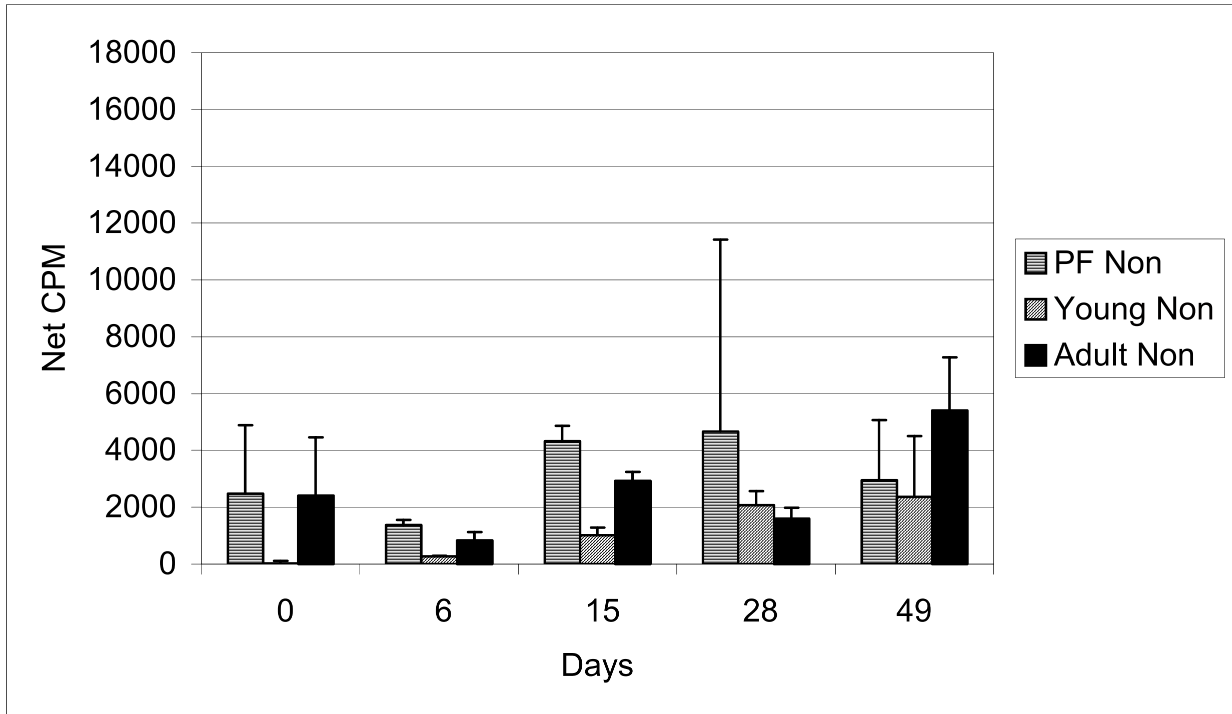


Fig. 2.12 Levels of circulating IgG (T) antibodies against the surface of freshly exsheathed cyathostome L_3, as measured by IFAT. PF: parasite-free. Non: non-challenged (two animals per group) (top chart). Ch: challenged (six animals per group) (bottom chart). Different letters above the columns indicate significant differences of IgG (T) antibodies when Challenged groups were compared. $P < 0.05$.



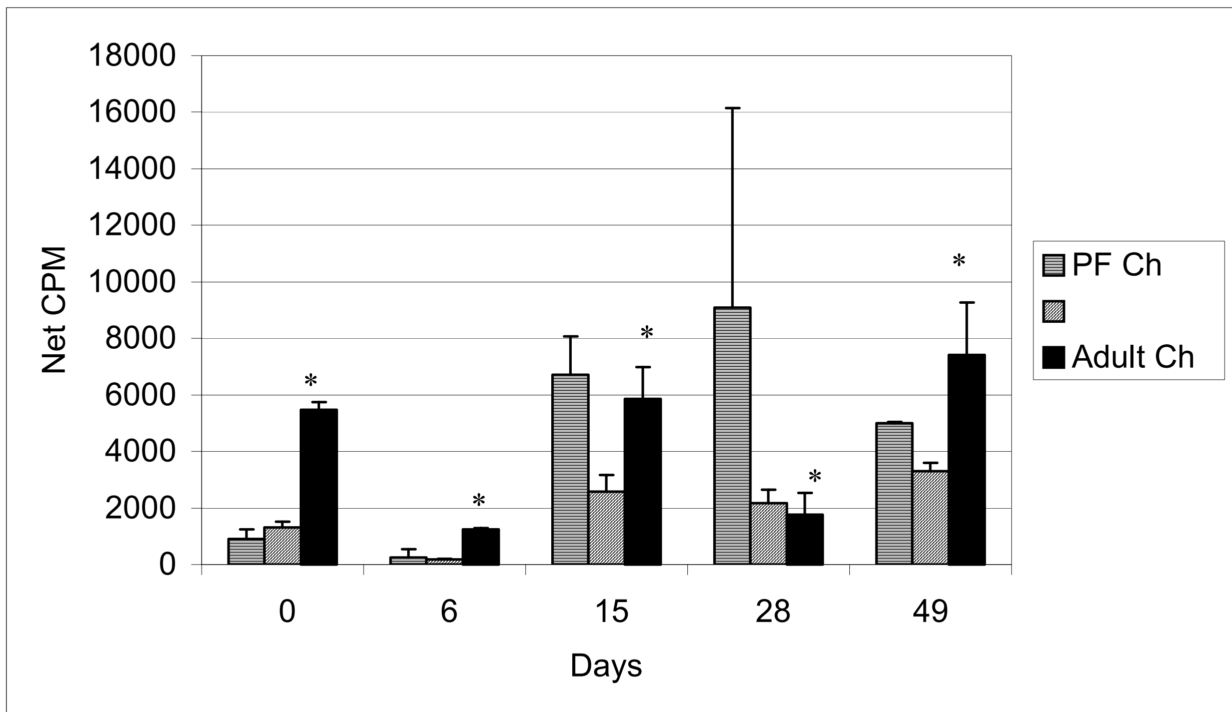
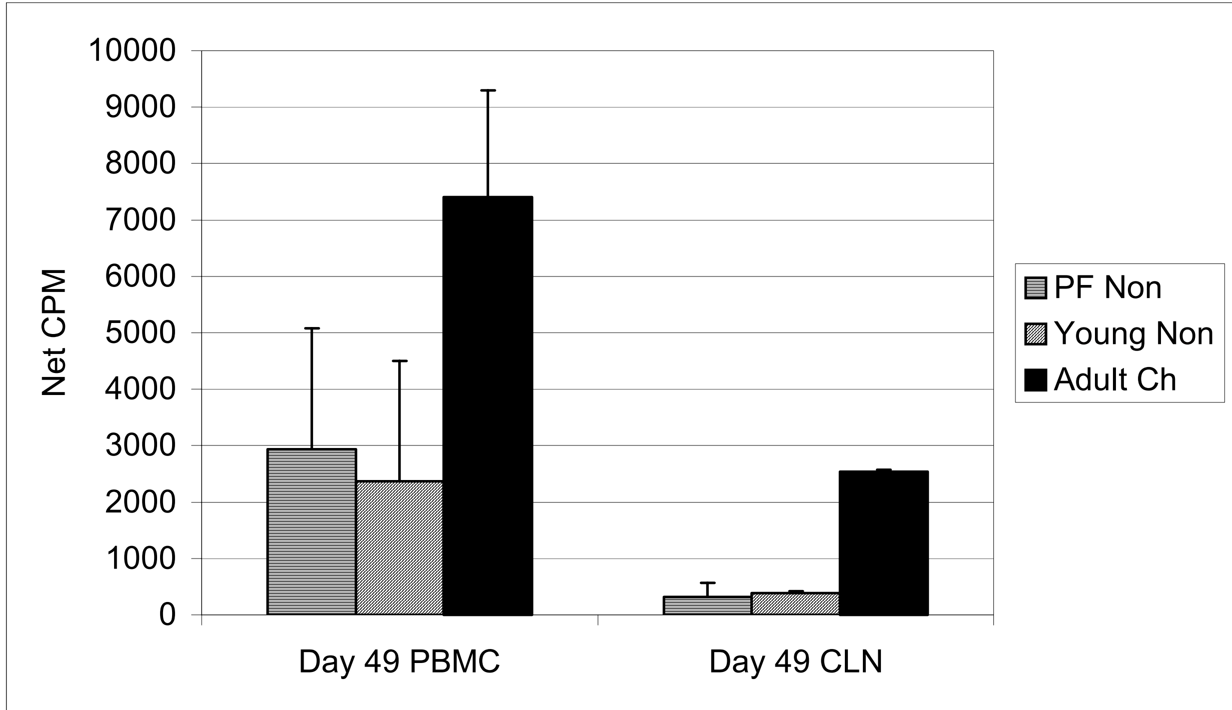


Fig. 2.13 Lymphoproliferative responses of circulating peripheral blood mononuclear cells (PBMC) cultured with 0.3µg/ml of *C. insigne* antigen. Results are shown in net counts per minute (CPM). PF: parasite-free. Non: non-challenged (two animals per group) (top chart). Ch: challenged (six animals per group) (bottom chart). The asterisk indicates that Adult Ch lymphoproliferative responses are significantly higher than those of PF Ch and Young Ch throughout the experimental period. $P < 0.05$.



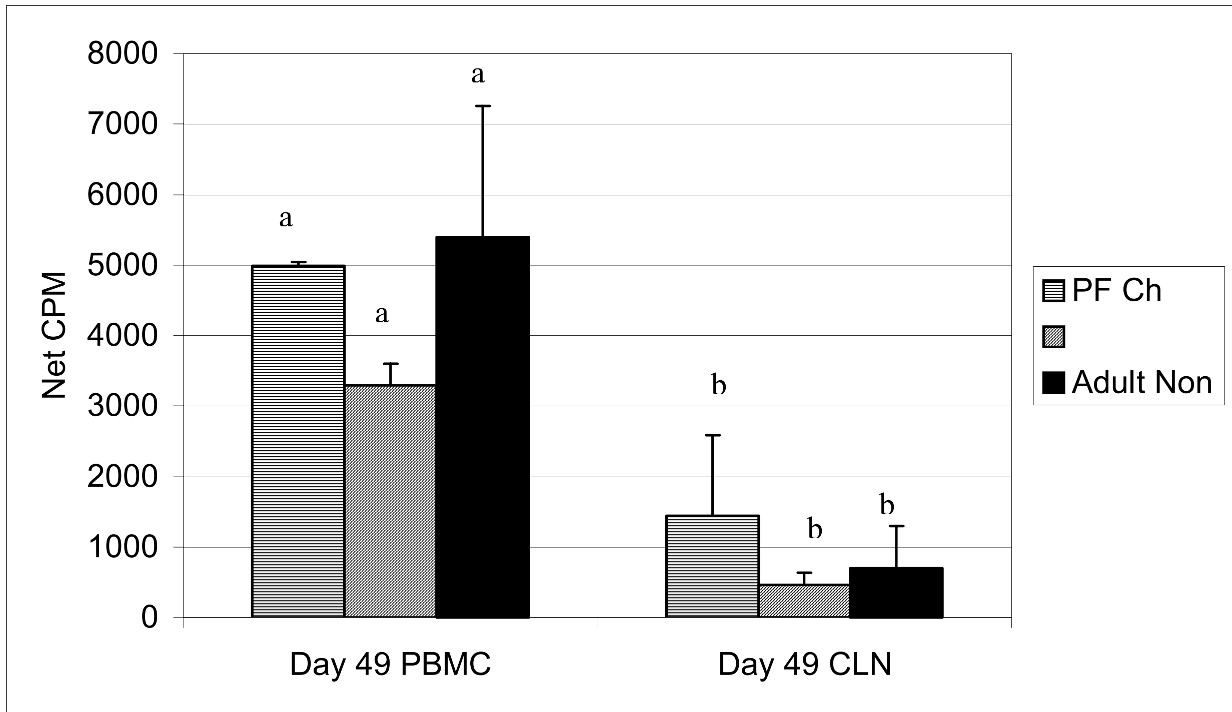


Fig. 2.14 Comparison of the lymphoproliferative responses of circulating peripheral blood mononuclear cells (PBMC) and cecal lymph node (CLN) lymphocytes, obtained on day 49, and cultured with 0.3µg/ml of *C. insigne* antigen. Results are shown in net counts per minute (CPM). PF: parasite-free. Non: non-challenged (two animals per group) (top chart). Ch: challenged (six animals per group) (bottom chart). Different letters above the columns indicate significant differences. $P < 0.05$.

No differences were seen in the lymphoproliferative responses of all groups when compared between days –56 and 0. During this period ponies were treated with anthelmintics and kept on a helminth free environment. Results are not shown.

At necropsy, the circulating lymphocytes (PBMC) had significantly higher proliferation rate than the lymphocytes obtained from the cecal lymph nodes (CLN) (Fig. 2.14). The analysis of the data did not show any statistical differences between groups.

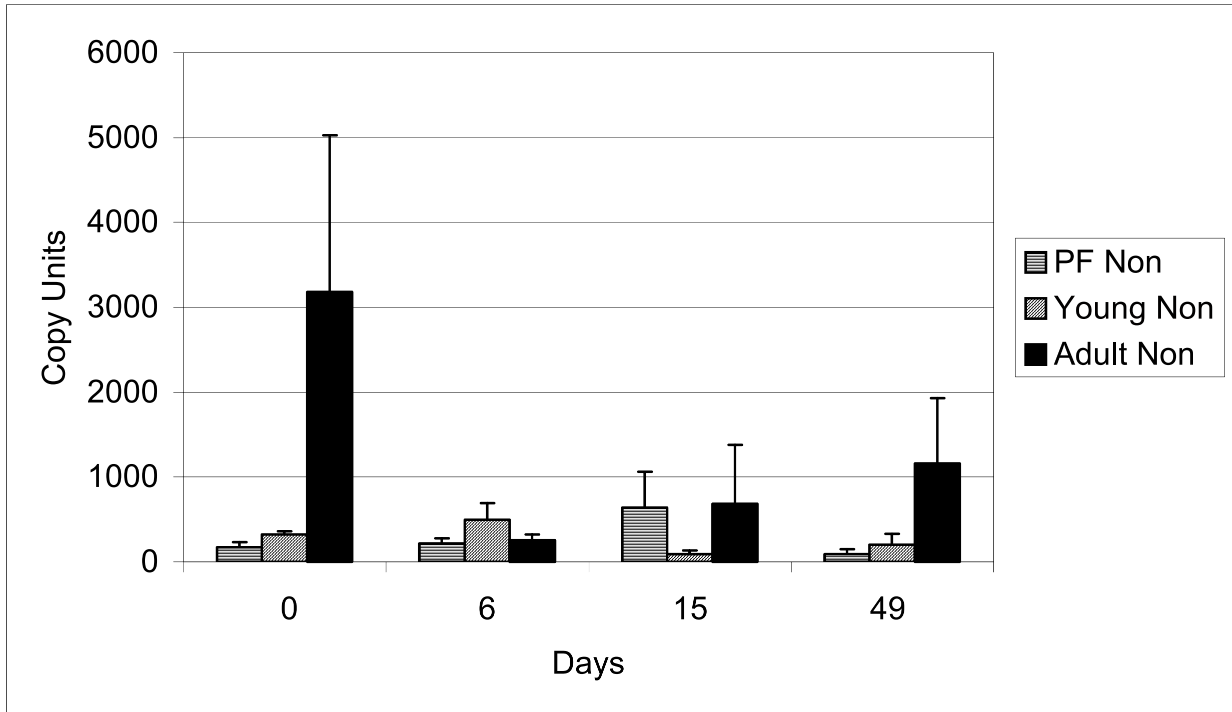
2.4.8 Quantitation of Equine Interleukin mRNA

Previously exposed ponies had the highest levels of IFN-γ throughout the experimental period.

A significant increase of IFN-γ in the Adult Ch and Young Ch ponies was seen when the data for the challenged groups was statistically analyzed (Fig. 2.15). The levels of IFN-γ for the PF Ch ponies decreased throughout the experimental period, whereas the levels increased in the previously exposed animals.

Adult Ch and PF Ch ponies had significantly higher levels of IL-4 mRNA than Young Ch ponies.

The levels of equine IL-4 mRNA for Adult Ch ponies were the highest, followed by the PF Ch animals with intermediate levels of this IL. Young Ch ponies had the lowest levels of IL-4. The statistical analysis of the data confirms these trends, where Adult Ch ponies had significantly higher levels of IL-4 than Young Ch ponies, but these levels were not significantly different from those of PF Ch ponies. Although the levels of IL-4 in the Young Ch group were the lowest no significant differences were seen between this group and the PF Ch animals.



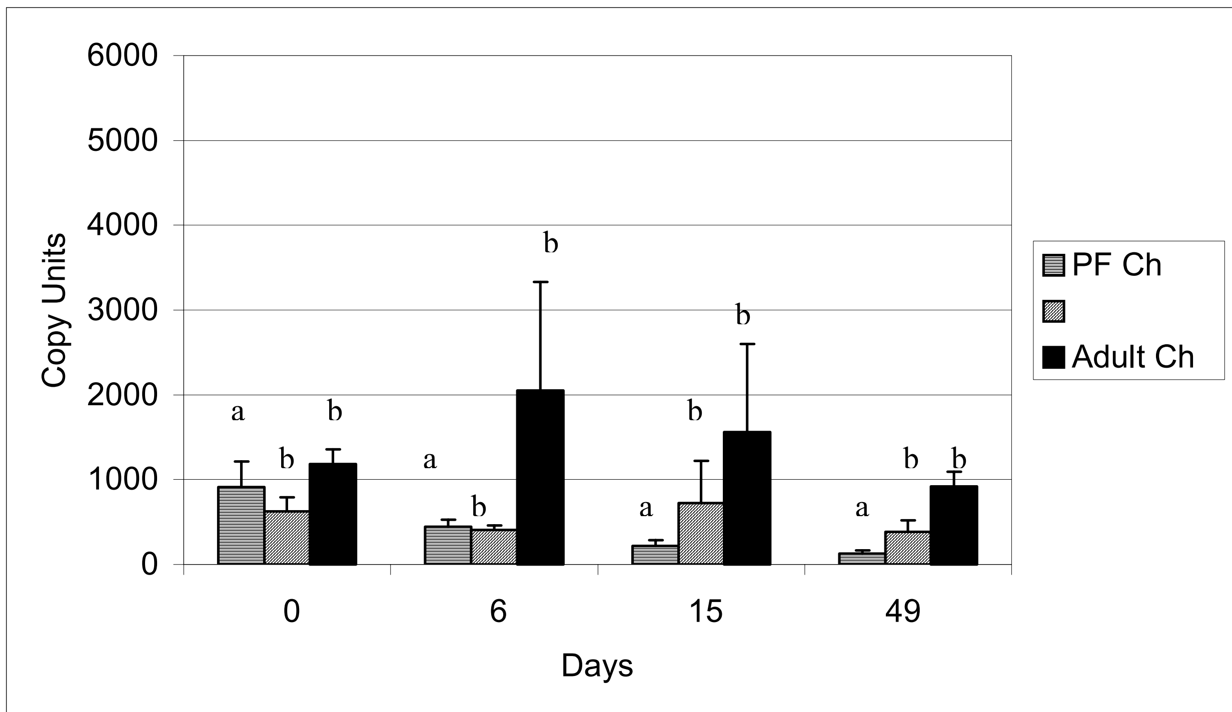
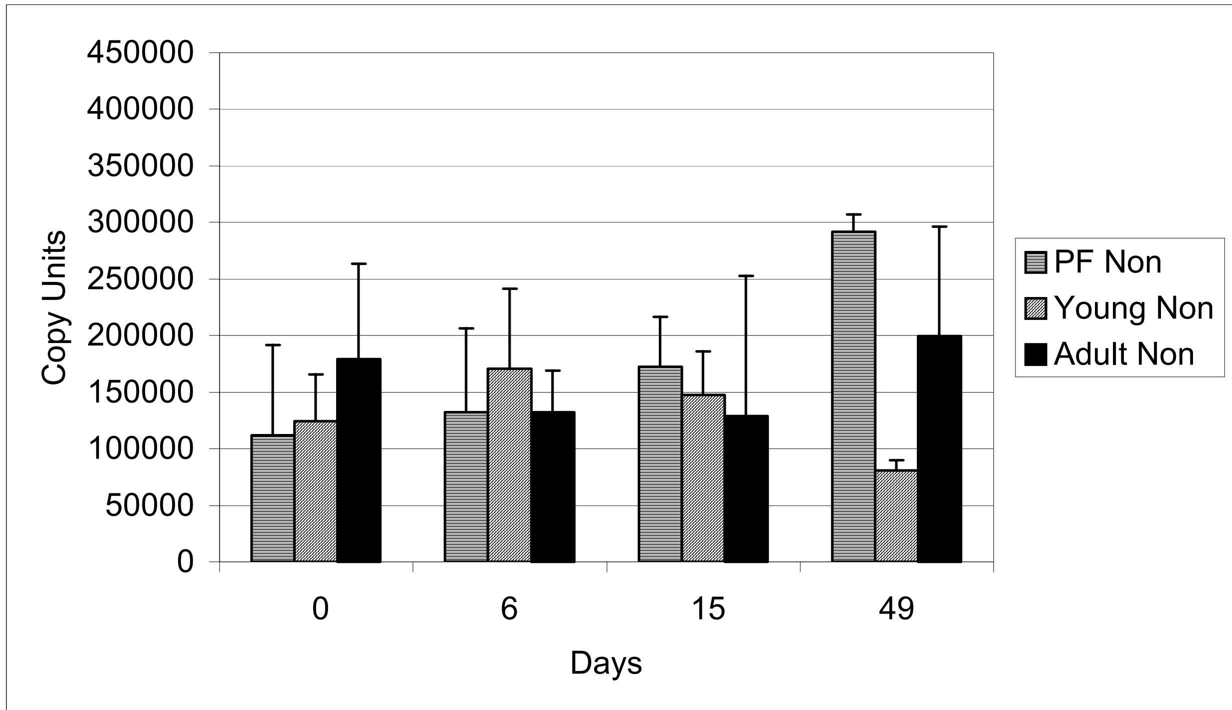


Fig. 2.15 Levels of IFN-γ mRNA from circulating peripheral blood mononuclear cells (PBMC) from days 0 to 49. Results are shown in Copy Units. The Y error bars represent the standard error of the mean. PF: parasite-free. Non: non-challenged (two animals per group) (top chart). Ch: challenged (six animals per group) (bottom chart). Different letters above the columns indicate significant differences. $P < 0.05$.



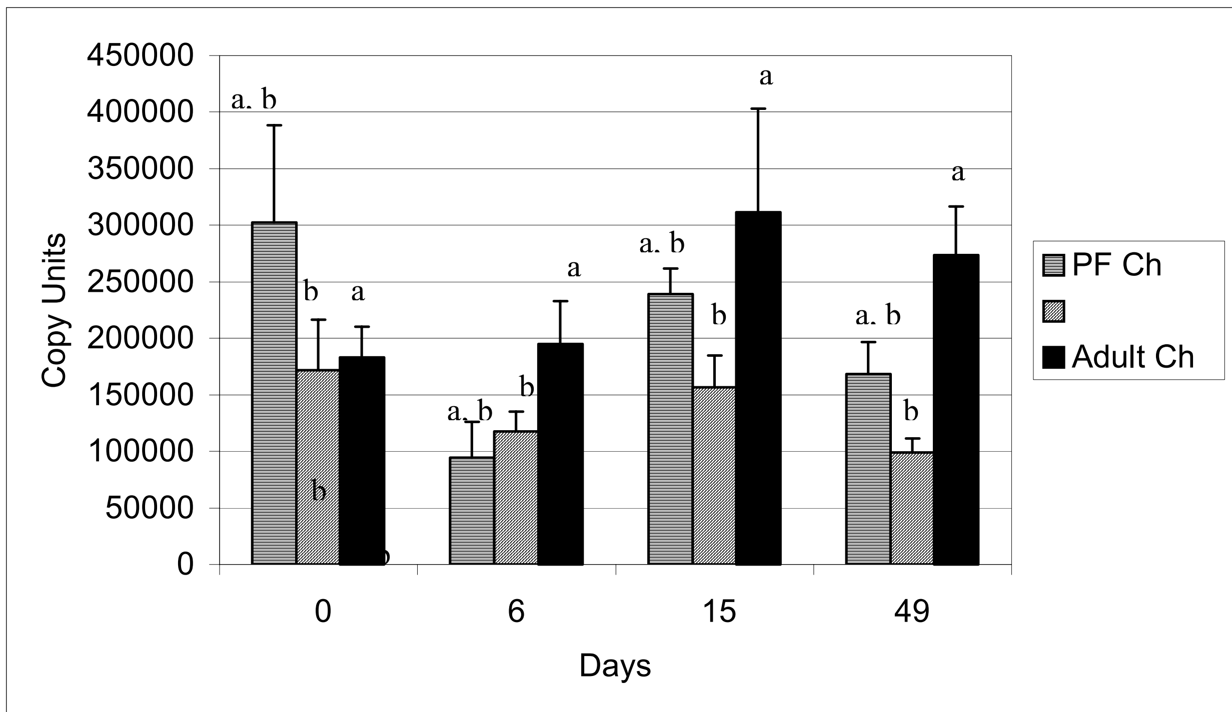


Fig. 2.16 Levels of IL-4 mRNA from circulating peripheral blood mononuclear cells (PBMC) from days 0 to 49. Results are shown in copy units (CU). The Y error bars represent the standard error of the mean. PF: parasite-free. Non: non-challenged (two animals per group) (top chart). Ch: challenged (six animals per group) (bottom chart). Different letters above the columns indicate significant differences. P < 0.05.

PF ponies had the highest levels of IL-13 overall.

The levels of mRNA for IL-13 in the PF Non ponies were markedly higher when compared to the other groups. This difference was mainly due to the extremely high levels of mRNA in one of the two non-challenged animals (Fig. 2.17). The response of this animal is so high in fact, that the levels of IL-13 for the PF Non are much higher than any of the other groups at day 15, and remained elevated when compared to those of the other groups through the end of the experiment. The cause of this rise is not known, especially taking into account that this particular pony did not have higher measurable levels of IL-4, IL-5, γ-IFN or β-actin. The statistical analysis of the challenged ponies showed that the PF Ch ponies had significantly higher levels of IL-13 than the Young Ch or Adult Ch at days 15 and 49 (Fig. 2.17).

Adult Ch ponies had higher levels of IL-13 than Young Ch ponies.

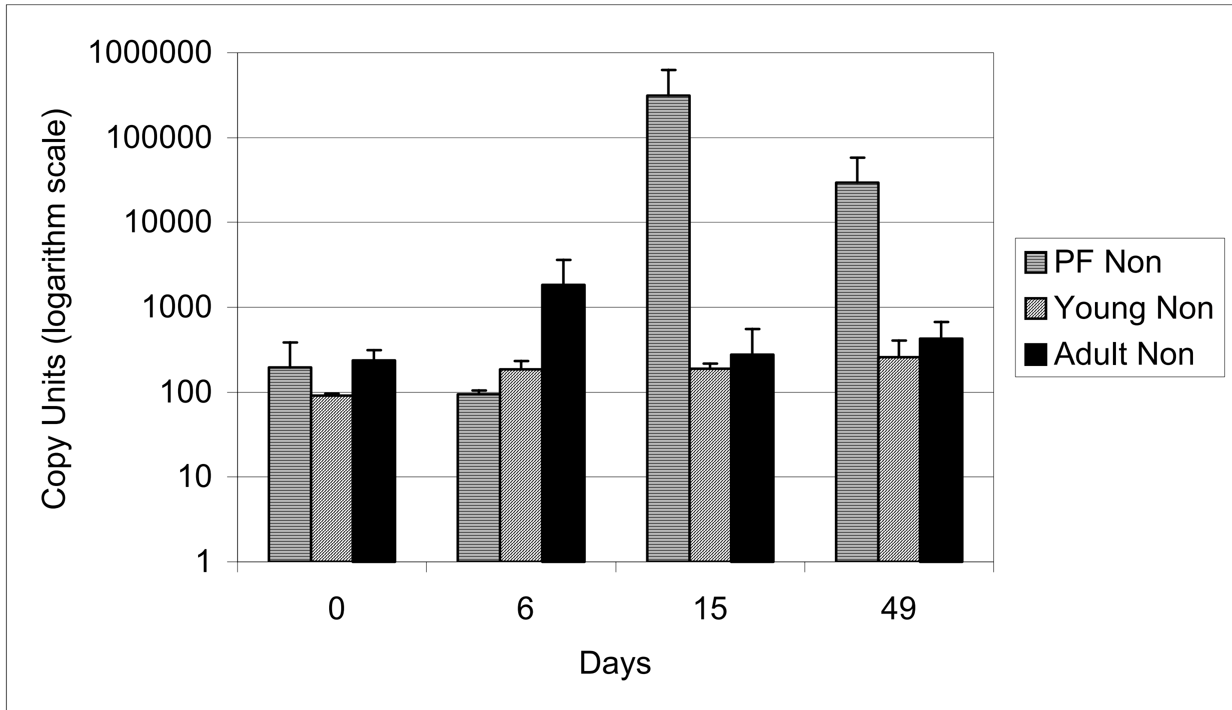
The levels of mRNA for IL-13 of the Adult ponies were higher than those of Young Ch (Fig. 2.17). The mRNA levels peaked on day 6 and decrease in the peripheral blood, although these levels were increased in the CLN at day 49 (data not shown).

The levels of IL-13 mRNA of the Non-challenged ponies was higher than of those of Ch ponies.

It is of interest to note that all Non-challenged groups expressed higher IL-13 mRNA levels than the Ch groups at some point during the experiment; examples are: Adult Non at day 6, Young Non at day 49 in the CLN (Fig. 2.17).

Adult Ch and Young Ch ponies had significantly higher IL-5 mRNA levels than PF Ch ponies.

The data presented in Figure 2.18 showed that Adult Ch ponies had higher IL-5 mRNA levels than Young Ch or PF Ch ponies, although significant differences between Adult Ch and Young Ch ponies were not seen when the data was statistically analyzed.



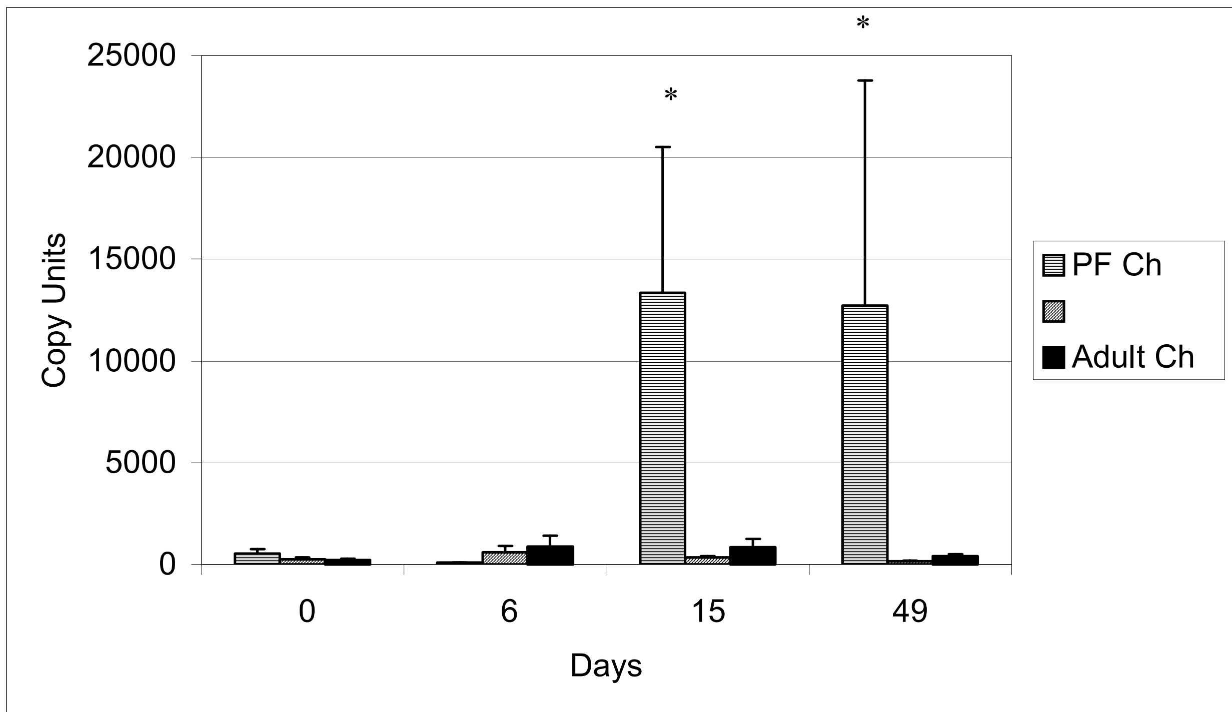
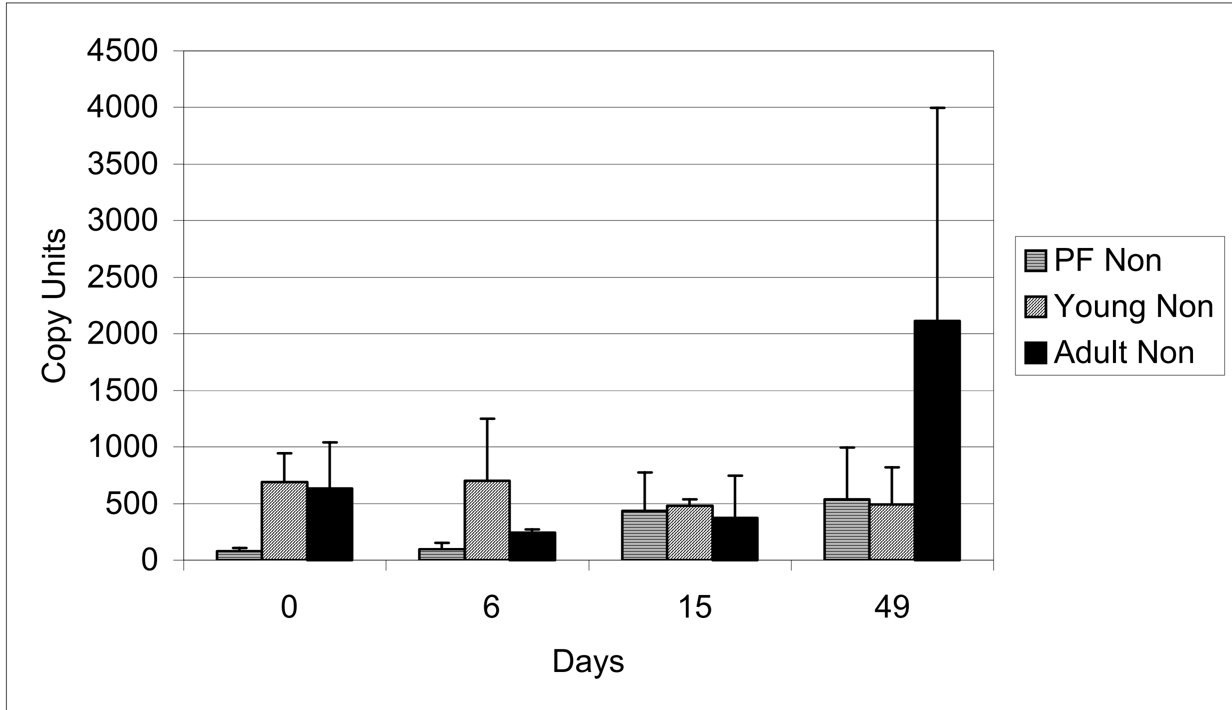


Fig. 2.17 Levels of IL-13 mRNA from circulating peripheral blood mononuclear cells (PBMC) from days 0 to 49. Results are shown in copy units (CU). The Y error bars represent the standard error of the mean. PF: parasite-free. Non: non-challenged (two animals per group) (top chart)(x axis in logarithm scale). Ch: challenged (six animals per group) (bottom chart). The asterisks indicate that the levels of IL-13 mRNA for the PF Ch group were significantly higher on days 15 and 49. $P < 0.05$.



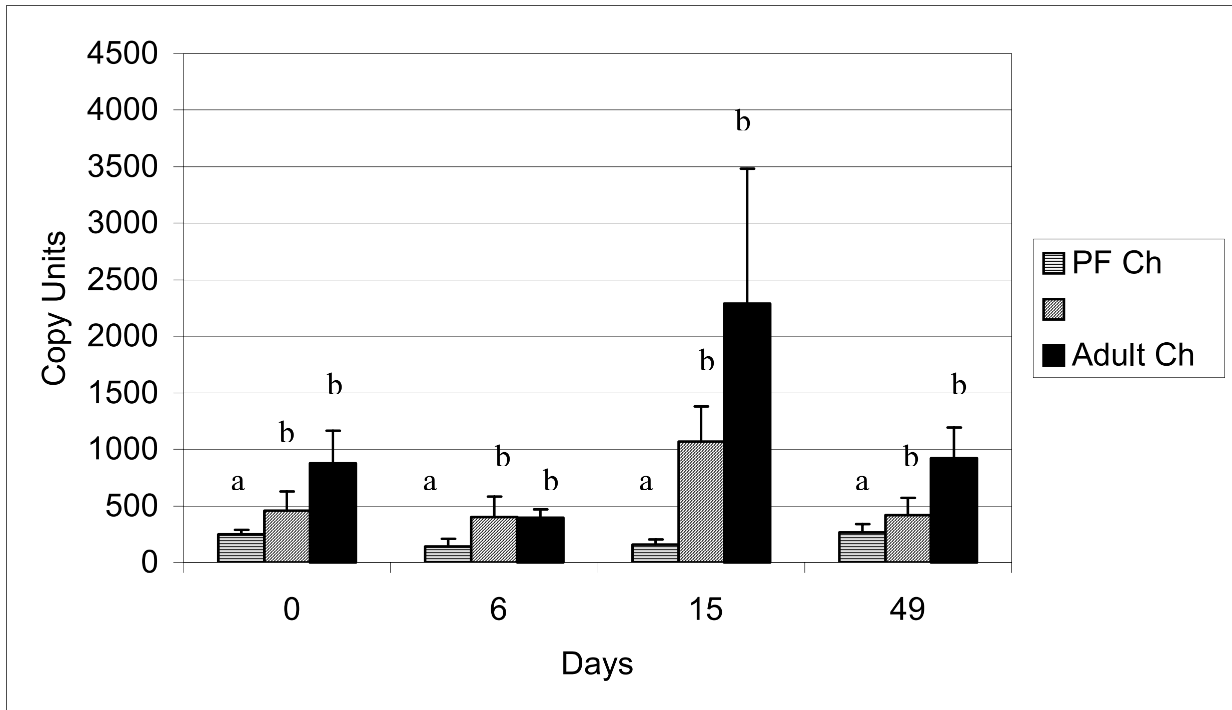


Fig. 2.18 Levels of IL-5 mRNA from circulating peripheral blood mononuclear cells (PBMC) from days 0 to 49. Results are shown in copy units (CU). The Y error bars represent the standard error of the mean. PF: parasite-free. Non: non-challenged (two animals per group) (top chart). Ch: challenged (six animals per group) (bottom chart). Different letters above the columns indicate significant differences. P < 0.05.

The levels of IL-5 mRNA obtained are similar to the levels of circulating eosinophils present in the same groups. PF Ch ponies produce increasing levels of IL-5 throughout the experiment, and this increase is gradual. Unusually high responses were seen on the Adult Non animals at day 49.

No significant differences were seen between circulating and cecal mRNA levels.

The systemic and cecal production of each interleukin's mRNA was compared at day 49 looking for differences suggesting a compartmentalized response, no statistically significant differences were found (data not shown).

2.5 Discussion

The hypothesis tested in this experiment, based on previous studies (Monahan et al., 1998) was that ponies would acquire resistance to cyathostomes with increasing exposure to cyathostome contaminated pastures. Thus, cyathostome-naïve animals would acquire the largest number of worms, followed in decreasing order by young and then adult ponies. The assumption behind this hypothesis was that helminth naïve ponies infected with cyathostomes would eliminate the infection using only innate immune responses. Whereas previously exposed ponies would eliminate the infection with acquired immune responses, and these would be more effective in ponies with longer exposure to cyathostomes as suggested by Klei and Chapman (1999). Three points can be made from the parasitological data recovered. First, the results showed that ponies previously exposed to cyathostomes, both the Young and Adult groups, had higher numbers of EL_3, than naïve animals, indicating a role of prior exposure to cyathostome contaminated pastures in the induction of hypobiosis. This is more clearly seen when percentages of each parasite stage are compared. Second, a lower number of DL, luminal L_4 and adult parasites were recovered from Adult ponies than from the Young or the PF. The greater exposure to these parasites by the Adult group suggests the acquisition of resistance to stages other than

EL_3. Finally, the total parasite numbers were unexpectedly greater in the Young ponies than in the PF or the Adult groups. This increase was due to EL_3 numbers when Young and PF were compared, whereas it was due to all stages when Young and Adults were compared. Together, these observations support the concept that horses do acquire resistance to cyathostomes, but the protective responses are slow to develop and their development varies depending on the degree of exposure of the animals to cyathostome contaminated pastures.

Previous studies of ponies exposed to cyathostome contaminated pastures have shown that numbers of hypobiotic EL_3 increase following pasture exposure, but do not confer protection following an controlled challenge (Chapman et al., 2002 a). The observations made during the current study confirm this finding. Further, the Young ponies used in the present study were age matched with the PF Ch ponies, indicating that previous cyathostome infections and not age elicits hypobiosis. Mechanisms associated with induction of hypobiosis to cyathostomes are unknown. However, immune mechanisms of resistance in conjunction with exposure of the larvae to external environment stimuli, such as temperature and humidity, the parasite's genetics, physiological and biochemical conditions of the host, and inter-specific competition have been postulated to influence the development of hypobiosis in trichostrongylid nematodes of ruminants (Gibbs, 1986). In cyathostomes, it has been proposed that hypobiosis occurs during all seasons of the year, but it is elevated during the winter months (Reinemeyer, 1986; Chapman et al., 2001, 2003). Thus, as with other nematodes it is likely that seasonal as well as acquired host factors play a role in cyathostome hypobiosis.

Reductions in adult parasite numbers (Foster, 1936; Love and Duncan, 1992; Chapman et al., 2002 b), and in parasite egg output in older equids (Love and Duncan, 1992; Klei and Chapman, 1999) have been widely noted among studies of naturally infected horses. These

observations have been used as evidence of acquired resistance. The analysis of the data obtained in this controlled challenge study showed that PF Ch and Young Ch ponies had similar numbers of DL and luminal stages despite differences in their histories of prior exposure to cyathostomes, whereas Adult Ch ponies had a 77.5% reduction of these stages. A possible explanation for this observation is that protective responses that affect developing larvae or luminal stages are different from those that induce hypobiosis. These responses also appear to be slower to develop, as reductions in parasite numbers were seen only in adult animals. The decrease in the number of cyathostome species found and the lower prevalence of species observed in resistant ponies have been suggested as another measurement of acquired resistance (Klei and Chapman, 1999). Although no differences in total parasite burdens were found between young and adult ponies experimentally challenged in previous reports (Chapman et al., 2002 a), adult ponies had decreased number of cyathostome species. Similar results were obtained in the present study, where the animals with longer prior exposure to cyathostomes had the lowest number of species. Therefore, suggesting that protective resistance eliminates the least common species.

A surprising finding in this experiment was that the Young Ch ponies had the largest total worm numbers. The likely explanation of this phenomenon is that young ponies had larger numbers of EL_3 and had not developed acquired resistant mechanisms against DL and luminal cyathostome stages. The higher rate of establishment observed in the Young Ch ponies when compared to Adult Ch ponies suggest the presence of more developed mechanisms of resistance to DL and luminal parasite stages in the latter group. Also, the adult ponies acquired a lower number of hypobiotic EL_3, possibly due to immune mechanisms targeted at the incoming L_3. It is possible that the reduced cyathostome L_3 establishment recorded for the Adult Ch ponies could be due to an immediate allergic type response that would reduce the invasion of the intestinal

mucosa by incoming L_3. The direct consequence of this allergic response may be similar to the "rapid expulsion" of L_3, a phenomenon described in sheep hyperimmunized by repeated infections with *Haemonchus contortus* larvae (Miller et al., 1985). The sheep became refractory to further infection. Although expulsion of L_3 was not measured in the present experiment in any of the challenged groups, it is possible that such an immune strategy to reduce cyathostome infections occurs in horses.

A few recent studies have attempted to associate immune effector mechanisms with acquired resistance. These included the measurements of cyathostome specific antibodies (Kara, 1996) and intestinal granulocyte responses in horses with different parasite burdens or exposure to cyathostomes (Collobert-Laugier, 2002). The data collected in this study attempted to extend these observations. The presence of intestinal mast cells in PF Ch, Young Ch and Adult Ch ponies seemed related to prior history of exposure to cyathostomes, therefore with acquisition of resistance. This suggestion is based on the absence of mucosal or submucosal mast cells in PF Ch ponies, and increased numbers of mast cells in the tissues of Young Ch and Adult Ch ponies. Furthermore, the presence of mast cells in same age animals (PF Ch vs. Young Ch) is likely due to prior cyathostome exposure. Adult ponies that had the longest exposure to cyathostome parasites had the highest intestinal mast cell counts in conjunction with the lowest numbers of DL, luminal L_4 and adult parasites suggesting an association between these cells and the reduction in parasite development and/or the removal of parasites from the lumen of the large intestine. The increase in mast cell numbers seen in the Adult Ch ponies is likely associated with increases in IL-4 mRNA measured in the same group of animals. This relationship between prolonged exposure to cyathostomes and increased numbers of mucosal mast cells in horses between older than 10 years of age also was observed by Collobert-Laugier et al. (2002). In that

study, the tissues were collected at an abattoir from large intestines of horses naturally infected with cyathostomes. It is possible that the increase in mast cell numbers observed in adult ponies or horses is related to the rapid expulsion phenomenon, as suggested by Miller et al. (1985) in sheep. Resistance was associated with massive infiltration of the mucosa by mast cells and globule leucocytes in sheep infected with *H. contortus*. The latter cell-type are partially degranulated mast cells often localized within the epithelium. Records of globule leukocytes are often described in ruminants (Miller et al., 1982; Balic et al., 2000) infected with gastrointestinal parasites, but their presence has not been documented in equids. It is possible that the failure identifying such cells is due to the lack of massive infiltrations of the intestinal epithelium by mast cells in horses, or to other morphologic characteristics that might need to be investigated using immuno- or histochemical techniques (Collobert-Laugier et al., 2002). Nevertheless, the significant increase of mast cells observed in adult ponies warrants further investigation in their role in the acquired resistance to cyathostomes.

Another cell type that had significant increases in numbers in the challenged ponies was the eosinophil. Tissue eosinophil counts in the cecum, dorsal colon, and ventral colon of ponies from the present experiment significantly increased after challenge. Furthermore, intestinal eosinophil counts in the Young Ch ponies were significantly higher than in the Adult Ch or PF Ch. Hence, the anamnestic responses of previously exposed animals upon cyathostome challenge in conjunction with concomitant elevations of IL-5 mRNA on the same dates suggests the possible involvement of eosinophils in an immune response to incoming L_3. Published data indicates that horses less than 10 years of age naturally infected with cyathostomes had significant associations between total larval or total worm counts and intestinal eosinophil counts (Collobert-Laugier et al., 2002). Even though cyathostomes do not have a migratory phase within

the host, *in vitro* studies with *S. vulgaris* L_3, a close relative of cyathostomes, have shown degranulation of equine eosinophils on the cuticular surface of *S. vulgaris* (Dennis et al., 1988) and immobilization of larvae *in vitro* (Dennis, et al., 1988). Eosinophils have been shown to kill *Haemonchus* spp. larvae in *in vitro* systems (Balic et al., 2000). Adult worms are not normally associated with eosinophilia in gastrointestinal infections of ruminants, but induce mast cell and goblet cell hyperplasia (Balic et al., 2000). Speculations such as if eosinophils are involved in EL_3 killing, could be made in cyathostome infections. It could be suggested that eosinophil mediated antibody-dependent cell-mediated cytotoxicity (ADCC) like events occur during mucosal migrations of developing larvae.

The production of antibodies directed against somatic and surface antigen of larval and adult cyathostomes has been investigated in recent years for different purposes. Some investigators have studied the possible use of antibodies directed against specific antigens of mucosal larval stages in order to develop diagnostic tools that would enable veterinarians to assess cyathostome infections in a non-invasive manner (Dowdall et al., 2002). Others have investigated the relationship between the different equine immunoglobulin isotypes to somatic or surface extracts of cyathostome L_3 or adults (Kara, a thesis, 1996). In the present study, levels of IgG and IgG (T) antibodies against somatic antigens of cyathostome L_3 were higher in Young Ch and Adult Ch animals. Challenge increased IgG (T) but not IgG. These results are similar to data obtained with pony sera of the controlled challenge described by Monahan et al. (1998, unpublished data). The high circulating levels of these immunoglobulins to cyathostome L_3 could be triggered by the presence of encysted larvae in the intestinal mucosa. The sharp rise of IgG (T) in naive animals after challenge and the overall higher levels of the same subisotypes in the Adult ponies suggest that IgG (T) is an important component of the immune response upon

acquisition of infection, although its role in protection is yet to be confirmed. Antibodies against the surface of freshly exsheathed L_3 showed that IgG and IgG (T) responses of Adult Ch were significantly higher than those of naïve animals. IFAT results from a previous study (Monahan et al., 1998; unpublished data) showed the same kinetic patterns for IgG and IgG (T) as those described in this experiment. The presence of high levels of IgG (T) in adult ponies both against somatic extracts of cyathostome L_3 and against their surface, would suggest once more a role in protection. Furthermore, the persistence of high levels of circulating antibodies against cyathostome L_3 than against somatic adult worm antigen extracts in challenges, as well as non challenged animals, would suggest that immune responses against incoming larvae might be more important than against adult cyathostomes. The specific roles of any of the equine immunoglobulin subisotypes still need to be investigated. Equine antibodies could be involved in the induction of hypobiosis in previously exposed ponies, in the blockage of larval invasion of the intestinal mucosa, or in other mechanisms of immunity. Alternately, these observations could be indirectly related to increases in Th2 type cytokines responses.

The increases of antibodies, mast cells and eosinophils in ponies infected with cyathostomes presented in this report are consistent with reports of increases of the same cell types and antibodies in ruminant (Miller et al., 1985; Balic et al., 2000) and rodent (Finkelman et al., 1997; Else and Finkelman, 1998) models of gastrointestinal nematode infections. In most gastrointestinal parasite infections a predominant Th2 type cytokine profile is elicited. The primary cytokine driving the Th2 type response is IL-4 (Urban et al., 1996; Garside et al., 2000; Finkelman et al., 1997; Else and Finkelman, 1998; Bancroft and Grencis, 1998). IL-4 has multiple effects on the immune system. If it is present early during a T cell response, it promotes the development of a Th2 type response phenotype, enhancing the production of IL-4 in a

positive feedback fashion (Paul, 4th edition, 1998). IL-4 and IL-13 independently enhance monocyte/macrophage and endothelial cell expression of adhesion molecules, increase macrophage expression of molecules associated with antigen presentation and T cell costimulation, stimulate production of eotaxin, suppress products of inflammatory mediators such as prostaglandins, reactive nitrogen, oxygen intermediates, IL-1α, IL-1β, IL-6, IL-12 and TNF-α, and stimulate the production of anti-inflammatory molecules (Finkelman et al., 1999). IL-13 enhances eosinophil responsiveness to chemokines, suppresses IL-2 induced proliferation of natural killer cells and their cytolytic activity and stimulates human B cells to switch to expression of IgG4 and IgE. IL-13 have many overlapping biological properties with IL-4, and it has been observed to induce IL-4 independent IgE synthesis. The overlap of activities between these two interleukins is partially due to the sharing of the type 2 IL-4 receptor (Finkelman et al., 1999). Activated T cells, mast cells, and basophils produce both cytokines, and dendritic cells and natural killer cells produce IL-13 (Finkelman et al., 1999). Both cytokines induce an allergic inflammation by stimulating mucus production, by inducing isotype switching to IgE (Paul, 1998), and by increasing the expression of chemokines and adhesion molecules that attract eosinophils and other inflammatory cells (Finkelman et al., 1999).

Studies of ponies challenged with the nematode parasite *S. vulgaris*, a close relative of cyathostomes, showed elevated levels of IL-4 and IL-13 mRNA (Swiderski, 1999; Edmonds, PhD dissertation, 2001). In the present experiment, measured levels of IL-4 mRNA from circulating lymphocytes were significantly increased in Adult Ch ponies, which was concurrent with higher counts of intestinal mast cells measured at necropsy. IL-4 and IL-13 have been deemed responsible for the exacerbated inflammatory responses of mice to infections with *T. spiralis* (Strait et al., 2003). The published data showed that anaphylaxis is primarily produced

by IL-4, but that both, IL-4 and IL-13, increase the sensitivity to vasoactive mediators secreted during an allergic response through exacerbation of vascular leakage (Strait et al., 2003). These actions are not limited to the vascular endothelium, since treatment of mice with IL-4 or IL-13 induces goblet cell hyperplasia (Dabbagh, et al. 1999), increase the ex vivo contractile response of jejunal smooth muscle to leukotrienes (Finkelman et al., 1997), and the ex vivo secretory response of jejunal epithelium to prostaglandin E_2 (Urban et al., 2001). The highly variable results of IL-13 measurement in the PF Non challenged ponies deter us from making any assumptions as to the role of this cytokine in the acquisition of protection in challenged ponies. However, the increased levels of IL-4 recorded for the Adult ponies suggests that this cytokine might be involved in a protective response in horses with longer exposure to cyathostomes.

Another elevated cytokine in Young Ch and Adult Ch ponies was IL-5. The increases of IL-5 in these groups of animals are indicative of the role of this cytokine in the anamnestic eosinophilia seen in these animals, but does not correlate with that of intestinal eosinophil counts at necropsy. The lack of correlation between intestinal eosinophils and IL-5 at the end of the experiment could be due simply to sampling schedule used. It would have been of interest to examine the mucosal eosinophil numbers at the height of the circulating eosinophilic response. It has been suggested that eosinophils play an important role in the elimination of nematode infections (Paul, 1998), but the only instances where eosinophils were found to be involved in the elimination of gastrointestinal nematodes was in infections of *Trichinella spiralis* (Maizels and Holland, 1998) and *Haemonchus contortus* (Balic et al., 2000). Peripheral eosinophilia has been correlated to increases of IL-5, the Th2 type cytokine responsible for the generation of eosinophils, in experimental mouse models (Else and Finkelman, 1998). However, treatment of mice with anti-IL-5 monoclonal antibodies, which eliminates eosinophilia, does not prevent the

expulsion of *N. brasiliensis*, *T. spiralis*, *T. muris*, or *H. polygyrus* (Else and Finkelman, 1998) from mice. However, the significantly increase of eosinophil counts in the intestinal tissues of ponies in this experiment after challenge are clearly part of an immune response to cyathostome infections which may or may not be important in protection.

Increases of peripheral eosinophils, intestinal mast cells and eosinophils, IgG (T) against surface antigens of cyathostome L_3, IL-4, and EL_3 counts were observed in Adult Ch ponies. This group had lower total number of worms, mainly due to a reduction of DL, luminal L_4, adult parasites. Fewer cyathostome species were also observed. All of these observations suggest that the immune mechanisms of resistance developed by Adult ponies confer protection against cyathostome infections. The same observations, related to the histories of prior exposure to these parasites in cyathostome contaminated pastures, also suggest that the acquisition of resistance is slow to develop, and it is targeted against each parasite stage present in the host. Results are not as clear as in inbred murine models of gastrointestinal infections however, the study of cyathostome infections in outbred horses or ponies warrants further research in this area.

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Chapter 3: Pasture Challenge

3.1 Introduction

Most of the evidence in acquired resistance of equids to cyathostome infections are based on field studies and necropsy data. Early observations (Foster, 1936) found that adult horses had fewer numbers of parasites than younger animals, making this report one of the earliest comments on the development of resistance by equids to cyathostome infections.

Other surveys have reported on the outcome of natural infections of animals of different ages and prior exposure to cyathostomes. Differences in larval acquisition and adult worm counts were also found in ponies older than 8 years of age when compared to ponies less than 4 years of age (Chapman et al., 2002 b and 2003). The adult ponies had lower total parasite numbers and fewer number of cyathostome species per animal implying that longer exposures to cyathostomes may elicit protective responses (Chapman et al., 2002 b, 2003). Grazing of a cyathostome contaminated pasture for a 5-week period by groups of helminth-naïve foals and previously exposed yearlings and adult ponies yielded a smaller worm burdens in the yearlings when compared to the foals (Love and Duncan, 1992 b). Adult ponies had lower parasite egg counts. Previously infected yearling ponies had higher numbers developing larvae than adult animals although they had comparable worm burdens (Love and Duncan, 1992 b). No attempts were made however, to distinguish the early larval stages (EL_3), considered the hypobiotic stage (Eysker et al., 1992), from the developing larval (DL) stages and it is not clear how to interpret this data.

Experimental challenge of ponies that had different grazing histories and helminth-naïve animals with cyathostome L_3 (Monahan et al., 1998; Chapter 2) also showed that adult ponies acquired fewer parasites. The reductions were seen in the developing and luminal stages. Even

though sterilizing immune responses have not been recorded in horses (Klei, 2000), the published literature suggests that horses with longer exposures to cyathostomes develop protective responses over the years if they are exposed to these parasites.

Short periods of grazing cyathostome contaminated pastures do not induce acquired resistance but increase hypobiosis. An experimental challenge of 100,000 cyathostome L_3 given to young and adult ponies, helminth-naïve or previously exposed to cyathostomes, showed that animals with prior exposure had significantly higher numbers of EL_3 and lower number of adult parasites (Chapman et al., 2002). Although these data demonstrates the significance of acquisition of responses in the induction of hypobiosis, other factors such as the climatic conditions could have induced infective L_3 into hypobiosis. To clarify which factors are responsible for this event further research would be needed.

Based on the reported findings (Chapter 2), the immune responses against cyathostomes were studied more in depth by experimentally challenging ponies of different ages and parasite exposure histories to a mixture of cyathostomes. Young and adult, previously exposed and helminth-naïve ponies were given 150,000 cyathostome L_3. Complete parasitological recoveries were compared with cellular and soluble mediators of the immune response. Results showed that adult ponies, with prior exposure to cyathostomes, had lower number of total worm counts than young previously exposed ponies or cyathostome-naïve animals. The differences seen in worm recoveries were largely due to increases in hypobiotic L_3 in the previously exposed ponies. These increases in hypobiosis were rapid in nature, as it has been shown that one season of pasture exposure to cyathostomes (Chapman et al., 2001), or 1-2 grazing seasons (Chapter 2) elicited this response. Adult ponies not only had lower numbers of parasite than young ponies, but also had decreased numbers of DL, luminal L_4 and adult parasites indicating that acquired resistance

against these stages is slower to develop than against EL_3. At the same time Adult ponies had higher mast cell numbers in the intestinal tissues at necropsy and increases in IL-4 mRNA levels throughout the experiment. Furthermore, the cyathostome challenge induced a clear anamnestic eosinophilia in previously exposed ponies, and a primary eosinophilic response in the naïve animals. The adult ponies also had significantly higher levels of IL-5 mRNA throughout the experimental period which corresponded to increases in eosinophil numbers. The presence of higher levels of IL-4 in the adult ponies together with the higher levels of antibodies against cyathostome L_3 surface or somatic antigens, increased intestinal mast cell counts and eosinophilia suggest that immune responses are active against cyathostome infections, although the specific mechanisms involved in the elimination of each parasitic stage are still unknown. While responses against specific stages were not detected the results confirmed that cyathostome infections elicit a Th2 type response in the horse.

To compare the results found in the experimental model to a more natural infection model, a pasture challenge was performed. Young and adult ponies, previously exposed or helminth naïve, were challenged naturally by grazing a cyathostome-contaminated pasture during 7 weeks. The rationale was that naïve ponies would acquire the largest numbers of parasites whereas the animals with longer prior exposure would show acquired resistance. Even though a pasture challenge is less well defined, it was felt that was a necessary step to confirm the findings of the experimental challenge. In an attempt to minimize differences in the processing of the samples the methods used were performed as described in Chapter 2. Correlations between parasitological data and protective immune mechanisms directed against the different parasite life stages were compared in order to validate the use of experimental challenges to study immune responses in cyathostome-infected horses.

3.2 Methods and Materials

3.2.1 Animals and Experimental Design

Mix breed ponies with different levels of exposure to cyathostomes were identified from a herd maintained on cyathostome-contaminated pastures. Levels of exposure were based on pony's age, which corresponded to the number of seasons of pasture exposure to cyathostomes. Previous studies suggest that resistance to cyathostome infections is attributable to age and previous exposure (Monahan et al., 1997). Eight 1.5-3 year old ponies (Young), with minimal exposure to cyathostomes, and eight 7-20 year old animals (Adult), with several seasons of exposure to cyathostomes, were chosen from this population. Eight 1-year-old animals, raised under helminth free conditions since birth, were used as non-exposed controls (Monahan et al., 1997). The design of this experiment closely resembles the study described in Chapter 2. The rationale was similar, it was hypothesized that the naïve animals would acquire the largest number of worms, followed by the young and the adult. However, in the current study a naturally acquired challenge was used. The objectives were to further characterize acquired resistance of equids to cyathostomes and identify the immune components associated with the post challenge parasite levels of the protective responses.

Eight weeks prior to pasture challenge, these twenty-four ponies were placed randomly in pairs within age groups, into box stalls. At day –56 all ponies were treated with 200µg/kg of ivermectin (Eqvalan®, Merial LLC, Iselin, NJ) plus 20mg/kg of oxibendazol (AnthelcideEQ, Pfizer Animal Health, Exton, PA) for five consecutive days to eliminate adult and larval cyathostome infestations as well as other nematode infections (Monahan et al, 1998; Chapman et al., 2002). Each group of eight animals was further subdivided into non-challenged (Non) animals and challenged (Ch) groups. The Ch groups consisted of six ponies each and the Non-

challenged of two ponies each. At day 0, all challenged ponies were placed on a cyathostome-contaminated pasture. Seven weeks later (day 49) these ponies were moved into stalls where they stayed until the termination of the experiment six weeks later on day 90. The Non-challenged ponies were not placed on pasture, they remained in the stalls throughout the length of the experiment. At day 90-post infection, all ponies were humanely sacrificed. Complete necropsies and parasite recoveries were performed at this time. The animals were fed hay, and Purina Horse Chow 100 (Ralston, Purina, N.C.) was provided twice a day. Water was available *ad libitum*.

3.2.2 Challenge

At day 0 the eighteen Ch animals were placed in a cyathostome-contaminated pasture for 7 weeks. The group of mares and foals that resided on the pasture, were left to act as pasture contaminators. The contaminator ponies were known to have only cyathostomes because they had been treated on regular basis (twice a year) with ivermectin, which eliminates the migrating stages of the large strongyles but does not remove encysted cyathostomes. Fecal cultures from the seeder ponies showed 99.9% cyathostome larvae. The remaining 0.1% were specimens of the genera *Oesophagodontus*, and *Triodontophorus,* that belong to the subfamily Strongylinae.

3.2.3 Pasture Larval Recovery

The cyathostome burden of the contaminated pasture was determined by direct examination of the grass (Baudena et al., 2000). Grass samples were collected at days –5, 10, 23, 37, 55, and 67. The 3.2-acre pasture contained a mixture of local grass species such as Dallas, Bahia, coastal Bermuda, and rye grass. The procedure followed was to walk a double W pattern through the pasture (Anonymous, 1977). Briefly, multiple samples were taken at 10 m intervals by clipping close to the ground without including roots in the sample. Approximately 260g of grass was collected at each time point, and wet/dry ratio determined by weighing 50g before and

following a drying process using a microwave oven. The remaining sample was soaked in tap water overnight in a #1 washtub with approximately 3-5 ml of dishwashing detergent added to reduce surface tension during immersion of the grass. Following the overnight soak, the grass was discarded, the liquid was strained to remove large particles of debris, and was returned to the tub for a minimum of 4 hr to allow larvae to settle. This was followed by a series of sedimentation and decanting steps until the volume was reduced to 250 ml. This final volume was concentrated by centrifugation. Infective larvae present in the pellet were recovered by a subsequent sugar flotation using a solution of specific gravity 1.20. Recovered L_3 were then washed and triplicate aliquots counted to determine the total number of L_3 recovered. This data was recorded as L_3 kg^{-1} dry herbage. The infective larvae were identified and categorized as small or large strongyles according to Soulsby (Soulsby, 7th edition, 1982).

3.2.4 Climatological Data

Temperature and rainfall measurements for the study period were obtained from the Southern Regional Climate Center. The most proximal recording station was located approximately 3 km from the pasture.

3.2.5 Samples Collected

Blood was collected by venipuncture on days –56, 0, 10, 14, 22, 30, 38, 43, 50, 58, 63, 72, 76, 85 and 90 into Vacutainers® containing EDTA for a complete blood count (CBC) or without additives to obtain sera.

On days –56, 0, 14, 38, 63 and 90 additional blood samples were obtained in Vacutainers® to which a sterile heparin sodium salt solution was added, to isolate peripheral blood mononuclear cells (PBMC). These PBMC were used in lymphoproliferation assays and to isolate mRNA for cytokine measurement.

Rectal fecal samples were obtained on days –56, 0, 31, 38, 44, 51, 59, 63, 73, 77, 86 and 90. The fecal samples were processed using a modified Stoll technique (Klei and Torbert, 1980). Results are presented as eggs per gram (EPG) of feces.

Ponies were humanely sacrificed and necropsy examinations were performed following a protocol previously described (Klei and Torbert, 1980; Monahan et al., 1998). Briefly, the large intestine was opened and the contents collected. Two or three 2x2 cm sections were taken from each organ for histological examination. The mucosal surfaces were then washed, and these washings were added to the contents. A ten percent aliquot of the mixed intestinal content was preserved with 10% formalin solution. Isolation and identification of developing and adult parasites were performed on a 1% aliquot of the total contents per animal.

After washing the luminal surfaces of the cecum, ventral and dorsal colons, each organ was separated and individually weighed. A 1% by weight portion of each organ was cut from the areas between the teniae bands for mucosal digestion. Larvae ere identified from an aliquot of digested mucosa with a stereomicroscope. Three 32-cm^2 samples were taken from the rest of the cecum, ventral colon and dorsal colon for transmural illumination (TMI) (Chapman et al., 1999).

3.2.6 Other Laboratory Procedures and Statistical Analysis of the Data

Methods and materials used to collect histological, pathological and immunological data have been described in Chapter 2. These procedures include: intestinal cell enumerations, parasites and antigen preparation, ELISA, isolation of equine PBMC, lymphoproliferation assays, RNA isolation and cDNA synthesis, detection of specific equine interleukin mRNA, and statistics.

3.4 Results

3.4.1 Parasite Recoveries

Young Ch ponies had the highest worm counts.

The Young Ch group had the highest numbers of total worms (Table 3.1). Statistical analysis showed that within Ch groups the Young had significantly higher numbers than the PF Ch and Adult Ch (Table 3.1). This difference did not correspond to the degree of prior exposure.

The Adult Ch ponies had approximately 3 times fewer worms than the Young Ch ponies (Table 3.1). Reductions were seen in DL (by digestion and by TMI) and luminal L_4, whereas both groups had similar numbers of adult worms.

Previously exposed ponies had significantly fewer DL and mature parasites than naive ponies.

Young Ch ponies had a 61% and Adult Ch ponies had a 45% reduction in the number of DL, luminal L_4 and adult worms when compared to PF Ch animals (Table 3.2). These results were obtained by subtracting the values obtained for the Non-challenged from the values of the Ch, and then comparing Young Ch and Adult Ch groups to PF Ch. The decreases were most clearly seen in the DL and adult worm populations. These reductions are not as marked as that seen in Chapter 2 for Adult Ch worm population.

A challenge infection seems to induce hypobiosis.

The comparison between the percentages of the different larval stages found in the Young and Adult Non challenged versus the Young and Adult Ch in Table 3.3 suggested that the challenge of previously exposed animals with cyathostome L_3 induced the incoming larvae to go

Table 3.1 Enumeration of the different cyathostome life cycle stages in the mucosa and lumen of the large intestine based on transmural illumination, digestion and recovery of larvae and adults from the lumen.

	EL_3[¶]	DL[¢]	TMI[£]	Luminal L_4[¥]	Adults	Total counts[§]
PF Non	206 ± 206[a] (0-411)	0 ± 0	0 ± 0	0 ± 0	0 ± 0[a]	206 ± 206 (0-411)
PF Ch	33,197 ± 10,140[a] (7,538 – 74,422)	7,109 ± 1,330[a] (1,883 – 11850)	5,779 ± 1,118[a] (2,947 – 10,897)	254 ± 61[a] (60 – 407)	372 ± 87[a] (148 – 739)	40,931 ± 10,806[a] (13,912 – 83,375)
Young Non	20,280 ± 17,761 (2,519 – 38,041)	5,656 ± 4,369 (1,287 – 10,024)	1,450 ± 1450 (0 –2,899)	39 ± 35 (4 – 73)	135 ± 102 (33 –236)	26,109 ± 22,266 (3,843 – 48,374)
Young Ch	131, 983 ± 39,143[b] (23,036 – 294,755)	8,521 ± 1,827[a] (2,482 – 13,868)	8,823 ± 3,434[a] (67 – 24,811)	208 ± 63[a] (6 – 431)	146 ± 43[a] (3-273)	140,857 ± 39,801a (28,499 – 301,884)
Adult Non	2,674 ± 1,467 (1,207 – 4,140)	2,410 ± 2,410 (0 – 4,820)	847 ± 847 (0 – 1,693)	8 ± 8 (0- 16)	133 ± 133 (0 – 265)	5,224 ± 4,017 (1,207 – 9,241)
Adult Ch	44,262 ± 21,590[a,b] (0 – 113,765)	6,441 ± 3,848[a] (0 – 23,376)	6,587 ± 4,361[a] (0 – 25,600)	208 ± 131[a] (0 – 803)	162 ± 90[a] (1 – 472)	51,074 ± 24,622[a] (1 – 138,356)

PF: parasite-free. Non: non-challenged (to animals per group). Ch: challenged (six animals per group). [¶]EL_3: hypobiotic early L_3, based on counts from digestion. [¢]DL: developing larvae, including mid- to late L_3 and early L_4, based on counts from digestion. [£]TMI: developing larvae counted with the transmural illumination technique. [¥]L_4: fourth stage larvae.
[§]Total counts = EL_3 + DL + luminal L_4 + adults.
This table shows the average number of the worms recovered for each pony group in each category, followed by the standard error of the mean and the range between parentheses. Different superscript numbers indicate significant difference with $P < 0.05$, only Challenged groups were compared.

into hypobiosis. It is also possible that the existing infection present in previously exposed ponies after the anthelmintic treatment may have helped to induce hypobiosis. The presence of luminal adult stages could have induced hypobiosis of the infective L_3 acquired while grazing the naturally cyathostome contaminated pasture.

A pasture challenge appears to induce hypobiosis in cyathostome naïve ponies.

Eighty-one percent of larvae in the PF Ch ponies were in the EL_3 stage. This is comparable to the 93.7% and 86.7% of found in the Young Ch and Adult Ch ponies respectively (Table 3.3). The data suggest that a short exposure (7 weeks) of PF ponies to a cyathostome contaminated pasture strongly drives the incoming larvae into a hypobiotic stage. Hypobiosis in the PF Ch group of the experimental challenge was much lower (51%) (Chapter 2), suggesting that the method of challenge affected the infection outcome. The percentage of larvae from pasture undergoing hypobiosis could have been influenced by a wide temperature range while on pasture (versus culture under monitored temperatures), the exposure to freezing temperatures in the external environment (vs. cold conditioning in the laboratory (4ºC)), and other unknown factors.

Non-challenged ponies had higher numbers of DL than their Challenged counterparts.

Even though there were only 2 animals in the Non Challenged groups, the data suggests that the elimination of the parasite burdens in the previously exposed animals with the anthelmintic treatment triggered the development of EL_3 (Table 3.3). This development was not hindered by the challenge as it occurred in the Ch ponies.

The anthelmintic treatment was not 100% effective.

The Non-challenged animals had a few residual cyathostomes after the anthelmintic treatment. One of the PF Non ponies had a very low number (411) of EL_3. This unusual situation

Table 3.2 Comparison between DL, luminal L_4 and adult worms in the Ch groups.

	DL[¢]	Luminal L_4[¥]	Adults	% Reduction
PF Ch	7,109	254	372	___
Young Ch	2,865	169	11	**61**
Adult Ch	4,031	200	29	**45**

PF: parasite-free. Ch: challenged (six animals per group). The results were obtained by subtracting the values obtained for the Non-challenged from the values of the Ch. [¢]DL: developing larvae, including mid- to late L_3 and early L_4, based on counts from digestion. [¥]L_4: fourth stage larvae. The % reduction was obtained by adding the values of DL, luminal L_4 and adult worms, and comparing the results obtained for the Young Ch or the Adult Ch to those obtained for the PF Ch.

Table 3.3 Percentages of cyathostome life cycle stages based on the total number of parasites obtained for each group.

	EL_3[¶]	DL[¢]	L_4[¥]	Adults
PF Non	100	0	0	0
PF Ch	81.1[a]	17.4[a]	0.6[a]	0.9[a]
Young Non	77.7	21.7	0.1	0.5
Young Ch	93.7[a]	6[a,b]	0.1[b]	0.1[b]
Adult Non	51.2	46.1	0.2	2.5
Adult Ch	86.7[a]	12.6[b]	0.4[b]	0.3[a,b]

PF: parasite-free. Non: non- challenged (two animals per group). Ch: challenged (six animals per group). [¶]EL_3: hypobiotic early L_3, based on counts from digestion. [¢]DL: developing larvae, including mid- to late L_3 and early L_4, based on counts from digestion. [¥]L_4: luminal fourth stage larvae. Different superscript letters indicate significant difference with $P < 0.05$, only Challenged groups were compared.

and break in PF status may have been due to the attempt to house the ponies of this experiment in a different barn that has been routinely used to rear and maintain PF ponies (Monahan et al., 1997; Chapter 2).

The number of cyathostome species recovered diminished with increased pasture exposure prior to challenge.

A total of 17 cyathostome species were found following pasture challenge when all groups were pooled together. These species are ranked by the number of parasites per pony, or intensity, in decreasing order (Table 3.4). Only 10 cyathostome species recovered at necropsy from Adult ponies, whereas Young animals had 11, and PF had 14. The prevalence of the first seven ranked species indicate that there were present in approximately 70 % of the animals. It is hypothesized that the most common species observed in the adult Ch ponies are better adapted to the mechanisms of acquired resistance that seem to develop with exposure or are more fit. Whereas a higher number of less common species are found in those animals without acquired resistance, such as the naive ponies (Klei, 2000; Chapman et al., 2003).

Parasites other than cyathostomes were recovered.

The tapeworm *Anoplocephala perfoliata* was found in 7 out of the 12 Ch previously exposed animals and in 5 out of the 6 PF Ch ponies (Table 3.5). The roundworm *Parascaris equorum* was found only in 3 of the PF Ch and 1 of the Young Ch animals. Third instars of *Gasterophilus intestinalis* were found in the stomachs of 3 Adult ponies. And the large strongyle *Strongylus edentatus* was recovered from one Adult Ch pony. Some *G. intestinalis* and *S. edentatus* specimens remained after treatment with IVM and OBZ. None of the anthelmintic drugs used are effective against *A. perfoliata*. The only difference seen with the non-cyathostome data shown in Chapter 2 is that the PF Ch ponies acquired *A. perfoliata* and *P. equorum* while grazing the pasture. The acquisition of these species is expected, given that the intermediate host of *A. perfoliata* is a mite that lives on pastures, and that the eggs of *P. equorum* are extremely resistance and are known to survive and be infective years after being deposited in the external environment (Austin et al., 1990).

Table 3.4 Cyathostome species identified from aliquots of the intestinal contents of the Challenged ponies.

Cyathostome species found in challenged ponies	**Rank** (Prevalence)			
	Total*	**PF**	**Young**	**Adult**
Cylicostephanus longibursatus	1 (13/18)	1 (6/6)	4 (4/6)	1 (3/6)
Cylicocyclus nassatus	2 (15/18)	2 (6/6)	1 (5/6)	3 (4/6)
Cyathostomum catinatum	3 (14/18)	4 (5/6)	6 (5/6)	2 (4/6)
Cylicostephanus goldi	4 (14/18)	6 (6/6)	2 (5/6)	4 (3/6)
Cylicostephanus calicatus	5 (13/18)	5 (6/6)	3 (6/6)	7 (1/6)
Cylicostephanus minutus	6 (13/18)	3 (6/6)	5 (5/6)	5 (2/6)
Coronocyclus coronatus	7 (14/18)	7 (6/6)	7 (6/6)	6 (2/6)
Cylicocyclus insigne	8 (6/18)	8 (5/6)	10 (1/6)	--
Cyathostomum pateratum	9 (6/18)	9 (5/6)	9 (1/6)	--
Poteriostomum ratzii	10 (6/18)	11 (3/6)	8 (2/6)	9 (1/6)
Coronocyclus labratus	11 (3/18)	10 (3/6)	--	--
Cylicocyclus ashworthi	12 (2/18)	12 (2/6)	--	--
Coronocyclus labiatus	13 (1/18)	--	--	8 (1/6)
Cylicocyclus elongatus	14 (1/18)	13 (1/6)	--	--
Petrovinema poculatum	15 (1/18)	--	11 (1/6)	--
Parapoteriostomum euproctus	16 (1/18)	14 (1/6)	--	--
Parapoteriostomum mettami	17 (1/18)	--	--	10 (1/6)

PF: parasite-free. *Total = PF Ch + Young Ch + Adult Ch
--: No adult cyathostome were recovered for the mentioned species.
Rank = cyathostome species ranked by intensity. Intensity = number of adult cyathostomes of the mentioned species obtained per pony.
Prevalence = number of ponies infected with the mentioned species of cyathostomes / number of ponies examined. Data shown between brackets.

Table 3.5 Numbers of non-cyathostome parasites recovered from the intestinal contents at necropsy.

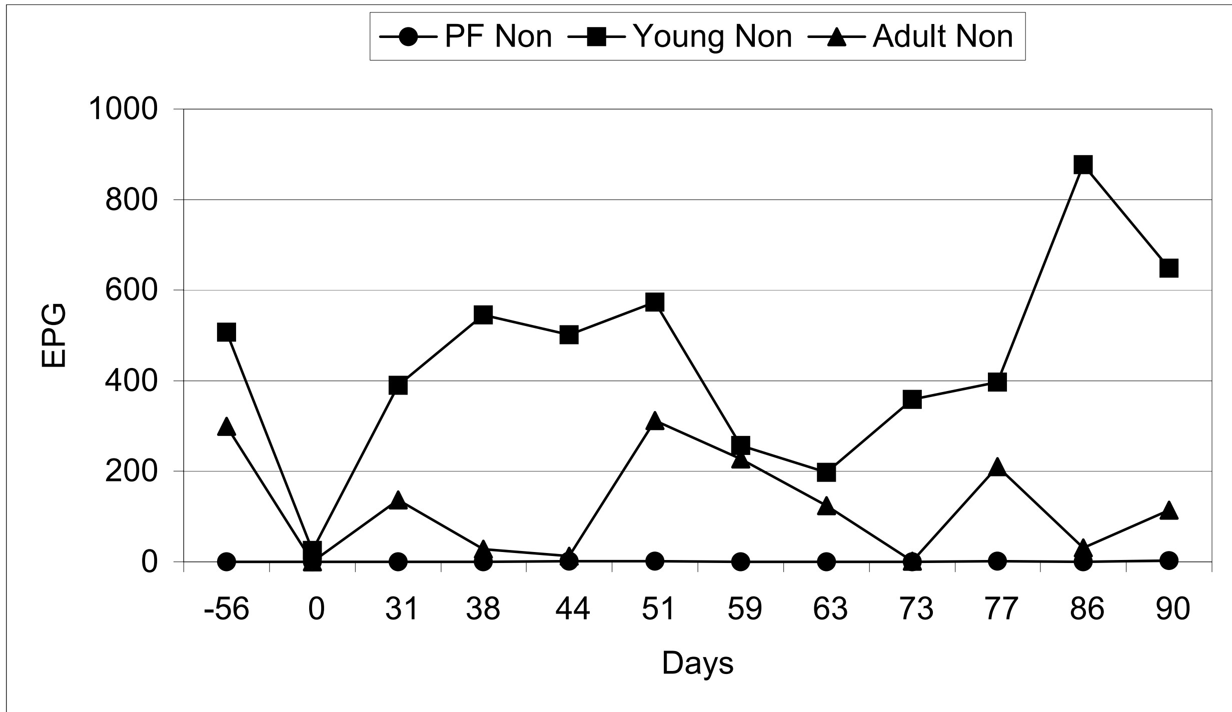
Group	Animal ID	Anoplocephala perfoliata	Parascaris equorum	Gasterophilus intestinalis	Strongylus edentatus
PF Non					
PF Ch	2-99	1	34 (immature)		
	13-99	2	1		
	19-99	1			
	21-99	1			
	31-99	1	1		
Young Non					
Young Ch	011	4			
	012		1		
	014	3			
	016	1			
Adult Non	037			3	
Adult Ch	034	7		20	21
	038			1	
	043	1			
	044	3			
	047	4			

PF: parasite-free. Non: non-challenged. Ch: challenged. Animal ID: pony identification number. *Anoplocephala perfoliata, Parascaris equorum, Strongylus edentatus*: adult or immature specimens. *Gasterophilus intestinalis*: third-stage instars.

3.4.2 Fecal Egg Counts

Adult Ch ponies had lower egg counts than Young Ch or PF Ch animals.

All previously exposed animals were shedding cyathostome eggs at day –56 (Fig. 3.1). The anthelmintic treatment eliminated the majority of the luminal parasites, therefore the egg production was reduced to zero by day 0. Although only on day 73 the PF Ch ponies presented significant FEC differences, the egg reappearance times were faster and the FEC higher in the Young and PF than in the Adult ponies. This observation suggests that immune responses may interfere with the parasite's egg production in the Adult ponies, as it has been suggested in publications (Chapman et al., 2002 b; Klei, 2000). Non-challenged ponies also



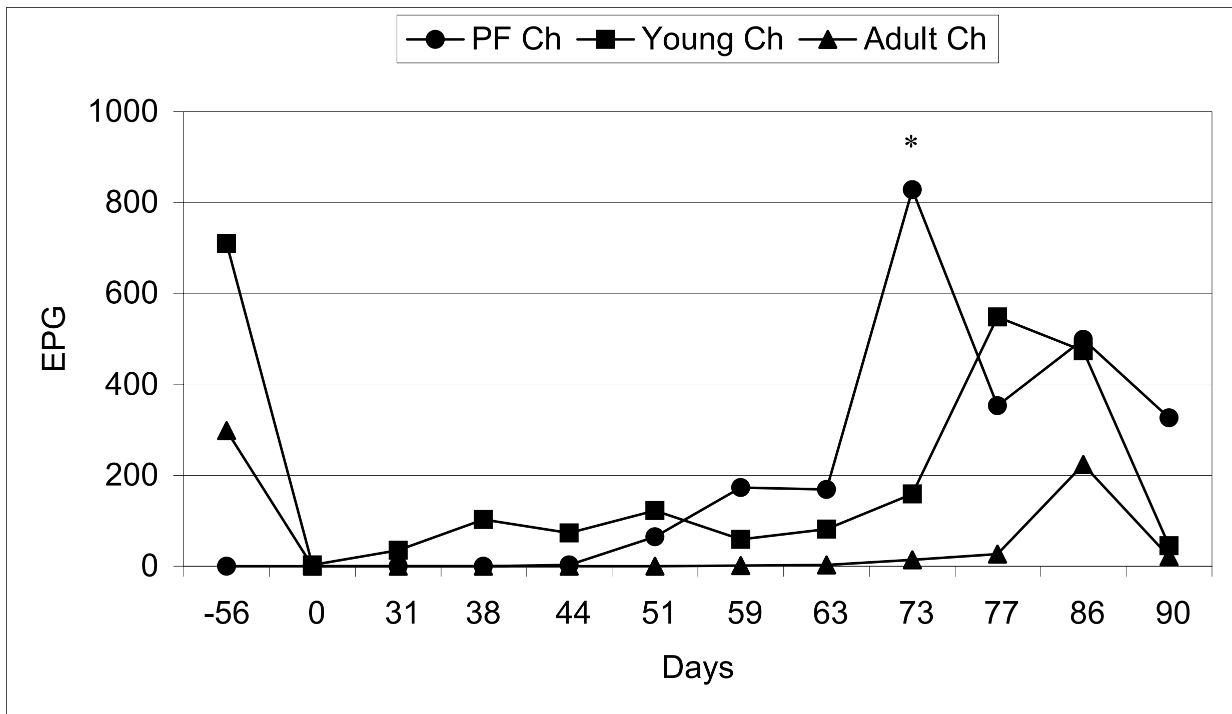


Fig. 3.1 Fecal egg counts presented as the average of eggs per gram (EPG) per group. PF: parasite-free. Non: non-challenged (2 animals per group) (top chart). Ch: challenged (6 animals per group) (bottom chart). The asterisk (*) indicates that the PF Ch animals had significantly higher FEC on day 73 than Young Ch or Adult Ch animals. $P < 0.05$.

had positive FEC. The high egg counts recorded in the Young Non challenged ponies are mainly due to one animal, even though both ponies eliminated parasite eggs. The kinetics of FEC in the latter group suggests that the elimination of the luminal parasites and the lack of reinfection stimulate prolificacy in these worms. Alternatively, the challenge could have stimulated the expulsion of the parasites by a self-cure phenomenon (Monahan et al., 1998). Whereas, the kinetics of FEC in the Adult Non challenged ponies suggests, that immune responses may interfere with the parasite's egg production.

3.4.3 Pasture Larval Recovery

Pasture L_3 fluctuated during the pasture phase of the experiment.

The counts of L_3 per kg of dry herbage were conducted during the pasture challenge period (Fig. 3.2). The likely explanation for the decrease in larval recoveries around day 37 is that freezing temperatures affected molting larvae. Cyathostome eggs have minimal development with temperatures below 10 ºC, and the most vulnerable stages to changing climatic conditions are the first and second stage larvae. Freezing temperatures most likely kill these stages, while morulated eggs and L_3 are more resistant. The combination of minimum temperature rise with 5 consecutive days of freezing (average: -1.7 ºC) likely induced development of the cyathostome eggs deposited on the pasture with a subsequent killing of the pre-infective larvae or the infective L_3. The low larval counts recorded at day –5 also correspond 8 days of freezing temperatures (average: -4 ºC).

The range of larvae each pony could have acquired while on pasture was calculated. The average weight of the 18 challenged ponies was 369 lbs or 168 kg. (median: 384 lbs or 175 kg.). It is estimated that a horse consumes approximately 2% of its live bode weight in dry matter per day (Cole, 1966). Therefore, these ponies would consume an average of 7.4 lbs or 3.4 kg of

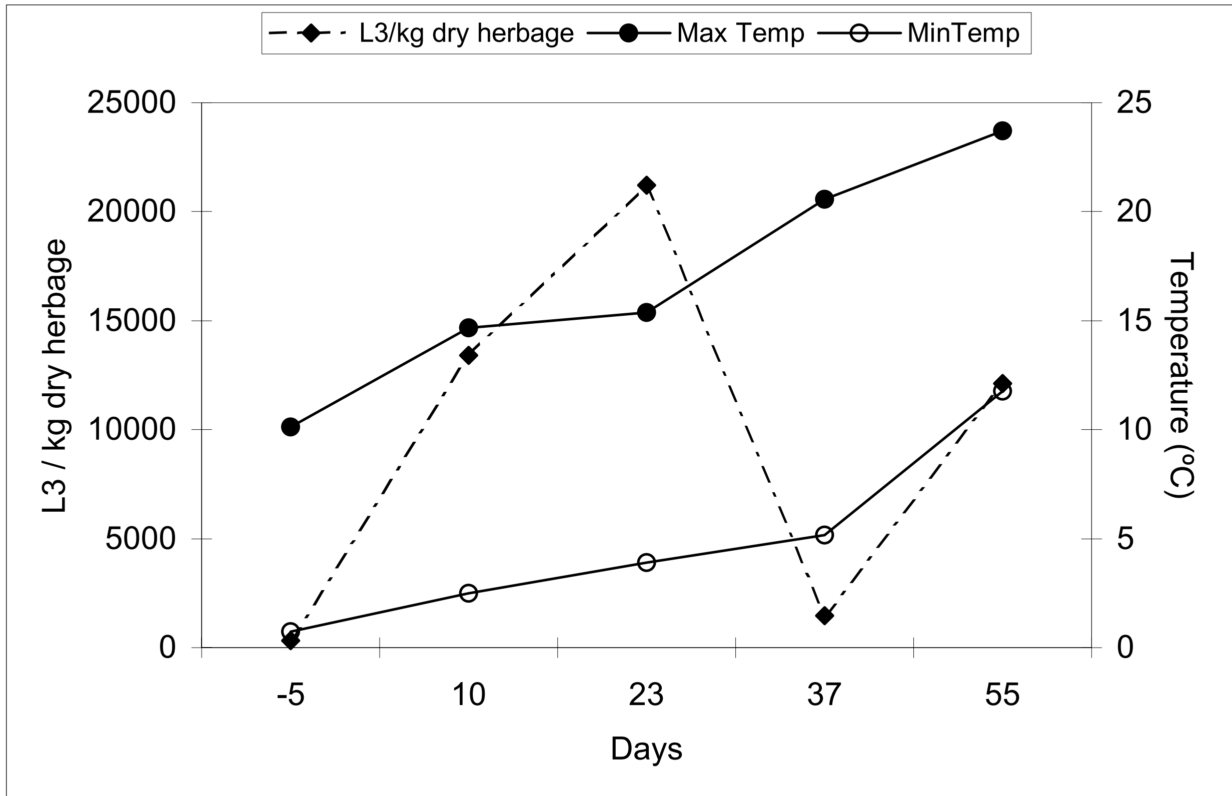


Fig. 3.2 Recoveries of L_3 from grass and maximum and minimum temperatures.
The dash line represents the recoveries of cyathostome L_3 per kg dry herbage. The solid lines represent the average temperatures in degrees Celsius, the closed circles are maximum temperatures, and the open circles are minimum temperatures.

herbage per day with a minimal larval consumption of 1,000 L_3 per day to a maximum of 72,000 of L_3 per day (average: 33,000 L_3 per day). Thus, the larval challenge ranged from 53,000 L_3 to 3,500,000 L_3 with an average of 1,600,000 L_3.

3.4.4 White Blood Cell Counts

Previously exposed ponies had higher peripheral blood eosinophilia than naïve ponies.

Eosinophils levels in Non-challenged animals were low following treatment. The PF Non had low eosinophil counts throughout the experimental period. The Young Ch and Adult Ch ponies showed higher eosinophilic responses than the PF Ch ponies (Fig. 3.3 top). The kinetics of the eosinophilic response was similar for both Young Ch and Adult Ch groups. Both groups

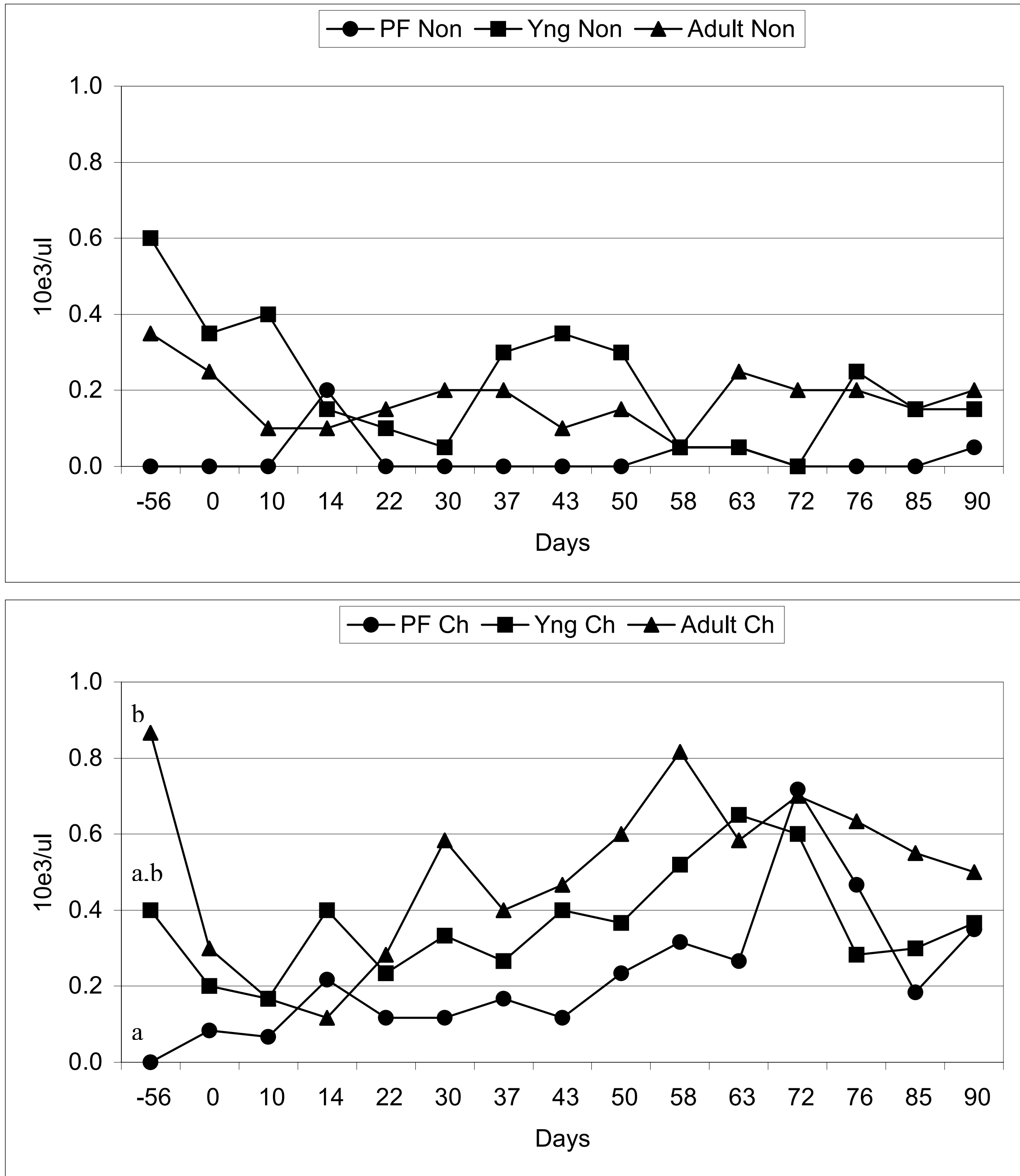


Fig. 3.3 Circulating eosinophil levels in the peripheral blood, presented as the average number of eosinophils counted per group. PF: parasite-free. Non: non-challenged (2 animals per group) (top chart). Ch: challenged (6 animals per group) (bottom chart). Different letters indicate significant differences between the Ch groups throughout the experimental period. P < 0.05.

showed anamnestic-like responses that started on day 10 for the Young and on day 14 for the Adult, with higher responses fort he latter group. The primary eosinophilic response seen in the PF Ch ponies peaks by day 72, two weeks after the Adult Ch peak and one week after the Young Ch peak. The statistical analysis of the eosinophil data showed that PF Ch ponies had significantly lower eosinophil counts than Adult Ch ponies. The Young Ch were not statistically different from the Adult Ch or the PF Ch, which indicated that this group had eosinophil levels higher than those of PF Ch and lower than those of Adult Ch ponies.

Young ponies had increased levels of circulating lymphocytes.

Levels of circulating lymphocytes were significantly higher throughout the experimental period in Young Ch ponies than in PF Ch and Adult Ch ponies (data not shown).

No differences were seen in any of the other parameters evaluated (WBC, white cell differential, hemoglobin, hematocrit, MCV, MCHC, PCV, plasma protein, and platelet counts).

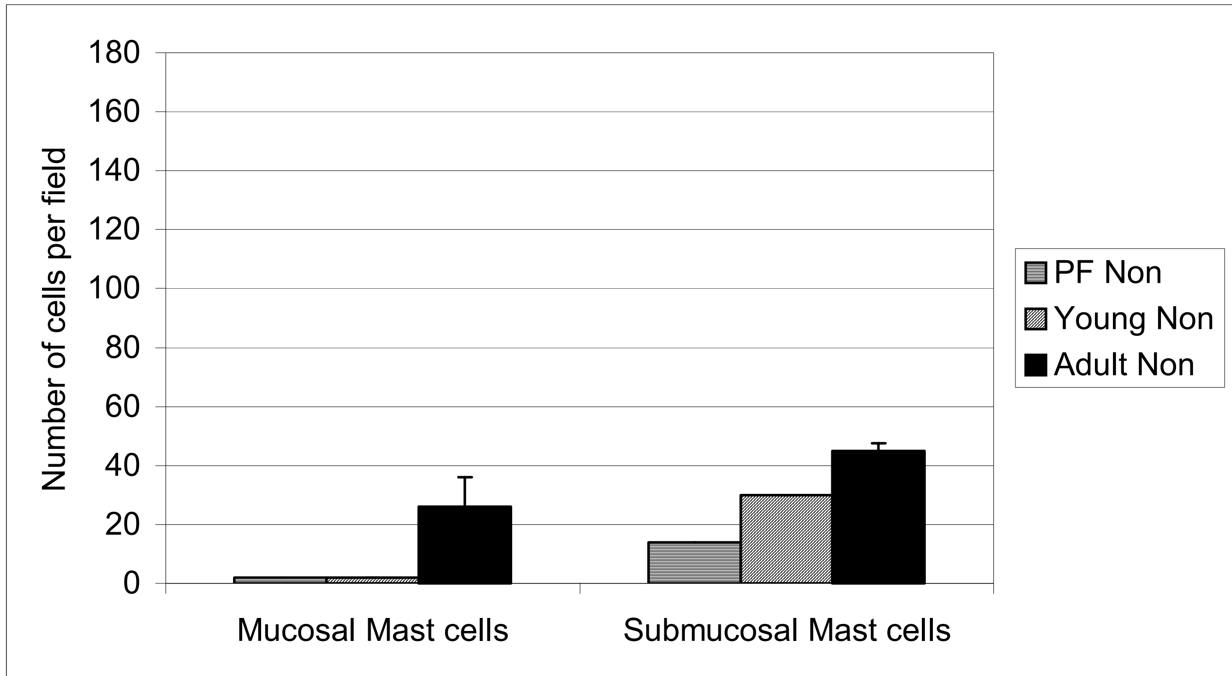
3.4.5 Intestinal Inflammatory Cell Responses

Previously exposed ponies had increased numbers of mucosal and submucosal mast cells.

Adult Ch and Young Ch ponies had significantly higher numbers of mucosal mast cells than the PF Ch (Fig. 3.4). Thus the ranking of groups by mucosal mast cell numbers corresponded to the level of pasture exposure to cyathostomes, with the Adults having the greatest numbers, followed by the Young and then the naïve. In all instances the Ch groups had greater numbers of mucosal mast cells than the Non-challenged groups.

Increased numbers of mucosal and submucosal eosinophils were found in challenged ponies.

Mucosal and submucosal eosinophil numbers in the Ch animals were markedly higher than in the Non-challenged animals in all exposure groups. Eosinophil counts were increased



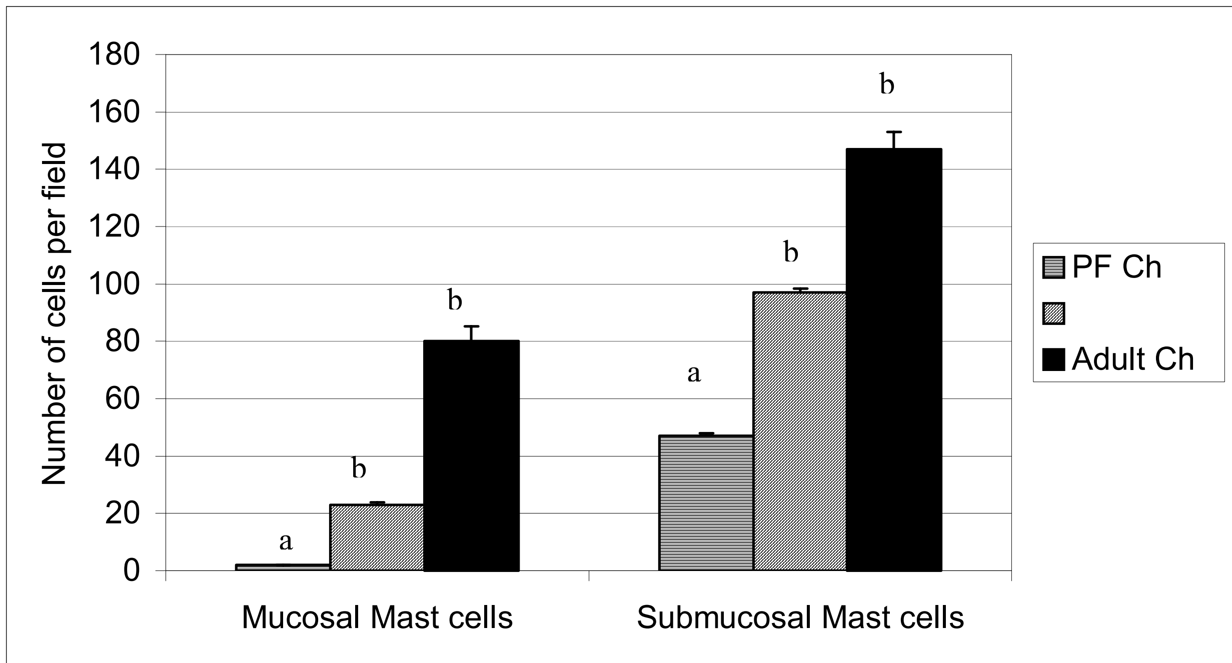
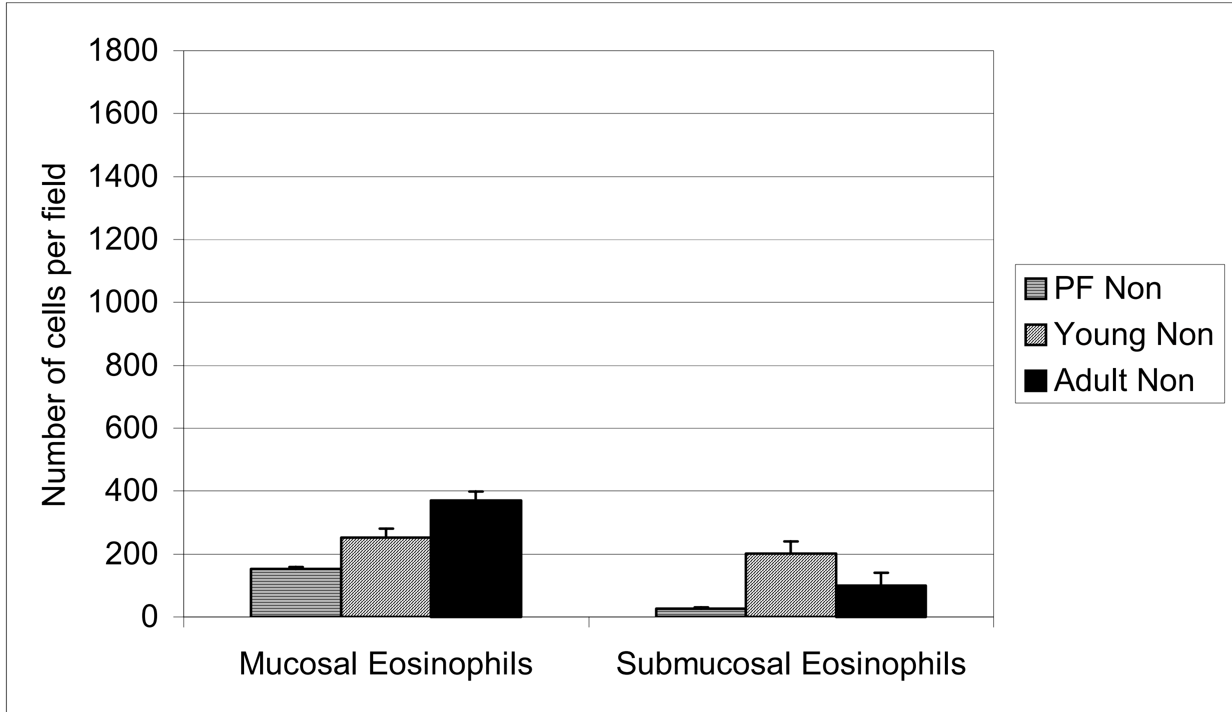


Fig. 3.4 Mucosal and submucosal mast cell counts from histological sections stained with Giemsa. PF: parasite-free. Non: non-challenged (two animals per group) (top chart). Ch: challenged (six animals per group) (bottom chart). The columns represent the sum of the averages of the number of cells counted per high power field obtained for the cecum, dorsal and ventral colons. The bars represent one standard error of the mean. Statistical analysis revealed differences per group. Different letters above the columns indicate significant differences. $P < 0.05$.



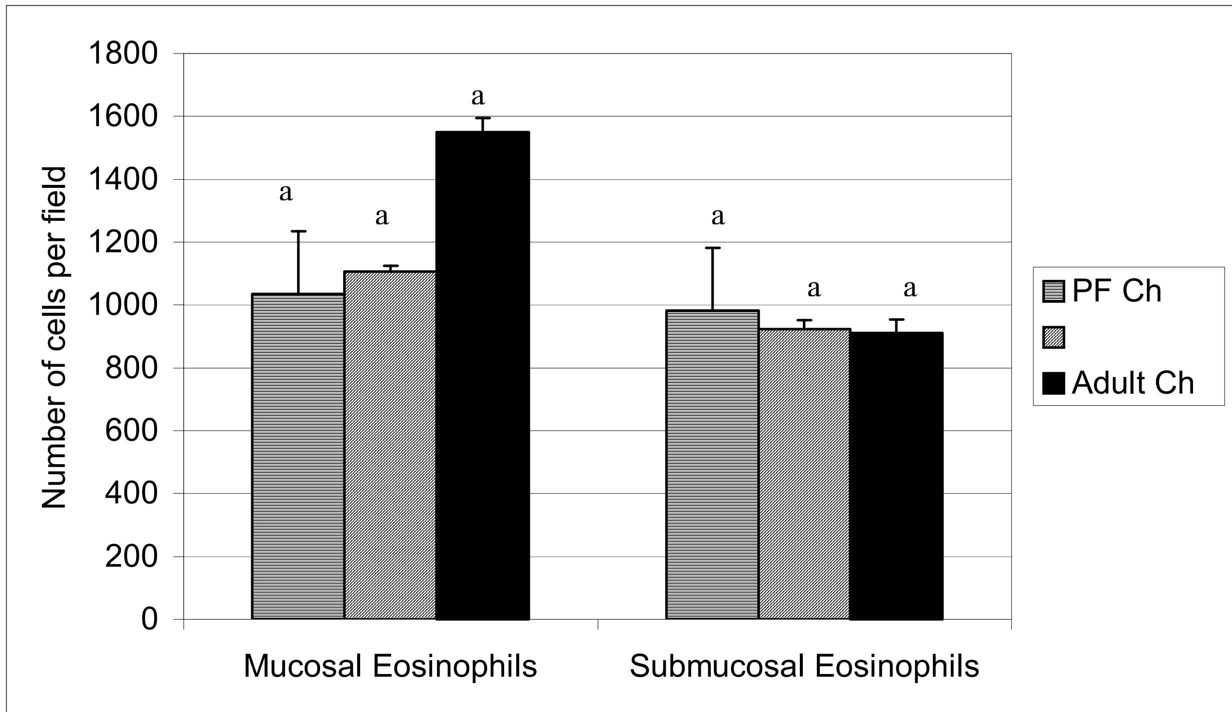


Fig. 3.5 Mucosal and submucosal eosinophil counts from histological sections, stained with Giemsa. The columns represent the sum of the averages of the number of cells counted per high power field obtained for the cecum, dorsal and ventral colons. The bars represent one standard error of the mean. Different letters above the columns indicate significant difference per group. P < 0.05.

in the mucosa of the Adult Ch group (Fig. 3.5), although statistical analysis of the data did not revealed any differences. In general, eosinophils were present in much larger numbers in all groups when compared to number of mast cells present in the same areas. These numbers corresponded to increases of EL_3 seen in all groups although they do not seem to correspond to the level of exposure. Larvae were not observed in the histological sections and thus the relationship between the eosinophils and the larvae were not noted.

3.4.6 Antibody Responses Measured by ELISA

PF ponies respond to challenge infection with an increased production of antibodies against somatic antigens.

In all instances the naïve animals had very low levels of IgG, IgG (T) and IgG (a) on days –56 and 0. PF Ch ponies had increased levels of IgG (Fig. 3.6), IgG (T) (Fig 3.7) and IgG (a) (Fig. 3.8) against adult somatic antigen after challenge. Significant increases were seen for IgG against somatic extracts of adult antigens at days 22 and 90.

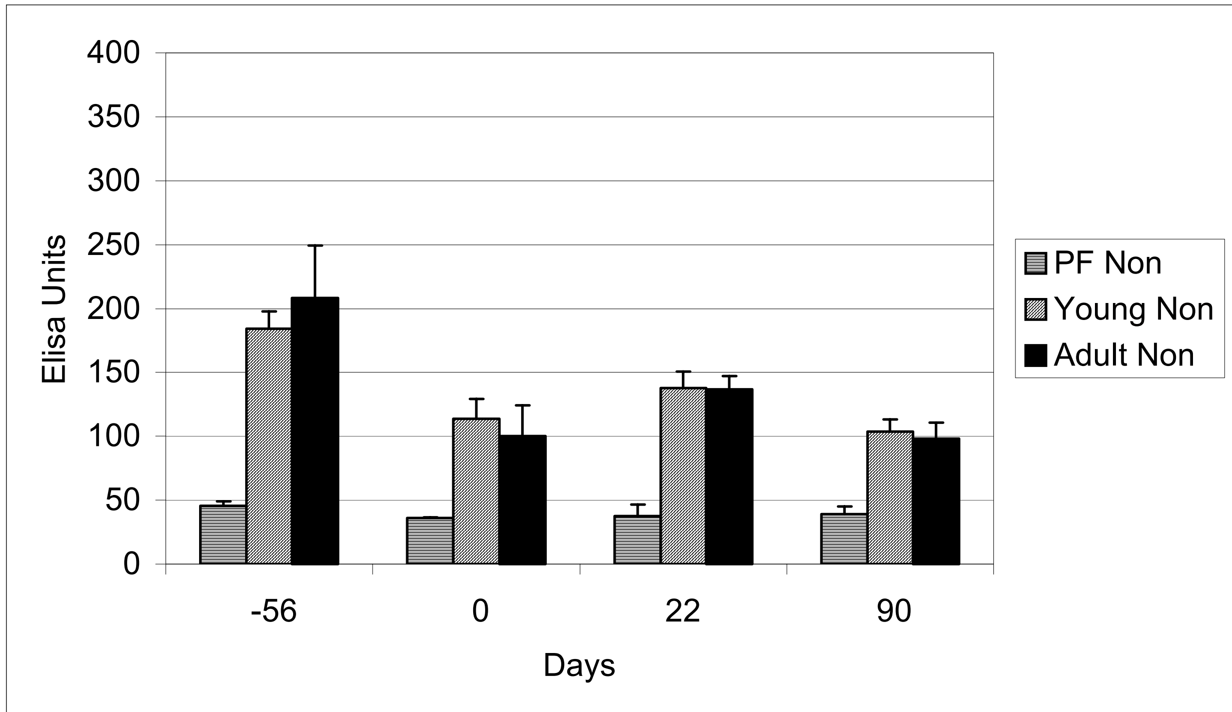
All isotypic antibodies against somatic adult antigens were elevated in Adult Ch ponies.

Even tough significant differences between groups were not found, the Adult ponies had higher levels of circulating IgG, IgG (T) and IgG (a) antibodies than Young or PF animals throughout the experiment. This observation suggests that a longer prior exposure to cyathostomes induced a quicker and higher antibody responses after challenge.

3.4.7 Lymphoproliferative Responses

The lymphoproliferative responses to C. insigne antigen increased throughout the experimental period.

Relatively high proliferative responses of the naïve animals on days –56 and 0, when animals had not been yet challenged, indicate a mitogenic activity of the *C. insigne* antigen (Fig. 3.9). The proliferative responses were suppressed on day 14. All Ch groups had increasing levels



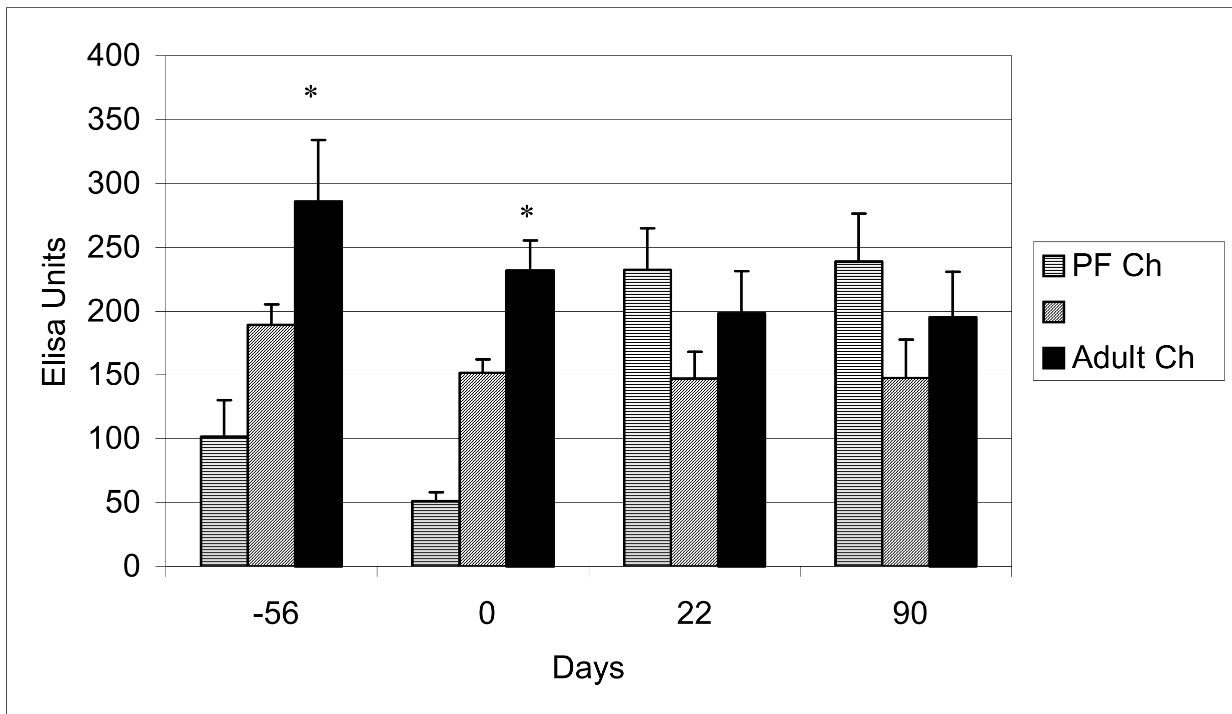
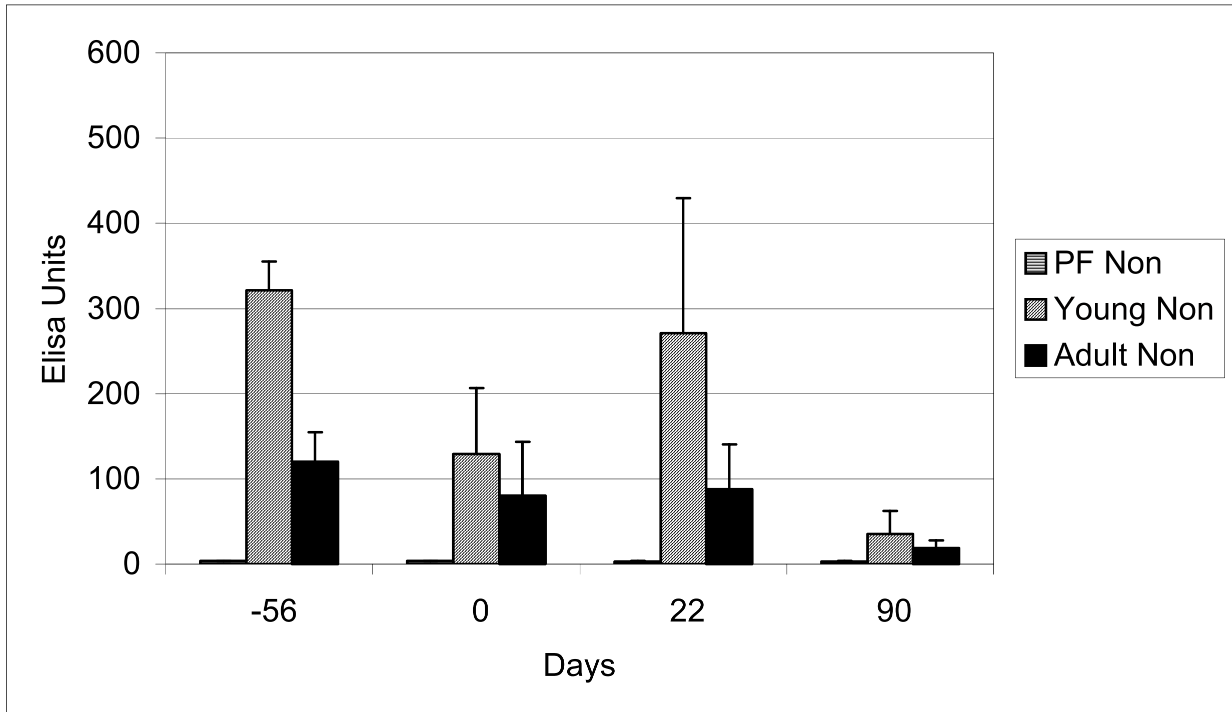


Fig 3.6 Levels of IgG antibodies against the somatic extracts of adult *C. insigne*.
PF: parasite-free. Non: non-challenged (two animals per group) (top chart). Ch: challenged (six animals per group) (bottom chart). * denotes significant increase of IgG antibodies in the Adult Ch group when compared to Young Ch and PF Ch animals on days –56 and 0. $P < 0.05$.



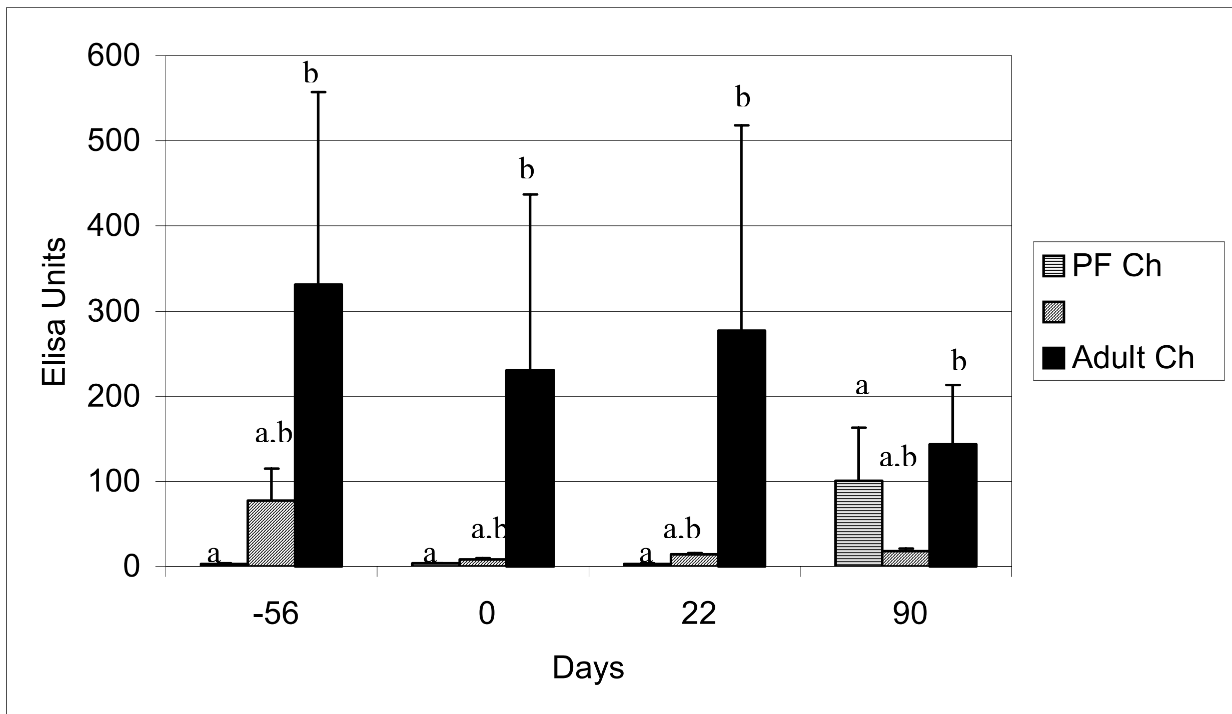
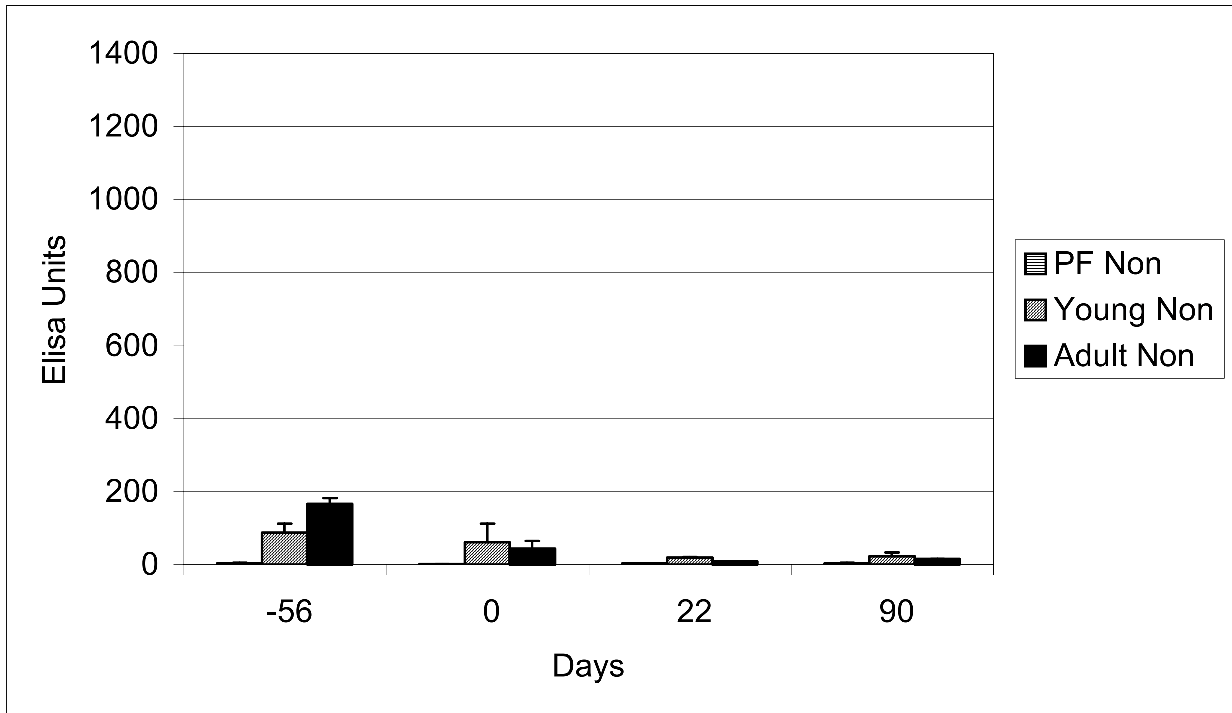


Fig. 3.7 Levels of IgG (T) antibodies against the somatic extracts of adult *C. insigne*. PF: parasite-free. Non: non-challenged (two animals per group) (top chart). Ch: challenged (six animals per group) (bottom chart). The analysis of the transformed data (log (x+1)) showed that the Adult Ch ponies had significantly higher levels of IgG (T) than the PF Ch (shown as different superscript letters), but not than the Young Ch ponies. $P < 0.05$



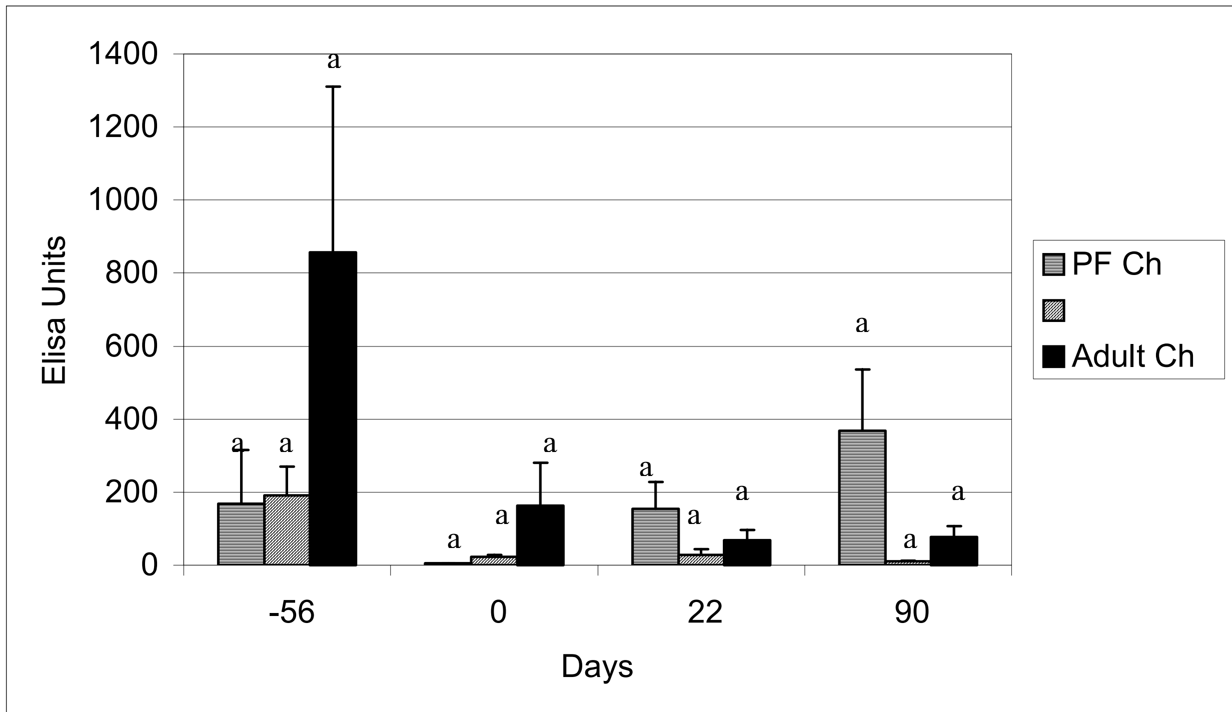


Fig. 3.8 Levels of IgG (a) antibodies against the somatic extracts of adult *C. insigne*. PF: parasite-free. Non: non-challenged (two animals per group) (top chart). Ch: challenged (six animals per group) (bottom chart). Same superscript letters indicate no significant differences of IgG (a) antibodies in the Ch groups. $P < 0.05$.

Increasing levels of proliferation from day 14 onwards. The stimulation of PBMC with PWM showed similar results to those of C. insigne (data not shown).

At necropsy, the circulating lymphocytes (PBMC) had a significantly higher proliferation rate than the lymphocytes obtained from the cecal lymph nodes (CLN) (Fig. 3.10).

3.4.8 Quantitation of Equine Interleukin mRNA

Levels of IFN-γ remained stable throughout the experimental period.

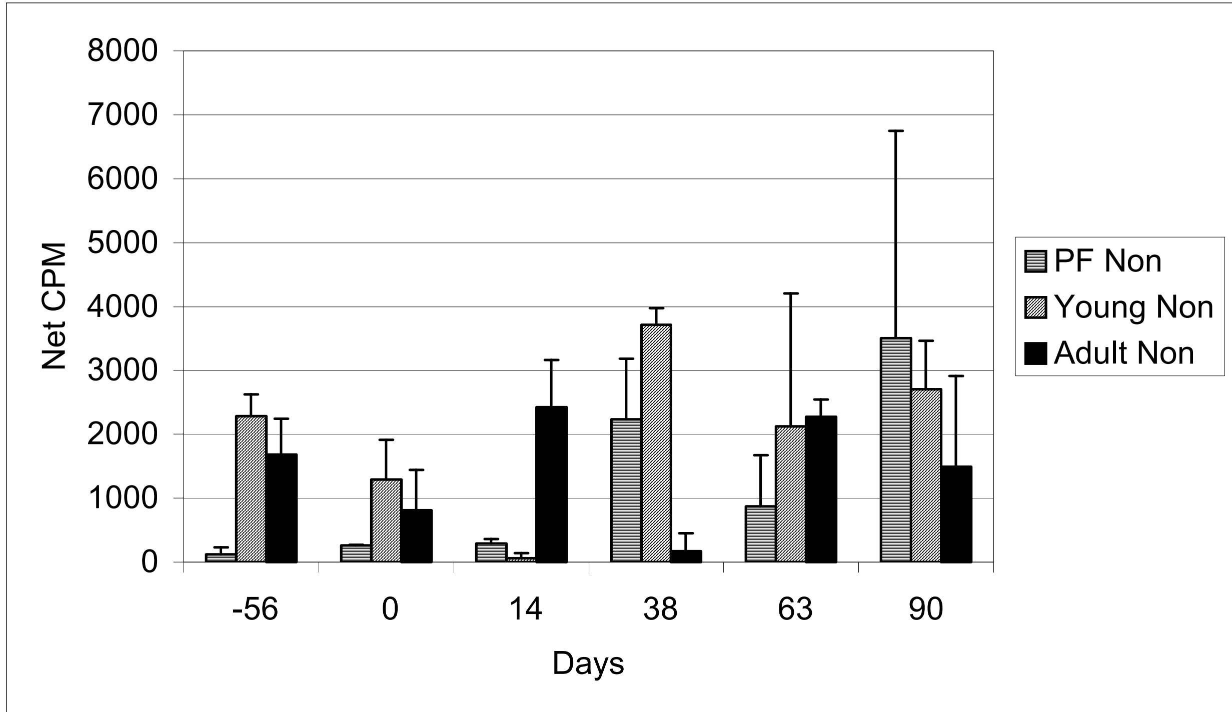
The statistical analysis of the data showed no differences after the initiation of the challenge (Days 0, 15 and 90) (Fig. 3.10). The data did not present trends of increase or decrease.

Adult Ch ponies had higher levels of circulating IL-4 mRNA than PF Ch ponies.

Levels of IL-4 mRNA were significantly higher in the Adult Ch ponies when compared to the PF Ch ponies, although no statistical differences were detected between Young Ch and Adult Ch ponies (Fig. 3.11). All groups had increased levels of IL-4 mRNA shortly after the initiation of the challenge (day 15) with these levels increasing until the end of the study. The Adult Ch ponies had the highest levels of IL-4 mRNA, followed in decreasing order by the Young Ch and the PF Ch ponies. This observation suggested that the levels of this interleukin were related to prior exposure to cyathostomes.

of proliferation from day 14 onwards, these increases were higher in the PF ch and adult Ch groups. The stimulation of PBMC with PWM showed results similar to those of *C. insigne* (data not shown).

At necropsy, the circulating lymphocytes (PBMC) had a significantly higher proliferation rate than the lymphocytes obtained from the cecal lymph nodes (CLN) (Fig. 3.10).



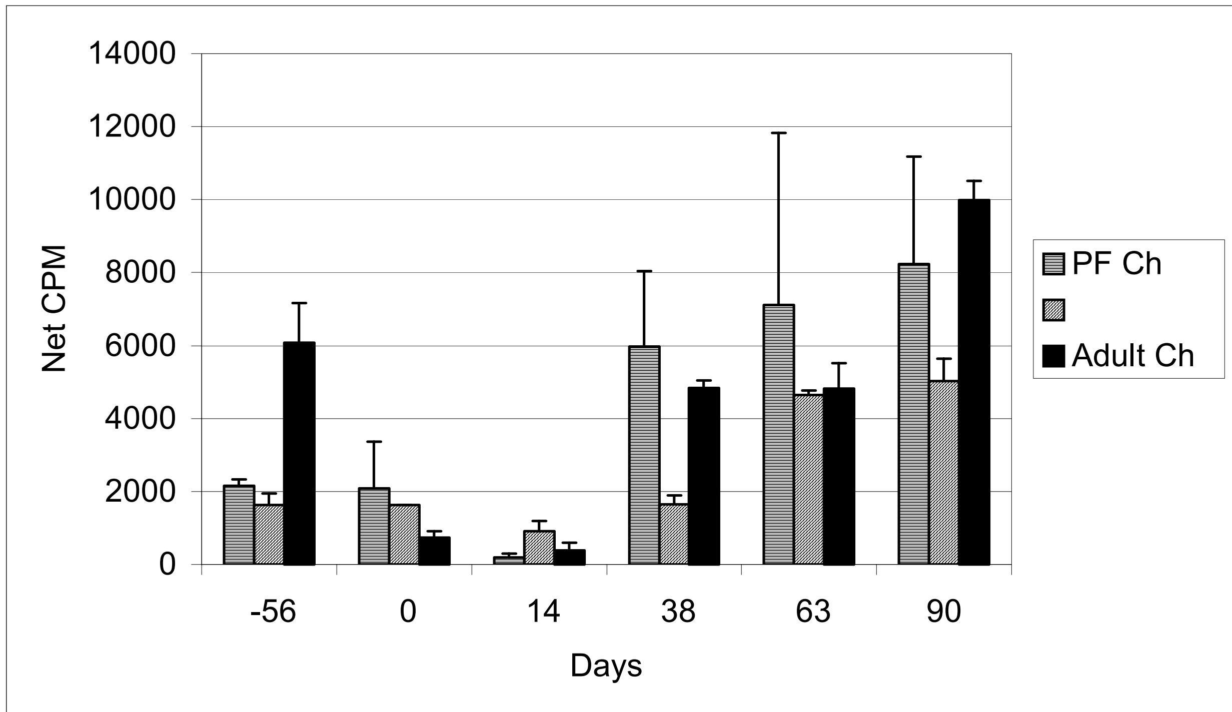
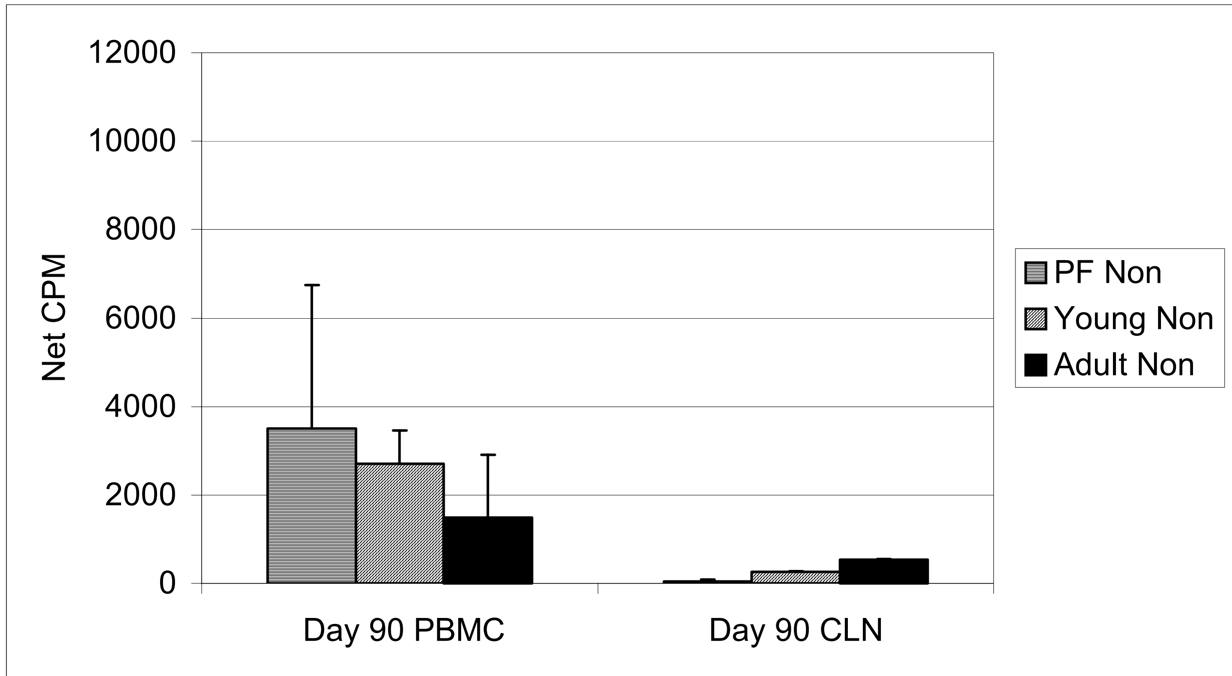


Fig. 3.9 Lymphoproliferative responses of circulating peripheral blood mononuclear cells (PBMC) cultured with 0.3µg/ml of *C. insigne* antigen. Results are shown in net counts per minute (CPM). PF: parasite-free. Non: non-challenged (two animals per group) (top chart). Ch: challenged (six animals per group) (bottom chart). No significant differences were detected with the statistical analysis of the data. $P < 0.05$.



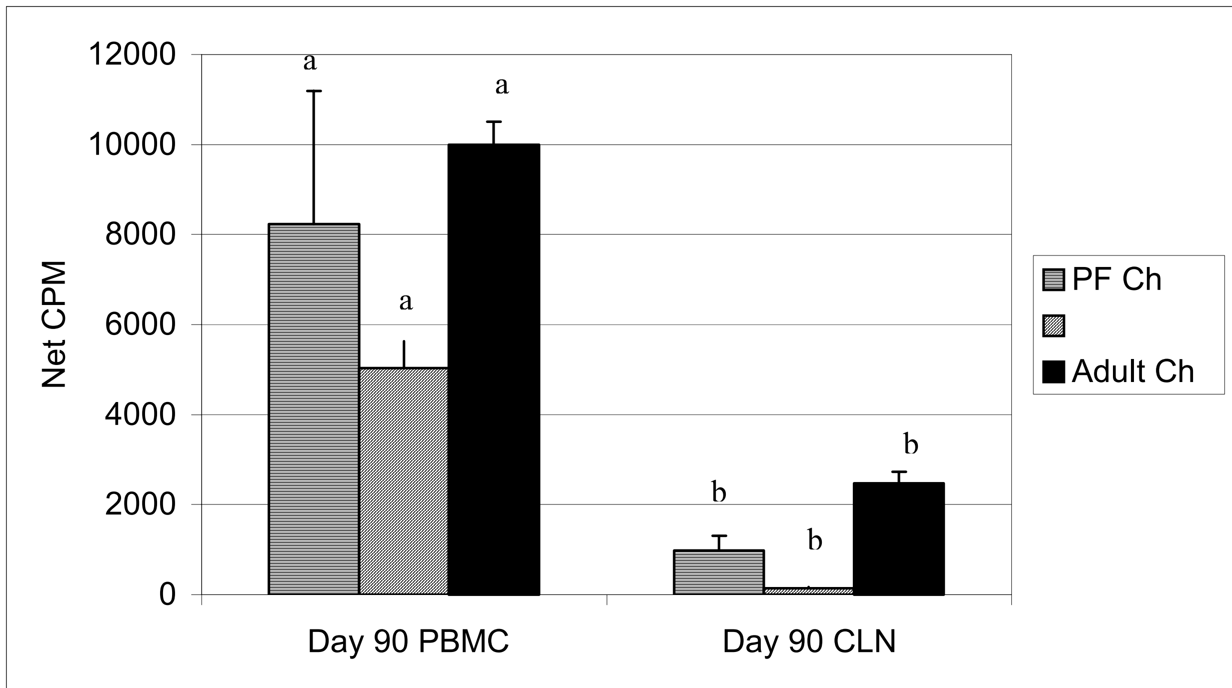
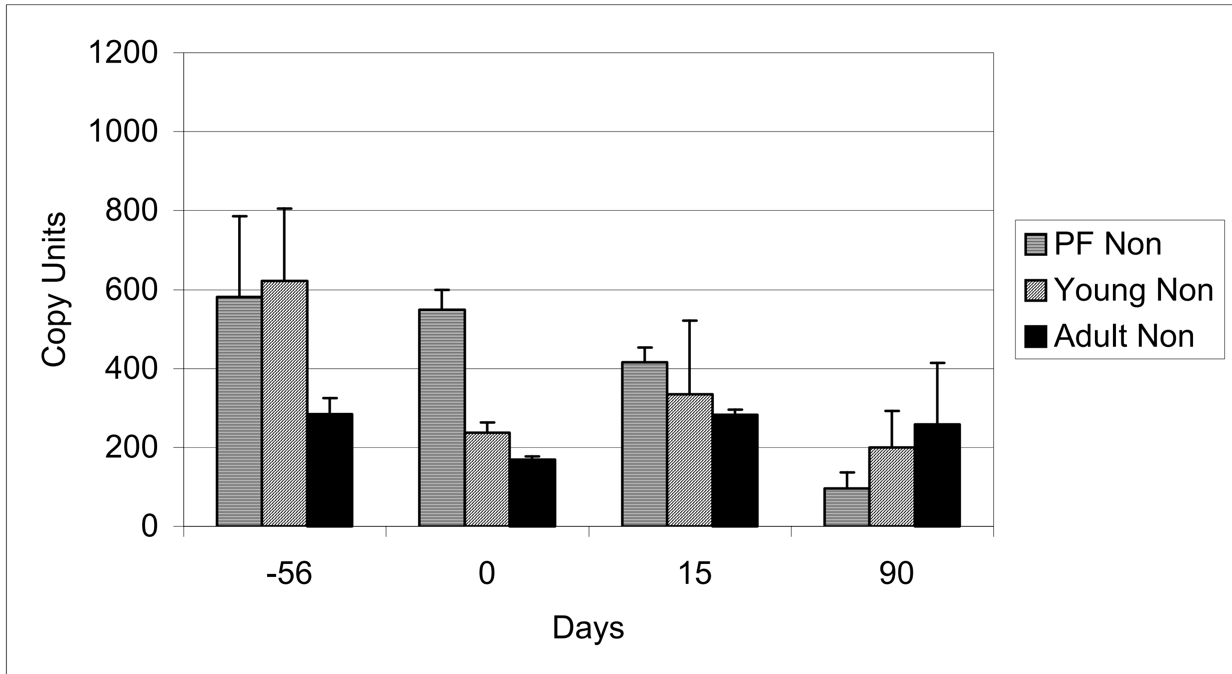


Fig. 3.10 Comparison of the lymphoproliferative responses of circulating peripheral blood mononuclear cells (PBMC) and cecal lymph node (CLN) lymphocytes, obtained on day 90, and cultured with 0.3µg/ml of *C. insigne* antigen. Results are shown in net counts per minute (CPM). PF: parasite-free. Non: non-challenged (two animals per group) (top chart). Ch: challenged (six animals per group) (bottom chart). Different letters above the columns indicate significant differences. $P < 0.05$.



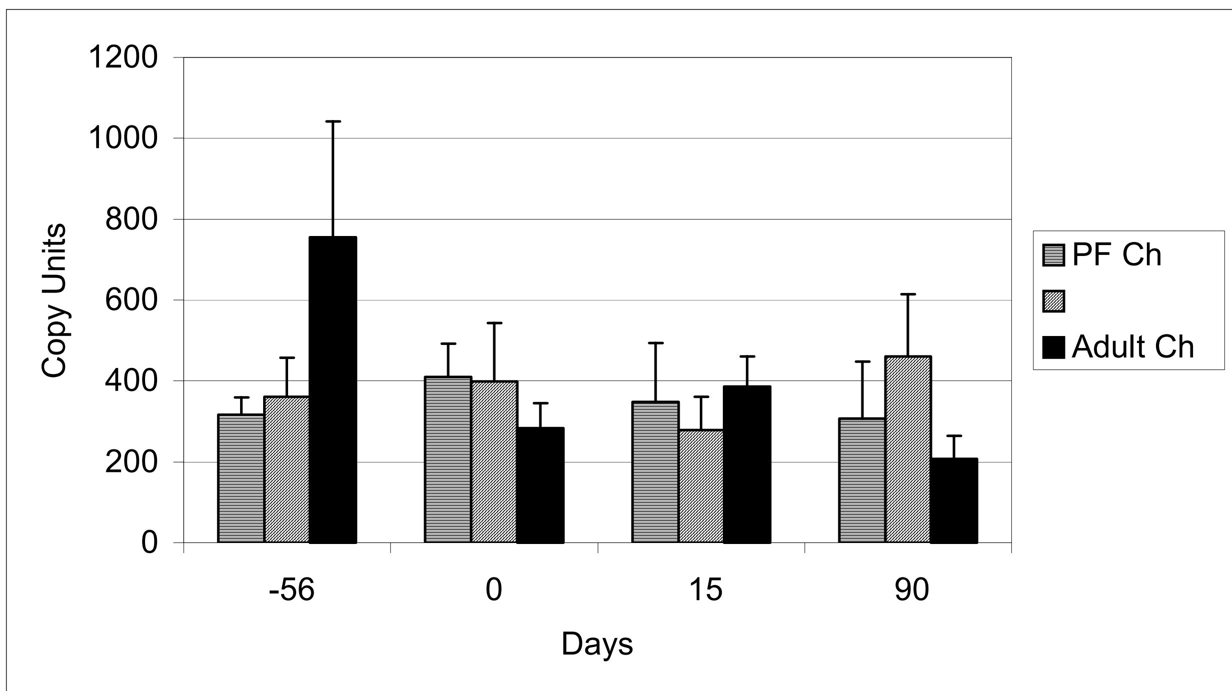
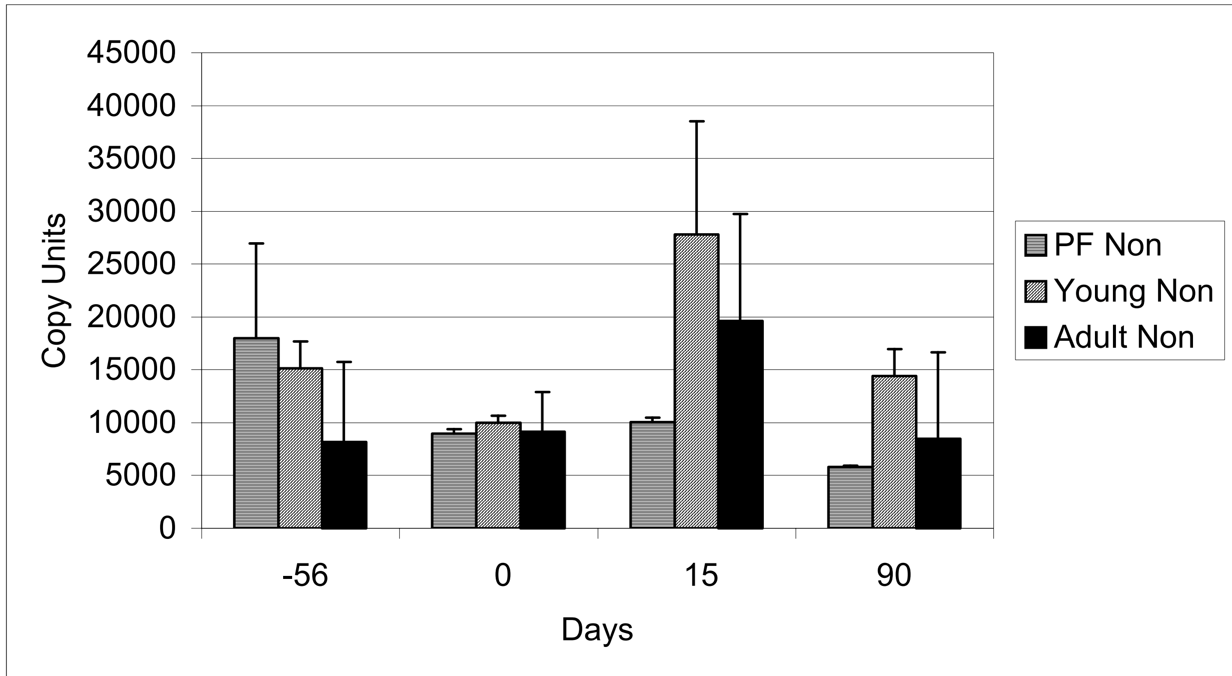


Fig. 3.10 Levels of IFN-γ mRNA from circulating from circulating peripheral blood mononuclear cells (PBMC) from days -56 to 90. Results are shown in Copy Units. The Y error bars represent the standard error of the mean. PF: parasite-free. Non: non-challenged (two animals per group) (top chart). Ch: challenged (six animals per group) (bottom chart). Statistical analysis showed no differences between the Ch groups. $P < 0.05$.



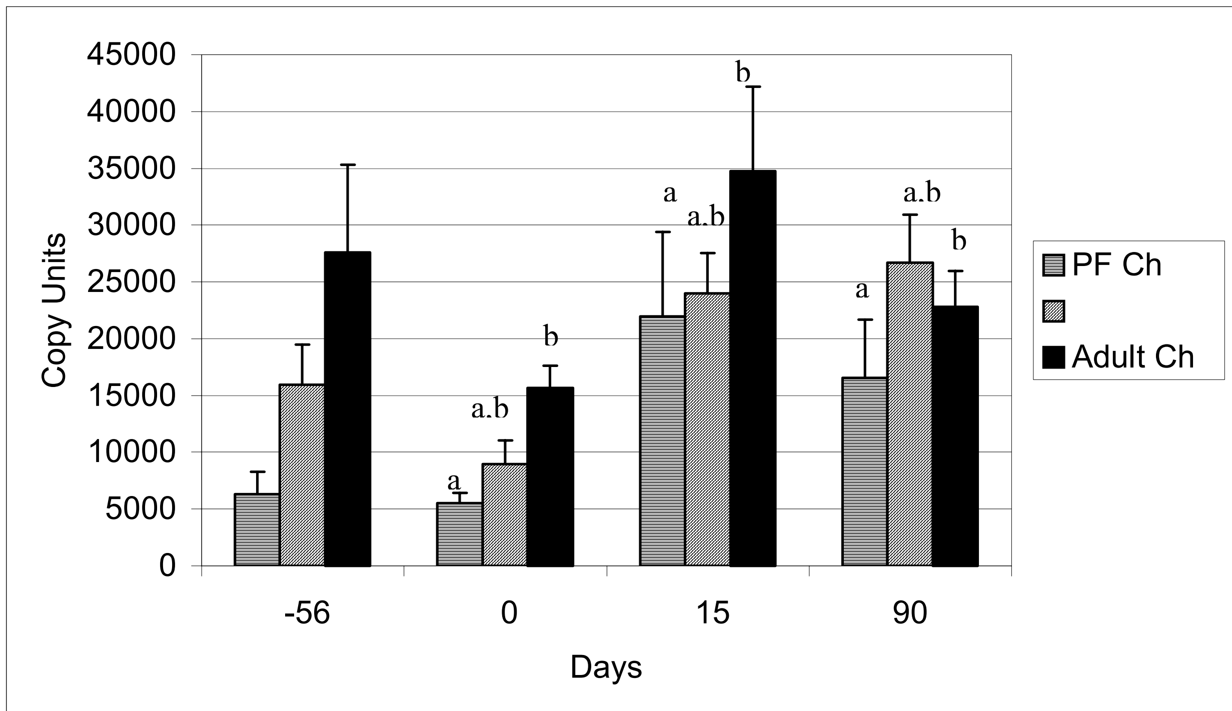
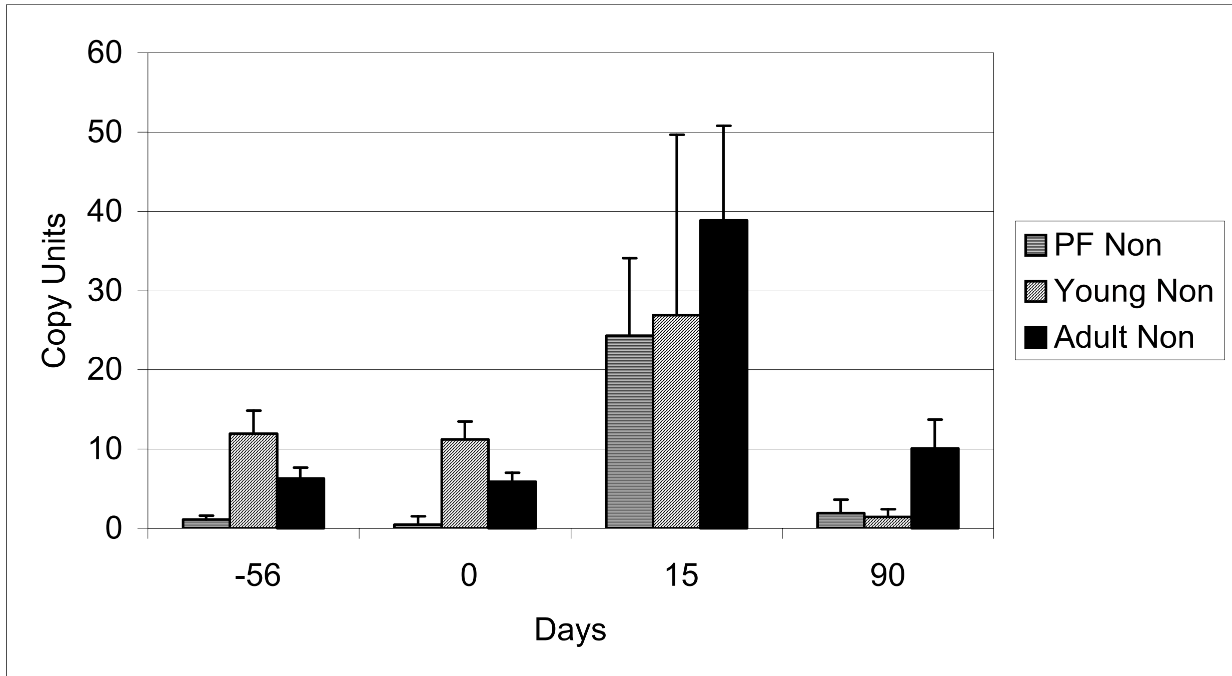


Fig.3.11 Levels of IL-4 mRNA from circulating from circulating peripheral blood mononuclear cells (PBMC) from days -56 to 90. Results are shown in Copy Units. The Y error bars represent the standard error of the mean. PF: parasite-free. Non: non-challenged (two animals per group) (top chart). Ch: challenged (six animals per group) (bottom chart). Different letters above the columns indicate significant differences. Only the data for days 0, 15 and 90 was analyzed for the Ch groups. $P < 0.05$.



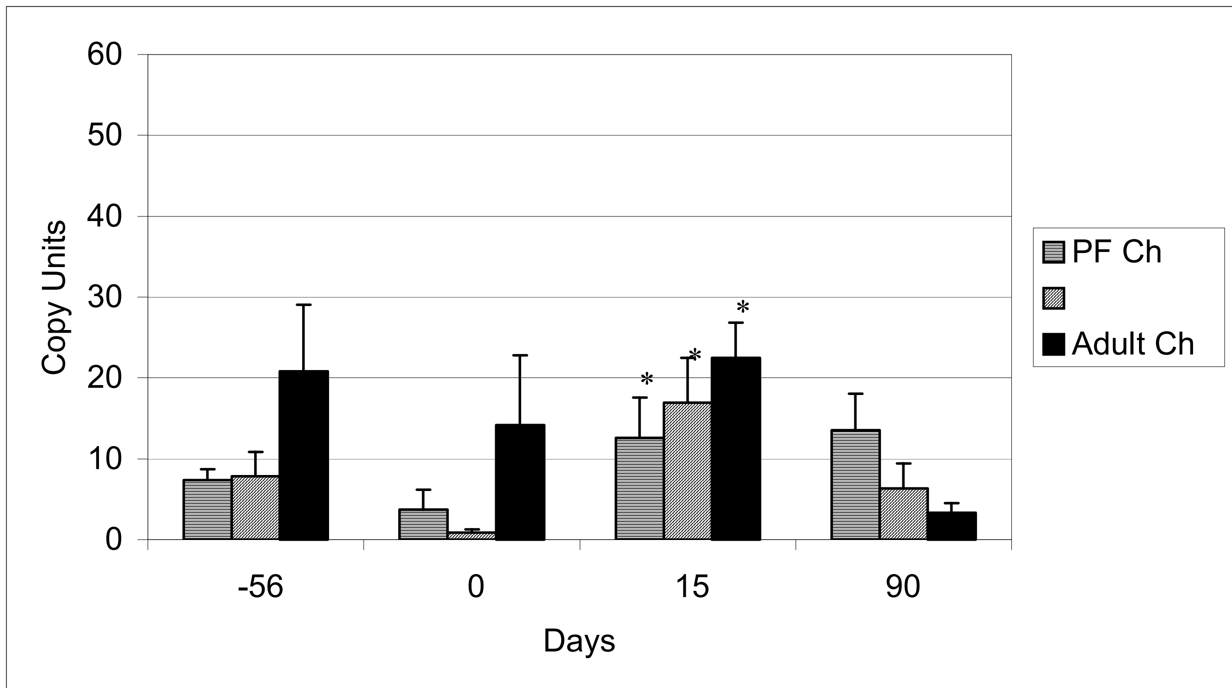
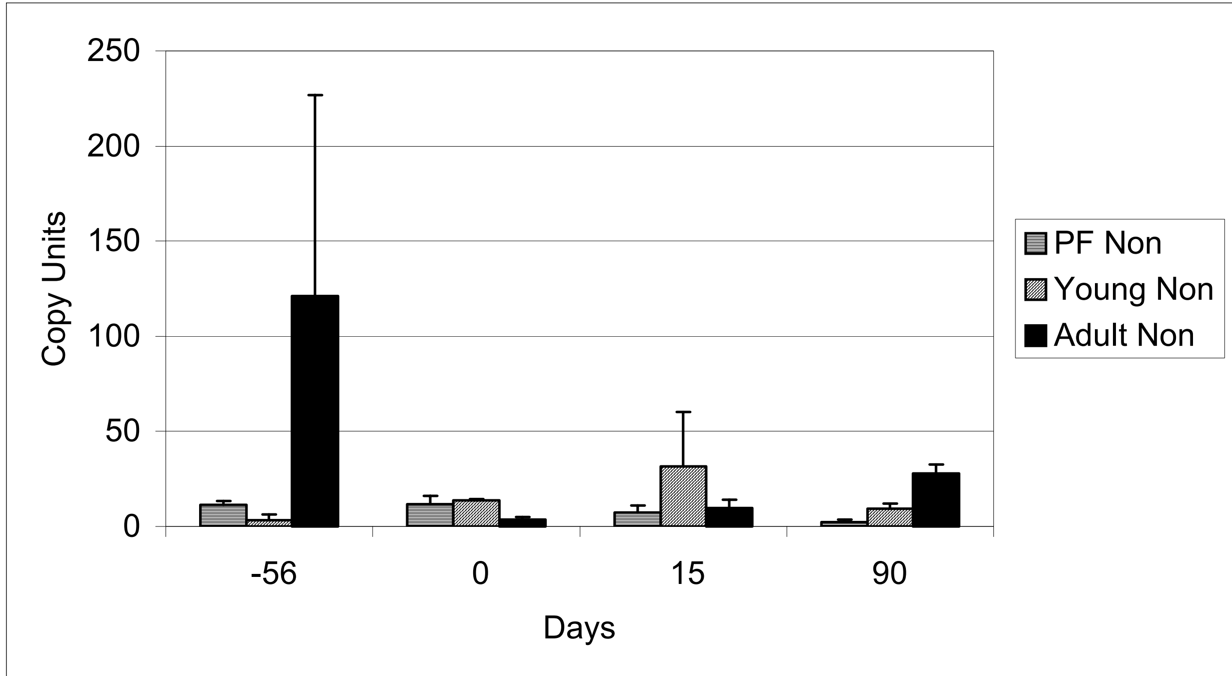


Fig.3.12 Levels of IL-13 mRNA from circulating peripheral blood mononuclear cells (PBMC) from days -56 to 90. Results are shown in Copy Units. The Y error bars represent the standard error of the mean. PF: parasite-free. Non: non-challenged (two animals per group) (top chart). Ch: challenged (six animals per group) (bottom chart). Only the data for days 0, 15 and 90 for the Ch groups was analyzed. The asterisks (*) indicate a significant increase of IL-13 mRNA on day 15 for the Ch groups. $P < 0.05$.



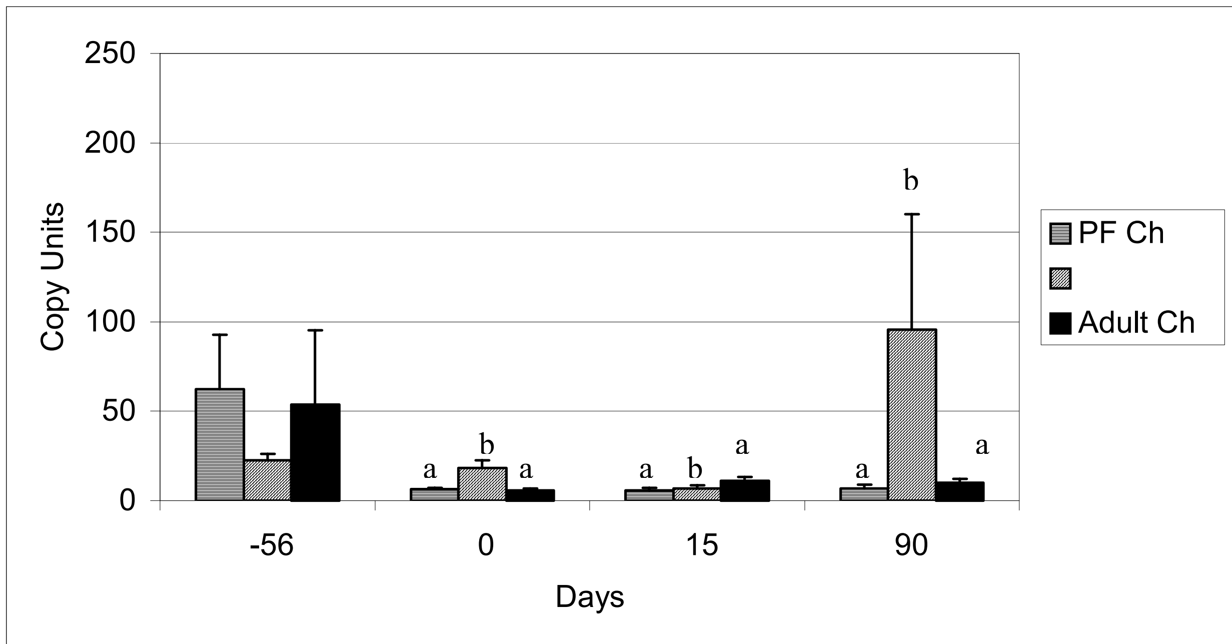


Fig.3.13 Levels of IL-5 mRNA from circulating from circulating peripheral blood mononuclear cells (PBMC) from days -56 to 90. Results are shown in Copy Units. The Y error bars represent the standard error of the mean. PF: parasite-free. Non: non-challenged (two animals per group) (top chart). Ch: challenged (six animals per group) (bottom chart). Different letters above the columns indicate significant differences. Only the data for days 0, 15 and 90 was analyzed for the Ch groups. The analysis of the transformed data (log (x+1)) showed that The Young Ch ponies had significantly higher levels of IL-5 mRNA than the PF Ch or Adult Ch groups. $P < 0.05$.

Significant increases of IL-13 mRNA were seen for all groups on day 15.

Increases in IL-13 were seen for all groups suggesting that challenge enhances the production of this cytokine (Fig. 3.12). However, the sudden rise of IL-13 mRNA in Non-challenged animals on day 15 cannot be explained.

The Young Ch ponies had significantly higher levels of IL-5 mRNA.

Results for IL-5 showed that PF Ch and Adult Ch ponies had significantly lower levels of IL-5 mRNA than Young Ch ponies after challenge (Fig. 3.13). The data points analyzed do not allow us to relate them to peripheral eosinophilia or with the levels of mucosal and submucosal eosinophils found in the intestinal tissues.

The data of each interleukin at day 90 was analyzed separately in order to detect differences between mRNA levels of circulating lymphocytes versus mRNA from cecal lymph nodes (data not shown). No statistical differences were found.

3.5 Discussion

The hypothesis behind this experiment was the same as that of Chapter 2. The mode of challenge was altered in order to compare the artificial experimental challenge used in Chapter 2 with a more natural acquisition of larvae, as in a pasture challenge. When the parasitological data was analyzed, slightly different results were obtained when compared to those discussed in Chapter 2. First, the exposure of animals to a pasture challenge increased the number of hypobiotic EL_3 irrespective of prior exposure. Second, Adult Ch and Young Ch ponies seem to show acquired resistance against cyathostome infections when compared to PF Ch animals. This resistance was seen in the decreased numbers of DL, luminal L_4 and adult worms recovered from the Young Ch and Adult Ch groups. Third, the differences in acquired resistance between Young Ch and Adult Ch animals is not as clear as it was with the experimental challenge. Results of the

experimental challenge study showed that only the Adult ponies acquired resistance. However, pasture challenge resulted in both groups have decreased numbers of developing and luminal parasite stages, suggesting the acquisition of resistance in both groups.

The higher number of hypobiotic larvae seen in the Young Ch ponies, compared to those in Adult Ch, is a phenomenon previously described in the literature (Love and Duncan, 1992; Monahan et al., 1998; Chapman et al., 2002, 2003). Although exposure of sentinel PF ponies to pasture challenges in cyathostome-contaminated pastures induced hypobiosis regardless of the season of the year (Chapman et al., 2001). The numbers of hypobiotic larvae were greater during the winter months (Chapman et al., 2001). Percentages of EL_3 obtained in a survey of naturally infected ponies were higher during the cooler months of the year in Louisiana (Chapman et al., 2003). Those results are consistent with the data obtained in this pasture challenge, where at least 80% of the acquired parasite burden was EL_3, regardless of their previous histories. If the PF Ch groups of the experimental (Chapter 2) and pasture challenges are compared it is clear that the pasture exposure induced a higher percentage of hypobiosis (55% in the experimental challenge versus 81% in the pasture challenge). The difference in acquisition of hypobiotic larvae could be due to the climatic conditions endured by the larvae while on pasture, or the size of the challenge, which was larger on pasture.

The constant acquisition of L_3 while on pasture also appeared to have effect on the egg production of the adult parasites. The comparison of the FEC of the challenged groups while on pasture indicated that parasite egg production was suppressed in the Young Ch and, especially, in the Adult Ch ponies during the first 7 weeks of the experiment, which corresponds to the pasture phase of the experiment. The intake of a large inoculum of infective L_3 could possibly elicit immune mechanisms of resistance that eliminate adult worms, therefore reduce FEC. Immune

mechanisms that eliminate adult nematodes associated with incoming L_3 have been demonstrated in sheep and calves infected with different gastrointestinal parasites (Balic et al., 2000). Expulsion of adult stages due to immune mechanisms induced by larval intake have been attributed to an immediate hypersensitive response of sheep against incoming larvae that can non-specifically affects the adult worms (Miller et al., 1985). Challenge of ponies with a mixture of strongyles has been similarly shown to reduce parasite egg production (Monahan et al., 1997). Decreases of cyathostome egg output in adult horses have been noted after challenge by Love and Duncan (1992), Klei (2000), and in Chapter 2. Reduction in fecundity of adult worms has been reported for several gastrointestinal nematodes of ruminants as a result of immune responses directed against the adult stages (Balic et al., 2000). The egg production of adult Ch ponies in the present experiment remained low after they were removed from the pasture, whereas the FEC of Young Ch and PF Ch ponies increased. This observation suggests immune responses that affect cyathostome egg production in horses could be similar to those reported for ruminants (Balic et al., 2000). The difference of FEC in Young Non Ch and Adult Non Ch ponies further suggest that Adult ponies have developed mechanisms of acquired resistance that affects egg production in a more effective manner than in Young animals. All of these observations support the contention that equids do acquire resistance to cyathostome infections, and that there are different immune mechanisms involved with the reduction in numbers of each parasitic stage.

Both Adult Ch and Young Ch groups had marked reductions of DL, luminal L_4 and adult parasites when compared to the naïve animals in the pasture challenge, this observation differs from that seen following experimental challenge (Chapter 2). A larger inoculum, such as the one ingested while on pasture, could have elicited responses that eliminated developing and luminal

stages in the Young Ch ponies. It is also possible that at least 2 grazing seasons are needed to produce these types of protective responses. The Young ponies used in this experiment were all 2 years of age and the Adult ponies were older than 7 years of age, therefore the Young had a minimum of two years of previous exposure to cyathostomes since they were kept on pasture at all times. However, this is the first report of acquired resistance to developing larvae and luminal stages in young animals, and the relationship between short exposure to cyathostomes and acquired resistance warrants further investigation.

The total number of cyathostome species recovered in all challenged animals was 17. The PF Ch ponies had 14 species and higher prevalence of this species than Young Ch, that 11 species present, or Adult Ch animals, that had 10. The decreased number of species found and the lower prevalence seen in the Adult Ch ponies is similar to previous reports (Foster, 1936; Chapman et al., 2001; Chapman et al., 2003; Chapter 2), suggesting that acquired resistance eliminates the less common species in this group of animals.

An unexpected observation is that PF Ch and Adult Ch ponies acquired a similar number of parasites, whereas Young Ch ponies had much larger number of worms. It is possible that the differences seen could be due to differences in grazing behavior. The cyathostome-exposed ponies used for this study had been kept on pasture prior to the initiation of the experiment. The naïve animals spent all of their lives in box stalls, since mother and offspring were brought indoors within the first 24 hours of birth (Monahan et al., 1997). After weaning the PF ponies were kept in stalls in pairs in order to maintain their PF status. When released on pasture they had not developed the grazing behavior seen in Adult or Young ponies, and it is likely that they ingested less grass. Therefore they may not have acquired the same number of L_3 as the other groups. Another confounding factor was that the majority of the naïve animals were intact males

between 12 and 18 months of age, an age at which they start to test their reproductive capabilities. These factors in conjunction with the lack of herd behavior (dominance ranking awareness) seen in the PF ponies could all have been key components of the lower larval intake seen in this group. It needs to be noted that no specific attempts were made to record the behavior of the ponies while on pasture. However, fights among the PF stallions, segregation of the different age groups, and establishment of dominance by adult and young ponies over the naïve animals resulted in the latter group being in the periphery of the dominant grazing herd, were observed. All of these observations suggest that the similarities in total worm counts observed between the PF Ch and the previously exposed ponies (Young and Adult) were maybe due to a reduced challenge of the PF group. Supporting this contention is that immune responses of the PF and the Adult groups are different. Levels of antibodies against somatic extracts of cyathostomes, peripheral eosinophil levels, intestinal tissue eosinophils and mast cells, and IL-4 were elevated in the Adult Ch ponies when compared to the PF Ch, suggesting the presence of acquire mechanisms of resistance in the Adult animals.

The mast cell counts in ponies following experimental or pasture challenges are strikingly similar. In both cases the animals with larger numbers of mast cells are the Adult Ch ponies, whereas the Non Ch had low numbers, suggesting that this cell type is related to acquired resistance in ponies with longer exposure to cyathostomes. The increasing numbers of mast cells found in the tissues of the PF Ch, Young Ch and Adult Ch ponies related to the histories of pasture exposure of these groups, suggests that infections with cyathostomes may be responsible for their increased presence in ponies with longer prior exposure to these parasites. This finding confirms the correlations of mast cells increases and total adult worm populations decreases found in naturally infected horses older than 10 years of age (Collobert-Laugier et al., 2002).

Therefore, it is likely that increased mast cell counts are associated with lower numbers of DL, luminal L_4 and adult worms and the higher levels of IL-4 mRNA in Adult Ch ponies. Furthermore, the increased numbers of mast cells in ponies could be compared to the mechanism of "immune exclusion" described for gastrointestinal infections in resistant sheep (Miller et al., 1985).

The acquisition of resistance in the Adult Ch ponies in this experiment has similarities to data found in an ovine experimental model of *Haemonchus contortus* infection (Balic et al., 2002). That study compared young sheep (10 months old) to wethers (2-3 years old) challenged in an equal manner. No larvae were found in the intestinal contents of the challenged young sheep. High eosinophil numbers were seen in intimate association with *H. contortus* L_4 in the abomasal mucosa of the wethers. The authors suggested that young sheep presented "immune exclusion" or "rapid expulsion", a phenomenon previously described in hyperimmunized sheep (Miller et al., 1985). Whereas wethers had a delayed rejection of the challenge. Similarities could be drawn, to a certain extent, with the Adult ponies used in this study. It is possible that the reduced cyathostome L_3 establishment recorded for the Adult Ch ponies could be due to a "immune exclusion" type response, that would reduce the invasion of the intestinal mucosa by incoming L_3. The direct consequence of this response would be the "rapid expulsion" of cyathostome L_3, however, expulsion of L_3 was not measured in the present experiment limiting our interpretation of this suggestion. On the other hand, Adult Ch ponies could have a "delayed rejection of the infection" type response where mast cells would be involved in the elimination of the infection, as it has been suggested for gastrointestinal infections of ruminants (Balic et al., 2000). The increased levels of IL-4 mRNA present in the Adult ponies would support this suggestion.

Eosinophils, another cell type associated with immune responses to nematode infections, were significantly elevated in challenged ponies. No clear relationships were established, however, between the numbers of eosinophils in the tissues and the resistance status of the host or larval burdens seen with increased numbers of intestinal eosinophils. Adult Ch ponies seemed to have higher intestinal eosinophilia, and in this case it is different from published reports (Collobert-Laugier et al., 2002) and the data collected in the experimental challenge (Chapter 2). The low levels of IL-5 mRNA obtained from these naturally challenged ponies do seem to relate to circulating eosinophil levels measured throughout the experiment. It is possible that the relationship was not present because far fewer measurements of IL-5 mRNA were done compared to eosinophil counts. Eosinophilia was present in all Ch groups, as an anamnestic response in previously exposed animals, and as a primary response in the PF group. The eosinophil levels seemed to correspond to the different levels of exposure present in the three groups with the highest eosinophilia corresponding to ponies with longer previous exposure to cyathostomes, although statistical differences were not seen.

Levels of IgG, IgG (T) and IgG (a) antibodies against somatic antigens of adult *C. insigne* were overall higher in Adult Ch ponies. These results were similar to those obtained in the experimental challenge (Chapter 2). PF Ch ponies did not have increases of IgG (T) and IgG (a) until day 90. The slower antibody responses seen in this group of animals could be due to the decreased intake of larvae when compared to the Young and Adult. These results are similar to results obtained with pony sera of the experimental challenge described by Monahan et al. (1998) (unpublished data). Although the specific role of any of the immunoglobulins in acquisition of resistance to cyathostome infections needs to be investigated.

The increases of antibodies, mast cells and eosinophils in ponies infected with cyathostomes presented in this report are likely related to increases of Th2 type cytokines as described for gastrointestinal parasite infection models in rodents (Finkelman et al., 1997; Else and Finkelman, 1998; Bancroft and Grencis, 1998) and ruminants (Miller, 1983; Miller et al., 1985; Meeusen and Balic, 2000). As previously described in Chapter 2, the principal cytokine responsible for the Th2 type response to gastrointestinal nematode infections is IL-4 (Urban et al., 1996; Finkelman et al., 1997; Else and Finkelman, 1998; Bancroft and Grencis, 1998; Garside et al., 2000). The present is the first report on cytokine levels of ponies naturally infected with cyathostomes. Although an increase in IL-4 was registered in a pony undergoing cyathostome larval expulsion when compared to a control animal, the nature of this data was anecdotal (Horohov et al., 1997). Even though the results obtained are not as clear as those obtained in the experimental challenge, this pasture challenge elicited increases of IL-4 in all challenged groups, especially Adult ponies. The increases of mast cells in the latter group are likely related to the increases of IL-4 mRNA reported. The IL-13 data was extremely different from the results reported in Chapter 2. The analysis of the IL-5 mRNA data lack the relationship between eosinophilia and IL-5 seen in the experimental challenge (Chapter 2). No relations could be established with circulating eosinophil levels measured throughout the pasture challenge experiment, and with eosinophil counts in intestinal tissues at necropsy. Nevertheless, the high eosinophils counts from all challenged animals suggest that this cell type may have a role in the induction of hypobiosis of the incoming cyathostome L_3 or in the delay of development of this stage. The analysis of all the cytokine data obtained in this pasture challenge suggests that natural infections of cyathostomes are similar to natural infections of *Ostertagia ostertagi* in

grazing cattle (Claerebout and Vercruysse, 2000; Gasbarre, 1997) regarding the production of a Th2 type cytokine profile.

The parameters measured in naturally challenged Adult ponies were similar to those described for the experimentally challenged Adult ponies (Chapter 2). In both cases the Adult Ch ponies had increases of peripheral eosinophils, intestinal mast cells and eosinophils, IL-4, and EL_3 counts. These findings in conjunction with decreases of total number of worms, of DL and luminal L_4, adult parasites and of cyathostome species suggest that the immune mechanisms of resistance developed in Adult ponies confer protection against cyathostome infections. The same observations, related to the histories of prior exposure to these parasites in cyathostome contaminated pastures, also suggest that the acquisition of resistance is slow to develop, and it is targeted against each parasite stage present in the ponies. Interestingly, decreases of developing and luminal cyathostomes were also noted in the Young Ch ponies. This was thought to be a characteristic of animals with much longer exposures to cyathostomes. The pasture challenge introduced more variability to the system, the number of infective L_3 ingested by the challenged ponies was greater, there was no control on the acquisition of this stage by the ponies, and the L_3 were exposed to environmental conditions that could have alter their phenotypic behavior. Nevertheless, ponies with longer prior exposure to cyathostomes, in particular Adult, had results similar to those discussed in Chapter 2. Therefore, comparisons of the data obtained for both challenges confirm the feasibility of using an experimental challenge to study the acquisition of protective responses under more rigorous conditions in cyathostome infections.

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Chapter 4: Summary

The hypothesis developed and tested in these experiments was that ponies would acquire resistance to cyathostomes with increasing exposure to cyathostome contaminated pastures. Thus, parasite-free (PF) animals would acquire the largest number of worms, followed in decreasing order by Young and then Adult ponies. The assumption behind this hypothesis was that helminth-naïve ponies infected with cyathostomes would eliminate the infection using only primary immune responses. Whereas previously exposed ponies would eliminate the infection with secondary or acquired immune responses, and these would be more effective in ponies with longer exposure to cyathostomes. The differences between the experiments described in Chapters 2 and 3 reside in the challenge. The experimental challenge consisted of the administration of a total of 150,000 cyathostome L_3 to each challenged pony over a period of 5 days. The pasture challenge consisted of 7 weeks of grazing a cyathostome contaminated pasture. In all other regards both experiments were carried out similarly.

The parasitological data recovered showed that ponies previously exposed to cyathostomes, both the Young and Adult groups, had higher numbers of EL_3, than naïve animals, when given 150,000 L_3. Whereas the exposure of ponies to a pasture challenge increased the number of hypobiotic EL_3 irrespective of prior exposure. This difference in the number of hypobiotic larvae, expressed as total counts by digestion or in percentage of the total number of worms acquired, suggests that continuous intake of infective L_3, as in a natural exposure, increases hypobiosis. The confirmation of this suggestion is the differences of EL_3 percentages between the PF Ch groups of the experimental (55%, Chapter 2) and pastures challenges (81%, Chapter 3). The data obtained in the experimental challenge suggests that induction of hypobiosis is immunologically regulated, since it is present in previously exposed ponies. On the

other hand, data from the pasture challenge would indicate that EL_3 induction develops with short grazing exposures to cyathostome contaminated pastures. It is possible that the induction of hypobiosis is am immune mediated mechanism and that immune responses against with EL_3 interfere with their development. Contrarily, the accumulation of massive numbers of hypobiotic larvae could have deleterious effects if they develop in unison as in cases of larval cyathostomiasis.

Another noticeable difference between challenges was the development of acquired resistance to developing and luminal stages of the cyathostome life cycle. In Chapter 2, a lower number of DL, luminal L_4 and adult parasites were seen in Adult Ch ponies when compared to the Young Ch or the PF Ch. Adult Ch ponies had 77.5% reduction of these stages. In Chapter 3, both Adult Ch and Young Ch animals had decreased numbers of DL, luminal L_4 and adult parasites (45% and 61% respectively). These data indicate that longer exposure to cyathostomes, as in the Adult ponies, increases the development of acquired resistance to developing and luminal parasites. The 7-week exposure that Young Ch ponies had in the natural challenge could have increased acquired responses against developing and luminal stages whereas the experimental 5-day challenge did not. Therefore, exposure to a natural challenge would increases acquired resistance.

A surprising finding was that the total parasite numbers of the Young were unexpectedly greater than in the PF and the Adult groups following both types of challenges. This increase was largely due to EL_3 numbers when Young and PF were compared, whereas it was due to all larval stages when Young and Adult ponies were compared. This finding is one of the most difficult to explain. We hypothesize that the differences of total parasite numbers observed in Chapter 2 were due to the manifestation of primary immune responses in conjunction with the absence of

an induction of hypobiosis in the PF ponies. This acquisition of resistance was manifested as reduction of all parasite stages in the Adults but not in the Young, because these responses are incompletely developed in the latter group. The pasture challenge, however, may have a confounding factor. It is possible that the PF ponies might not have acquired the same challenge as the Young or Adult ponies due to their grazing behavior. Nevertheless, the higher rate of cyathostome establishment observed in the Young Ch ponies when compared to Adult Ch ponies could be due to the presence of a greater degree of resistance to incoming L_3 and developing and luminal parasite stages in the Adult group.

The following diagrams aim to explain the differences between the protective responses of PF, Young and Adult ponies against the different cyathostome stages developing in the large intestine of these animals.

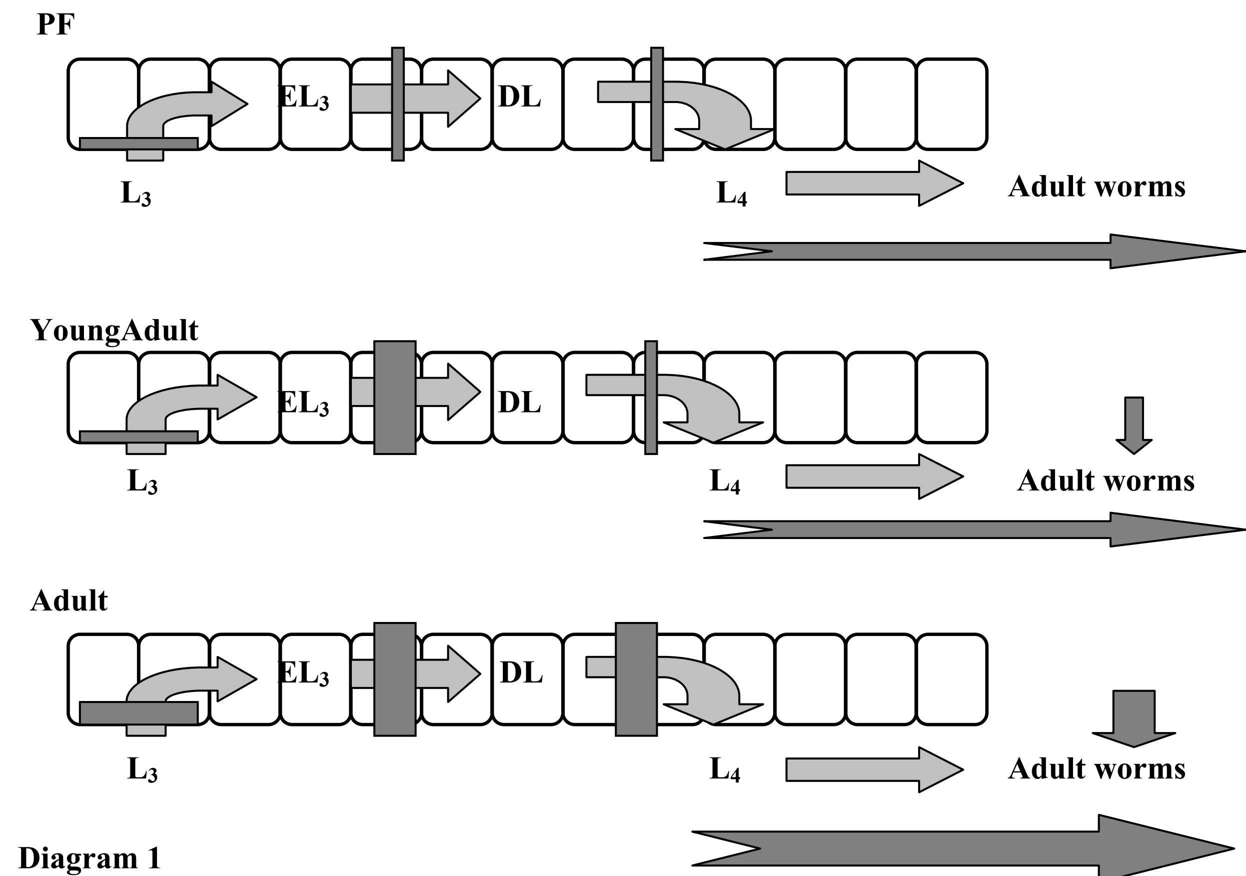

Diagram 1

Diagram 1 presents the invasion and development (represented with the light gray arrows) of the cyathostome L_3 to late DL in the mucosa of the equine large intestine (represented as cubes with rounded corners), and further development to the adult stage in the intestinal lumen. The dark gray blocks and arrows represent the primary or secondary immune responses that affect invasion, development and adult worms. In the case of the PF animals (Diagram 1, top) it is hypothesized that the primary immune responses elicited by the 5-day challenge prevent some L_3 from invading the mucosa, other responses affect the development from L_3 to EL_3, EL_3 to DL, and from DL to L_4. The big dark gray arrow below the L_4 and adult worms stages would indicate the presence of protective responses that increase the elimination of these luminal stages. The responses of ponies with prior exposure to cyathostomes, Young and Adult (Diagram 1, middle and bottom), differ from those of PF in that there are responses that prevent the EL_3 from developing, which was manifested as significant increases of EL_3 in these two groups (see above). The Young and Adult animals also have decreased FEC probably due to immune responses that affect the viability of the adult worms and / or decrease their egg production as well. The Adult ponies seemed to also have protective responses that decrease the number of DL that reach adulthood.

Diagram 2 (see below) presents the same drawings of invasion and development, the difference resides in the magnitude of the protective responses (dark gray blocks) elicited by the pasture challenge. In the case of the 7-week challenge it is hypothesized that there is in increase in the protective responses that prevent the development of EL_3 to DL in all groups. This assumption it is supported by the increased percentages of EL_3 found in the PF Ch, Young Ch and Adult Ch groups (see above). The groups with prior exposure to cyathostomes, Young and Adult (Diagram 2, middle and bottom), also seemed to have protective responses that decreased

the number of EL_3 that reached adulthood, those responses that eliminated luminal stages, and of those protective responses that reduced the viability and / or the egg production of adult worms. Differences between the Young and Adult groups resided in the increased protective responses of the Adult Ch ponies that blocked the invasion of the mucosa by the cyathostome L_3.

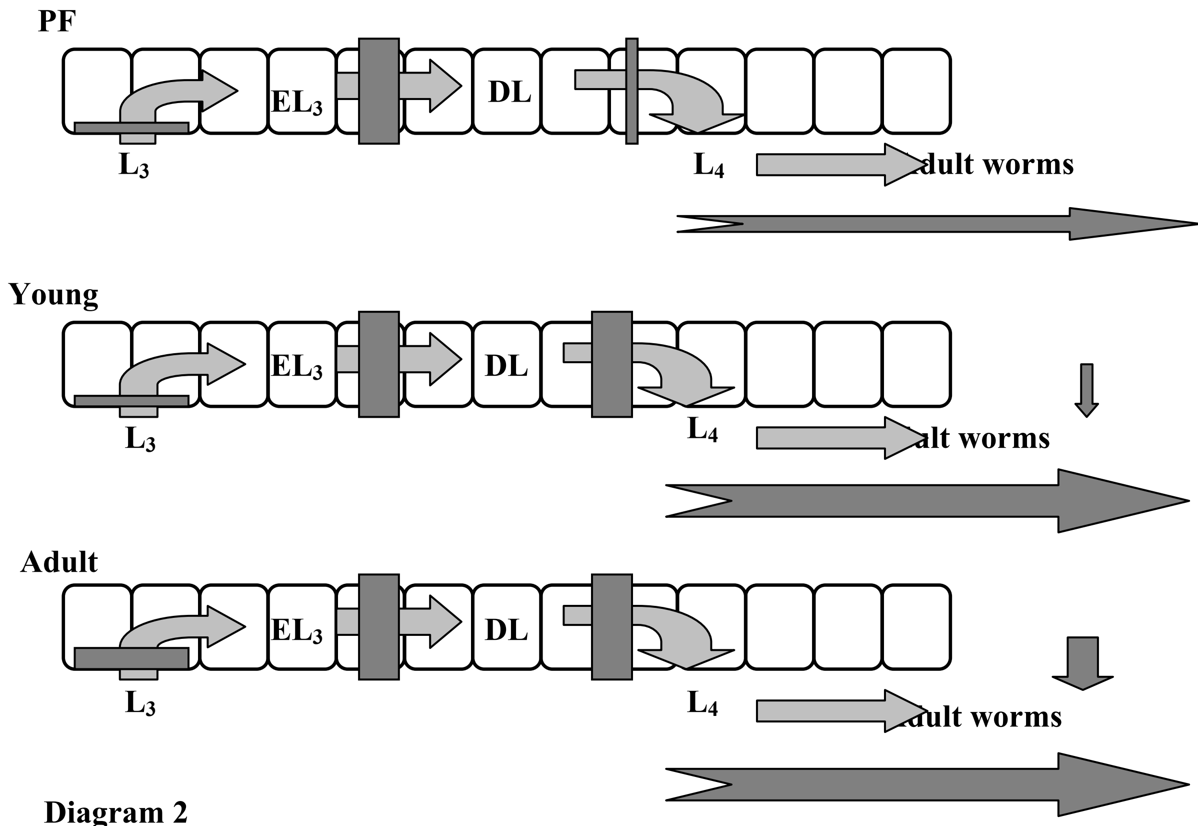

Diagram 2

The prevention of the invasion of the intestinal mucosa by the cyathostome L_3 could be due to a mast cell independent primary response, such as seen for *Nippostrongylus brasiliensis* infections in rodents. In this scenario the parasites release antigens that induce Th2 cells to release IL-4 and IL-13. Both cytokines produce goblet cell hyperplasia with consequent mucus hypersecretion, and IL-4 increases gut motility and net fluid secretion. These events promote the expulsion of the mucin-entrapped worms. This type of response could likely happen in the PF Ch

animals, and would explain the lower establishment rates in these animals. An example of mast cell dependent primary or secondary response has been detected in rodents infected with *Strongyloides* spp. In this case the parasite release antigens that induce Th2 cells to produce IL-3 and IL-9, which stimulate mast cell growth and degranulation, increasing intestinal permeability, smooth muscle contraction and net fluid secretion. On reinfection with the same species, a secondary response can be mounted almost immediately by mast cells armed with pre-formed immunoglobulin E (IgE) synthesized by parasite-specific B cells. The reaction described is also known as immediate hypersensitivity Type 1 or IgE mediated hypersensitivity. These types of responses could be present in any of the pony groups used in these studies, especially in the Adult group.

The lower FEC present in animals with prior exposure to cyathostomes, especially in the Adult ponies, indicates that these animals developed mechanisms of resistance to the adult parasites. We hypothesize that immune responses directed to the adult worms could reduce their feeding and/or reproductive abilities with a consequent reduction of the adult population and/or egg production. Adult Ch ponies also had fewer cyathostome species and a lower prevalence of species in both types of challenges suggesting, therefore, that protective resistance eliminates all the cyathostome species.

The pattern of mast cell counts of both experimental and pasture challenges were similar. In both cases the animals with larger numbers of mast cells were the Adult Ch ponies, suggesting that this cell type is related to acquired resistance in ponies with longer exposure to cyathostomes. The increasing numbers of mast cells found in the tissues of the PF Ch, Young Ch and Adult Ch ponies related to the histories of pasture exposure of these groups, suggests that infections with cyathostomes may be responsible for their increased presence in ponies with

longer prior exposure to these parasites. Therefore, it is likely that increased mast cell counts are associated with lower numbers of DL, luminal L_4 and adult worms and the higher levels of IL-4 mRNA in Adult Ch ponies. It has been postulated that mast cells could be involved in the "weep and sweep" mechanism in conjunction with IgE in other hosts.

Eosinophils, another cell type associated with immune responses to nematode infections, were significantly elevated in challenged ponies, although no clear relationships were established between resistance status of the host or larval burdens and this increase in numbers of intestinal eosinophils. Adult ponies had higher peripheral eosinophilia in both challenges, but the data collected at necropsy, regarding eosinophil levels in the intestinal tissues, was not consistent with circulating eosinophil levels. Intestinal eosinophil counts were more elevated in the Adult pasture challenged ponies than in the experimentally challenged ponies. The scenario was reversed for Young Ch ponies, with higher levels in the experimental challenge. Whereas PF Ch ponies had eosinophil counts comparable to those of Adult Ch ponies for the most part. Therefore, a clear relationship between this cell type and resistance to cyathostomes could not be made.

Levels of IgG, IgG (T) and IgG (a) antibodies against somatic antigens of adult *C. insigne* were overall higher in Adult Ch ponies in both experiments. Levels of IgG and IgG (T) antibodies against somatic antigens of cyathostome L_3 were higher in Young Ch and Adult Ch animals. Antibodies against the surface of freshly exsheathed L_3 showed that IgG and IgG (T) responses of Adult Ch were significantly higher than those of PF Ch animals. Furthermore, the persistence of higher levels of circulating antibodies against surface and somatic cyathostome L_3 antigens than against somatic adult worm antigens in Challenged, as well as Non-challenged animals, would suggest that immune responses against incoming L_3 are more important than

against adult cyathostomes. The data indicates that IgG (T) is consistently increased following cyathostome infection. Another observation of interest is that antibodies in PF Ch ponies clearly increased after experimental or natural challenge. Although the specific role of any of the immunoglobulin subisotypes still needs to be investigated, it is possible that they may play a role in the induction of hypobiosis in previously exposed ponies, or in the blockage of larval invasion to the intestinal mucosa. The role of equine immunoglobulins in antibody-dependent cell-mediated cytotoxicity (ADCC) in cyathostome infections has not been investigated, therefore relationships between immunoglobulins and immune mechanisms of resistance cannot be made.

The granulocytic and antibody increases recorded in both experiments are consistent with a Th2 type cytokine profile as described for gastrointestinal parasite infection models in rodents and ruminants. The measurement of IL-4, IL-13 and IL-5 was performed to confirm this profile, whereas the measurement of IFN-γ was performed to evaluate a Th1 cytokine profile. These results are the first to report on cytokine measurements of cyathostome-infected ponies. The hallmark cytokine for a Th2 type response, IL-4, was elevated in ponies with longer exposure to cyathostomes. The disparity in the results of IL-13 levels measured in both challenges, especially concerning the PF ponies, does not allow us to speculate on its role in resistance to cyathostome infections. The data obtained on IL-5 imply a relationship between peripheral eosinophilia and this cytokine. Overall, the results obtained in the experimental challenge are more consistent with those obtained in gastrointestinal infections of rodents. Whereas the results obtained in the pasture challenge resemble the outcome seen in naturally acquired gastrointestinal nematode infections of ruminants.

The se experiments allowed us to study the kinetics of immune responses in helminth naïve animals versus to that in ponies with long or short prior exposures to these parasites. Both

types of challenges showed that Adult ponies expressed acquired resistance as reductions of total number of worms, of DL, luminal L_4, adult parasites and of the number of cyathostome species found. The immune parameters measured in Adult ponies that corresponded to the observed decreases were increases of peripheral eosinophils, intestinal mast cells and eosinophils, IL-4, and EL_3 counts. These observations suggest that the immune mechanisms of resistance developed in Adult ponies, conferring protection against cyathostome is slow to develop and it is targeted against each parasite stage present in the host. The data obtained for the Young Ch ponies suggests that cyathostomes play a role in the immunosuppression of mechanisms of resistance in ponies with short exposures to cyathostome contaminated pastures. These results warrant further research in the area, especially in the difference between immune mechanisms of helminth naïve ponies and animals with short exposures to cyathostome contaminated pastures.

Further research in the area would be directed towards the study of induction of hypobiosis, the specific role of granulocytes and antibodies in acquired resistance to cyathostomes, and the role of specific cytokines in these responses. The new research efforts should be carried out utilizing experimental challenges in order to have a better control of the experimental conditions.

Vita

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