Chapter 1

General introduction

Relativeren moet je leren.
In het beste geval word je door een wetenschappelijke studie minder dom. (C. Palmen)

INTRODUCTION

Parasitic nematode infections are still one of the major causes of production losses in grazing cattle in temperate regions of the world, with the majority of these infections involving the intestinal lumen dwelling nematode *Cooperia oncophora* and its abomasal counterpart *Ostertagia ostertagi.* Although nematode parasitism is common in animals of all age classes, calves, entering the first grazing season, are the most susceptible to nematode infections. Therefore, most work on nematode infections in cattle refer to calves.

In general*, C. oncophora* has not received much attention compared to other nematodes such as *Dictyocaulus viviparus*, *O. ostertagi* and others. This is mainly caused by the mild pathogenicity of the worm. In natural conditions, clinical parasitism as caused by *C. oncophora* is rarely seen. Infection has been associated with production losses [179, 183] but the use of effective anthelmintics (anti worm drugs) during the last decades has reduced its clinical importance. Increasing concerns over the presence of drug residues in food animals, the incidence of anthelmintic resistance and the escalating costs of the development of new anthelmintics all suggest that alternative control strategies must be developed. The most likely alternative for anthelmintics would be a vaccine, but this requires an improved understanding of the immune response, together with a more detailed knowledge of the parasites themselves.

In the first part of this introduction, the life cycle, diagnosis and control of *C. oncophora* will be discussed. The second part will focus on the host and more specifically on the host-parasite interactions as observed during infection. Features of host immunity will be discussed from the immunological and the parasitological point of view.

1. THE PARASITE: *C. ONCOPHORA*

C. oncophora belongs to the trichostrongylid nematodes of ruminants (Phylum Nemathelminthes, Class Nematode). It is a small (10-12 mm), pink worm that preferentially resides in the small intestine of cattle but sheep and goats are also susceptible. Female worms lay eggs of the strongyle type: being oval, thin-shelled, colourless and medium sized (+/-50 μm).

1.1. Life cycle

A first detailed life cycle of *C. oncophora* was only described in the early sixties [121]. Under natural conditions, animals are infected by intake of infective third-stage larvae (L3) with the grass. The L3 exsheath in the abomasum and, subsequently moult to fourth stage larvae (L4) in the small intestine, i.e. the final habitat of the worm. A last moult takes place 10 days after ingestion, resulting in fifth-stage larvae (L5) that are the young adult stage. At this time point, male and female worms can clearly be differentiated based on their

morphology. The first gravid females occur around day 14 after infection and a few days thereafter eggs can be detected in the faeces. Eggs pass to the pasture with the faeces and develop into first stage larvae (L1) which hatch and moult to become second stage (L2) and L3. The time-span for this development merely depends on the weather conditions. Moist conditions are also necessary for L3 to migrate actively from the faecal pat to the grass where they are eaten by the host. L3 are very resistant to weather conditions that prevail in Western-European countries; they can overwinter on pasture and by doing so give rise to new generations of this species the next year.

1.2. Diagnosis, Pathogenesis and Epidemiology

1.2.1. Diagnosis

Accurate non-invasive diagnosis of infection and differentiation of infection among different trichostrongylids is an important component of proper management and efficient, economical drug treatment. The available diagnostic techniques and their usefulness for estimating nematode exposure has been reviewed recently [75].

Until now, *in vivo* diagnosis of gastro-intestinal (GI) nematode infections relies mainly on the detection of parasite eggs in the faeces of infected animals. The most widely applied method is the McMaster method [100]. The advantage of this method is that it is an easily applicable and simple technology method but the sensitivity and reproducibility are rather low. The detection limit of the McMaster assay is often between 25 and 50 eggs per gram faeces (EPG) and in cattle values less than 10 EPG are often reported [39]. The Wisconsin sugar flotation [50] technique has a higher sensitivity but is more labour intensive. Recently, a new method that uses both salt- and sugar solutions for flotation of nematode eggs was described [159].

Since eggs of many common genera are indistinguishable, differentiation of eggs in mixed infections must be performed by faecal egg counts followed by faecal cultures (LPG) for the recovery and identification of infective larvae. LPG has a higher sensitivity than EPG and allows differentiation of the species but a disadvantage of larval cultures is that yields are never 100% and, particularly when for some reasons the yield is low, the proportion of larvae developing may differ between species [75].

The golden standard of diagnosis of nematode parasitism remains the enumeration and differentiation of worms at necropsy. This method provides an accurate estimate of the level of infection and simultaneously allows differentiation of the involved species. An obvious drawback of this method is that animals have to be killed and this is far too expensive for diagnostic purposes at farm level. There is no continuous reliable correlation between EPG/LPG and the number of adult worms. Whereas in animals with a first nematode-

exposure, EPG is a good value to estimate worm burden, upon challenge this relationship decreases; immunity resulting in a reduced fecundity might affect this relationship and in *Teladorsagia circumcincta* infected sheep worm fecundity declines as worm number increases [212]. Furthermore, various nematode species greatly differ in egg-laying capacity [57] and this might disturb the relationship between egg-counts and the performance of the animals. This implies that whatever test is used, one should be aware of the limitations involved.

Beside parasitological diagnosis, there is a growing field of other means of diagnosis based on molecular or immunological tools. A PCR-based technique was developed that allows the identification and relative quantification of *O. ostertagi* eggs in a mixed faeces culture [248]. The *C. oncophora* 14.2 kDa ELISA has shown to be a sensitive tool to detect the exposure level in both naturally [185, 99] and experimentally infected calves [247]. These are only a few of the available examples but despite their relative success, new methods are not widely applied and commercially available and people stick to the conventional way of diagnosis.

1.2.2. Pathogenesis

Clinical parasitism following *C. oncophora* is very rare under natural conditions and calves, entering their first grazing season form the age class that is most susceptible to nematode infections. Obvious signs of disease are loss of appetite, dull hair coat, diarrhoea, and weight gain depression. Experimental trickle infections during six weeks with a daily dose of 10,000 *C oncophora* L3 in calves at 3 months of age revealed that despite the development of a good immunity, the damage to the intestinal mucosa resulted in considerable pathophysiological changes [9]. This was evidenced by increased plasma protein losses and stunting and fusion of the villi, together with an excessive production of mucus. However, by week 12 after infection there was substantial repair which might account for the somehow contradictory results of Coop et al. [47] who found little pathological damage following *C. oncophora* infections and no larval penetration of the mucosa. In the latter experiment, calves were necropsied at a much later time point after infection and it is likely that full recovery of disease had occurred by then.

1.2.3. Epidemiology and Prevalence

The most important GI nematodes of cattle in Western Europe are *O. ostertagi* and *C. oncophora*. Calves infect themselves after turnout with overwintered larvae and faecal egg output starts 3 weeks thereafter. Between approximately 5 and 12 weeks after turnout a correlation exists between faecal egg output and initial infection levels but, due to the onset of developing immunity this correlation is lost in the later phase of the grazing period [176].

Following the initial infections a midsummer increase of pasture infectivity occurs from two months after turnout onwards. Because overwintered pasture infectivity diminishes rapidly in spring, a delay in turnout diminishes the level of initial infection and, this effect is enhanced when the pasture is mown before turnout [31]. *C. oncophora* is far more profilic than *O. ostertagi*. Hence, it dominates the faecal egg output in the first grazing season [31]. However, the generation of immunity against *C. oncophora* is faster, and therefore in older animals *O. ostertagi* is the predominant type isolated from larval cultures [31].
Recently, our lab performed a large survey to investigate the presence of nematode eggs in faeces of grazing cows in the Netherlands (± 1,400 faeces samples collected between June and September 2000 and ± 4,000 in the same period in 2002). In 70% of the investigated animals in 2000 eggs were found in the faeces, while in 2002 50% of faecs samples contained eggs (Ted Mes, personal communication). Based on coprocultures, it was shown that only 15% of the infected animals were infected with *C. oncophora*. These results are consistent with abattoir surveys in the Netherlands [33] and Belgium [2] that revealed a prevalence of nematode eggs in faeces in more than 80% of the animals. In these surveys abomasa, blood and faeces were examined from +/- 110 dairy cows with known grazing history. Based on larval identification *Ostertagia* spp. and *Trichostrongylus* spp. were the most prevalent, with only 16% of the larvae being *Cooperia* spp. in Belgium and 4% in the Netherlands. These prevalence data only apply to adult cows and give no information on the importance of *C. oncophora* in young animals as all FGS calves (if not treated) pick up *Cooperia* [127]. The conclusions are two-fold: (i) the dominant genera in adult cows are still *Trichostrongylus* spp. and *Ostertagia* spp. as has been described in earlier reports [34] and, (ii) the observations support the more effective built up of acquired immunity against *C. oncophora* compared to *Ostertagia* ssp. and *Trichostrongylus* spp..

1.3. Parasite control

Many parasite control strategies aim at minimizing pasture infestation levels during the first grazing season (FGS). Not many farmers used to combat GI parasitism in the second year or in adult cattle, because in these age categories clinical disease does not often occur and strategic measures are more difficult to implement. Minimizing larval challenge during the FGS is beneficial for growth performance but it may also result in slower built up of immunity thereby affecting production parameters in the second year [179, 233]. The advent of anthelmintic resistance has led to renewed interest in non-chemical means of controlling helminth infections of livestock [238]. Among the methods under investigation are: genetic host resistance, improved nutrition, biological control, grazing management, and vaccination. Correctly integrated combinations of these approaches, along with occasional use of anthelmintics may provide the best approach for sustainable helminth control. This

combination of control techniques is termed integrated parasite management (IPM). All factors that might contribute to IPM will be briefly reviewed below.

1.3.1. Treatment

Nowadays in Europe, most parasite control is protective in its orientation, based on a regular and suppressive use of anthelmintics (99% protection) in the form of programs or sustained release. Liver flukes and GI nematodes are nearly always controlled by a combination of anthelmintic drugs and pasture management. Although this practice can be extremely effective, it is not sustainable as the situation is threatened by the increased evidence of anthelmintic resistance in worms and by societal concerns over the presence of drug residues in food animals. Resistance is a major concern in the sheep trichostrongylids, but it appears to be also spreading in cattle nematodes (reviewed in [228]). Anthelmintic resistance in *Cooperia spp.* has been reported against benzimidazoles (BZ, thiabendazole (TBZ), oxibendazole, oxfendazole) and macrolide lactone (ML) e.g. ivermectin (IV) and moxidectin (MD)) [238]. Most concerns surround IV resistance and the reports on this type of resistance are associated with frequent treatment of young cattle crowded on moist pastures [80]. One approach to reduce treatment frequency would be to measure infection or the risk of infection and treat tactically when the infection level reaches a threshold.

1.3.2. Vaccination

Anti-parasitic drugs are very effective, relatively cheap, and easy to administer (e.g. oral dosing and pour-on) and have accordingly set very high benchmarks by which vaccines will be judged. Conventional vaccine approaches in viral or bacterial diseases are aimed as a weapon to abolish infection completely. Regarding GI nematodes of ruminants, it is more appropriate to consider a vaccine as an epidemiological tool to maintain low-level pasture contamination [249]. Using this approach, minor infections would boost immunity and avoid the development of clinical symptoms or production losses. Mathematical models suggest that parasite vaccines may not have to achieve the efficacy of anti-parasitic drugs to offer substantial benefit to their users but could be used to prevent clinical pathology in animals and/or reduce the build up of parasites on pasture, either applied alone or along-side biological control or grazing management [21].

There are no commercially available vaccines for the control of helminth infections in ruminants, with the notable exception of that for the lungworms *D. viviparus* and *D. filaria*. The vaccine against *D. viviparus* was developed following the discovery of Jarret et al. [124] that two doses of 1,000 larvae, attenuated by irradiation, induced up to 98% protection against challenge with the parasite. Based on this success, attempts were made to develop a similar vaccine for other nematode infections such as *Ancylostomum caninum* in dogs [164]

and *Haemonchus contortus* in sheep [205]. The sheer number of larvae required, and disadvantages such as the cost of production, quality control and limited shelf life have precluded other irradiated nematode vaccines from commercial availability.

There are two types of antigens associated with nematode parasites: (1) soluble excretory and/or secretory products (ESP); and (2) those fixed at external surfaces or within the parasite (the so-called somatic antigens). Some of the ESP and exposed somatic antigens induce an immune response in the host during the course of infection and are designated 'natural antigens', while antigens that do not induce an immune response during infection are designated 'hidden antigens' (reviewed in [170]). Vaccination trials against *H. contortus*, *T. circumcincta*, *T. colubriformis* with cuticular collagens, ESP, and other putative natural antigens have resulted in substantial reduction in egg output and worm burden (reviewed in [69]). Considerable effort has also been applied to developing strategies based on the use of hidden antigens, especially gut molecules, as vaccine (reviewed in [167, 170]). Very few of these attempts are approaching a commercial product for GI nematodes, for reasons ranging from difficulties in reproducing the effect with recombinant proteins to consolidation in the animal health industry. One of the problems encountered is that all of the so far putative candidates contain glycoconjugate moieties that may carry essential protective (and parasite-specific) epitopes that cannot be properly synthesized in these protein expression systems [230].

1.3.3. Alternatives

High quality and equilibrated nutrition of cattle has shown to significantly reduce their susceptibility of animals to GI nematode infection [48]. Attempts to use biological control agents such as nematophagous fungi to limit larval population on herbage have yielded encouraging laboratory results but have not yet been commercially applicable [144, 218]. Furthermore, limiting pasture contamination can also be achieved effectively by alternate or rotational grazing [10, 76, 77]. The effectiveness of the latter method is highly influenced by factors such as the interval between grazing periods on one pasture and by mowing of the pastures [76, 77]. However, given the limited amount of grazing space for cattle in many countries, alternate grazing is not always feasible.

1.3.4. Genetic resistance and responder types

An increasing attractive adjunct for control of GI nematodes would be the identification of host genes that influence acquired or innate resistance to the parasites. The genetic background of animals has been reported to be a significant factor in how a host responds immunologically to infection and several studies demonstrated that EPG values of pastured cattle are strongly influenced by host genetics [146, 91, 134]. In addition, EPG values are not

normally distributed and within a herd, only a small percentage is responsible for the majority of parasite transmission [7, 94]. This pattern strongly suggests that genetic management of a small percentage of a herd could considerably reduce overall parasite transmission. This over-dispersion of EPG is used as one of the major characteristics to define responder types following *C. oncophora* infection.

Primary infection of 3-month-old calves with 100,000 L3 *C. oncophora* infection (which are followed during 6-7 weeks) have been used to detect genetic differences among cattle in their resistance to GI nematode infections. The choice of parasite species, age of calves and larval dose was made to simultaneously minimize environmental effects on infection and maximize host response differences. It was found that at the age of 6 months calves are less susceptible to infection with *C. oncophora* [135] which results in a lower among calves variability in response than at 3 months of age. Younger animals might be even more susceptible but under natural conditions calves are not put on pasture before 3 months of age. The larval dose was selected following the observation that with a lower (e.g. 10,000 or 20,000 L3) or higher dose (e.g. 200,000 or 500,000 L3) the variation between calves appeared to be much smaller. These experimental infections have the potential to discriminate between three major responder types based on parasitological variables (EPG, worm counts) (fig. 1) and the speed by which the parasite is expelled from the host [231].

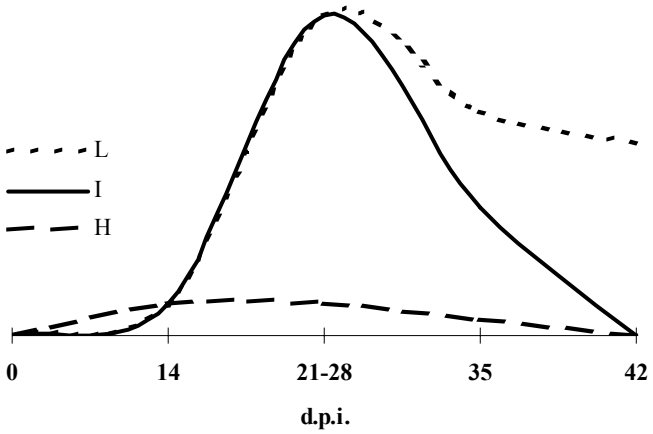

FIGURE 1 SCHEMATIC REPRESENTATION OF EGG OUTPUT PATTERN OF HOST RESPONDER TYPES FOLLOWING PRIMARY *COOPERIA ONCOPHORA* INFECTION IN 3-MONTH-OLD CALVES. Days after infection (d.p.i.) are depicted on the X-axis. L=low responders (± 40% of the population), I=intermediate responders (± 60% of the population) and H=high responders (± 2% of the population).

High responders, a small proportion of the host population, seem refractory to infection and have very low or no egg output and the worm burden at necropsy is low. *Low* responders show high EPG which is continued for weeks, and hence they have high worm burdens at necropsy. In *Intermediate* responders the EPG is initially similar to that in *Low* responders but 4 to 5 weeks after infection, the EPG starts to decline rapidly. These animals usually

show intermediate worm burdens at necropsy, but these can still range from low to high numbers.

Although a classification into responder types is to some extent an oversimplification of reality, it may be useful in studying and identifying relevant immune mechanisms. In addition, the existence of three major types of responder is not restricted to *C. oncophora*. Based on the result from an extensive breeding program, Gasbarre et al. [89] demonstrated that calves on pasture could also be separated into three types related to their EPG values: (i) *Type I* never demonstrated EPG values and is comparable to the *High* responders following experimental *C. oncophora* infection. (ii) *Type 2* showed rises in EPG values through the first two months of the test and then the EPG fell and remained at very low level, hence their EPG pattern is similar to our *Intermediate* responder animals (iii) *Type 3* maintained high EPG levels throughout the test, and resembles the *Low* responder animals. Although these pasture infections were mixed infections, the predominant species involved was *O. ostertagi*. A secondary infection experiment of these three types revealed that based on the EPG values, type 1 and type 2 animals were immune, while type 3 animals continued to shed eggs in their faeces [89]. Based on these results, the authors suggested the following categorization of the animals: innately immune (type 1 and *High* responders), acquired immune (type 2 and *Intermediate* responders) and immunologically non responsive (type 3 and *Low* responders).

The similarity in responses in a group of animals, despite the difference in nematode species and experimental versus natural infection, suggests that the occurrence of responder types is not restricted to one parasite species or one cattle breed and is promising in view of the identification of genes involved in resistance to GI nematodes. But we have to remain aware that resistance of a host to one species cannot always be extrapolated to another species. In addition, the genetic trait for resistance might compete with traits for maximal production.

2. THE HOST AND ITS DEFENCE

The development of well-defined rodent laboratory models of GI nematode infections has made significant contributions to the understanding of immunity to infection at three levels: (i) the type of T cell responses that controls and regulates the effector response (ii) the events in initiating a particular type of T cell response and (iii) the effector mechanisms responsible for worm expulsion. However, rodents are often not the natural host of the parasites studied and each host-parasite system has its own specificities. Hence, extrapolation of these studies to infection in the natural large animal host might be misleading. In the following sections, the emphasis will lay on results obtained from GI nematode infections in ruminants and data gathered from rodent models will be briefly outlined where needed.

2.1. Intestinal immune system

The gut is the major route of entry for a variety of antigenic materials, including infectious organisms, environmental contaminants, and food. Equilibrium has to be reached between tolerance towards the extensive range of nutritional antigens and the generation of effective immunological protective mechanisms against potentially harmful micro-organisms such as parasites. The intestine is protected by gut associated lymphoid tissues (GALT) consisting of Peyer's patches (PP), isolated follicles, lymphocytes within the epithelium (IEL) and within the lamina propria (LPL) of the mucosa.

The PP are lymphoid aggregates in the jejunum, ileum and cecum. In young ruminants, ileal PP consist predominantly of B cells and 1% T cells. In mature animals more αβ T cells are present, but still 70% of the cells are B cells. The majority of these T cells are CD4+ Th cells (twice as much CD4+ than CD8+ cells) with an equal Th1: Th2 distribution. In contrast the population of IEL consists mainly of CD8+ αβ T cells and other lymphocytes are rare, resulting in a CD4:CD8 ratio of 1:8. Half of the LPL are αβ T cells, with a CD4:CD8 ratio of 2:1 and the T-helper cell populations appear to be skewed towards a Th2 phenotype.

The third major T cell subpopulation is the γδ T cells, which are very prominent in ruminants as compared to other species [111]. The general principles that apply to activation of αβ T cells through their TCR are profoundly different than those that apply to γδ T cells. They do not see antigens presented on autologous MHC molecules nor do they respond specifically to protein peptides (reviewed in [109]).

The role of γδ T cells in infected ruminants is still not very clear, however, they might serve as regulatory cells in intestinal immune responses. It has been suggested that food proteins are presented to villous epithelial cells, rather than via M cells to organized lymphoid tissues [140] and that this alternate antigen presentation pathway induces suppressive T lymphocytes in the intestinal mucosa [29]. Suppresive γδ T cells have been demonstrated in cattle, both in blood and in mammary gland secretions [45, 175]. Depletion of γδ T cells in sheep infected with *T. colubriformis* reduced egg excretion and worm counts, suggesting that also in helminth infected ruminants γδ T cells might be suppressive [153]. The authors suggested that protection was affected by loss of cytokine production, leaving other cytokines free to induce different mechanisms. This is consistent with the recent observation that by early secretion of IFN-γ, bovine γδ T cells might contribute to an inflammatory response and establish a Th1 cytokine environment, which is important for the clearance of bacteria and other intracellular pathogens [13]. In nematode infections however, this would ablate the induction of a protective Th2 response.

2.1.1. Antigen presentation

The first step in the induction of the immune response is the uptake and the presentation of antigens. Both the adult and larval stages of nematodes secrete antigens in their environment. These are referred to as excretory/secretory products (ESP) some of which are likely to interact with antigen presenting cells (APC) initializing the immune response. Four major cell types are thought to present antigen within gut associated lymphoid tissues: dendritic cells (DC), macrophages, B cells and epithelial cells [172]. In ruminants the classical presentation of antigen by epithelial cells may not occur since these cells do not express MHC class II molecules [101, 186].

DC may contribute to the Th1 and Th2 dichotomy in the immune response as different types of DC capable of inducing specifically Th1 or Th2 cells have been described [43]. However, recent reports suggest that the 'DC1-DC2' idea is likely oversimplified, indicating that DC remain largely plastic and that the direction of Th1 or Th2 responses is determined by factors such as the state of DC maturation, the antigen dose and stimulation of DC by pathogen-derived products [30]. There is as yet no information available on the main cell type involved in nematode antigen presentation *in vivo*. *In vitro* assays demonstrated the ability of schistosome egg glycolipid to elicit cytokine responses by human monocytes [229]. *In vivo*, the glycan determinants can probably also instruct DC to induce Th2-polarized responses [155].

At least two types of bovine DC (called afferent lymph veiled cells) with distinct antigen presenting capacities have been described [114] but their role and contribution in *C. oncophora* infections remains unexplored.

2.1.2. Involvement of cell mediated immune response (CMI)

Attempts to define the inductive requirements for parasite immunity have come from the *in vivo* use of monoclonal antibodies (Mabs) in rodent models. These studies demonstrated the overall importance of T-lymphocytes for protection against GI nematodes in rodents infected with *Trichinella spiralis* [106], *Nippostrongylus brasiliensis* [129], *Heligmosomoides polygyrus* [222] and *Trichuris muris* [139]. In these models, the T cell subset to which protection has been ascribed is CD4+ cells. Similar studies involving treatment with anti-CD4 Mab have been attempted in ruminants when reagents became available, but the most convincing studies regarding the role of CD4+ cells in protection against GI nematodes in ruminants have come from passive transfer experiments. Transfer of gastric lymph lymphocytes from resistant lambs to their genetically identical uninfected twin conferred protection against subsequent infection with *H. contortus* [207] and *O. circumcincta* [206]. Studies involving prolonged administration of Mabs are limited in cattle by the development of anaphylactic

reactions to murine Ig within 1 week [113]. Consequently, most studies report on the kinetics of cellular changes, in peripheral blood, tissues and draining lymph nodes.
Marked differences in frequencies of lymphocyte subpopulations have been observed in the peripheral blood, draining lymph nodes and tissues following GI nematode infections in ruminants (reviewed in [15]). Reactions taking place during larval development and adult infections significantly differ, as well as reactions induced by distinct nematode species (reviewed in [15]) emphasizing that the effector mechanisms of parasite expulsion are very complex and that the contribution of a specific lymphocyte subpopulation might differ depending on the host-parasite system, and the infection and resistance level of the animals.

2.1.3. Th1-Th2 dichotomy

The seminal observation that murine and human CD4+ cells could be segregated in T-helper 1 (Th1) and T-helper 2 (Th2) based on the cytokines they secrete [165, 189], has provided a basis for understanding the underlying cell regulatory mechanisms controlling resistance to infection. Th1 cells produce IFN-γ, lymphotoxin and interleukin 2 (IL-2) whilst Th2 cells secrete IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. A naïve T cell can differentiate into either a Th1 or Th2 cell type, passing through an intermediate stage where it has an unrestricted cytokine profile (often referred to as Th0). The differentiation pathway is influenced by a number of factors, the most potent of which seems to be the immediate cytokine environment a T cell experiences at the time of antigen presentation. Thus, IL-12 promotes the development of Th1 cells whilst Th2 cells can develop in the presence of IL-4.
Cytokines released by Th1 cells promote macrophage activation, which results in delayed-type hypersensitivity and promotes the production of opsonizing antibodies. These mechanisms are particularly important in the clearance of intracellular organisms. In contrast, the characteristics of helminth infections such as eosinophilia, mastocytosis, IgG1 and IgE production are all mainly controlled by Th2 cytokines [81]. The induction of mastocytosis is regulated by a variety of cytokines including IL-3, IL-4, IL-9 and the growth factor stem cell factor [149]. IL-5 is a key factor in the growth and differentiation of eosinophils [193] whereas IL-4 and IL-13 are involved in isotype switching to IgG1 and IgE [216].
Although not as clear cut as in mice, Th1 and Th2 responses have also been described in cattle [36]. Studies conducted by Brown et al. [37, 35] revealed that the majority of Th cell clones specific for *Babesia bovis* and *Fasciola hepatica* showed a less restricted cytokine profile and more resembled the Th0 like profile. Regarding GI nematodes in cattle, the immune response to *O. ostertagi* has been studied most extensively. Soon after infection with *O. ostertagi*, primed lymphocytes leave the draining lymph nodes, enter the peripheral circulation and home to the tissues immediately surrounding the parasite [4]. The immune

response in the abomasum is in many ways similar to that seen in other nematode infected mammalian hosts, with high levels of expression of IL-4 in lymphocytes from the draining lymph nodes and in lymphocytes isolated from the mucosa [5; 41]. But unlike other models, the immune response also elicits the expression of IFN-γ, implying that in *O. ostertagi* infected calves the immune response is not stereotypic Th2 like.

2.2. Effectors

2.2.1. B cells and Antibodies

B lymphocytes are ultimately responsible for the generation of protective antibodies following infection and hence both components of the humoral immune response are intimately linked. Beside their role as antibody secreting cells (ASC), B cells also act as antigen presenting cells and might contribute to the priming and maintenance of T cell responses.

While the antibody response to GI nematode infections in ruminants has been studied extensively, there is only very little information concerning other functional contributions of B cells during infection. Therefore, both aspects will be discussed in separate sections.

2.2.1.1. B cells

A redundant role for B cells and antibodies in the expulsion of *T. muris* was suggested following the observation that transfer experiments with immune CD4+ cells from infected BALB/c mice to SCID mice (which lack both T and B cells) conferred resistance [66]. However, infection of B cell deficient μMT mice (which have a disrupted immunoglobulin μ chain gene) with *T. muris* revealed that B cells are required for resistance to infection, i.e. during a primary *T. muris* infection the B cells appear to be important for the development of a protective Th2 immune response (by cytokine action and costimulation) [28]. A critical role for B lymphocytes in primary and memory anti-filarial immunity was also demonstrated in *Brugia pahangi* infection in mice [173, 174].

Infection with *H. contortus* in sheep and *O. ostertagi* in calves is associated with an increase in abomasal lymph node weight and a concurrent increase in the proportion of B cells [16, 90]. Concurrent herewith, the frequency of B cells in the abomasal mucosa increases. Although related to infection, the functional properties of these B cells have not yet been clarified.

2.2.1.2. Antibodies

Nematode infections constitutively induce the production of parasite-specific antibodies both in the peripheral blood and at mucosal surfaces and the elevation of antibody titres is more pronounced after a secondary infection than after a primary infection (reviewed in [161,

[15]). To date, most studies examining the kinetics of the antibody responses during nematode infections have used crude worm preparations of different nematode developmental stages. This makes it difficult to compare results from different laboratories, as the antigen constitution of crude worm preparations highly differ depending on the different extraction methods used.

i) IgG1 and IgG2

A functional distinction can be made between IFN-γ dependent Th1 antibody isotypes and IL-4 dependent Th2 related isotypes [1]. For cattle, it has been shown that IgG1 and IgA can be classified as Th2 associated isotypes, as opposed to IgM and IgG2 that are associated with a Th1 response [73, 36].

Consistent with the induction of a Th2 response in all infection models examined IgG1 was the predominant serum antibody isotype over IgG2 [40, 97, 112, 176]. In the same studies IgA antibodies were generally low in the serum of infected animals but IgA is a mucosae-associated Ig and peripheral titres might not have a predictive value for its role in infection.

The role of increased systemic antibody titres during *C. oncophora* infection is not entirely clear, but antibody titres correlate with various parasitological parameters of resistance [135]. Primary infection with *C. oncophora* induced significant increases in IgG1 titres against *C. oncophora* adult crude worm antigens whereas no IgG2 titres and a minor increase in IgA titres are observed [176]. In addition, western blot analysis revealed that the *C. oncophora* specific antibody response was mainly directed against low molecular weight antigens [55, 176, 232] and that the recognition of these antigens could be used to distinguish susceptible and resistant calves [231].

ii) IgA

IgA responses are typically associated with GI nematode infections and have been more observed in the local mucosal tissues, mucus, draining lymph nodes and in lymph than in serum (reviewed in [15]). The number of IgA secreting cells is generally increased in the mucosae of animals bred for resistance to GI nematodes [98]. Although the exact function of IgA is not defined, there is a strong negative correlation between worm length and IgA levels in the gastric lymph of *T. circumcincta* infected sheep, suggesting that mucosal IgA could interfere with the feeding processes of parasites [204]. In addition to a direct effect on the worms, IgA might also interact with eosinophils [60]. IgA/antigen complexes can bind through the Fc-α receptor on eosinophils and provoke the release of anti-inflammatory mediators which have a detrimental effect on the worm population.

iii) IgE

The production of IgE against worm allergens and parasite non-specific IgE during GI nematode infections in rodents and man is well documented [123]. Strikingly, in many individuals the total serum IgE level correlates roughly with both disease severity and protective immunity to parasites.

IL-4 is the most important cytokine mediating IgE synthesis but human and murine B cells also synthesize IgE in response to the closely related cytokine IL-13 [216]. IgE synthesis has also been observed in the absence of IL-4; cross-linking of CD40 (expressed on the surface of B cells) alone is sufficient to elicit polyclonal IgE responses, but when combined with signals from the B cell antigen receptor the response becomes antigen specific [78]. Although the exact mechanisms are not entirely elucidated, the polyclonal generation of IgE following parasite infection suggests that nematode products might have some activities or structure homology with cytokines responsible for the induction of IgE [187]. Recent reports demonstrated the ability of parasite products to potentiate IgE synthesis independently of infection [61, 217].

The development of different Mabs which specifically recognizes ovine and/or bovine IgE has allowed study of the role of IgE responses in ruminants [201, 136, 137]. Sheep IgE levels increased after infection with *T. colubriformis* [200], *H. contortus* [136], and *T. circumcincta* [119]. One of these studies revealed a correlation between total serum IgE and the decrease in worm counts following primary infection with *H. contortus* in sheep [136]. Data of *O. ostertagi* infections in cattle were less consistent but generally indicated a slight increase in serum IgE levels following infection [219, 11]. Recently, a correlation between total serum IgE levels and protection was demonstrated in calves re-infected with *D. viviparus* [137].

2.2.2. Eosinophils

Eosinophils and mast cells are the major effector cells typically associated with helminth infection. Eosinophils develop in the bone marrow and are constitutively released at a low rate in the blood circulation; they normally comprise only a small fraction of circulating leucocytes (<1-5%). Most of the eosinophils are found in tissues, predominantly those at surfaces of the body that interact with the external environment e.g the gut. Their half-life in blood is about 18 hours, but in tissues they are believed to survive for several days [125]. During helminth infections eosinophils are released more rapidly from the bone marrow, their survival in tissue is enhanced [202] and the rate of bone marrow eosinophilopoeisis increases substantially.

The early stages of differentiation are controlled by the cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-3, while the later stages of differentiation and maturation are under the influence of IL-5, which is predominantly produced by activated

T-cells and mast cells. The high conservation in both sequence [158] and biological activity [214, 215] of IL-5 between rodent, human and ruminant species supports its unique role in the generation of eosinophilia. However there is a minor population of IL-5 independent eosinophils that develops and functions in the absence of functional receptors for IL-5, GMCSF and IL-3.

The primary function of eosinophils is considered to be the defense against organisms that are to large to be phagocytosed, such as parasitic helminths. Under the influence of a Th2 environment they respond to chemoattractants and other signals by homing into inflammatory or helminth-infected sites, where they become activated and degranulate. Degranulation of eosinophils is generally initiated by cross-linking of surface receptors. Eosinophils have receptors for various components of the complement pathway and immunoglobulin isotypes, including IgG and IgA and low affinity receptors for IgE. Of particular importance for GI nematode infections, is the expression of receptors for the secretory component of IgA on the surface of eosinophils. Binding of secretory IgA provides the most potent stimulation for degranulation of eosinophils [143, 166]. In addition, eosinophils might bind and respond to carbohydrate ligands expressed on the parasite surface, such as the Lewis^x-related molecules and cell adhesion molecules similar to selectins that have, for example, been demonstrated on schistosomula.

The ability of eosinophils to kill a variety of parasites *in vitro* (reviewed in [157]) has led to the suggestion that eosinophils are anti-parasite effector cells. Despite an increased eosinophilia during intestinal nematode infection, a protective role for this population *in vivo* has not yet been identified. Treatment of mice with anti-IL-5 Mabs to ablate eosinophilia does not prevent the expulsion of *N. brasiliensis, T. spiralis, T. muris* or *H. polygyrus* (reviewed in [63]) which suggests that eosinophils are not the only effectors involved in the expulsion of helminths from the gut. In contrast, there is some evidence for an eosinophil-mediated protective immune response operating against the migratory tissue dwelling larval stages of two other nematodes, *Angiostrongylus cantonensis* [194] and *Strongyloides venezuelensis* [138].

Similarly, eosinophilia occurs in nematode infected ruminants [38, 15] but a functional role for this cell population is not well defined. Indirect evidence for the contribution of eosinophils in protection was provided by the significant correlations that exist between genetic variations in susceptibility to infection and the magnitude of the eosinophil response. Independently selected lines of sheep, bred for an increased resistance to nematode infections showed greater eosinophil responses after infection compared to random-bred or low-responder flocks [54, 95, 38]. This correlation was only observed after priming of sheep with natural or experimental infection. Similarly, the presence of eosinophil-potentiating activity (EPA) in the gastric lymph of sheep infected with *O. circumcincta* was inversely correlated with worm burden, but only early after secondary and not after primary infections [213]. In

contrast, an increase in eosinophil numbers was observed after primary infection of sheep with *Nematodirus battus* and this concurred with the rejection of adult worms [245]. Simultaneous infections with *O. ostertagi* and *C. oncophora* evoked a small rise in blood eosinophils [133], but the distinct contribution of both nematodes to the eosinophilia was not investigated.

2.2.3. Mast cells

Tissue mast cell hyperplasia is one of the characteristics of the mammalian host response to nematode infections [161, 82]. In rodents, the recruitment of mast cells is dependent upon the release of T-cell derived cytokines such as IL-3, IL-4, IL-9 and IL-10. The growth of mast cells is mainly regulated by stem cell factor (SCF). SCF is also known as *kit*-ligand because it binds to the c-*kit* receptor, a tyrosine kinase which is abundantly expressed on the mast cell surface [85].

The effect of mast cell activation might be directly anti-parasitic by the secretion of low molecular weight granule mediators e.g. histamine, 5-hydroxytryptamine, mast cell proteinase, prostaglandins, and leukotriens that have a negative effect on worm survival [192]. Indirectly, the granule chymases that are secreted in the gut lumen can contribute to an increased mucosal permeability and thus facilitate the translocation of plasma proteins such as humoral parasite-specific antibodies into the gut lumen [22]. Activation of mast cells and the consequent release of mediators are commonly associated with protection [192, 162] but these processes might also have detrimental effects on the host [86].

The most convincing data on the protective role of mucosal mast cells have been generated from mast cell-deficient mice that have a mutation affecting tyrosine kinase activity of the *c-kit* protein (reviewed in [162]). Data using this experimental model suggested that expulsion of *T. spiralis* or *Strongyloides ratti* is to some extent mast cell dependent [168, 169]. Treatment of *T. spiralis* infected mice with a monoclonal antibody for the receptor of SCF depleted intestinal mast cell populations and completely abrogated the protective immune response [104]. However, worm expulsion might also occur in the absence or with very low numbers of mast cells (reviewed in [192]). For examples, the expulsion of *N. brasiliensis* occurs without a significant contribution of mast cells and while *T. spiralis* infection in mice requires mast cells for adult worm expulsion, the rejection of *T. spiralis* in rats seems to be mast-cell independent.

Similarly several studies suggest that mast cells are specifically activated during the rejection of nematodes in sheep [116, 72], whereas other observations show that sheep can express high levels of resistance without any obvious increase in mast cell numbers [118]. Hence, the functional contribution of this cell population to protective immunity against nematodes is not very clear. The number of mast cells and globule leucocytes were

determined in calves primary and secondary infected with *C. oncophora*, but results were not conclusive and no clear effect of infection on these populations was observed [84].

2.2.4. Mucus and motility of the gut

Increased mucus production, increased fluidity in the gut lumen and increased motility of the gut are often associated with the host's response to GI nematodes in both rodents and ruminants (reviewed in [192] and [162]). Direct effect of mucus on nematodes was demonstrated using an in vitro larval migration assay [59]. The intestinal mucus of sheep rendered immune to *T. colubriformis* contained significantly more larval migratory inhibitory (LMI) activity in comparison to mucus derived from nematode-free sheep. These mechanisms appeared to be not species-specific as in the same experiment it was demonstrated that the 'immune' mucus exhibited LMI also against *H. contortus, Nematodirus spatigher* and *T. circumcincta* larvae [59]. Similarly, mucus from calves immunized with *O. ostertagi* showed in vitro LMI activity [46].

Although hyperplasia of goblet cells and mucus secretion are often considered as being immunological non-specific mechanisms there is growing evidence that during enteric infections the host develops an integrated response involving the coordinated actions of all tissues in the gastro-intestinal tract (reviewed in [225]). Infection with *T. spiralis* results in increased fluid secretion into the lumen of the small bowel as well as increased intestinal propulsive activity and more rapid intestinal transit. A specific recruitment of the immune response in these events was demonstrated by the attenuation of these processes in athymic rats [108, 234]. Recently, a putative role for the parasite specific response including CD4+ cells, IL-5 and eosinophils as well as *c-kit* dependent cells in the regulation of motility disturbance associated with *T. spiralis* infection was proposed [223, 224, 226, 227].

3. EFFECTS OF THE IMMUNE RESPONSES ON *C. ONCOPHORA*

There are a range of parasitological parameters that can be used to assess host responsiveness, and within each host-parasite system the effect of the host's immune response on the worms might differ. Furthermore, terms such as establishment, fecundity or survival can be defined in many ways making it difficult to compare data from one research group to another. Under the traditional view of the host immune response, all effects on the worms are a direct result of attack by effector cells and molecules of the host immune response. Based on the observation of *S. ratti* infection in rats, an alternative was proposed for the way in which worm survival and/or fecundity might be limited [236]. The author proposed that the reduction in the fecundity and survival of worms in immune hosts was at least partly a result of the energy expended by a parasite to protect itself against immune attack. Which extreme reflects the reality is not known, but as parasites have evolved to an

obligatory parasitic phase within a host, it is likely that both alternatives should be considered when interpreting the altered morphology of survival of the worms within an infected host.
A brief overview is given of the parasitological variables which are used throughout this thesis. In view of the clarity, this section will focus only on *C. oncophora* and a short definition of each of the variables will be included.

3.1. EPG and ratio EPG

EPG is defined as the number of eggs per gram faeces as measured by a modified McMaster method with a sensitivity of 50 EPG. The cumulative EPG is defined as the sum of the EPG values during a specified experimental period and within this period EPG was determined every two days.
Ratio EPG is a parameter derived from the mean EPG pattern observed in intermediate responders after primary infection with 100,000 L3 *C. oncophora* (fig. 1). Following infection a peak in egg output occurs around day 21-28 p.i.. Shortly thereafter, the EPG decreases as a consequence of acquired immunity and around day 35-42 p.i. the egg output is very low or zero. We defined ratio EPG as being the ratio: mean EPG day 35-42 p.i./mean EPG day 21-28 p.i. This parameter reflects the effect of the host immune response on both the expulsion of the worm population and worm fecundity (see section 3.2. and 3.3). The reduction in egg output at day 42 p.i. is less pronounced in low responders than in intermediate responders and this will consequently result in a distinct ratio EPG. A threshold in ratio EPG is set at 0.4 which indicates a reduction in egg output of at least 60 % as observed in intermediate responders. Animals with a ratio EPG >0.4 and a high cumulative EPG are considered as low responders and animals with a ratio EPG <0.4 are considered as intermediate responders. High responders may show a ratio EPG taking any value, but show a significantly lower peak in egg excretion by day 21-28 p.i., concurrent with a low cumulative EPG.

3.2. Establishment

The parasite must establish itself and there is ample evidence to show that the success of establishment is lower in immune as opposed to naïve hosts (reviewed in [161, 192, 15]). In the current study we defined establishment as the ability of the worms to establish as (juvenile) worms beyond the L4 stage. It is important to differentiate this way of defining with a definition of establishment based on the larval stages. Indeed, measurement of establishment based on the larval stages might result in a higher outcome as compared to our definition. Immunity can affect both, and while the infective larvae might reach their niche, their development can be hampered or delayed. The arrested development of the larvae obviously

will result in a lower adult worm burden. We chose the definition based on the adult stages because all the other parameters used also refer to the adult worm population.

3.3. Sex ratio, Worm length and Worm fecundity

Sex ratio is defined as the percentage male worms (number of male worms/sum of male and female worms). In animals primary infected with *C. oncophora* before the development of acquired immunity an equal proportion of male and female worms prevails (50% of each sex). As a result of immunity male worms are commonly expelled first [3, 51] and the sex ratio decreases. Hence, this parameter can be used to assess worm expulsion.

Worm length and fecundity can both be affected as manifestations of resistance to adult nematode parasites (reviewed in [15]). Worm length is commonly used to ascertain effects on growth and development of a parasite population while an effect on the reproduction capacity of the worms is measured by worm fecundity. Worm fecundity is defined in this thesis as the number of eggs per female worm. Such a definition allows differentiation among effects on the production of the eggs and effects on the egg-laying machinery of the worms which is not feasible with a definition of worm fecundity based on the egg output in the faeces.

4. SCOPE OF THE STUDY

GI nematode infections in ruminants are commonly treated with effective anti-parasitic drugs, but in view of the development of resistance, there is a need for alternative means of parasite control. A likely alternative for anthelmintics would be a vaccine, but this requires an improved understanding of the immune response, together with a more detailed knowledge of the host-parasite interactions. We used *C. oncophora* infections in calves as a model to investigate the mechanisms involved in the immune response against GI nematodes.

Development of acquired immunity following *C. oncophora* infection in calves is relatively fast. Using experimental infections with 100,000 L3 in 3-month-old calves, animals can be differentiated in *High*, *Intermediate* and *Low* responders. Until now, this differentiation relied solely on parasitological variables as EPG and worm counts at necropsy. In **Chapter 2** the systemic immune response following a primary infection with 100,000 L3 *C. oncophora* was characterized. We investigated whether calves classified into different responder types based on parasitological variables also feature different immune responses. **Chapter 3** reports on the local immune responses in these primary infected animals. A detailed analysis of the immunological and parasitological events which coincided with worm expulsion was performed to identify possible effectors. The comparison of

Intermediate and *Low* responder animals allowed us to identify putative mechanisms involved in worm expulsion.

Based on the data from primary infected animals, we knew that acquired immunity is developed in *Intermediate* but not in *Low* responders. Under natural conditions, animals are re-infected after turn out during the second grazing season. Hence, it is important to know whether the immunity generated during primary infection is long-lasting and protective against re-infection. To address this question, animals primed with 30,000 or 100,000 L3 larvae were re-infected with 100,000 L3. The housing period was mimicked by keeping the animals worm-free during a period of 2.5 months. The parasitological features of the re-infection experiment are described in **Chapter 4.** We investigated two different issues; the effect of the infection dose on the development of immunity against re-infection and whether the more resistant phenotypes are sustained after re-infection. Until now responder types can only be distinguished following infection. Hence, at some time points only a few animals per group were analysed.

The immune response was compared between animals that were primary and secondary infected. The role of B cells and antibodies in the peripheral blood and in small intestine is described in **Chapter 5.** Based on the results from primary infections (**Chapter 2**), a more detailed phenotypic and functional analysis of the B cell population was done at day 28 p.i.. **Chapter 6** describes the distinct kinetics in the T cell and effector responses among primary and secondary infected animals.

The findings described in this thesis are discussed in **Chapter 7**.