Chapter 2

Characterisation of host responder types after a single *Cooperia oncophora* infection: kinetics of the systemic immune response

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Er is hier. Er is tijd
Om overmorgen, iets te hebben achtergelaten,
Daar moet je vandaag voor zorgen,
Voor sterfelijkheid.
(H. de Coninck)

ABSTRACT

After primary infection with 100,000 third stage larvae of the intestinal nematode *C. oncophora* in 3-month-old calves a high variability in egg output and worm counts is observed. Based on this variability infected animals can be divided in different responder types. The three major phenotypes can be classified as high, intermediate and low responder animals. We investigated whether calves classified into different responder types show different immune responses during infection. Peripheral blood eosinophil counts and flow cytometric analysis of different lymphocyte subsets of the blood did not reveal major differences between infected and control animals, nor between responder types. However, the levels of *Cooperia*-specific IgG1 and IgA during primary infection are significantly higher in intermediate responders than in low responders. In the intermediate responders, isotype specific responses were negatively correlated with parasitological parameters expressing worm expulsion and influence on worm fecundity. Total serum IgE levels were elevated in most of the infected animals. A quantitative positive relationship between worm counts and total serum IgE levels was observed. Based on the observed correlations, we propose a role for the humoral response against the maintenance of the infection in the gut.

INTRODUCTION

Variation in immune responses to nematodes in cattle has been reported to depend on the infection dose [3, 130], age [8, 135] and sex [146]. Apart from these, genetic background has been reported to be a significant factor in how a host responds immunologically to infection [3, 146, 132]. For *Cooperia oncophora* a dose of 100,000 infective third stage larvae (L3) at a host age of three months has proven to result in large differences in parasitological variables (eg. faecal egg output and worm burdens) between calves.

With the primary infection protocol for *C. oncophora* it is possible to distinguish between three major responder types within the wide variation in response. This is based on cluster analysis of the faecal egg output and the necropsy results such as total worm burden and the sex ratio of the harvested worms, at days 35-42 post infection (p.i.) [231]. These major phenotypes can be classified as high, intermediate and low responder animals. High responders, a small proportion of the host population, show almost no egg output (EPG) and the worm burden at necropsy at day 42 p.i. is low. Low responders show high egg output which is continued for weeks and hence have high worm burdens at necropsy. In intermediate responders egg output at first is similar to that in low responders, but around day 28-35 p.i., EPG's start to decline rapidly. These animals usually show intermediate worm burdens at necropsy, but these can still range from low to high numbers. It is likely that differences between the host responder types are at least partly immunologically based. For example, Albers [3] showed that calves showing high faecal egg counts had lower antibody titres against crude worm antigens than calves showing low faecal egg counts. Although a classification of hosts into distinct responder types is to some extent an oversimplification of reality, it may be useful in studying and identifying relevant immune mechanisms. The utility of having different responder types to study immune mechanisms in a parasite-host model has been demonstrated extensively in laboratory animal models [25, 68, 65, 169] and in sheep [96]. These studies have provided much information on immune mechanisms involved in parasite rejection and expulsion. However, various immune mechanisms are frequently linked to a specific parasite-host system. Hence, results cannot always be easily extrapolated to parasite infections in other animals. We started to investigate whether calves classified into different responder types based on parasitological variables feature different immune responses during a primary *C. oncophora* infection. The present study reports on the systemic responses observed.

MATERIALS AND METHODS

Infective larvae

C. oncophora L3 larvae were obtained by coprocultures using standard procedures [32] and kept in water at 10°C until use. Larvae used for infection were less than 3 months old. The larvae used

were from the Wageningen laboratory strain which has been maintained by regular passage through donor calves since 1967. This strain is known to have a shorter prepatent period (14 days) than field strains and contains 30% *C. surnabada* [3, 84].

Animals and experimental design 1
Twelve female Holstein-Friesian calves were raised under helminth-free conditions on a commercial farm and purchased at 3 months of age. Throughout the experiment the animals were kept indoors at the animal facility of the Faculty of Veterinary Medicine in Utrecht. Animals were housed in one group, and were fed hay and water ad lib and concentrates at a maximum of 500 gr per day. Prior to infection animals were checked for general health conditions and faeces were collected to test the worm-free status of the animals.
Eight calves were infected orally with a single dose of 100,000 *C. oncophora* L3 larvae and four calves were kept as non-infected controls. Faeces were collected from the rectum once a week during the first two weeks of the infection and thereafter three times every week. Two blood samples, one for serum and one on heparine, were collected from the jugular vein one week before infection and weekly thereafter from day 7 to 42 p.i.. At day 42 p.i. calves were necropsied.
This experiment was part of a larger one with 36 animals. Twenty-four animals were infected and 12 animals served as uninfected controls. Necropsy of 8 infected and 4 non-infected calves was performed at days 14, 28 and 42 p.i.. Because the calves slaughtered at the earlier time points could not yet be allocated to a certain responder type, immunological data are not included in this paper.

Animals and experimental design 2
In order to support some of the results a supplementary group of 24 animals from a previous experiment was included in the analysis of the humoral response. Those animals were from an experiment carried out in 1993 at the University of Wageningen (Ploeger et al., unpublished results). Twenty–four, male Holstein-Friesian calves were infected with a single dose of 100,000 L3 *C. oncophora* larvae at 3 months of age. Before infection the animals were reared under helminth-free conditions at the animal husbandry facility of the Wageningen University. Faeces was collected from the rectum once a week from days 0 to 14 p.i., and two times a week thereafter. Blood samples for serum were collected two times a week from the jugular vein. Serum had been stored at - 20°C until use in this experiment. Necropsy of the infected animals was performed 42 days after infection. Only the serological data (isotype specific ELISA's) from this experiment were carried out by us, all parasitological data were gathered independently at the University of Wageningen were.

Parasitology
The numbers of eggs per gram faeces (EPG) were determined with a modified Mc Master technique (with a sensitivity of 50 EPG). After slaughter of the animals, intestinal washings were done according to the methods described by Eysker and Kooyman [74]. To obtain a more detailed insight in the localisation of the worm burden in the intestine in relation to the population dynamics (expulsion process) the small intestine was divided into six different segments in experiment 1 (table 1). All segments were processed separately. Worms were counted in 2% aliquots of the intestinal washings. In experiment 2 intestinal washings were done similarly, however the small intestine was not subdivided in different segments. Parasitological data from this experiment always refer to the entire small intestine starting from behind the pylorus to the ileo-caecal junction.

Isolation of peripheral blood lymphocytes
Peripheral blood monomorphonuclear cells (PBMC) were isolated using a density gradient technique with Ficoll-Paque (Pharmacia Biotech, Germany). Cells were resuspended in RPMI-1640

(Gibco BRL, Paisley, UK) supplemented with penicilline (500 units/ml), streptomycin (0.5 mg/ml) (P/S); 10% foetal calf serum (FCS) and glutamine (2mM) (complete medium) at a concentration of $2*10^6$ cells/ml and kept on ice until staining.

TABLE 1. Identification of the different segments in which the small intestine was divided at necropsy

Segment	Localisation	anatomical nomenclature
S1	first meter after the pylorus	duodenum
S2	next 5 meters	proximal jejunum
S3	next 5 meters	middle jejunum 1
S4	next 5 meters	middle jejunum 2
S5	next 5 meters	distal jejunum
S6	last part of the small intestine	ileum

Flow cytometric analysis of lymphocyte subset in the peripheral blood

Monoclonal antibodies to different bovine surface antigens used in this experiment are described in table 2. Irrelevant mouse serum was used as negative control.

Hundred microliter (µl) of cells at a concentration of $2*10^6$ cells/ml were incubated with the optimal concentrations of antibodies. After washing twice, goat-anti-mouse-FITC (Becton-Dickinson, San Jose, USA) was added to the cells. All incubations were performed on ice, in the dark, for 30 minutes with a total volume of 50 µl. All dilutions and washing steps were done in ice cold phosphate buffered saline (PBS) supplemented with 4% BSA (fraction V, Sigma) and 0.1% NaN_3 (FACS buffer).

After the final incubation step cells were washed twice and fixed for 10 minutes on ice with 100 µl of 0.1% paraformaldehyde in PBS. Cells were washed and resuspended in 100 µl FACS buffer and stored at 4°C in the dark until FACS analysis. Analysis was done on a flow cytometer (FACS-Calibur, Becton-Dickinson) either on the same day or one day after staining.

TABLE 2. List of monoclonal antibodies used in this experiment

Antigen identified	Mab (Clone)	Reference	Application
Bovine CD3	MM1A	Vet Immunol Immunopathol 1993; **39**: 85	FACS analysis
Bovine CD2	ILA42	Vet Immunol Immunopathol 1993; **27:** 43	FACS analysis
Bovine CD4	ILA11	J immunol 1986; **136**: 4385	FACS analysis
Bovine CD8	ILA105	ECACC (http://fuseii.star.co.uk)	FACS analysis
Bovine TCR1-N12	CACT61A	Vet Immunol Immunopathol 1993; **39**:161	FACS analysis
Ovine MHC II	VPM54	VMRD, Pullman, WA, USA	FACS analysis
Bovine BB-2	BAQ44A	Immunology 1989; **68**: 365	FACS analysis
Bovine IgG1	K372g6	Sigma, St Louis, MO, USA	ELISA
Bovine IgG2	BG-27	Sigma, St Louis, MO, USA	ELISA
Bovine IgA	K842F9	Serotec, Oxford, UK	ELISA
Ovine IgE	IE7	Parasitology 1997; **114**:395	ELISA

Leucocyte differentiation
A bloodsmear on a glass slide was made once a week from blood collected on heparine. The slides were Giemsa stained. Of each calf 2 bloodsmears were made and 200 cells were counted on each slide.
The total number of leucocytes per ml blood was counted with an automatic cell counter (Celltrac) after lysis of the red blood cells with 4% saponin. Each count was performed at least in duplicate. Results were expressed as follows: Absolute number of eosinophils = number of eosinophils counted/200 x number of leucocytes/ml or as the % of cells: e.g. % of eosinophils = number of eosinophils counted/ 200.

Isolation and preparation of *Cooperia*-specific antigens
C. oncophora L3 were exsheated by incubation in 0.1% hypochlorite solution in water under continuous shaking at 37°C. Exsheathment was monitored microscopically. Exsheated larvae were washed twice in PBS (3:1 v/w) and subsequently centrifuged at 2,000 x g for 10 minutes. Then the larvae were homogenised using a mortar and pestle which were kept in liquid nitrogen for 5 minutes prior to use. Droplets of the larval suspension were pelleted into powder which was collected and kept on ice until fluid again. The extract was centrifuged at 15,000 x g at 4°C for 20 minutes and the supernatant containing the L3 antigen extract was collected for further use.
Adult *C. oncophora* worms were obtained from donor calves at day 18 p.i. by a modified Baermann technique. Worms were washed at least three times in PBS supplemented with 2% P/S and stored at -80°C until antigen preparation. Worms were grinded with a dounce homogenizer and ice cold PBS (2:1) was added. The extract was centrifuged at 15,000 x g at 4°C for 20 minutes. Supernatant was collected and one volume of a stock protease inhibitor Complete Mini solution (1 tablet in 10 ml MilliQ) (Promega, Madison, USA) was added to 7 volumes of supernatant.
Cooperia adult excretory/secretory products (ES) were obtained as has been described before for *Haemonchus contortus* [16].
All supernatants were sterilised by filtration (low protein binding membrane, 0.45 μm, Corning) and checked for bacterial contamination. Protein concentration was determined by the Bradford assay. The antigen extracts were stored at -80°C until further use.

ELISA

Cooperia-*specific antibody isotype specific ELISA*
The *Cooperia*-specific elisa as used by Poot et al. [178] was modified to be used for the detection of IgA, IgG1 and IgG2 isotype specific antibodies against *Cooperia* L3, Ad and ES antigen. Elisa plates (Greiner high binding, cat 655093) were coated overnight at room temperature with 100 μl/well of antigen (5 μg/ml) diluted in carbonate-bicarbonate buffer (pH=9.6). All subsequent incubations were done with 100 μl for 1 hour at 37°C and between each step plates were washed at least three times with MilliQ containing 0.05% Tween. After coating, wells were blocked with 100 μl of PBS containing 0.1% gelatine (blocking buffer). All sera were diluted in blocking buffer (1:100) and incubated in duplicate. On each plate a standard positive and standard negative serum control were included. The standard positive serum was obtained from a calf repeatedly infected with *C. oncophora*. Following incubation with monoclonal mouse anti bovine IgG1 (1:1,000), IgG2 (1:2,000) and IgA (1:100) antibodies (table 2), plates were incubated with alkaline phosphatase conjugated goat-anti-mouse antibodies (DAKO, Denmark). Finally 100 μl P-nitrophenyl phosphate disodiumsalt substrate solution (PNPP, Pierce, USA) was added to each well. The enzymatic reaction was allowed to proceed for 30 minutes at room temperature after which plates were further incubated at 4°C. Plates were read on an automated ELISA reader

(CERES UV 900 C) at a density of 405 nm after 1½ hour total incubation (IgG1) or after overnight (14-16 hours) incubation (IgG2 and IgA). Optical densities were recalculated to a percentage of the OD value of the positive reference serum (PROD).

$$PROD = \frac{\text{Average of (OD sample-OD blanco)}}{\text{Average of (OD standard positive serum-OD blanco)}}$$

The blanco is the average of the OD of wells with no serum incubation.

Total IgE ELISA

The total serum IgE was measured using the monoclonal anti-ovine IgE (IE7) in a sandwich ELISA as described for *H. contortus* [136]. The cross-reactivity and specificity of this monoclonal for bovine IgE has been demonstrated recently [137].

Analysis

Data were analysed by comparing between different groups using the Kruskal-Wallis and the Mann-Whitney test. Significant results are depicted as follows: * ($P<0.05$), ** ($P<0.01$) and *** ($P<0.001$). A value of $P<0.1$ is defined as a tendency and marked with $^{+}$.

The following parasitological variables were used as a measure of host responsiveness. The sex ratio was defined as the percentage male worms (= (number of male worms/sum of male and female worms) x 100). As male worms are expelled first [3, 48, 229], this parameter is useful as an indicator of worm expulsion. Based on the distribution of worms in the gut at days 14, 28 and 42 p.i. (data not shown) we used the sex ratio in S2, which is the predilection site of the adult worm population. For experiment 2 we used the sex ratio in the worm burden of the whole small intestine.

The ratio EPG was defined as the ratio: mean EPG of week 5-6 p.i. (end in the egg excretion pattern)/mean EPG week 3-4 p.i. (peak of egg excretion pattern). This parameter features the effect of the host immune response on both the expulsion of the worm population and worm fecundity. Analysis revealed that the parameter ratio EPG is significantly positively correlated with the number of eggs per female worm (exp 1: $R=0.82$ ($n=8$ and $P<0.01$); exp 2: $R=0.67$ ($n=24$, $P<0.001$)).

Finally, to confirm our hypothesis that the humoral immune response in intermediate responders has an effect on the worm infection in contrast to what we expect in the low responders, we correlated the individual antibody titres with the corresponding EPG values during the patent phase of the infection (day 21-day 42 p.i.). For this analysis weekly serological data and corresponding EPG values from all the animals were included. This resulted in a correlation analysis with $n=8$ for the low responders and $n=20$ for the intermediate responders in experiment 1 and $n=51$ for the low responders and $n=144$ for the intermediate responders in experiment 2. All correlations were evaluated with Pearson's correlation coefficient.

RESULTS

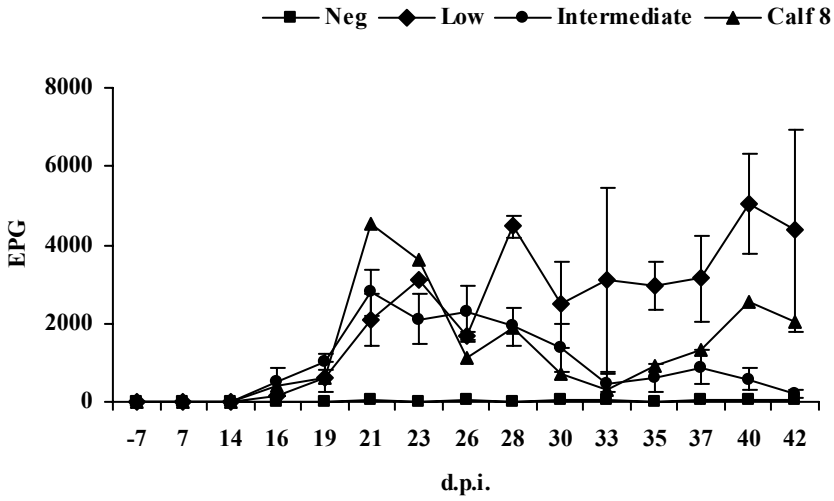
Classification into responder types

Experiment 1

Based on the faecal egg output (fig. 1), the necropsy results and the ratio EPG (table 3) we could classify the animals of experiment 1 into two low and five intermediate responders in this experiment. None of the infected calves could be classified as a high responder. In all

animals the egg output started between days 14 and 16 p.i., and a peak was reached from day 21 to day 28 p.i.. The low responders maintained this high EPG throughout the infection. In the intermediate responders a decrease in egg output was observed, with an individual variation in the rate of decrease. In those animals the egg output was reduced by 90% of the peak at day 42 p.i.. In calf 8, classified neither as a low nor as an intermediate responder, initially a 90% reduction in EPG occurred but thereafter the egg output increased again.

FIGURE 1 The EPG pattern (+/- SD) of the different responder types after infection with 100,000 L3 *Cooperia.*: (■) control animals, (◆) low responders, (●) intermediate responders, (▲) calf 8. The classification into responder types was done based on the EPG and the necropsy results as depicted in table 3.



Necropsy of *Cooperia* infected calves at day 14 and day 28 p.i. demonstrated that in the early phase of the infection *Cooperia* preferentially homes in the proximal jejunum (fig. 2) with a 1:1 ratio of male and female worms.

TABLE 3. Individual necropsy results including the percentage of the worm burden in the different segments. The corresponding sex ratio for each segment is written between brackets. The ratio EPG of the infected animals is included.

		% of total burden in each segment (% males)						Ratio EPG
Respondertype	Calf	S1	S2	S3	S4	S5	S6	
Low	2	5 (90)	65 (50)	11 (29)	4 (28)	8 (21)	7 (13)	1
	4	0.1(100)	40 (60)	54 (41)	2 (67)	1 (40)	3 (47)	1.7
Intermediate	3	13 (53)	38 (44)	4 (37)	26 (54)	10 (54)	9 (55)	0.3
	5	21 (45)	65 (45)	10 (23)	2 (73)	1 (86)	1 (100)	0.4
	9	26 (60)	52 (42)	5 (48)	4 (33)	3 (17)	10 (37)	0.3
	10	14 (32)	10 (28)	3 (35)	10 (27)	33 (54)	30 (68)	0.1
	11	37 (5)	51 (7)	3 (0)	0 (0)	0 (0)	7 (47)	0
Calf 8	8	12 (68)	47 (56)	10 (32)	13 (33)	6 (24)	11 (35)	0.6

Almost no worms were detected in the distal gut. The necropsy results of day 42 p.i. (table 3, fig. 2) showed a relocation of the worm population with a shift to the distal gut.

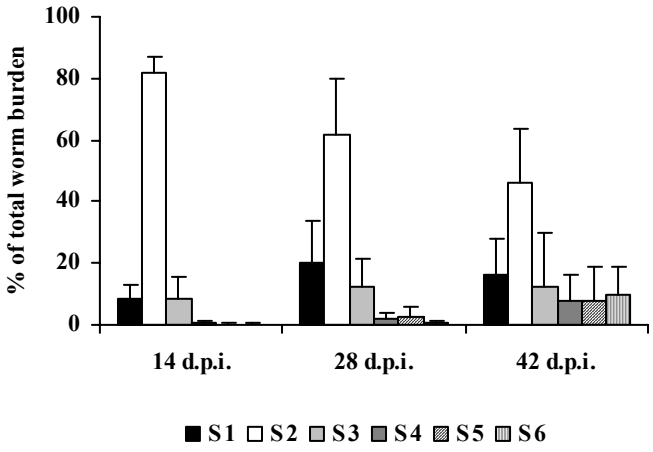
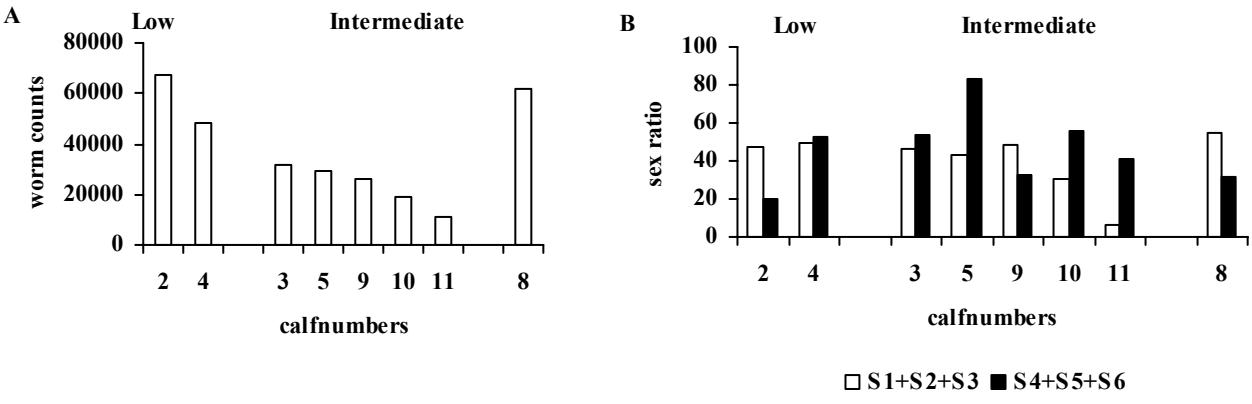


FIGURE 2 The segmental distribution of the worm population throughout the small intestine at days 14, 28 and 42 p.i. is given (n=8 at each time point).

In the intermediate responders the worm counts in the proximal half of the small intestine (S1+S2+S3) at day 42 p.i. showed a reduction of at least 25% compared to the low responders (fig. 3A). The worm burden in the proximal gut of calf 8 was higher than that of the low responders.

Both responder types also differed in the worm sex ratio in the different segments of the small intestine. Whereas in the low responders the sex ratio in the distal gut was lower or equal to the sex ratio in the proximal gut, four out of five intermediate responders showed a higher sex ratio in the distal gut, indicating a shift of the male worm population to the distal gut (fig. 3B). Together, the necropsy data indicated an ongoing expulsion of the worm population in the intermediate responders but not in the low responders.

FIGURE 3 The cumulative worm counts of the proximal segments S1, S2 and S3 at 42 DPI are depicted for the different responder types (A). The difference in sex ratio in the proximal (S1+S2+S3 □) and the distal gut (S4+S5+S6 ■) is illustrated for the different responder types (B).



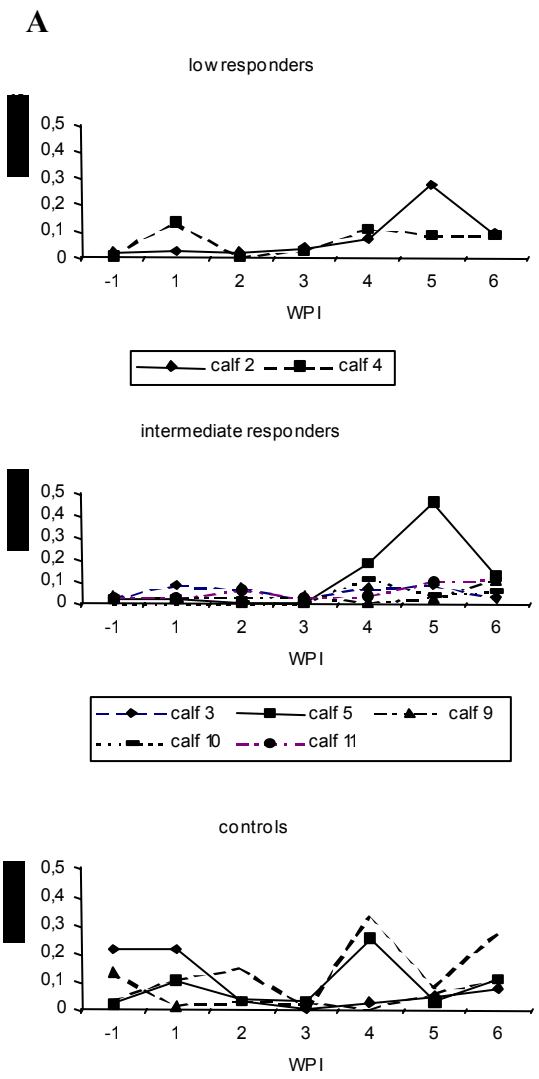
Low worm numbers were recovered from the control animals slaughtered at day 42 p.i. (range: 200-1750) due to cross contamination during the experiment. Because the number of worms was low, egg output was minimal (50-150) and infection only could have occurred late in the experiment, this finding is considered inconsequential for comparison between control and infected calves.

Experiment 2

This experiment comprised a group of 24 calves which were all infected with 100,000 *C. oncophora* larvae. Classification in low and intermediate responders was done based on the EPG pattern, the ratio EPG and the necropsy results (total worm counts and sex ratio at day 42 p.i.). A threshold for the ratio EPG was set at 0.4, which implied that animals which had a reduction in their egg output of at least 60% by day 35-42 p.i. were classified as intermediate responders. Animals with a ratio EPG higher than 0.4, were classified as low responders. This classification resulted in 6 low and 16 intermediate responders. Analysis of the necropsy results revealed significantly lower worm counts in the intermediate responders (mean +/- SD= 23,976+/-25,745) compared to the low responders (mean +/- SD= 61,810+/-34,279) (P=0.015). Also the sex ratio in the intermediate responders was lower than in the low responders (29+/-13% versus 47+/-1%; P=0.05), emphasizing the ongoing expulsion of the worm population in the intermediate responders. Two animals had a similar EPG pattern as calf 8 from experiment 1 and could not be classified as any of the responder types (calf 34 and 39). Therefore, they were analysed separately.

Leucocyte differentiations

Throughout the infection peripheral eosinophil counts were highly variable both in infected (range absolute numbers 13 – 272*10^3 eosinophils/ml) and non infected animals (range absolute numbers 19- 250*10^3 eosinophils/ml) which did not allow us to interprete the results in view of the infection. Before infection calf 1 (control animal) had the highest number of peripheral blood eosinophils. This dropped to basal level (23*10^3 eosinophils/ml) by week 2. Twenty-eight days after infection we found that the percentage of eosinophils in four infected animals (calf 4, 5, 8 and 11) was higher than in negative control animals. At day 35 and day 42 p.i. a higher percentage was found in 5 (2, 4, 5, 8 and 11) and 6 infected animals (2, 4, 5, 8, 9 and 11), respectively. However, this pattern was not found for the absolute number of eosinophils. The course of the absolute number of eosinophils during the infection is depicted for the individual animals in fig. 4A.



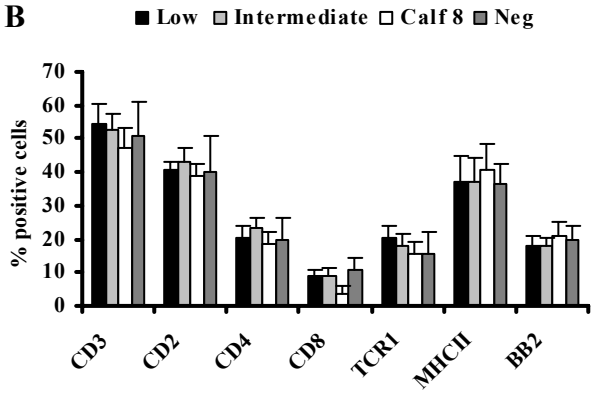


FIGURE 4 Weekly fluctuations of absolute number of peripheral blood eosinophils in the infected and control animals are depicted. Data are mean of duplicates. The absolute number was obtained by multiplying the obtained percentage with the number of leucocytes (A). The mean percentage (+/- SD) of the lymphocyte subsets of the blood circulation during the whole period of the experiment was calculated and compared between the different responder types and the control animals (B).

Flow cytometric analysis of lymphocyte subsets in the peripheral blood

Figure 4B represents the mean % of lymphocyte subsets obtained by flow cytometric analyses throughout the infection. The results depicted were obtained by calculating the mean value of the weekly average % for the different responder types. In all animals CD3+ cells accounted for the largest population with no significant differences between infected and control animals. In both control and infected animals the percentage of CD4+ cells remained within the normal range (infected: 13.8-27.3%; control: 14.9-23.4%) throughout the infection. The number of CD4+ and CD8+ cells together was continuously lower than the number of CD2+ cells which indicates the presence of a distinct CD2+ population which is negative for CD4 and CD8 (data not shown). During the whole course of the experiment

the number of CD8+ cells in the control animals was consistently lower compared to the infected animals.
Infection did not appear to affect the MHCII+ and BB2+ subsets of the PBMC's. In the control animals the BB2+ subset ranged from 12.8 to 21.9% compared to 11.8 to 22.9% in the infected animals. Similarly, the MHCII+ subsets in the control animals ranged from 27.3 to 51.5 % whereas the range in the infected animals was from 24.2 to 46.6%.

ELISA

Cooperia-*specific antibody isotype specific ELISA experiment 1*
Exposure to a primary *C. oncophora* infection with 100,000 L3 resulted in high levels of ES-specific IgG1 in all infected animals (fig. 5C). The L3 and Ad-IgG1 response was different for both responder types (fig. 5A and 5B). The *Cooperia* Ad-specific IgG1 in the intermediate responders was significantly higher compared to the low responders from day 35 p.i. onwards (P=0.05). The *Cooperia* L3-specific response revealed a higher variability in response, albeit 42 days after infection in the low responders we did measure lower levels of IgG1 (PROD 15.5 and 37.7 for calf 2 and 4 respectively) compared to the intermediate responders (PROD range 41.9-90.6).
Throughout the infection no anti-parasite IgG2 antibodies were demonstrable in the infected animals (data not shown).

TABLE 4. Pearsons correlation coefficient between the serum *Cooperia*-specific IgG1, IgA, total IgE and parasitological parameters expressing worm expulsion (sex ratio S2 and ratio EPG), exposure to the parasite (total worm burden and worm burden S2), and immunity against worm fecundity (EPG patent infection (day14- day 42 p.i.) and ratio EPG). Significant results are written as follows: *: P<0.05; **: P<0.01; ***: P<0.001. A tendency is defined as P<0.1 and depicted with no superscript. "none" is defined as R value corresponding to P>0.1.

					EPG patent infection		Wormburden	
	Isotype	Antigen	sex ratio	ratio EPG	Low	Intermediate	Total	S2
Experiment 1	IgG1	L3	-0.67	-0.65	+0.77*	-0.61**	none	none
		Ad	-0.56	-0.85*	none	-0.67**	none	none
		ES	-0.62	none	+0.49*	-0.72**	none	none
	IgA	L3	none	none	none	none	none	none
		Ad	none	none	+0.86*	-0.57**	none	none
		ES	none	none	+0.77	-0.72**	none	none
	tot IgE		none	none	none	none	+0.57	+0.6
Experiment 2	IgG1	Ad	-0.36	-0.48*	none	-0.31**	none	ND
	IgA	Ad	none	none	0.2	0.11	none	ND

During the patent phase of the infection a significant negative correlation between the *Cooperia*-specific IgG1 titres and the EPG was found in the intermediate responders (table 4). On the contrary, this correlation was positive in the low responders and calf 8. For calf 8, this relationship included only four data points and was therefore excluded from the analysis. Moreover Ad- and L3-specific IgG1 titres at day 42 p.i. were negatively correlated with the ratio EPG ($P<0.05$ and $P<0.1$ respectively) and the sex ratio ($R=-0.56$ and $P<0.1$ respectively), both parameters related to host responsiveness to the infection. A tendency to a negative correlation between ES-specific IgG1 and the sex ratio was also observed.
A slight increase in serum *Cooperia* L3-, ES- and Ad-specific IgA levels was observed in the intermediate responders from day 21 p.i. onwards (fig. 5B). Calf 8, neither an intermediate nor a low responder showed the most pronounced increase in *Cooperia*-specific IgA, starting from day 28 p.i. onwards. Low responders did not show any *Cooperia*-specific IgA response.

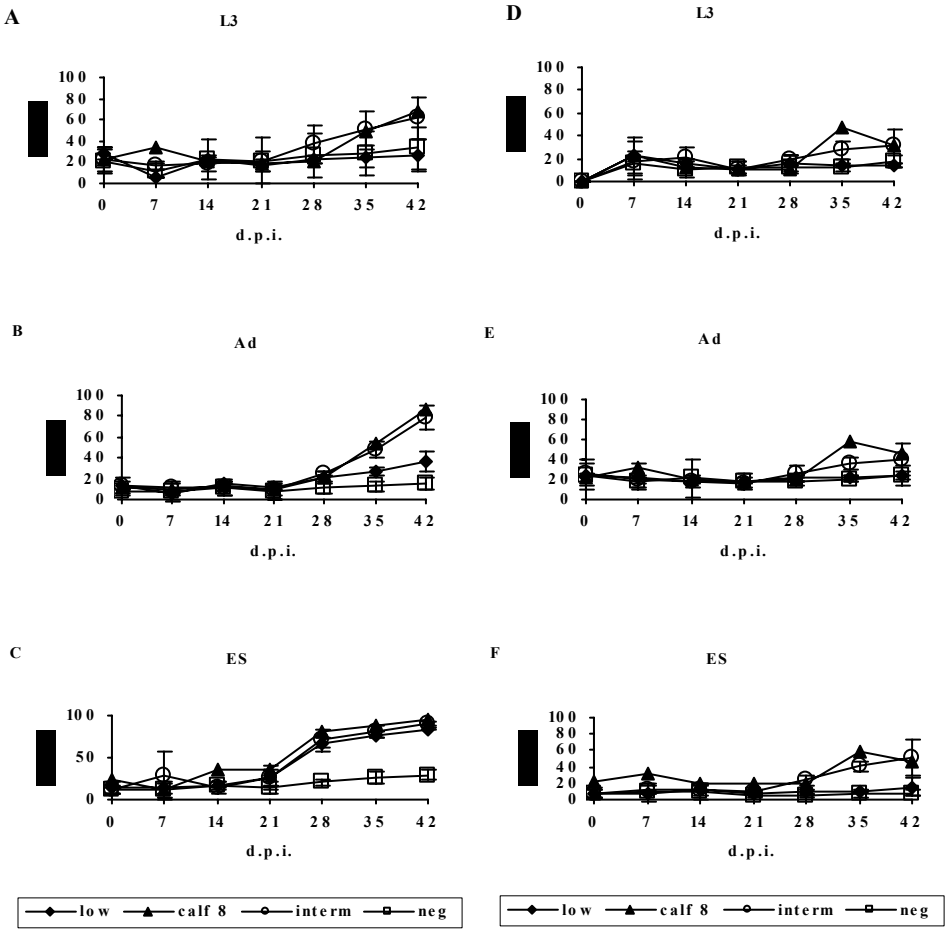


FIGURE 5 *Cooperia*-specific IgG1 (A-C) and IgA (D-F) titres against L3 crude worm antigen (L3), adult worm crude worm antigen (Ad) and adult excretory/secretory products (ES). All animals except control animals were infected with 100,000 L3 *Cooperia* at day 0. Data shown are the mean of duplicates +/- SD and given as the percentage of the OD value of the positive reference serum (PROD).

The serum IgA response of the intermediate responders to *Cooperia* ES and Ad showed the same correlation with the EPG as was seen with IgG1 (Ad ($P<0.05$) and ES ($P<0.001$)). Similar to the IgG1 titres, for both antigens a positive correlation was found in the low responders ($P<0.05$).

Cooperia-*specific antibody isotype specific ELISA experiment 2*

In an attempt to confirm some of the observed correlations between the *Cooperia*-specific humoral response and parasitological variables, isotype specific ELISA's were performed with sera from animals of experiment 2. Only the reaction with *Cooperia* Ad antigen was tested as this seemed to reveal the highest differences between low and intermediate responders.

In all infected animals the *Cooperia*-specific IgG1 increased from day 28 p.i. on, with the increase being higher in the intermediate than the low responders. This increase resulted in more elevated IgG1 level in the intermediate responders at day 42 p.i. (PROD range: 70-103 versus 66-95 in the low responders ($P=0.22$)). Similar to what we observed in experiment 1 infection did not induce the production of *Cooperia*-specific IgG2.

Compared to experiment 1 the Ad-specific IgA titres in all animals were higher (PROD exp 1 at day 0 p.i. = 13.6-45.4 versus PROD exp 2 at day 0 p.i.: 17.5-63.8). In the intermediate responders at day 39 p.i. a non-significant increase in *Cooperia* IgA was measured, whereas for the low responders this was measured at day 35 p.i.. In both responder types by day 42 p.i. the mean IgA levels dropped to baseline level (data not shown). Strikingly, calf 34 and 39, which showed a similar EPG pattern as calf 8 from experiment 1 had significantly increased *Cooperia* Ad IgA at day 42 p.i. ($P<0.05$, data not shown).

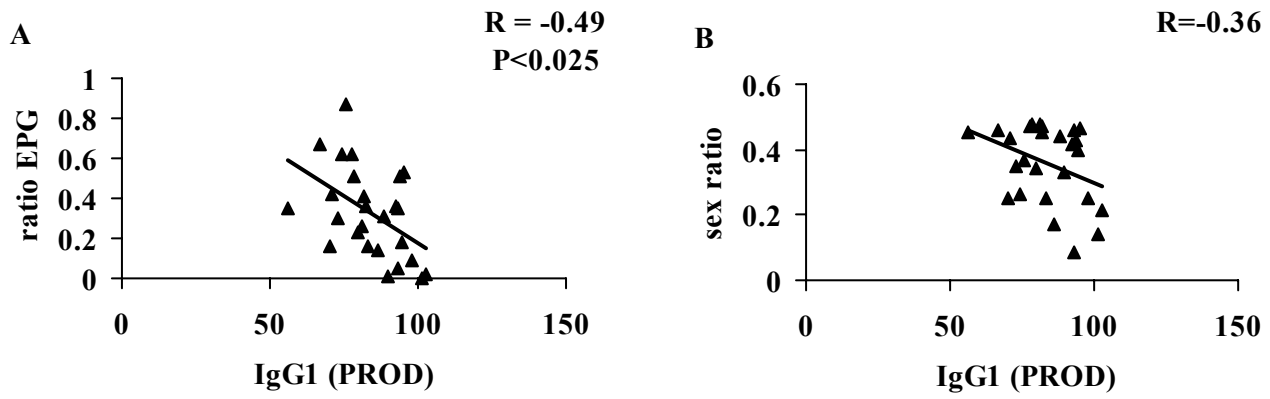


FIGURE 6 Plots of *Cooperia* Ad-specific serum IgG1 versus ratio EPG (A) and sex ratio (B) of the animals of experiment 2. Data of both intermediate and low responders are included.

The correlations between the isotype specific antibody response and parasitological variables showed a similar pattern as in experiment 1, albeit that the R values were lower (see table 4). The negative correlation between *Cooperia* Ad-specific IgG1 titres with the ratio EPG (R=-0.49, P<0.025) and with the sex ratio (R=-0.36, P=0.1) are depicted in fig. 6 A and B, respectively. Figure 7A and 7B show the relationship between the *Cooperia* Ad IgG1 and IgA titres and the EPG during the patent phase of the infection**.**

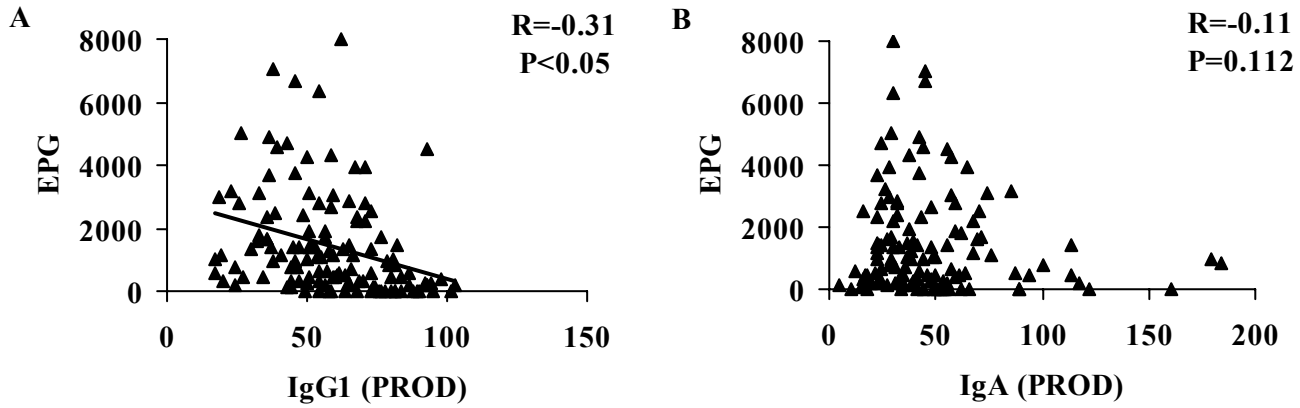


FIGURE 7 Plots of the correlations between *Cooperia* Ad-specific serum IgG1 (A) and IgA (B) with the EPG during the patent phase of the infection (day 14 to 42 p.i.). Data are derived from animals of experiment 2.

Total IgE titres

In all but two infected animals (calf 5 and 9) a marked rise in serum total IgE level was found during infection from day 14 p.i. onwards (fig. 8).

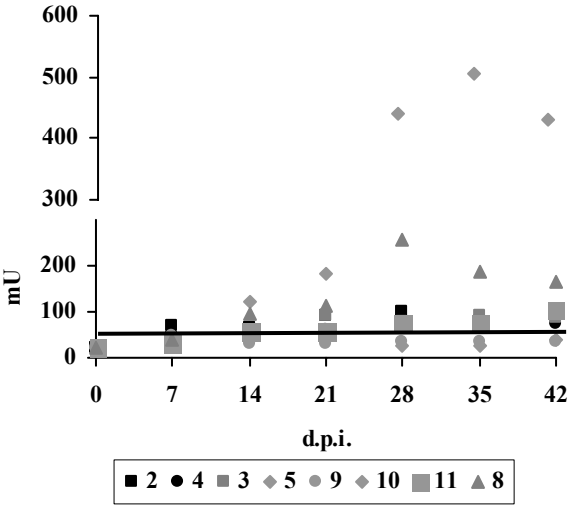


FIGURE 8 Individual values of total serum IgE levels for all infected calves. Data were transformed as described previously [136] and are expressed as mU. The cut off value (—) was set at: overall mean + 1 x SD of the serum total IgE of the four control animal

The difference in total IgE levels was individual and not related to the responder types. Forty-two days after infection, in the infected animals a positive linear correlation was present between the total worm burden as well as the worm burden in S2 and the total serum IgE level ($R=0.57$ and $P<0.1$, respectively) (table 4). No correlation was present with the sex ratio and the ratio EPG.

DISCUSSION

After primary infection of 3-month-old calves with *C. oncophora* we could distinguish three major responder types based on parasitological variables [231]. In this study, the systemic immune response of two of these responder types was investigated. Based on the EPG and the necropsy results we classified the infected calves of experiment 1 in two low and five intermediate responders. Calf 8 did not fit the classification and was analysed separately. The intermediate responders expelled their worm population before or around time of necropsy (day 42 p.i.), whereas the low responders and calf 8 did not. This was evidenced by a shift from the proximal to the distal gut in the adult worm population of the intermediate responders, as well as a higher sex ratio in the distal gut. This is similar to laboratory animal models in which it has been demonstrated that worms are redistributed proximal and distal to their predilection site just before they are expelled [161]. We classified the animals of experiment 2 in 6 low and 16 intermediate responders based on both their necropsy results and the EPG. We could not monitor the dynamics of the worm population throughout the small intestine as no segmental division had been made. However, the lower worm burden and sex ratio in the intermediate responders are an indication for an ongoing expulsion as found in experiment 1.

A slight increase was present in the number of peripheral blood eosinophils in six out of eight infected animals (experiment 1). However the same fluctuations were observed in the non infected controls. In all animals a weekly fluctuation in this cell population occurred. No evidence of a different quantitative peripheral blood eosinophil response between the intermediate and low responder types was present. Although eosinophils are known to be associated with helminth infections the different reports are not conclusive concerning their role during a parasite infection [157, 24]. To get more conclusive data on the role of eosinophils during a *Cooperia* infection we have to look at the local eosinophil response as well as the functional activity of the detected eosinophils.

The present study showed no major changes in different lymphocyte subsets in peripheral blood due to the infection based on analysis of the different subpopulation over time, (data not shown). During the entire course of the experiment the control animals showed a lower percentage of CD8+ cells. However, this was also observed prior to infection and can be due either to biological differences between the animals or to a haplotype dependent affinity

of the anti-bovine CD8 monoclonal used in this study. The cyclic pattern in the percentage of lymphocyte subsets has been described before and was attributed to physiological effects [243]. No changes were observed in the B-cell population nor in the MHCII+ and the CD4+ populations. The higher number of CD2+ cells compared to the sum of CD4+ and CD8+ cell could be attributed to a subset of the TCR1+ γδ T cells which is CD2+WC1- and occurs in the blood circulation [246]. Previous studies of gastrointestinal nematode infections in ruminants reported an increase in CD4+ cells, B cells and γδ T cells early after infection [5, 101, 154]. However, those cellular changes occurred in local tissues and draining lymph nodes. Data from experiments in which the systemic cellular immune response after gastrointestinal nematode infections was studied, lack consistency. This suggests that parasite-specific cells in blood are recirculating from the mucosal sites where the parasites are located. Thus, monitoring the local events during infection might yield more evidence of a role for the cellular immune response.

Cooperia-specific IgG1 and IgA titres were measured in the infected animals of experiment 1 from day 21 p.i. onwards. Except for the ES-specific IgG1 levels, the intermediate responders consistently produced higher titres of parasite specific antibodies than the low responders. This was also true for experiment 2, although the quantitative difference was not as big. The induction of *Cooperia*-specific IgG1 after a primary infection has been described before [176]. Similar findings were reported for other gastrointestinal nematodes in ruminants such as *O. ostertagi* in calves [150, 112, 88] or *H. contortus* in sheep [97, 195]. However, the finding that IgG1 levels after a primary *Cooperia* infection enable to differentiate between responder types is new.

In both experiments the parasite-specific humoral immune response was significantly correlated to parasitological parameters of infection. At day 42 p.i. a negative correlation is present between the *Cooperia*-specific IgG1 titres and the sex ratio in S2, and also with the ratio EPG, parameters linked to the expulsion and the fecundity of the worms. Furthermore, as can be expected based on their different EPG pattern, both specific IgG1 and IgA titres of the intermediate responders were significantly negatively correlated with the EPG during the patent infection while a positive correlation was found for the low responders. Those correlations were all significant in experiment 1 ($P<0.05$). In experiment 2 the *Cooperia* specific IgA showed the same tendency although not significant ($P=0.1$). Our results suggest an effector role for L3 and Ad specific serum IgG1 during a primary *Cooperia* infection. The role of ES-specific IgG1 seems to be more related to the level of the infection as in all infected animals similar levels were detected.

In both experiments we could not measure a significant correlation between serum IgA titres and parasitological parameters expressing worm expulsion. Throughout the infection the IgA levels remained low or were absent. However, a role for IgA is not excluded as it is

known to be a mucosae-associated Ig and peripheral titres might not have a predictive value. Moreover, both IgG1 and IgA seem to have an association with the egg output and hence the fecundity of the worms. A negative correlation between IgA and fecundity has also been reported in sheep infected with *Teladorsagia circumcincta* [212]. The higher levels of IgG1 and IgA in intermediate responders than in low responders emphasises a possible role for the humoral response in the host protective mechanisms.

In this study we could not detect *Cooperia*-specific IgE using an isotype specific Elisa with *Cooperia* Ad and ES antigen (data not shown). The difficulty to measure parasite specific IgE has been reported before [188]. This can be attributed to a lack of sensitivity of our assay or to competition of the low % of serum IgE with the more abundant isotypes such as IgG1. Measurement of total serum IgE revealed an increase in some of the infected animals. No difference was seen between the intermediate and low responders and in two infected animals (one intermediate responder and one low responder) we could not measure any serum IgE. Surprisingly, the rise in total serum IgE started as early as day 14 p.i., 7 to 14 days before the induction of *Cooperia*-specific antibodies. The knowledge that a Th2 skewed response leads to the transcription of epsilon and gamma mRNA by a common IL-4 dependent mechanism, implies that the increase of IgE early after infection is induced by a different mechanism [78]. However, regulation of IgE responses does not always require antigen specific responses, as crosslinking of the CD40 molecule on the B cell alone is sufficient to elicit a polyclonal IgE response [49]. This could be an explanation for the early onset of IgE production in the infected animals. The total serum IgE level was positively correlated with the total worm burden as well as the worm burden in S2 of the infected animals. On the contrary, no correlation was present with parasitological parameters expressing host responsiveness. Therefore, our data suggest rather a quantitative than a qualitative relation between IgE and the level of parasitosis in the infected animals. Similarly, in a study in which calves were infected with different doses of the intestinal nematode *Cooperia punctata*, the total serum IgE levels were positively correlated to the level of exposure of the animals (F. N. J. Kooyman, unpublished observation).

Taken together, our results showed that a primary infection with *Cooperia* in calves induced a type 2 shift in the immune response. Although the Th1/Th2 paradigm is not so clear in ruminants [36], the bias of the serological immune response towards IgG1 and IgE is commonly associated with a Th2 profile. Moreover, the results suggest that intermediate and low responder calves differ in their ability to initiate a Th2-type response. Based on the observed correlations, we propose an effector role for the humoral response against *Cooperia* infections in the gut. A study of the local responses is ongoing to address the possible mechanisms involved in the regulation of an effective local response against *C. oncophora*. The finding that the different responder types based on parasitological variables

also feature a different immune response is very interesting as this also provides the opportunity to study the influence of genetic components of the host response.

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