Immunity in calves against *Dictyocaulus viviparus* following a low primary infection

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SUMMARY

Thirty Holstein Friesian heifer calves, 3 months of age, were used to evaluate whether a low primary infection with *Dictyocaulus viviparus* would result in immunity against challenge infection 5 or 12 weeks later. Fifteen calves were experimentally infected with 30 larvae at day 0, while the other calves remained as uninfected controls. On day thirty-five 6 primary infected (G1) and 6 non-infected calves (G2) were challenged with 2000 larvae. These groups were necropsied on day 70. On day 84 the remaining 9 primary infected (G3) and 9 non-infected (G4) calves were similarly challenged and necropsied on day 119. Respiratory signs developed in most calves from approximately 2 weeks after challenge, but signs were more severe in G2 and G4 than in G1 and G3. The low primary infection resulted in significantly reduced faecal larval excretion (> 80 %) and worm burdens (> 70 %). In the primary infected groups the proportion of female worms in the worm burden was increased, suggesting that part of the developed immunity affected survival of the worms after their establishment in the host. Worm lengths and worm fecundity were also significantly reduced. All observed effects were stronger following challenge on day 84 than on day 35, but the effect of the duration of a primary infection (contrast between G1 and G3) was only statistically significant in the case of mean faecal larval excretion on the day of necropsy ($P < 0{\cdot}05$) and fecundity ($P < 0{\cdot}05$).

Key words: *Dictyocaulus viviparus*, immunity, primary infection.

INTRODUCTION

Dictyocaulus viviparus infections are a common cause of respiratory disease in cattle in the temperate areas of the world, particularly in calves of dairy cattle. A shift, however, towards lungworm disease in older age classes, particularly in dairy cows, has been demonstrated in the UK (David, 1993, 1997). The same trend is suspected to occur in other countries in Western Europe, though no hard data are available to support this. The reasons for this shift of lungworm disease to older age classes are not known, but it may be associated with the extensive use of chemoprophylactic systems against parasitic gastroenteritis in the first and second grazing seasons. Because of the suppression of lungworm infections through these systems young cattle might not be able to build up immunity against these infections and, as a consequence, be unprotected against lungworm as adults.

To enable the design of rational control methods in young and adult cattle it is necessary to understand the population dynamics of *D. viviparus* infections in detail. Recently a first simulation model for the population dynamics has been developed for *D. viviparus* infections (Ploeger & Eysker, 2000). In the course of the modelling process many gaps in our knowledge were identified. The present paper describes the first experiment (1) to validate this model with new independent sets of data and (2) to gain more insight in the development of immunity following a low primary infection. The hypothesis was that a low primary infection with *D. viviparus* does not result in development of immunity against a challenge infection, irrespective of the interval between primary and challenge infection.

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MATERIALS AND METHODS

Animals and parasite strain

Thirty Holstein Friesian heifer calves were used. When they arrived on 1 April 1999 from a single supplier at the age of 3 months they had already been weaned. The *D. viviparus* used was from the Intervet vaccine strain. Larvae arrived in 2 batches that were both cultured in November 1998.

Experimental design

After 1 week of adaptation the calves were randomly divided into 4 groups of 6 (G1), 6 (G2), 9 (G3) and 9 (G4) calves. On day 0 all calves of G1 and G3 were experimentally infected with 30 L3 of *D. viviparus*, whereas calves of G2 and G4 served as non-infected control groups. The calves of G1 and G2 were experimentally infected with 2000 L3 of *D. viviparus*

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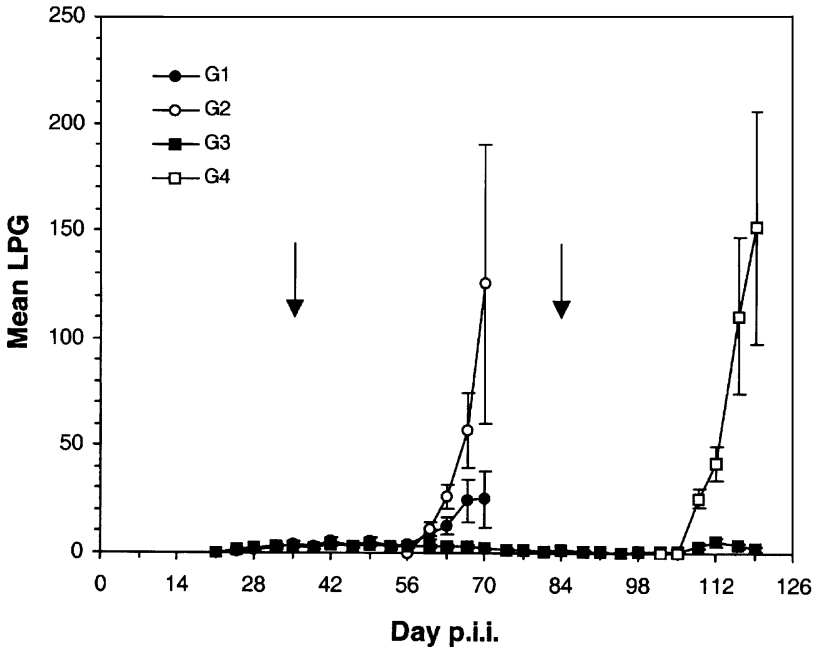

Fig. 1. Mean faecal larval excretion per group. Vertical bars represent the standard error. Arrows indicate the time of the challenge infection for groups G1 and G2 (day 35) or G3 and G4 (day 84).

on day 35 and necropsied on day 70. The calves of G3 and G4 were challenged with 2000 L3 of *D. viviparus* on day 84 and necropsied on day 119. From day 0 to day 112 six calves of both G3 and G4 were also infected experimentally twice a week with *Cooperia punctata*. The reason for this was that we also wanted to examine whether in the presence of *D. viviparus* infections an ELISA with a specific recombinant low molecular weight protein of *C. oncophora* (Poot *et al*. 1997) could be used for serodiagnosis of *C. punctata* infections. Infection doses with *C. punctata* for pairs of calves in either group were 310, 1250 and 5000 larvae, respectively. The other 3 calves in these groups served to check if any unexpected interaction between the 2 parasitic infections would occur. The *C. punctata* infections appeared to have no consequences for the population dynamics of the *D. viviparus* infections discussed in this paper. The results of the *C. punctata* part of the experiment will be presented elsewhere.

One calf in G2 died on day 67 from a bacterial complication of lungworm disease and another in G3 was killed *in extremis* on day 106 while suffering from the same. These calves were omitted from further analysis.

Observations

Faecal larval counts from 30 g of faeces were determined from each calf twice a week using the Baermann method.

Necropsy was performed using the methods described earlier (Eysker, Boersema & Hendrikx, 1990). The length of 25 intact male and female worms, or as many worms as present, was measured from each calf. Parasite fecundity was measured in 2 ways. First, for each calf the numbers of larvae per gram faeces (LPG) at necropsy was divided by the number of female worms recovered. Subsequently the results for G3 and G4 were multiplied with 1·19 to adjust for the fact that these calves were 49 days older at necropsy than those of G1 and G2 and hence produce more faeces. Secondly, after length measurement a female worm was transferred to a 14 ml vol. Falcon tube containing 6 ml of a 2% sodium hypochlorite solution. This disintegrated the entire worm liberating the eggs from the uterus. After at least 8–10 min and thorough mixing by repeatedly inverting the tube, 3 samples of 20 μl were pipetted on top of a saturated sugar solution ($\sim$ 3–5 drops) in wells of a 96-well microtitreplate. After filling a complete microtitreplate (32 worms in triplicate samples), eggs concentrated at the top of the saturated sugar solution were either immediately counted under a dissecting microscope (magnification 20 to 32 $\times$) or scanned at 10 $\times$ magnification using a Coolpix 950 digital camera (Nikon) attached to a Nikon binocular (SMZ800). Scans were stored at normal density in JPEG format and eggs were counted later visually on computer screen using any of a number of standard graphic programs.

The respiration rates of the calves were monitored weekly.

Analysis

Statistical analysis was performed with 2 ANOVA analyses. In the first the main effect was group (G1–G4) followed by assessment of contrasts between groups and the second was on 2 main effects;

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Table 1. Worm counts and measurements after necropsy and LPG at day of necropsy

Calf	Total no. of worms	No. of females (%)	LPG at day necropsy	Mean worm length ± S.D. (mm)		Fecundity LPG/female	Mean eggs in uterus ± S.D.*§
				Females	Males		
G1: 30 L3 day 0–2000 L3 day 35–necropsy day 70							
753	3	2 (67)	0·30	–‡	–	0·1500	–
757	12	8 (67)	4·53	26	19	0·5667	17·0
770	289	120 (42)	63·67	44·1 ± 19·5	31·6 ± 2·7	0·5306	58·6 ± 15·2
771	56	29 (52)	0·67	30·4 ± 5·3	22·0 ± 1·2	0·0230	26·5 ± 15·6
773	62	36 (58)	15·03	–	–	0·4176	–
779	446	246 (55)	66·87	43·3 ± 2·5	29·4 ± 3·4	0·2718	64·2 ± 11·9
Mean	145	73 (57)	25·18	35·9	25·5	0·3266	41·6
reduction†	70·3 %		80·0 %	24·3 %	25·9 %	38·5 %	49·3 %
G2: 2000 L3 day 35–necropsy day 70							
752	338	150 (44)	55·77	46·0 ± 4·0	32·7 ± 2·3	0·3718	63·0 ± 23·5
754	700	313 (45)	106·77	53·3 ± 3·6	36·7 ± 2·9	0·3422	113·7 ± 20·8
756	385	190 (49)	29·50	39·8 ± 7·5	31·0 ± 5·9	0·1553	46·4 ± 21·0
765	451	219 (49)	56·47	47·3 ± 4·3	37·0 ± 6·8	0·2578	97·9 ± 17·7
781	573	250 (44)	381·67	50·5 ± 2·7	34·5 ± 3·4	1·5267	89·5 ± 12·9
Mean	489	224 (46)	126·04	47·4	34·4	0·5307	82·1
G3: 30 L3 day 0–2000 L3 day 84–necropsy day 119							
759*	118	78 (66)	0·13	42·9 ± 7·0	27·4 ± 4·1	0·0020	46·6 ± 16·6
762	1	1 (–)	0·00	–	–	0·0000	–
768	8	6 (75)	0·07	–	–	0·1321	–
769*	41	22 (54)	6·70	38·0 ± 7·0	30·9 ± 4·1	0·3619	50·6 ± 16·9
774*	129	67 (52)	8·87	31·1 ± 5·2	25·6 ± 3·7	0·1573	27·4 ± 9·9
775	7	6 (86)	0·23	27·5 ± 3·3	–	0·0462	19·1 ± 11·4
777	7	3 (43)	0·47	–	14·8 ± 1·7	0·1849	–
778	37	22 (59)	1·43	24·3 ± 1·4	18·9 ± 1·4	0·0774	15·6 ± 7·5
Mean	43	26 (62)	2·24	32·8	23·5	0·1204	31·9
reduction†	93·7 %		98·5 %	32·5 %	34·8 %	76·8 %	62·0 %
G4: 2000 L3 day 84–necropsy day 119							
758	499	256 (51)	140·00	41·8 ± 5·3	33·3 ± 4·4	0·6500	57·1 ± 8·6
764	1033	507 (49)	536·67	52·3 ± 5·1	38·5 ± 2·2	1·2581	100·6 ± 26·0
766	1284	613 (48)	260·00	52·6 ± 3·3	38·1 ± 2·6	0·5041	93·6 ± 19·0
767	526	283 (54)	66·20	47·4 ± 3·8	37·0 ± 2·7	0·2780	89·5 ± 15·9
772	390	184 (47)	95·73	50·9 ± 3·4	34·2 ± 2·4	0·6184	81·9 ± 18·1
776	510	227 (45)	61·83	46·1 ± 5·3	35·7 ± 3·2	0·3238	89·2 ± 23·9
780	718	326 (45)	49·75	45·3 ± 3·8	35·8 ± 3·3	0·1805	85·8 ± 16·9
782	1184	621 (52)	160·07	52·3 ± 3·3	36·4 ± 2·3	0·2030	73·8 ± 22·3
783	10	3 (30)	1·63	–	–	0·6456	–
Mean	684	336 (47)	152·43	48·6	36·1	0·5179	83·9

* Some (1–6) worms from the primary infection may be included.
† Percentage reduction compared to the challenge control group necropsied on the same day.
‡ Not done, because worms were not sufficiently intact for accurate measurements or were not present.
§ Values represent the counts made in the 20 μl samples. To obtain the actual number of eggs values have to be multiplied by 300.

primed yes/no and challenge day 35/84 and interactions between these effects. When appropriate, transformations were applied to homogenize variance between groups. In addition linear and non-linear regression was applied to relate worm length with fecundity and worm burden and repeated measures analysis was done on respiration rate.

RESULTS

Faecal larval excretion

The faecal larval excretion is shown in Fig. 1. Table 1 lists the individual faecal larval counts on the day of necropsy. All primary infected calves from G1 and G3 had patent infections between days 21 and 28 and faecal larval counts increased subsequently until day 35. After the challenge infection on day 35 calves of G2 started to shed larvae on day 56 and subsequently faecal larval counts increased rapidly. Faecal larval excretion in the primary infected calves of G1 also increased following challenge but to a much lower level than in G2. On the day of necropsy (day 70) mean faecal larval counts were lower in G1 than in G2 (see Table 1; $P < 0{\cdot}005$). Mean faecal larval counts in G3 calves declined to zero in most animals between days 84 and 105. After the challenge

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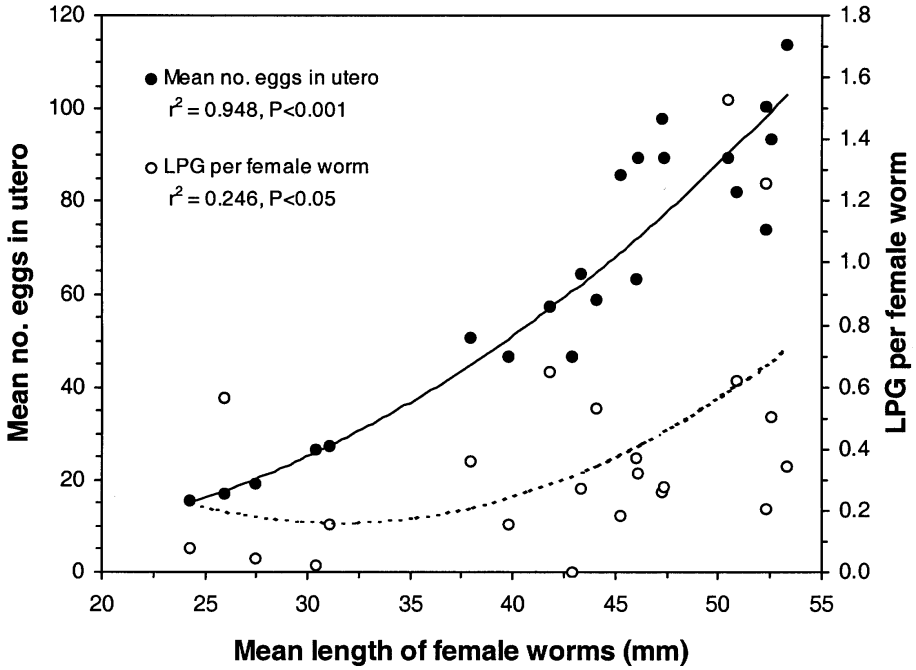

Fig. 2. The association between female worm length and fecundity measured as the number of eggs in the uterus or as the number of larvae excreted per gram of faeces (LPG) per female worm. Data represent means per calf.

infection in G3 and G4 on day 84 overall patterns in mean faecal larval counts were similar to those seen in G1 and G2. However, mean faecal larval counts remained significantly lower in G3 than in G1 after challenge ($P < 0{\cdot}05$). The contrast between G3 and G4 on the day of necropsy was highly significant ($P < 0{\cdot}001$). No statistically significant differences were found in mean LPG after challenge between G2 and G4. The interaction between the main effects 'primary infection' and 'day of challenge infection' bordered on being statistically significant ($P = 0{\cdot}0502$).

Worm counts

The necropsy data are given in Table 1. The low primary infection resulted in significantly reduced worm counts after challenge in G1 and G3 compared with G2 and G4, 70·3% and 93·7% reduction respectively ($P < 0{\cdot}005$). Though the mean worm count was lower in G3 compared with G1, the difference was not significant. The contrast between G2 and G4 was also not significant. Similar results were obtained with respect to the proportion of female worms in the total worm burden. The primary infected calves showed higher proportions of female worms (contrast G1–G2: $P < 0{\cdot}05$; contrast G3–G4: $P < 0{\cdot}005$). None of the other contrasts was significant.

Worm length and fecundity

Both male and female worms were smaller in groups G1 and G3 compared with those from G2 and G4 (see Table 1). For female worms the contrast G1–G2 bordered on significance at $P < 0{\cdot}10$ and by contrast G3–G4 was significant at $P < 0{\cdot}005$. For male worms the respective contrasts were both significant at $P < 0{\cdot}05$ and $P < 0{\cdot}005$. The aim of measuring 25 worms per calf was not met for all calves. This was not only due to limited numbers of worms recovered in some calves, but was particularly due to the fact that many worms were not intact or broke easily when handled. In total, 392 male and 403 female worms were measured. Primary infection resulted in a reduced fecundity of the worms established from the challenge infection (see Table 1). For fecundity measured as LPG per female worm this was clear after the challenge on day 84 (contrast G3–G4: $P < 0{\cdot}01$). However, the effect was not significant after the challenge on day 35. The contrast between G1 and G3 (effect of duration of primary infection) was significant ($P < 0{\cdot}05$). Similar results were obtained with fecundity measured as number of eggs in the uterus. In this case the significance of contrasts were G1–G2 $P < 0{\cdot}05$, G3–G4 $P < 0{\cdot}005$ and G1–G3 not significant. Of the 403 female worms measured 362 could be used to count eggs in the uterus. Fig. 2 shows the relationship between mean length of female worms recovered per calf and the 2 measures for fecundity. Clearly, there is a close association between length and number of eggs in the uterus. This was also found on an individual basis for all 362 worms in the data set using a simple power function ($r^2 = 0{\cdot}684$). Based on the LPG per female worm the relationship was far less close, though still statistically significant (Fig. 2). The correlation between mean number of eggs in the

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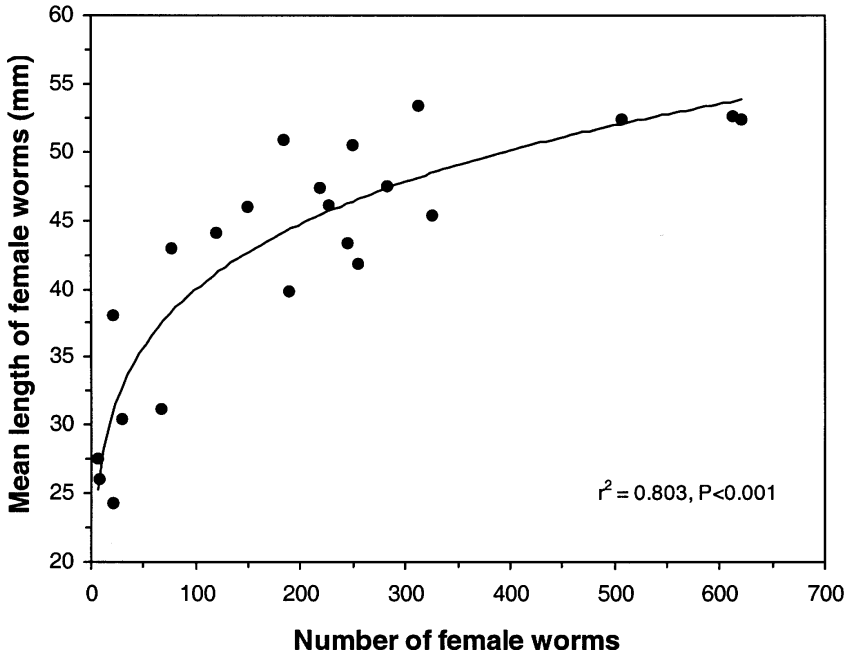

Fig. 3. The association between female worm burden and length of female worms. Data represent means per calf.

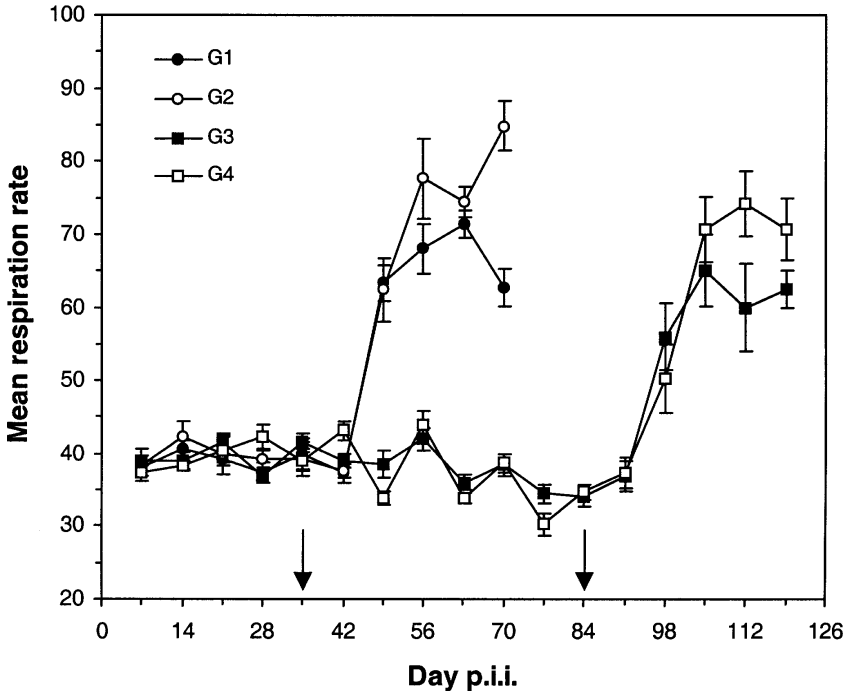

Fig. 4. The mean respiration rate per group. Vertical bars represent the standard error. Arrows indicate the time of the challenge infection for groups G1 and G2 (day 35) or G3 and G4 (day 84).

uterus and LPG/female worm was 0·438 ($n = 22$, $P < 0·05$). Fig. 3 depicts the relationship between female worm burden and mean length of female worms. Again a strong and close association was observed.

Respiration rate

The mean respiration rates of the groups of calves are given in Fig. 4. The primary infections with 30 larvae did not result in any increase in respiration rate. The challenge infections resulted in increased rates in all groups from 14 days after challenge onwards. There was a trend that this increase was more pronounced in G2 and G4 than in G1 and G3 ($P = 0·068$), but significant differences were only observed between G1 and G2 on day 70 ($P < 0·001$). Nevertheless, calves from all groups developed serious disease following the challenge infection. Repeated measures analysis showed that the increase

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in respiration rate was more pronounced after challenge infection on day 35 than on day 84 ($P < 0{\cdot}05$).

DISCUSSION

Among others, Michel and co-workers (Michel, 1962; Michel *et al*. 1965) demonstrated that high primary infection doses (over 3000 L3) result in a very strong immunity against challenge infection. The present experiment clearly proves for the first time that much lower primary infections generate a substantial degree of immunity, a degree that increases with duration of infection. Hence, the hypothesis that a low primary infection with 30 larvae of *D. viviparus* will not result in development of immunity against a challenge infection has to be rejected. In the present study primary infection resulted in lower worm burdens (> 70 % reduction), worm length (25–35 % smaller), fecundity (38–77 % reduction), faecal larval excretion (> 80 % reduction) and a higher proportion of female worms in the total worm burden 35 days after challenge (10–15 % higher). These effects were stronger after the challenge on day 84 compared to challenge 7 weeks earlier. The increased proportion of female worms suggests that part of the developed immunity affected worm survival in the lungs. This may be a similar phenomenon as observed for *C. oncophora* infections. Male *C. oncophora* worms are expelled earlier by the host than female worms (Albers, 1981; Kloosterman, Ploeger & Frankena, 1991). The observed effect on fecundity is at least partly considered to be a reflection of the reduced worm length. There was a very close association between length of worms and the number of eggs found in the uterus, both on mean values per calf as on values for individual worms. In many host–parasite systems, host immunity not only results in reduced worm burdens but also in smaller worms (Albers, 1981). This was also found in the present study. For *Teladorsagia circumcincta* Stear & Bishop (1999) showed that worm length and fecundity of female worms are correlated. Earlier Ractliffe & LeJambre (1971) demonstrated an increase in rate of egg production with parasite growth for several species of intestinal nematodes. However, the percentage reduction in fecundity was much larger than that in worm length. In the absence of knowledge on the exact relationship between worm length (parasite growth) and eggs in the uterus in non-immune hosts, a specific effect of immunity on the reproductive capacity of female worms cannot be ruled out.

Two interesting observations concerning the fecundity measures were (1) that the number of eggs present in the uterus of female worms was found to be in the range of 20000 to 30000 in the challenge control calves of groups G2 and G4, and (2) that the association between worm length and fecundity was far less clear when LPG per female worm was considered compared to the number of eggs *in utero*. Ploeger & Eysker (2000) made an estimate for fecundity in the range of 11000 L1s produced per female worm per day. This was based on a combination of data on faecal larval excretion and worm burdens some 5–6 weeks following primary infection. The highest estimate was 25000 L1s per female worm per day. If, in the present study, LPG per female worm is recalculated to total number of L1 produced per female worm per day average estimates lie in the range of 6000 to 7000 which is 4- to 5-fold lower than the observed number of eggs in the uterus. Explanations to combine these figures together include that development time of eggs in the uterus takes days and/or that many eggs may never get excreted as L1 in the faeces and become lost somewhere in the process of passage from lungs and through the gastrointestinal tract. That the process of passage has some influence is indicated (1) by a weak but significant correlation between both LPG per female worm and the mean number of eggs in the uterus and (2) by a less clear association of mean female worm length with LPG per female when compared with the numbers of eggs in the uterus.

The results of the groups receiving only the challenge infection (G2 and G4) indicate that within the age range present in the experiment no age-related immunity could be detected. This confirms the observations of Michel *et al*. (1965). Finally, although a substantial degree of immunity was induced by the primary infection, it was insufficient to protect calves from lungworm disease after challenge. Similar observations were made in field trials (Eysker *et al*. 1992, 1993*a*, *b*). In those trials calves received a primary dose of 20 L3 followed by natural challenge. The primary infection resulted in increasing pasture infectivity from 4 to 5 weeks later, subsequently resulting in high worm burdens approximately 2 months after the primary infection. At that time many calves developed lungworm disease in varying degrees of severity. This indicates that although faecal larval excretion after a natural challenge may be reduced as a result of a low primary infection, there is still a substantial risk for development of clinical disease.

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