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AN ANTHELMINTIC TEST FOR GASTRO-INTESTINAL NEMATODES OF CATTLE

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ABSTRACT

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Suitable experimental groups of calves for controlled anthelmintic tests were created by repeatedly dosing susceptible worm-free animals orally with infective larvae of *Haemonchus placei, Ostertagia ostertagi, Oesophagostomum radiatum* and *Cooperia* spp. (*C. pectinata* plus *C. punctata*) and giving a single percutaneous dose of *Bunostomum phlebotomum*. Calves were infested in such a way that at treatment the worms were either present as third stage larvae, fourth stage larvae or fifth and adult stages. Enough calves were infested to enable the data to be interpreted by the non-parametric method.

Optimal results were achieved by testing compounds against a specific stage of development. A combined test was evolved where two groups of 11 calves were treated when the worms were at different stages of development but only a single group of 9 control calves was used. For more accurate worm counts delaying the slaughter of calves for 3 to 4 weeks after administering the final dose of infective larvae is advocated. Nylon grit gauze with 500 micron apertures allows worms to migrate more easily into the filtrate of the ingesta than nylon mesh with 225 micron apertures in which they tend to become trapped.

INTRODUCTION

This paper describes the application of the non-parametric method (NPM) of evaluating anthelmintics, originally developed for use in sheep, to tests in cattle. Groeneveld & Reinecke (1969) showed that the worm populations in a group of sheep do not have a normal distribution and, moreover, that in a controlled anthelmintic test the distribution of the worm burdens in treated animals differ from that in untreated controls. They therefore devised the NPM, which makes full allowance for this markedly skew distribution of worms in a flock of sheep. Subsequently modifications suggested by C. J. Clark (Imperial Chemical Industries, Macclesfield, Cheshire, England, personal communication, 1969) have been incorporated in this test.

Before the larval anthelmintic test is carried out with any species or combination of species of worms in the same host, 20 to 22 worm-free lambs are artificially infested. At least 11 of these lambs are treated and 9 act as undosed controls while any extra animals either serve as larval viability controls or as substitutes for animals that die prior to treatment.

In South Africa during the last 3 years hundreds of larval anthelmintic tests, involving more than 2 500 sheep, have been carried out and the results analysed by by the NPM. It is accepted as a standard method of evaluating any anthelmintic used for all stages of development of the parasitic nematodes of sheep.

Many techniques in the tests in cattle differed fundamentally from those already described for sheep by Reinecke (1966 a, b; 1968), Reinecke & Anderson (1967) and Reinecke, Snijders & Horak (1962). A detailed description of each step is therefore given.

ISOLATION OF PURE STRAINS OF NEMATODES

The following methods were used to isolate pure strains of the common nematodes of cattle:

(1) *Haemonchus placei:* Adult female worms, collected from cattle at the Johannesburg abattoir, were cut into pieces with a pair of scissors. The eggs obtained were then placed in worm-free calf faeces, incubated and third stage larvae harvested.

(2) *Ostertagia ostertagi:* This species was isolated from faecal cultures containing a mixture of infective larvae of *O. ostertagi, Cooperia oncophora, Oesophagostomum radiatum* and *Trichostrongylus* spp. obtained from several calves. A worm-free calf was infested with this mixture and faeces collected from it 3 to 5 weeks later, were cultured. The relative number of *O. ostertagi* in this culture was determined and 11 000 larvae of this species were dosed to a second worm-free calf. Four days later this calf was dosed with levamisole at 7,5 mg/kg *per os*. Cultures subsequently prepared from its faeces were a mixture of *O. ostertagi* and *C. oncophora*. A third calf was dosed daily for 6 days until 18 000 infective larvae of *O. ostertagi* had been administered to it and then, 5 days after the last dose, it was treated with levamisole at 15 mg/kg. It yielded a pure strain of *O. ostertagi* which was used to infest other worm-free calves.

(3) *Bunostomum phlebotomum:* A culture was made from a faecal specimen containing eggs of *B. phlebotomum* and the number of infective larvae of this species was estimated on a percentage basis. Three calves were infested percutaneously by pouring the larval suspension, concentrated in 5 ml of water, onto a shaved circular area on the loins. The individual calves were infested with 500, 1 000 and 2 000 infective larvae respectively and 2 months later *B. phlebotomum* eggs were present in their faeces. These three calves still developed mixed infestations but another calf subsequently infested with larvae harvested from their faeces developed a pure infestation.

(4) *Oesophagostomum radiatum:* Larvae from a mixed culture were harvested and approximately 5 ml of the larval suspension was pipetted into a centrifuge tube, which was then placed in chipped ice in a 50 ml beaker. Slides were also chilled in the ice, then removed, dried rapidly with paper tissues and placed under a stereoscopic microscope. A few drops of the cold suspension were taken up with a fine glass pipette and drawn along a slide. The chilled larvae are very lethargic and the long tail sheath of *O. radiatum* is easily recognisable. These larvae were removed individually and transferred to a plastic specimen bottle containing de-ionized water. This process was repeated until 308 infective larvae of *O. radiatum* had been collected and these were used to infest a wormfree calf. Thirty-eight days later eggs of *O. radiatum* were present in its faeces.

(5) *Cooperia* spp.: A faecal culture from a calf yielded a mixed infestation of *H. placei, O. radiatum, C. pectinata*

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and *C. punctata*. A dose estimated to contain 5 000 infective larvae of *Cooperia* spp. was given to a worm-free calf and faeces were collected from the 14th to the 19th day after infestation. Only *Cooperia* spp. were present in cultures made from these faeces and they were used to infest a worm-free calf.

Subsequently a pure strain of *C. oncophora* was established using the same technique.

References in this paper to *Cooperia* spp. usually imply a mixture of *C. pectinata* and *C. punctata*. In Experiment 4, however, *C. oncophora* mixed with *O. ostertagi* was used instead.

Numerous attempts to transfer live worms collected at autopsy to worm-free calves by surgical means were unsuccessful.

Maintenance of Pure Strains of Nematodes

Weaned worm-free bull calves were each infested with a single species. When worm eggs appeared the faeces were immediately collected for making larval cultures.

The infestation and egg production of 62 calves over a period of 2 years are summarized in Table 1.

This was not a controlled experiment and these observations are recorded merely to show the difficulties of maintaining pure strains in donor calves. With reference to the larval doses (Table 1), in the case of *B. phlebotomum* a single dose of infective larvae is placed on the skin. With the other species the infective larvae are divided into three equal doses administered orally at 2 to 3 day intervals.

O. ostertagi is the most difficult species to maintain as it produces low egg counts for a few weeks only. Michel & Sinclair (1968) have advocated the use of immunosuppressant drugs to stimulate egg production. A calf with a mass of 84 kg infested with *O. ostertagi* was therefore injected intramuscularly with 30 mg prednisolone acetate (Delcortinal, Scanpharm, Copenhagen) daily for 5 days a week, but it died after 5 weeks from secondary bacterial infection. This treatment maintained the egg output at 250 to 300 e.p.g. for 4 out of the 5 weeks. Untreated calves only have a sporadic egg count of 50 or 100 e.p.g.

Frequently *B. phlebotomum* also gives low egg counts for short periods, probably due to poor methods of percutaneous infestation. An improved method of infestation is fully described later in Experimen 1.

Little reliance can be placed on the calf host as a continual source of infested faeces from which to culture infective larvae. Since as many as 100 000 infective larvae of a single species may be required in the anthelmintic trials, all the faeces from a donor must be collected once it is infested and either stored at 4°C in the refrigerator or cultures made and the larvae stored.

Larval Cultures

The eggs in faeces may be stored in the refrigerator at 4°C for 6 to 8 weeks. Eggs of *Cooperia* spp. remain alive for more than a year in a refrigerator (K. C. Kates, Beltsville Parasitology Laboratory, Maryland, U.S.A., personal communication, 1963).

Faeces were collected, cultures made and larval doses prepared as described by Reinecke (1968).

Infective larvae of *H. placei, Cooperia* spp. and *O. radiatum* readily migrate up the inner surface of the glass jars. The infective larvae of *O. ostertagi,* however, migrate sporadically and must be harvested two or three times a day for 3 to 5 days.

The method of Roberts & O'Sullivan (1950) is used to harvest infective larvae of *B. phlebotomum*. Larvae are identified according to the description of Keith (1953).

After counting, the larvae are concentrated by decanting and the suspension poured into flat-sided glass medicine bottles of 120 ml capacity. The depth of water must not exceed 5 mm or the air will not contain sufficient oxygen. A maximum of 0,25 million infective larvae (5 000 larvae per ml) is stored in each bottle. The bottles are kept flat on their sides in a cupboard.

This technique of larval storage differs from that advocated by the Veterinary Laboratory, Weybridge, England (Anon., 1971), in that they store their larvae at 4°C in refrigerators, but at Onderstepoort this has not been as successful as storage at room temperature. (Storage in refrigerators has not been thoroughly tested in this laboratory).

With the exception of *B. phlebotomum,* which remains alive and fully infective for only a few weeks, other species can be stored for at least 3 to 4 months providing fungi, protozoa or putrefactive bacteria do not contaminate the cultures. According to workers at Weybridge, *O. ostertagi* survives at least 6 to 12 months if stored in these bottles at 4°C.

A judicious combination of larval storage and the storage of eggs in faeces in the refrigerator ensures that sufficient infective larvae are available when required for anthelmintic trials.

Experimental Animals

Donor calves

Bull calves, predominantly Friesians, are kept initially in stalls with concrete floors and fed milk substitutes and lucerne hay from the age of 7 to 10 days. They are then treated with levamisole at 20 the 30 mg/kg and 7 to 10 days later with thiabendazole at 200 mg/kg live mass. They are transferred to special cages 2 m high with expanded metal floors 1,4 m² in area and raised 50 cm above ground level. Water is provided in a small drinking container and a semicircular feeding trough protrudes into the cage from the front next to the door of the pen. The pen is cleaned daily with a strong stream of water which washes faeces and spilled feed through on to the concrete floor below.

Ideally donor calves should be bred and reared as described above but as this is expensive the calves described below can if necessary be used as donor calves.

Table 1 Infestation of "donor" calves

Species	No. of larvae dosed	Minimum prepatent period (days)	Maximum eggs per gramme	Period when max. egg count was recorded (weeks)	Total period when egg count was recorded (weeks)
H. placei	3 000 – 6 000	22	100 – 900	1 – 4	4 – 10
O. ostertagi	20 000 – 30 000	23	50 – 300	2 – 4	2 – 7
**Cooperia* spp.	5 000 – 20 000	14	100 – 1 250	2 – 9	7 – 14
B. phlebotomum	3 000	52	50 – 300	2 – 5	5 – 10
O. radiatum	1 000 – 2 000	36	50 – 1 400	1 – 5	6 – 12

**C. pectinata* and *C. punctata*

Worm-free calves

These are derived from two sources:–

(1) Weaned calves: These may be purchased from farmers raising veal on the battery system. Although there is no guarantee that they are fully susceptible or worm free, they are satisfactory. Only one animal (Calf 87 in Experiment 5) out of a total of 60 control calves was partially resistant to experimental infestation.

(2) Suckling calves: The only advantage in using suckling calves is that they are cheaper initially than weaned calves. Rearing them was extremely time-consuming and despite treatment there was a 10 to 15% mortality.

All the calves were vaccinated against paratyphoid and treated with double doses of anthelmintics, e.g. levamisole at 15 mg/kg followed 7 to 10 days later by thiabendazole at 200 mg/kg.

Calves were housed in pens with concrete floors, which were washed with water and scrubbed with brooms daily. A lean-to roof provided shelter and in the

FIG. 1 The aeration apparatus for mixing the digests in the small waterbath

winter canvas on the fences protected the calves from the wind. Sterile hay or sawdust acted as bedding. They were fed sterilized lucerne hay *ad libitum* as well as 0,5 to 1,5 kg of a high-protein high-energy concentrate (calf pellets) per calf per day.

Autopsy Procedures

Unless otherwise stated the following procedure described below was used to recover the helminths at autopsy from the experimental animals.

The gut was divided into three parts, viz. the abomasum, the small intestine and the caecum plus colon, which were handled separately. Each part was opened and the ingesta washed into a modified Baermann apparatus containing physiological saline and placed in a waterbath (Reinecke, 1967). After 1 hour the filtrate of the ingesta and the residue of each part were heated to 60°C and subsequently treated as described by Reinecke (1968), except that the residues were washed through 100 and not 400 mesh sieves. If any worms were trapped in the nylon mesh, the mesh was placed in a specimen jar for later examination.

The mucosa and muscular layers of the gut were scraped off the serosa with a butcher's knife and the former homogenized in a blender. The serosa of the abomasum was discarded but that of the small intestine and the caecum and colon was chopped into 5 mm^2 pieces and treated as a unit. Pepsin solution, consisting of pepsin scales 2% m/v and HCl 3% v/v, was added to the homogenized gut wall in 4 l jars. These jars were placed in a waterbath at 50°C and air bubbled through it to keep the gut wall in suspension (Fig. 1). Digestion of the abomasal wall was complete after 45 minutes. The intestinal wall was digested for 1½ hours, then the supernatant was decanted; the residue was blended once again and digested with fresh pepsin HCl for a further hour. When digestion was complete the specimens were treated in the same way as the ingestal filtrates.

The trachea and bronchi were cut open with scissors and the lungs washed with a strong stream of water. The lung washings were treated in the same way as the ingestal filtrates. The lungs were cut into pieces (1 cm^3) and placed in a trap with saline. The nylon mesh was placed on top of the lung cubes and a piece of plastic diamond mesh on top of the nylon, to push the lung tissue below the surface of the saline. These were placed in a waterbath at 40°C for 4 hours. The lung cubes were then placed on a sieve (5 mm aperture) and adherent worms washed off with a strong stream of water. Thereafter these specimens were also treated in the same way as ingestal filtrates.

If pure* nylon gauze was used in the traps a solution consisting of 600 ml 10 N HCl and 400 ml deionised water was poured over the mesh. The nylon dissolved, leaving a clear solution containing the trapped worms. By a process of repeated sedimenting and decanting, the HCl was diluted to the point at which it could be poured on to stainless steel mesh sieves and the worms collected. If there are impurities in the nylon a white glutinous precipitate forms and the worms cannot be recovered. In these cases the cloth was placed in a specimen jar and formalin added.

Estimates of Worm Burdens

If 1 000 or more worms were present in a specimen their number was estimated as described by Reinecke (1968), but when less than 1 000 were present total counts were made. If possible 120 worms were collected from each specimen and placed in 10% formalin. When less than 120 specimens of any species were present all of them were collected. The larval stages of the worms were identified according to Sprent (1946) for *B. phlebotomum;* Andrews & Moldonado (1941) for *O. radiatum,* Veglia (1915) for *H. placei* and Keith (1967) for C. *pectinata.* These identifications were used to estimate the stage of development and the total number of each species present.

When the worm burdens of all the calves in an experiment had been determined the number of the median of the controls was checked and recounted as well as the count above and below it. The counts below the reduced value of the median in the treated group were also recounted.

Larval Anthelmintic Test (Modified Non-Parametric Method)

The test is similar to that described for the common nematode parasites of sheep (Reinecke, 1966 a, b; 1968; Reinecke & Anderson, 1967). The data are analysed by the non-parametric statistical method of Groeneveld & Reinecke (1969) as modified by C. J. Clark (Imperial Chemical Industries, Macclesfield, Cheshire, England, personal communication, 1969), referred to hereafter as the modified NPM.

*The specification of pure nylon cloth was kindly supplied by the South African Bureau of Standards and is as follows:–
(1) Plain woven cloth
(2) Mass per square metre (free from filling) 30 g
(3) Filling 0,1%
(4) Ply of yarns: one ply warp and weft
(5) Threads per 25,5 mm: for warp 104 and for weft 97
(6) Fibre composition both warp and weft: Nylon 6 continuous multifilament

Table 2 The moults and the prepatent periods of the parasitic nematodes of cattle

Species	Age in days at the: –			Author
	3rd Moult	4th Moult	Minimum prepatent period	
H. placei	1½ – 2	14	*26 – 28(22)	Bremner 1956
O. ostertagi	3 – 4	10 – 11	*25	Rose 1969
O. ostertagi	3	10	*23	Douvres 1956
T. axei	4 – 6	10 – 14	*21	Douvres 1957
C. pectinata	2 (3)	8	13 – 14(14)	Keith 1967
B. phlebotomum	(8)	(21 – 25)	52 – 56(52)	Sprent 1946
N. helvetianus	8 (5)	15 (14 – 16)	*21 – 26	Herlich 1954
O. radiatum	8 – 9	19	*35 – 41(36)	Andrews & Maldonado 1941

*Prepatent periods derived from other sources
( ) = Unpublished observations

In these anthelmintic tests worm-free calves are infested in such a way that the efficacy of compounds against all the parasitic developmental stages can be assessed. The days on which the moults occur must be known before the experiments can be carried out because the third moult is regarded as part of the third stage and the fourth moult as part of the fourth stage (Table 2). Five of the six experiments described below are designed to fulfil these biological requirements as well as the non-parametric method of statistical analysis of results. One trial (Experiment 4) compares the efficacy of two dosage rates of one compound with that of another compound.

As hook-worms are refractory to repeated infestation the method of Gibson (1964) is used to test the efficacy of compounds against *B. phlebotomum*. In this method worms are either in the third, fourth or adult stage at the time of treatment. Throughout these trials this procedure was followed except in Experiments 3 and 5, in which the *B. phlebotomum* were immature fifth stage worms instead of sexually mature adults. In the case of other species, when the calves were treated they had been repeatedly infested to produce worms at various stages of development (Reinecke, 1966 a, b; 1968; Reinecke & Anderson, 1967).

In Experiment 1 the materials and methods are described in detail. Thereafter only the modifications are described.

TABLE 3 Experiment 1. Experimental design

Day	No. of infective larvae dosed to each calf			
	B. phlebotomum	*O. radiatum*	*H. placei*	*Cooperia* spp.
—8	—	258	—	—
—7	965	233	—	—
—6*	—	244	—	—
—5	—	232	—	—
—4	—	210	—	—
—3	—	242	1 479	1 458
—2	—	250	1 480	1 948
—1	—	284	1 528	1 452
Total	965	1 953	4 487	4 858
0	Treated Calf 11 to 21 inclusive with mebendazole at 20 mg/kg Killed Calf 1: Day 0 Control			
+10	Killed Calf 2 to 6 inclusive: Controls			
+11	Killed Calf 7 to 10 inclusive: Controls			
+12	Killed Calf 11: and 13 to 16 inclusive: treated on Day 0 Calf 12: died treated on Day 0			
+13	Killed Calf 17 to 21 inclusive: treated on Day 0			

*On Day —6 one Calf in this experiment died but no worm counts *post mortem* were made

EXPERIMENT 1 THIRD STAGE LARVAE

Materials and methods

Twenty-one weaned dairy calves reared and maintained under worm-free conditions were used and infested as indicated in Table 3.

The required number of infective larvae of *B. phlebotomum* were pipetted into a centrifuge tube and three doses in excess of those required prepared. These tubes were left in an upright position overnight and on the day of infestation the supernatant fluid was removed by suction with a pipette until the larvae were suspended in 5 ml. The contents of one tube were poured into a counting chamber, examined for motility, Lugol's iodine added and the larvae counted.

Each calf was percutaneously infested on Day —7 by the methods previously described. The remaining doses were checked for motility and the larvae counted in one tube The mean of the number in this tube and of the one counted prior to dosing was recorded as the number of larvae dosed percutaneously.

In Experiment 5 the clipped area on the loins was thoroughly scrubbed with hot water and cleaned with cotton wool. The centrifuge tubes containing the infective larvae concentrated in 2 ml of water were warmed in beakers at a temperature of 30°C and one was then inverted on the clipped area on each calf and held in position for at least 2 minutes. The tube was rinsed with warm water (30°C) and this was also placed on the clipped area. Infective larvae for Experiment 6 were obtained from cultures prepared from infected faeces stored at 4°C. Ten days prior to infestation some 20 cultures were made and the larvae harvested 8 days later. After the larvae had been applied to the loins a dry paper tissue was placed over this area and its edges thoroughly wetted to induce it to stick to the hair. Most of the tissues fell off after 5 minutes though a few remained on the loins for up to 15 minutes.

In Table 3 the design of this experiment is summarized.

RESULTS

Larvae were in the third stage on the day of treatment as indicated by the worms present in Calf 1 (Table 4). With the exception of the very low numbers of *B. phlebotomum,* the infestations of the other species in the control calves, although they vary markedly, are quite adequate for an anthelmintic test. *Cooperia* spp. were recounted in Calf 2 and the total reduced from 1 737 to 1 520. In Calf 6 the original estimate was 1 909 but when recounted the total was 1 678. The latter is the median.

Estimation of anthelmintic efficacy

The efficacy is assessed by the non-parametric method of Groeneveld & Reinecke (1969) as modified by Clark (personal communication, 1969). His modifications can be summarized as follows:

(1) The median of the controls rather than the lower limit of the median is used to indicate the worm burdens of the controls.

(2) Simulation studies have shown that if this median is reduced by 75% after treatment there is no chance that compounds which produce an 80% reduction in worm burdens (or less) in 80% of the treated herd will be graded Class A.

(3) At the 90% confidence limit when 11 animals are treated the gradings are as follows:

Class A: more than 80% effective in more than 80% of the treated herd. This is estimated by multiplying the control median by 0,25 and only one of 11 treated animals may exceed this figure.

Class B: more than 60% effective in more than 60% of the treated herd, which is estimated by multiplying the control median by 0,4. Three out of 11 treated animals may exceed this figure.

Class C: more than 50% effective in more than 50% of the treated herd, which is estimated by

TABLE 4 Experiment 1. Worms recovered at autopsy

	Calf No.	*H. placei*				*Cooperia* spp.					*O. radiatum*				*B. phlebotomum*		
		Stage of development			Total	Stage of development				Total	Stage of development			Total	Stage of development		Total
		L_3*	L_4	5		L_3	L_4	5	A		L_3	L_4	5		L_3	L_4	
Controls																	
Killed on Day 0	1	318	19	0	337	156	33	3	0	192	397	13	0	410	11	0	11
Killed on Day +10	2	0	10	858	868	0	38	1218	264	1520	115	467	0	582	0	3	3
Killed on Day +10	3	0	10	681	691	0	136	922	25	1083	117	278	0	395	0	2	2
Killed on Day +10	4	0	40	1228	1268	0	400	2222	49	2671	134	733	0	867	0	2	2
Killed on Day +10	5	0	33	1446	1479	0	98	2426	0	2524	105	1020	0	1125	0	3	3
Killed on Day +10	6	0	70	962	1032	0	56	1477	145	1678	45	663	61	769	0	5	5
Killed on Day +11	7	0	85	1814	1899	0	4	2629	136	2769	63	995	0	1058	0	10	10
Killed on Day +11	8	0	7	550	557	0	14	805	0	819	0	698	0	698	0	19	19
Killed on Day +11	9	0	17	834	851	0	18	982	0	1000	14	246	0	260	0	58	58
Killed on Day +11	10	0	0	1074	1074	0	2002	162	0	2164	8	909	0	917	0	62	62
Treated on Day 0 with mebendazole at 20 mg/kg																	
Killed on Day +12	11	0	0	1	1	0	29	54	2	85	0	1014	0	1014	0	49	49
Died on Day +12	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Killed on Day +12	13	0	8	27	35	0	67	117	1	185	2	729	0	731	0	84	84
Killed on Day +12	14	0	0	0	0	0	13	57	9	79	0	539	0	539	0	3	3
Killed on Day +12	15	0	0	5	5	1	31	12	2	46	41	300	0	341	1	77	78
Killed on Day +12	16	0	0	9	9	1	13	2	0	16	0	141	0	141	0	2	2
Killed on Day +13	17	0	0	0	0	0	79	36	18	133	50	581	0	631	0	25	25
Killed on Day +13	18	0	0	5	5	0	14	116	0	130	6	465	0	471	0	4	4
Killed on Day +13	19	0	0	40	40	0	19	70	15	104	42	676	0	718	0	11	11
Killed on Day +13	20	0	0	37	37	0	53	95	8	156	16	505	0	521	0	31	31
Killed on Day +13	21	0	0	12	12	0	8	85	2	95	6	139	0	145	0	37	37

*L_3 - Third stage larvae L_4 - Fourth stage larvae 5 - Fifth stages A - Adults

TABLE 5 Experiment 1. Anthelmintic efficacy. (The data of Calf 1 are not included as it acted as an indicator control)

H. placei		*Cooperia* spp.		*O. radiatum*		*B. phlebotomum*	
Controls	Treated	Controls	Treated	Controls	Treated	Controls	Treated
557	0	819	0	260	0	2	0
691	0	1000	16	395	141	2	2
851	0	1083	46	582	145	3	3
868	1	1520	79	698	341	3	4
1032	5	*1678*	85	*769*	471	5	11
1074	5	2164	95	867	521	10	25
1268	9	2524	104	917	539	19	31
1479	12	2671	130	1058	631	58	37
1899	35	2769	133	1125	718	62	49
	37		156		731		78
	40		185		1014		84
1032 × 0,25 = 258 0/11 exceed 258 Class A		1678 × 0,25 = 419,50 0/11 exceed 419,50 Class A		769 × 0,5 = 384,5 8/11 exceed 384,5 Class X		Numbers too low for analysis	

multiplying the control median by 0,5, and 4 out of 11 treated animals may exceed this figure.

Class X: Ineffective i.e. it does not even comply with Class C.

Worm burdens are ranked and anthelmintic efficacy summarized in Table 5. It is essential that a very accurate count be made of the control median and any figure close to it. In this experiment, for example, *Cooperia* spp. were recounted in Calf 2 and the total reduced from 1 737 to 1 520. In Calf 6 the original estimate was 1 909 but when recounted the total was only 1 678. The latter is the median of the controls.

This median is reduced to 419,5 by multiplying by 0,25. This represents a 75% reduction and since the highest burden in the treated calves is 185 (Calf 13), mebendazole easily attains a Class A for this species. Similarly it reaches Class A against *H. placei* but fails against *O. radiatum,* for which it is graded as Class X. Because very few *B. phlebotomum* are present in the controls its efficacy cannot be assessed.

In the controls the worms recovered from the digested gut wall were interesting. Fifth stage *H. placei* were dominant in the abomasal digests, from 30 to 253 worms of this species being found. The digested intestinal mucosa was negative for *O. radiatum* in only one specimen; from the others 10 to 136 worms, predominantly third stage larvae, were recovered. The serosal digests were usually negative, and the maximum was three worms recovered from one specimen. In nine out of the ten controls the mucosa of the gut wall contained between 5 and 115 *Cooperia* spp., mainly in the fifth stage. The serosa was negative in four calves and a maximum of three *Cooperia* spp. was recovered in the other six. It is clear that little purpose is served in digesting the serosa of the gut wall.

In most of the controls some *H. placei* were trapped in the nylon mesh used for the abomasal ingesta; similar observations were made for *Cooperia* spp. in the small intestine but in only three calves were a maximum of three *O. radiatum* trapped in the nylon mesh used for the ingesta of the caecum and colon.

EXPERIMENT 2. FOURTH STAGE LARVAE

This experiment was planned to test the efficacy of mebendazole against fourth stage larvae of the same helminth species.

Materials and methods

Twenty-three weaned calves were used. On Day—40 they were dosed with levamisole at 15 mg/kg and on Day—31 with thiabendazole at 200 mg/kg. One calf died but no autopsy was carried out. The remaining 22 calves were dosed with larvae treated and slaughtered according to the schedule shown in Table 6.

TABLE 6 Experiment 2. Experimental design

Day	No. of infective larvae dosed to each calf			
	B. phlebotomum	*O. radiatum*	*H. placei*	*Cooperia* spp.
—21	—	232	—	—
—19	—	250	—	—
—17	—	219	—	—
—16	—	—	554	—
—15	—	300	530	—
—14	2 192	—	475	—
—13	—	256	492	—
—12	—	—	504	—
—11	—	248	608	—
—10	—	—	548	763
— 9	—	284	500	829
— 8	—	—	610	734
— 7	—	—	785	896
— 6	—	—	392	525
— 5	—	—	505	830
— 4	—	—	506	453
Total	2 192	1 789	7 009	5 030
0	Treated Calf 33 to 43 inclusive with mebendazole at 20 mg/kg Killed Calf 22 to 30 inclusive: Day 0 controls			
+3	Killed Calf 33 to 38 inclusive: treated on Day 0			
+4	Killed Calf 31 & 32: Day +4 controls Killed Calf 39 to 43 inclusive: treated on Day 0			

At autopsy the wall of the small intestine and the caecum and colon were minced in an electric mincing machine instead of scraping off the mucosa before digestion. All the other procedures at autopsy were carried out as described for Experiment 1.

Results

These are summarized in Table 7. The stage of development at treatment is indicated by the controls killed on Day 0. By far the largest number of *O. radiatum* were fourth stage larvae and as this is the stage desired on the day of treatment the design fulfilled the object of the experiment. Eight out of nine calves killed on Day 0 had more fourth stage larvae of *H. placei* than fifth stage worms but the latter were always present. On Day 0 only 5 of 9 Day 0 controls had more fourth stage larvae

TABLE 7 Experiment 2. Worms recovered at autopsy

Group	Calf No.	*H. placei*				*Cooperia* spp.			*O. radiatum*				*B. phlebotomum*		
		Stage of development			Total	Stage of development		Total	Stage of development			Total	Stage of development		Total
		L_4	5	A		L_4	5		L_3	L_4	5		L_3	L_4	
Controls															
Killed on Day 0	22	716	611	0	1327	944	1094	2038	28	567	0	595	0	3	3
Killed on Day 0	23	900*	587	0	1488	1556*	987	2544	0	153	6	159	0	8	8
Killed on Day 0	24	1199	449	0	1648	1337	1508	2845	104	727	7	838	0	3	3
Killed on Day 0	25	1057	286	0	1343	1211	874	2085	11	203	0	214	13	15	28
Killed on Day 0	26	859	862	0	1721	857	793	1650	71	354	1	426	0	1	1
Killed on Day 0	27	941	501	0	1442	1252	781	2033	76	517	0	593	0	22	22
Killed on Day 0	28	1102	388	0	1490	1066	1133	2199	54	378	0	432	1	11	12
Killed on Day 0	29	1453	712	0	2165	1403	1377	2740	55	663	0	718	0	39	39
Killed on Day 0	30	840	133	0	973	1456	980	2436	52	375	0	427	0	1	1
Killed on Day + 4	31	604	825	0	1429	499	1621	2120	35	316	12	363	0	6	6
Killed on Day + 4	32	281	807	0	1088	415	1030	1445	36	390	33	459	0	19	19
Treated on Day 0 with mebendazole at 20 mg/kg															
Killed on Day + 3	33	0	0	0	0	209	567	776	16	46	0	62	0	0	0
Killed on Day + 3	34	0	2	0	2	301	286	587	29	73	0	102	0	0	0
Killed on Day + 3	35	0	7	13	20	294	412	706	48	20	0	68	1	3	4
Killed on Day + 3	36	1	20	0	21	304	205	509	11	18	0	29	0	0	0
Killed on Day + 3	37	0	1	0	1	371	956	1327	53	186	0	239	0	0	0
Killed on Day + 3	38	7	15	0	22	330	252	582	5	8	0	13	0	0	0
Killed on Day + 4	39	0	7	0	7	229	267	496	21	154	0	175	0	0	0
Killed on Day + 4	40	0	1	0	1	298	844	1143	11	141	0	152	0	1	1
Killed on Day + 4	41	8	1	0	9	237	1690	1927	5	33	0	38	0	0	0
Killed on Day + 4	42	0	1	0	1	363	586	949	54	75	0	129	0	0	0
Killed on Day + 4	43	0	1	0	1	269	413	682	34	83	0	117	0	0	0

*Including one third stage larva

TABLE 8 Experiment 2. Anthelmintic efficacy. Only Calf 22 - 30 inclusive are included as controls

H. placei		*Cooperia* spp.		*O. radiatum*		*B. phlebotomum*	
Controls	Treated	Controls	Treated	Controls	Treated	Controls	Treated
973	0	1 650	496	159	13	1	0
1 327	1	2 033	509	214	29	1	0
1 343	1	2 038	582	426	38	3	0
1 442	1	2 085	587	427	62	3	0
1 488	1	*2 199*	682	*432*	68	*8*	0
1 490	2	2 436	706	593	102	12	0
1 648	7	2 544	776	595	117	22	0
1 721	9	2 740	949	718	129	28	0
2 165	20	2 845	1 143	838	152	39	0
	21		1 327		175		1
	22		1 927		239		4
1 488 × 0,25 = 372 0/11 exceed 372 Class A		2 199 × 0,5 = 1 099,5 3/11 exceed 1 099,5 Class C		432 × 0,4 = 172,8 9/11 exceed 172,8 Class B		Numbers too low for analysis	

than fifth stage worms of *Cooperia* spp. Controls killed on Day +4 had more fourth stage larvae of *O. radiatum* than fifth stage worms. This tendency was, however, reversed for *H. placei* and *Cooperia* spp. both of which had more worms in the fifth stage than fourth stage larvae. Once again worm burdens of *B. phlebotomum* were completely inadequate for trials of this nature.

In the digested abomasal wall *H. placei* was always present and from 17 to 185 worms were recovered from the controls. Two calves yielded no *Cooperia* spp. and in the others, from 8 to 332 worms were present; the controls were all positive for this species. The digested gut contained from only 1 to 104 *O. radiatum,* mostly third stage larvae. It is probable that thorough washing of the gut wall would remove most, if not all, of the *Cooperia* spp., witch do not normally occur there.

Anthelmintic efficacy is summarized in Table 8. There is no doubt that mebendazole warrants a grading of Class A for its efficacy against *H. placei.* For *Cooperia* spp. the median count was 2 199 (Calf 28) and both this and the worms from calves with lower numbers were recounted (Table 8). In the treated group the *Cooperia* spp. from Calf 33 numbered 776 when recounted while in Calf 42 the check revealed 949 worms. The median 2 199 × 0,5 = 1 099,5 and both 949 and 776 fall far below this. Therefore eight out of 11 results comply with the requirements for Class C.

The median for *O. radiatum* as well as the two worm burdens below it were recounted. The treated group contained nine calves with less than the control median (432 × 0,4 = 172,8) and it therefore falls in Class B.

No conclusions could be drawn regarding the efficacy of this compound against *B. phlebotomum* as there were too few worms present.

EXPERIMENT 3. FOURTH STAGE LARVAE, FIFTH AND ADULT STAGES

In this experiment the efficacy of mebendazole was tested against fifth stage and adult worms. The fourth stage larvae were also included to make the test more comprehensive.

Materials and methods

Twenty-three weaned dairy calves were used. With the exception of Calf 53 all calves were dosed with thiabendazole at 200 mg/kg on Day —66. All the calves were subsequently dosed on Day —60 with levamisole at 15 mg/kg.

The number of infective larvae of each species and the days on which they were dosed are summarized in Table 9. One calf died on Day —41 but no autopsy was performed. Calf 44, which died on Day —27, served as a viability control. Calf 54 accidentally received an additional larval dose of 250 *O. radiatum,* 1 480 *H. placei* and 1 948 *Cooperia* spp. on Day —40 (i.e. the same num-

TABLE 9 Experiment 3. Experimental design

Day	No. of infective larvae dosed to each calf			
	B. phlebotomum	*O. radiatum*	*H. placei*	*Cooperia* spp.
—59	1 096 dosed to 18 calves		—	—
—54	1 096 dosed to a further 3 calves		—	—
—45	—	106	—	—
—42	—	107	—	—
—41	1 275 and 200 dosed to Calf 65 only			—
—40	(Calf 54 accidentally dosed with the larvae used in Experiment 1 on Day —2			
i.e.	—	250	1 480	1 948)
—39	—	111	—	—
—36	—	110	—	—
—33	—	106	335	—
—30	—	115	306	—
—27	—	117	312	—
—27	Calf 44 died used as a larval viability control			
—24	—	125	309	—
—21	—	133	339	338
—19	—	130	301	391
—18	—	—	276	281
—17	—	131	404	387
—16	—	—	346	378
—15	—	114	261	326
—14	—	—	328	330
—13	—	120	297	375
—12	—	—	297	268
—11	—	125	352	320
—10	—	—	348	303
— 9	—	129	342	291
— 8	—	—	298	260
— 7	—	—	346	338
— 6	—	—	411	329
— 5	—	—	407	346
— 4	—	—	358	420
Total	1 096	1 779	6 973	5 681
0	Treated Calf 55–65 inclusive with mebendazole at 20 mg/kg			
+1	Killed Calf 45–53 inclusive: Day +1 controls			
+3	Killed Calf 55–60 inclusive: treated on Day 0			
+4	Killed Calf 61–65 inclusive: treated on Day 0 Killed Calf 54: Day + 4 control			

TABLE 10 Experiment 3. Worms recovered at autopsy

Group	Calf No.	*H. placei*				*Cooperia* spp.				*O. radiatum*					*B. phlebotomum*	
		Stage of development			Total	Stage of development			Total	Stage of development				Total	Stage of development	Total
		L_4	5	A		L_4	5	A		L_3	L_4	5	A		A	
Controls																
Died on Day —27	44	134	0	0	134	0	22	3	25	0	30	0	0	30	95*	95
Killed on Day +1	45	420	152	187	759	190	302	260	752	2	65	113	155	335	1	1
Killed on Day +1	46	151	227	271	649	232	367	418	1 017	5	100	87	81	273	69	69
Killed on Day +1	47	359	156	150	665	569	474	432	1 475	0	44	63	103	215	37	37
Killed on Day +1	48	229	171	160	560	427	305	621	1 353	16	171	77	35	299	19	19
Killed on Day +1	49	425	739	424	1 588	480	1 137	1 637	3 254	21	174	120	102	417	111	111
Killed on Day +1	50	228	126	136	490	171	307	517	995	0	36	74	5	115	8	8
Killed on Day +1	51	252	285	308	845	377	684	976	2 037	0	103	78	63	244	2	2
Killed on Day +1	52	380	532	398	1 310	682	624	1 306	2 612	5	174	78	44	301	7	7
Killed on Day +1	53	362	659	352	1 373	464	639	1 234	2 337	2	334	115	0	451	27	27
Killed on Day +4	54	574	394	1 747	2 715	551	483	3 438	4 472	66	109	65	35	275	13	13
Treated with mebendazole at 20 mg/kg																
Killed on Day +3	55	0	0	0	0	204	127	15	346	16	37	0	0	53	13	13
Killed on Day +3	56	0	3	0	3	118	155	10	283	4	52	0	0	56	8	8
Killed on Day +3	57	0	0	0	0	181	188	27	396	0	13	0	0	13	60	60
Killed on Day +3	58	0	0	0	0	36	11	4	51	13	21	0	0	34	19	19
Killed on Day +3	59	0	0	0	0	65	13	0	78	9	14	0	0	23	47	47
Killed on Day +3	60	0	0	0	0	147	164	23	334	15	67	0	0	82	44	44
Killed on Day +4	61	0	0	0	0	202	64	25	291	30	54	0	1	85	13	13
Killed on Day +4	62	0	0	0	0	158	217	45	420	6	45	0	0	51	130	130
Killed on Day +4	63	0	0	1	1	51	74	11	136	1	14	0	0	15	2	2
Killed on Day +4	64	0	0	0	0	100	93	4	197	3	61	0	0	64	15	15
Killed on Day +4	65	0	0	0	0	233	370	68	671	5	100	0	0	105	17	17

*These were 5th stage worms

TABLE 11 Experiment 3. Anthelmintic efficacy. Only Calf 45 to 53 inclusive included as controls

H. placei				*Cooperia* spp.				*O. radiatum*				*B. phlebotomum*	
L_4		5th & A		L_4		5th & A		L_4		5th & A		A	
Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated
151	0	262	0	171	36	562	13	36	13	79	0	1	2
228	0	306	0	190	51	785	15	44	14	112	0	2	8
229	0	331	0	232	65	824	85	65	14	115	0	7	13
252	0	339	0	377	100	906	89	100	21	122	0	8	13
359	0	*498*	0	*427*	118	*926*	97	*103*	37	*141*	0	*19*	15
362	0	593	0	464	147	1 660	142	171	45	168	0	27	17
380	0	930	0	480	158	1 873	165	174	52	171	0	37	19
420	0	1 011	0	569	181	1 930	187	174	54	222	0	69	44
425	0	1 163	0	682	202	2 774	215	334	61	268	0	111	47
	0		1		204		262		67		0		60
	0		3		233		438		100		1		130
359 × 0,25 = 89,75 0/11 exceed 89,75 Class A		498 × 0,25 = 124,5 0/11 exceed 124,5 Class A		427 × 0,5 = 213,5 1/11 exceed 213,5 Class C		926 × 0,4 = 370,4 1/11 exceed 370,4 Class B		103 × 0,5 = 51,5 5/11 exceed 51,5 Class X		141 × 0,25 = 32,25 0/11 exceed 32,25 Class A		Numbers are too low for analysis	

bers and species of larvae as were given to calves on Day —2 in Experiment 1 (Table 3); it was subsequently kept as a Day +4 control and its worm burdens were not used for the analysis of efficacy.

At autopsy the nylon mesh cloths were not dissolved in acid but were placed in formalin; the adherent worms were removed for confirmation of their identity microscopically.

Results

These are summarized in Table 10. In the controls the numbers of fourth stage larvae of *H. placei, Cooperia* spp. and *O. radiatum* were considerably lower than the numbers of fifth and adult stages. The number of adult *B. phlebotomum* was again too low for subsequent efficacy analysis.

When compared with the results in Experiments 1 and 2, large numbers of worms were trapped in some of the nylon cloths. In Calf 51, 292 *H. placei* and 219 *Cooperia* spp.; in Calf 52, 309 and 150, and in Calf 54, 969 and 343 respectively were trapped in nylon mesh cloths. In the other autopsies the cloths were either free of worms or not more than 30 were trapped. This seems to indicate that worms were destroyed when the nylon was dissolved with HCl in the previous experiments.

In the controls, 8 to 266 *H. placei* were recovered from the abomasal wall while 8 to 105 *Cooperia* spp. and up to 66 *O. radiatum* were recovered from digested intestinal walls. Three of these calves were negative for larval stages of *O. radiatum*.

As indicated in Table 11, mebendazole maintains its efficacy against fifth stage and adult *H. placei* and reaches Class A against adult *O. radiatum*. It failed, however, to meet the minimum requirements for Class C against fourth stage larvae of *O. radiatum* and is therefore graded Class X, i.e. ineffective. This is considerably worse than the analysis in Experiment 2, when it was graded Class B. Probably the design of Experiment 3 discriminates against the anthelmintic.

For *Cooperia* spp. the Class C classification is maintained for fourth stage larvae, as was the case in Experiment 2 and it improved to Class B against fifth stage and adult worms. In the controls the median worm count for both *Oesophagostomum* and *Cooperia* and those below it were recounted and in the treated calves five of the highest values were recounted.

Discussion

The time taken to count, transfer the worms to specimen bottles and then identify the species and stage of development microscopically in the 11 controls varies from 8,00 to 17,25 hours per calf (mean 10,7 hours). In the treated animals the time required varies from 3,2 to 5,6 hours (mean 4,0 hours). To check the total number of *O. radiatum* present in the digest and caecal and colonic ingesta with a stereomicroscope may take 6 hours, whereas a recount of either *Cooperia* spp. from the small intestine or *H. placei* from the abomasum should not take more than 3 hours.

The three experiments described above showed that calves can readily be infested experimentally at regular intervals with *H. placei, Cooperia* spp. and *O. radiatum* and develop adequate worm burdens for the larval anthelmintic tests. Moreover, the efficacy of the compound can be assessed by the Modified NPM.

TABLE 12 Experiment 4. Experimental design

Day	No. of infective larvae dosed to each calf		
	O. ostertagi	*C. oncophora*	*O. radiatum*
—10.	336	394	—
— 9.	474	514	—
— 8.	520	562	296
— 7.	695	668	355
— 6.	444	667	335
— 5.	480	664	218
— 4.	918	782	216
— 3.	—	—	200
— 2.	—	—	239
— 1.	—	—	255
Total	3 867	4 251	2 114
0.	Killed Calf 66 Day 0 control Treated Calf 70, 71 & 72 with mebendazole at 30 mg/kg Treated Calf 73, 74 & 75 with mebendazole at 40 mg/kg Treated Calf 76, 77 & 78 with levamisole at 5 mg/kg		
+17.	Killed Calf 67: Day +17 control Killed Calf 70: treated on Day 0 with mebendazole at 30 mg/kg Killed Calf 73 & 74: treated on Day 0 with mebendazole at 40 mg/kg		
+18.	Killed Calf 68: Day +18 control Killed Calf 71: treated on Day 0 with mebendazole at 30 mg/kg Killed Calf 76 & 77: treated on Day 0 with levamisole at 5 mg/kg		
+19.	Killed Calf 69: Day +19 control Killed Calf 72: treated on Day 0 with mebendazole at 30 mg/kg Killed Calf 75: treated on Day 0 with mebendazole at 40 mg/kg Killed Calf 78: treated on Day 0 with levamisole at 5 mg/kg		

TABLE 13 Experiment 4. Worms recovered at autopsy and anthelmintic efficacy

Group	Calf No.	*O. ostertagi*					*O. oncophora*				*O. radiatum*			
		Stage of development				Total	Stage of development			Total	Stage of development			Total
		L_3	L_4	5	A		L_4	5	A		L_3	L_4	5	
Controls														
Killed on Day 0	66	459	2 104	0	0	2 563	1 210	540	0	1 750	420	0	0	420
Killed on Day +17	67	0	81	706	2 420	3 207	15	51	2 021	2 087	7	276	1 241	1 524
Killed on Day +18	68	0	0	350	1 669	2 019	16	49	2 235	2 300	20	458	780	1 258
Killed on Day +19	69	0	2	203	2 356	2 561	0	57	1 851	1 908	0	298	1 160	1 458
Treated														
Treated on Day 0 with mebendazole at 30 mg/kg														
Killed on Day +17	70	0	40	806	857	1 703	0	3	59	62	0	358	367	725
Killed on Day +18	71	0	10	504	1 149	1 663	0	0	14	14	0	295	654	949
Killed on Day +19	72	0	0	354	1 540	1 894	0	0	64	64	0	165	960	1 125
Treated on Day 0 with mebendazole at 40 mg/kg														
Killed on Day +17	73	0	0	876	1 499	2 375	0	0	40	40	3	216	1 129	1 348
Killed on Day +17	74	0	0	322	1 159	1 481	0	1	3	4	30	215	227	472
Killed on Day +19	75	0	9	327	1 821	2 157	0	1	40	41	3	62	1 034	1 099
Treated on Day 0 with levamisole at 5 mg/kg														
Killed on Day +18	76	0	60	713	1 547	2 320	0	0	1	1	3	208	279	490
Killed on Day +18	77	0	0	356	1 448	1 804	0	0	21	21	0	229	168	397
Killed on Day +19	78	0	0	280	1 604	1 884	0	3	2	5	7	141	689	837
		Anthelmintic efficacy												
Control median (excluding Calf 66)		2 561					2 087				1 458			
		2 561 × 0,5 = 1 280,5					2 087 × 0,25 = 521,75				1 458 × 0,5 = 729,0			
Hypothetical grading if more calves were dosed Treated														
Mebendazole at 30 mg/kg		3/3 exceed 1 280,5 Class X					0/3 exceed 521,75 Class A				2/3 exceed 729 Class X			
Mebendazole at 40 mg/kg		3/3 exceed 1 280,5 Class X					0/3 exceed 521,75 Class A				2/3 exceed 729 Class X			
Levamisole at 5 mg/kg		3/3 exceed 1 280,5 Class X					0/3 exceed 521,75 Class A				1/3 exceed 729 Class X			

Experiment 4. Dose Determination

Certain problems remained to be solved. The small larval stages are difficult to find in large masses of ingesta and digested gut and the process of recovery is extremely laborious. When the worms can be seen macroscopically they are more easily recovered and efficiency increases. It follows that it is also easier to check the worm counts under these conditions.

The results with mebendazole in previous experiments showed that this compound is not very effective against the larval stages of either *O. radiatum* or *Cooperia* spp. and it was thought that if the dose were increased the results might improve. It also was felt that anthelmintic tests should be carried out on *O. ostertagi*. With this in view a dose determination trial was planned, in which the dose of mebendazole was increased to (a) one-and-a-half times and (b) twice the therapeutic dose and it was compared with levamisole. The tests were carried out against third stage larvae of *O. radiatum* and fourth stage larvae of *O. ostertagi* and *Cooperia* spp.

With the exception of the Day 0 control (Calf 66) killed on the day of treatment, slaughter was delayed for 2 weeks to allow the worms to grow to the fifth or adult stage, when they are more easily seen and recovered *post mortem*.

Materials and methods

Thirteen weaned Afrikaner calves were each dosed with 50 ml of a 7,5% m/v levamisole solution, i.e. from 29 to 42 mg/kg.

A mixed culture of infective larvae of *O. ostertagi* and *C. oncophora* in almost equal proportions was dosed to these calves from Day —10 to Day —4 and *O. radiatum* from Day —9 to Day —1 (Table 12). On Day 0 these calves were divided into four groups (Table 12) as follows:

(i) Undosed controls; (ii) treated with mebendazole at 30 mg/kg dosed with a stomach tube; (iii) treated with mebendazole at 40 mg/kg dosed with a stomach tube; (iv) treated with levamisole at 5 mg/kg injected intramuscularly.

At autopsy the gastro-intestinal tract was divided into the following portions: abomasum, duodenum, proximal small intestine, distal small intestine, caecum plus proximal colon and distal colon plus rectum. The ingesta of each portion of the intestinal tract was as described previously. In the control calves the abomasum and various parts of the small intestine were digested separately but the entire wall of the caecum and colon was digested as a unit. In the treated calves only the ingesta of each portion of the intestine were kept separate, while the wall of the intestinal tract was pooled for digestion.

Worms were recovered microscopically from Calf 66 and the digesta of the other calves. However, the intestinal ingesta of all the other calves (Calf 67 to 78 inclusive) were examined macroscopically on flat plastic trays.

The nylon cloths were placed on a board with a dull green surface and the trapped worms counted. If possible 10 worms were removed for subsequent identification.

The larval stages of *O. ostertagi* were identified according to the descriptions by Douvres (1956) and Rose (1969).

These data and the estimation of efficacy are summarized in Table 13.

The autopsy on Calf 66, killed on the day of treatment, revealed third stage larvae of *O. radiatum,* third and fourth stages larvae of *O. ostertagi* plus fourth stage larvae and fifth stage *O. oncophora* (Table 13). With the exception of *O. ostertagi,* there was a marked increase in the number of worms recovered at autopsy from the controls killed later on Days +17, +18 and +19. Only 420 *O. radiatum* were recovered from Calf 66 but the lowest burden of this species in the three controls killed subsequently was 1 258 in Calf 68 (Table 13). Calf 66 yielded 1 750 *C. oncophora* while the burdens of this species ranged from 1 908 to 2 300 worms in the other three controls.

Anthelmintic efficacy: Neither compound had any effect on *O. ostertagi.* At dosage rates of 30 and 40 mg/kg, mebendazole appeared to have little if any effect on *O. radiatum* but levamisole seemed to have some efficacy against third stage larvae of this species. The best compound against *C. oncophora* was levamisole but mebendazole was also highly effective at both dosage rates (Table 13).

Worm distribution: .Total worm counts were made in the controls and the distribution of the species in the various parts of the gut expressed as a percentage (Table 14). The highest percentage of *O. ostertagi* was present in the digested abomasal wall, though in Calf 66 (killed on the day of treatment) 23,6% of the worms were recovered.from the filtrate of the proximal half of the small intestine. With this species no useful object is served in doing a separate examination of the duodenum. Only in Calf 69 were 5,2% of the *O. ostertagi* recovered from the duodenum and the nylon used in the separation of the filtrate and residue from this organ.

Most of the *C. oncophora* were present in the proximal half of the small intestine but up to 10% were from the filtrate of the duodenum. Although 69,7 to 90,0% were present in the filtrate of the proximal half of the jejunum, in one animal (Calf 69) an alarmingly large percentage (19,7%) were trapped in the nylon mesh.

Table 14 Experiment 4: Worm distribution in the control calves

Site of recovery	Range expressed as a percentage		
	O. ostertagi	*C. oncophora*	*O. radiatum*
	%	%	%
Abomasum			
wall disgest	33,2–84,4	0	0
ingesta filtrate	0,9–32,8	0,0–0,002	0,0–0,02
ingesta residue	0,0– 2,4	0	0
nylon mesh	0,01– 0,3	0	0
Duodenum			
wall digest	0,0– 0,4	0,0–0,03	0
ingesta filtrate	0,0– 4,0	1,3–10,0	0,0–0,02
ingesta filtrate	0,0– 0,6	0,0–0,02	0
nylon mesh	0,0– 0,2	0,0–0,14	0
Small intestine			
proximal ingesta filtrate	0,0–23,6	69,7–90,0	0
distal ingesta filtrate	0	0,0– 3,7	0
proximal ingesta residue	0	6,1–21,8	0
distal ingesta residue	0	0,0–11,7	0,0– 0,4
nylon mesh	0	0,4–19,7	
Caecum & colon			
proximal ingesta filtrate	0		
	0	0	86,0–97,9
distal ingesta filtrate	0	0	0,0– 7,7
proximal ingesta residue	0	0	1,2–12,4
distal ingesta residue	0	0	0,0– 0,4
nylon mesh	0	0	0,0– 2,0
Small intestine, colon & caecum			
Wall digest	0,0–0,04	0,0– 0,1	0,02–99,0

The experiment was designed so that on the day of treatment third stage larvae of *O. radiatum* would be present in the intestinal wall and this accounted for the fact that 99% were recovered from the digests of the small intestine and caecum in Calf 66. In the other calves, however, it ranged from 0,02 to 6,9%. Once the worms entered the gut lumen *O. radiatum* accumulated in the ingesta of the caecum and in the proximal part of the colon up to the end of the *ansa spiralis*. In the three calves slaughtered on Day +17, +18 and +19 respectively, however, there were as many as 7,7% in the filtrate and 0,4% in the residue of the ingesta from the distal part of the descending colon.

Macroscopic recovery of worms: The specimens from Calf 66 were examined under the stereoscopic microscope because the worms were still in the early larval stages and too small to be easily seen macroscopically. The worms were recovered macroscopically from the other 12 calves killed on Day +17, +18 and +19, except from the digested abomasal wall and the digests of the rest of the intestinal tract, from which they were recovered microscopically. They were all identified microscopically. The entire process of worm recovery and identification was completed for these 12 calves in 14 days, i.e. a little more than 10 hours per calf. However, the three controls with large numbers of worms may take as long as 15 hours per autopsy while the treated calves with low worm burdens take as little as 5 hours to examine. None-the-less counting and removing large worms without using a microscope reduces the working time by at least 50 to 60% compared with that taken for the microscopic examinations in previous experiments (e.g. Experiment 3).

A 1/20 aliquot was taken from each specimen and examined microscopically to check whether any worms had been missed in the macroscopic examination. This confirmed the results of the previous examination and proved that the macroscopic examination is completely satisfactory. Despite this, if any small worms are present in a particular specimen it should be examined with a stereoscopic microscope to avoid any possible error.

Comment: Three modifications were introduced in the trial. Firstly, if the worms can be seen macroscopically they can be recovered quicker and with less tedium. It is therefore essential to delay slaughter for as long as possible to give the larval stages time to develop into adults. Secondly, species such as *C. oncophora* are almost entirely confined to the proximal half of the jejunum and although most of them migrated through the nylon mesh into the filtrate many remained trapped in this mesh. Thirdly, *O. radiatum* was distributed throughout the entire length of the colon and it was impossible to discard the last half of the descending colon, as is the case in sheep, because up to 7% of this species occurred there (Table 14).

Experiment 5: Third Stages Larvae, Fifth and Adult Stages

It was still necessary to work out an anthelmintic test for *O. ostertagi*. Turner, Kates & Wilson (1962) and Reinecke (1966) have shown that if sheep are simultaneously infested with infective larvae of *Haemonchus contortus* and *Ostertagia circumcincta* the two species interact with each other to the detriment of the former and uniform worm burdens are not produced. It is possible that *H. placei* and *O. ostertagi* in calves may also react with each other similarly and it would therefore be unwise to attempt to mix these two species in the same host. If, therefore, experiments are repeated only one of these two species should be used. In the experiments described below *O. ostertagi* was used as nothing was known about the methods of artificial infestation of this species for anthelmintic tests of this nature.

In the first three experiments described the design was unsatisfactory because there were numerous gaps in the infestation period. The most unsatisfactory species was *B. phlebotomum* and strenuous efforts had to be made to improve the methods of infestation because the controls had very low worm burdens.

Two further experiments were therefore planned in attempts to solve some of the problems encountered and the following species were used: *O. ostertagi, B. phlebotomum, O. radiatum* and a mixture of *Cooperia pectinata* and *C. punctata,* hereafter referred to as *Cooperia* spp. The main object of the trials was to develop a method of testing anthelmintics against *O. ostertagi:* secondly, to improve the experimental design for the other species mentioned, and thirdly, to improve the method of infestation of *B. phlebotomum*.

Materials and methods

Twenty-four weaned dairy calves were used, varying in age from 5 to 9 months at the commencement of the trial. On Day –42 each calf was dosed orally with thiabendazole at 200 mg/kg, followed on Day —41 with

Table 15 Experiment 5. Experimental design

Day	No. of infective larvae dosed to each calf			
	O. radiatum	*B. phlebotomum*	*O. ostertagi*	*Cooperia* spp.
—40	108	3 232	—	—
—39	96	—	—	—
—38	109	—	—	—
—37	108	—	—	—
—36	95	—	—	—
—35	138	—	—	—
—34	136	—	—	—
—33	131	—	—	—
—32	157	—	—	—
—31	113	—	—	—
—30	105	—	—	—
—29	109	—	—	—
—28	149	—	—	—
—27	120	—	—	—
—27	Calf 79 died Day —27 larval viability control			
—26	118	—	—	—
—25	101	—	—	—
—24	125	—	—	—
—23	121	—	—	—
—22	117	—	—	—
—21	112	—	—	—
— 3	—	—	675	863
— 2	—	—	1 913	1 489
— 1	—	—	1 000	1 072
Total	2 368	3 232	3 588	3 424
	The following calves were only dosed on one day with the same batch of infective larvae used for the other calves			
— 3	10 000	Calf 80	2 700	2 254
— 2	10 000	Calf 81	3 826	2 978
— 1	10 000	Calf 82	2 120	2 144
0	Calf 92 to 102 inclusive dosed with levamisole at 5 mg/kg			
+ 3	Calf 80, Day —3 control: Calf 81, Day —2 control and Calf 82, Day —1 control killed			
+22	Calf 83 to 91 inclusive killed, Day +22 controls			
+23	11 Calves treated on Day 0 killed			

levamisole injected intramuscularly at the dosage rate of 15 mg/kg. Details of infestation, treatment and slaughter are summarized in Table 15. A single dose of infective larvae of *O. radiatum, O. ostertagi* and *Cooperia* spp. was administered to Calf 80 on Day –3, Calf 81 on Day —2 and Calf 82 on Day —1 respectively (Table 15).

At autopsy worms were recovered from the four larval viability controls (Calves 79 to 82 inclusive) as described

FIG. 2 Intestinal washing apparatus

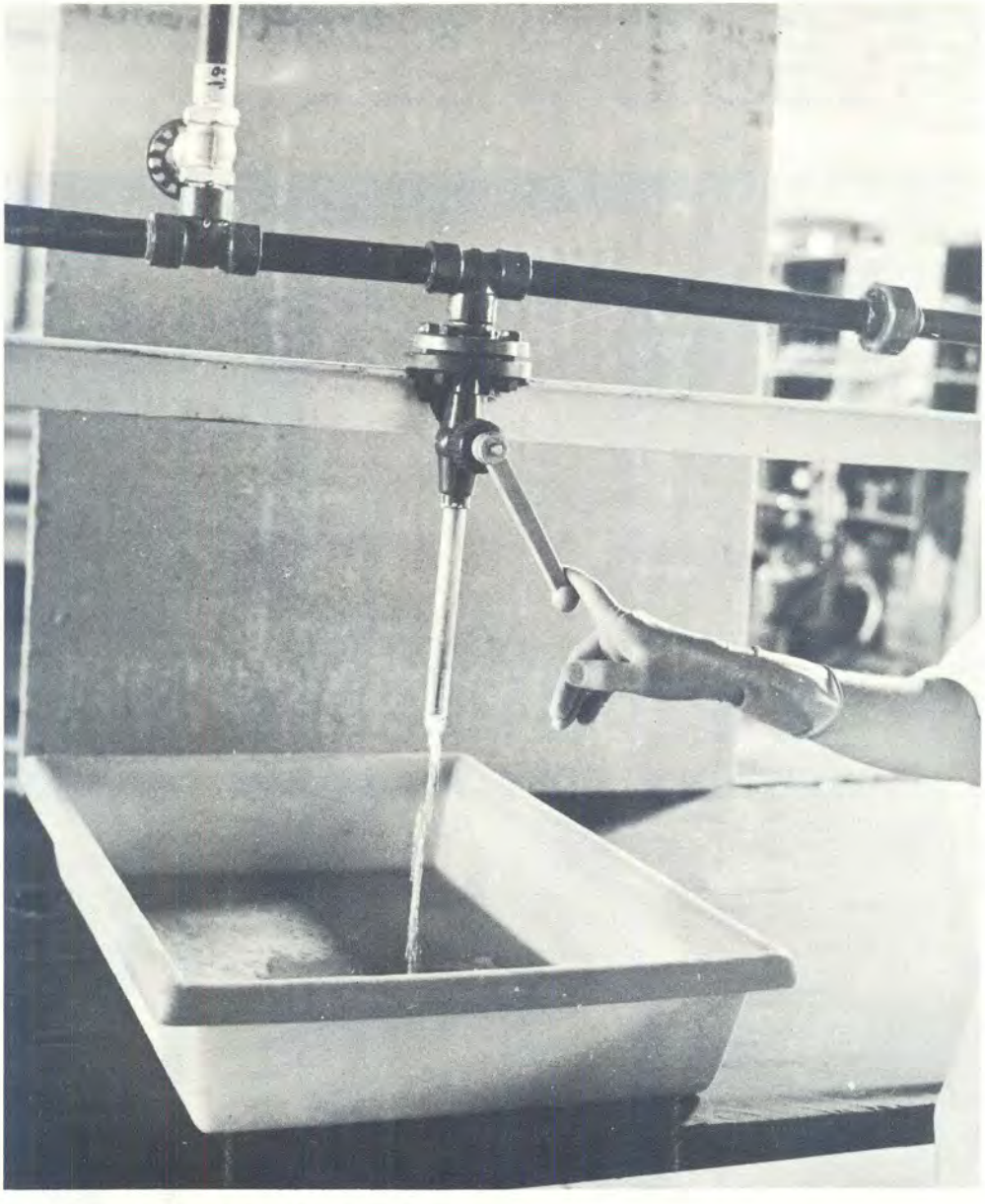

FIG. 3 Operating the stop-cock of the intestinal washing apparatus

for Calf 66 in Experiment 4. All the specimens were counted using the stereomicroscope and identified with the standard microscope. Although Calf 79 died on Day —27 and the other three were killed on Day +3, most of the worms were in the third larval stage.

At autopsy of the remaining nine control and 11 treated calves the abomasum and duodenum were treated as a unit. The proximal small intestine consisted of the first 10 m of the jejunum, and the distal small intestine of the rest of the jejunum and ileum. The caecum and colon constituted one unit. These four specimens were dealt with separately but the wall of the entire jejunum, ileum, caecum and colon was minced and digested together.

As a preliminary test showed that 300 ml of 2% m/v pepsin and 10 N HCl 3% v/v required 4 to 6 hours to digest 100 g minced gut at 50°C, the concentration was increased to 3% m/v which digested the gut in 2 hours.

In this and the subsequent experiment the Intestinal Washing Apparatus (Fig. 2) was used when the intestinal tract was processed. This consists of 2 plastic reservoirs, 250 l capacity, which are filled with physiological saline at 40°C. These reservoirs are on platforms 2,3 m above the floor. Plastic pipes lead to 6 stop-cocks above the workbench and deliver a flow of saline to wash the gut while it is being opened (Fig. 3).

A framework of metal mesh was made to fit into the sink used for sieving (Fig. 4). This provided a platform for the bucket while the liquid is poured into the sieve and another platform for the specimen jar, which is then at the correct level for transferring the specimens with a stream of water.

Nylon cloth with apertures of 225 microns was used in the traps for all calves with the exception of three controls; i.e. Calves 83, 84 and 85. In these autopsies a stiff nylon grit-gauze [Simon-MacForman (Pty) Ltd., Benrose, Johannesburg] with apertures of 500 microns was used. As this was too stiff to lie flat on the plastic grid it was placed on top of the cross bars under the grid. Its edges were clipped on to the upper edges of the

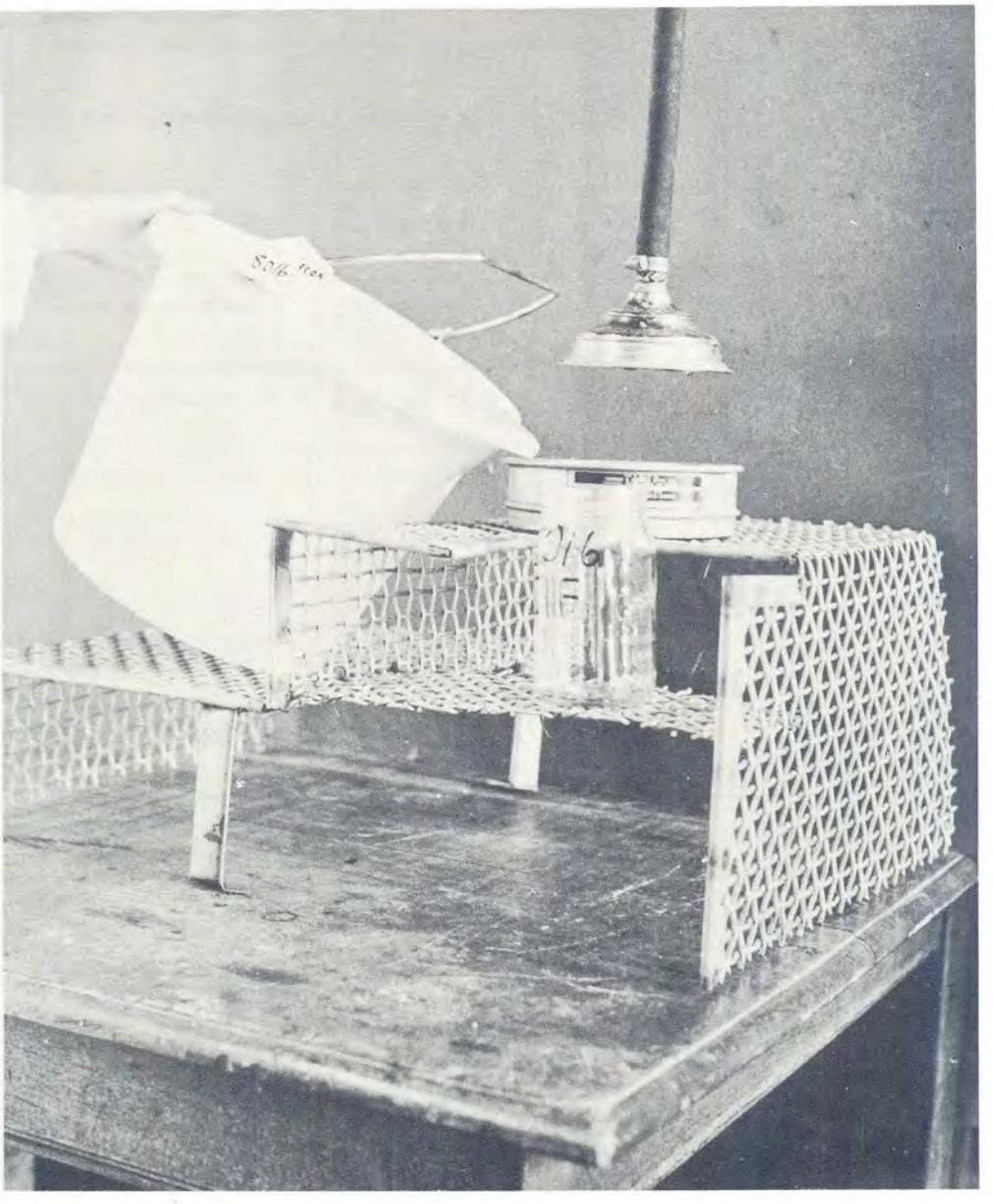

FIG. 4 Apparatus to assist sieving

trap with the type of spring clips used for paper files. Any worms that were trapped after removal of the nylon cloths were left *in situ*, and cloth washed off and placed with the trapped worms in a bottle with formalin.

With the exception of the digested gut wall, which was examined microscopically, all specimens were examined macroscopically to recover and count the worms. As filtrates of the small intestine of Calf 87 contained a few stunted fifth stage and minute fourth stage larvae of *Cooperia* spp., the entire residues and filtrates of its small intestine were examined microscopically and total counts carried out.

When the experiment was finished the median of the controls and the count below it were checked, as well as the reduced count of the treated calves immediately below the value of the reduced median.

Results

The larval viability control that died on Day —27 (Calf 79) contained a few third and fourth stage *O. radiatum* and fourth stage larvae of *B. phlebotomum,* indicating that the viability of the initial larval doses was low. The number of infective larvae of *Cooperia* spp. dosed to Calf 80, (the Day —3 larval viability control) was estimated to be 2 978. This figure was apparently wrong because 3 319 fourth stage larvae were recovered 4 days later at autopsy (Table 16).

With this exception fewer *Cooperia* spp. were recovered from the other two larval viability controls than from most of the controls killed on Day +22 and considerably more *O. ostertagi* from the 9 controls killed on Day +22 than from those killed on Day +3 (Table 16). Although only 6 to 176 *B. phlebotomum* were recovered this was an improvement on previous experiments and these numbers are adequate to determine the median of the controls and assess anthelmintic efficacy.

Worm Distribution: The distribution of worms is summarized in Table 17. In each calf the total number of each species was used to estimate the percentage distribution in the various specimens collected at autopsy.

TABLE 16 Experiment 5. Worms recovered at autopsy

Group	Calf No.	*O. ostertagi*				*Cooperia* spp.				*O. radiatum*					*B. phlebotomum*	
		Stage of development			Total	Stage of development			Total	Stage of development				Total	Stage of development	Total
		L_4	5	A		L_4	5	A		L_3	L_4	5	A		A	
Controls		Larval viability control Day —27														
Died on Day —27	79	0	0	0	0	0	0	0	0	172	140	0	0	312	(10 L_4)	10
		Larval viability control Day —3														
Killed on Day +3	80	365	0	0	365	3 319	0	0	3 319	2 855	5	0	0	2 860	0	0
		Larval viability control Day —2														
Killed on Day +3	81	270	0	0	270	1 595	0	0	1 595	3 642	0	0	0	3 642	0	0
		Larval viability control Day —1														
Killed on Day +3	82	400	0	0	400	1 162	0	0	1 162	1 802	0	0	0	1 802	0	0
Controls																
Killed on Day +22	83	0	362	1 798	2 160	0	0	1 588	1 588	0	0	83	1 443	1 526	107	107
Killed on Day +22	84	3	10	1 436	1 449	0	516	1 392	1 908	0	0	140	881	1 021	176	176
Killed on Day +22	85	0	6	1 773	1 779	0	161	2 030	2 191	0	16	94	1 471	1 581	109	109
Killed on Day +22	86	0	0	1 859	1 859	0	17	2 007	2 024	0	0	49	374	423	43	43
Killed on Day +22	87	12	12	1 039	1 063	76*	124	2	204	0	258	372	91	721	42	42
Killed on Day +22	88	30	0	1 526	1 556	2	0	2 194	2 196	0	62	545	375	982	6	6
Killed on Day +22	89	23	1	1 451	1 475	0	0	2 303	2 303	0	60	72	331	463	6	6
Killed on Day +22	90	14	14	2 208	2 236	46	123	1 260	1 429	0	8	40	1 302	1 350	98(i)	99
Killed on Day +22	91	9	23	1 287	1 319	0	0	2 388	2 388	0	1	456	419	876	22	22
Treated with levamisole at 5 mg/kg																
Killed on Day +23	92	0	0	805	805	0	0	128	128	0	12	0	8	20	0	0
Killed on Day +23	93	0	0	79	79	0	0	38	38	0	24	64	0	88	0	0
Killed on Day +23	94	0	2	680	682	0	0	18	18	0	1	8	0	9	0	0
Killed on Day +23	95	0	2	195	197	0	0	13	13	0	2	3	0	5	0	0
Killed on Day +23	96	0	2	232	234	0	0	19	19	0	1	6	1	8	0	0
Killed on Day +23	97	1	0	390	391	0	0	7	7	0	1	1	0	2	0	0
Killed on Day +23	98	0	2	600	602	0	0	27	27	0	9	0	3	12	0	0
Killed on Day +23	99	1	1	365	367	0	0	12	12	0	10	7	0	17	0	0
Killed on Day +23	100	3	3	524	530	0	0	14	14	0	0	2	0	2	0	0
Killed on Day +23	101	9	0	558	567	0	6	8	14	0	3	1	0	4	0	0
Killed on Day +23	102	0	0	294	294	0	0	10	10	0	3	5	0	8	0	0

*Including two third stage larvae (i)One fifth stage

TABLE 17 Experiment 5. Worm distribution in the control calves

Site of recovery	*O. ostertagi*		*Cooperia* spp.		*O. radiatum*		*B. phlebotomum*	
	Calf 83, 84 & 85	Other calves	Calf 83, 84 & 85	Other calves	Calf 83, 84 & 85	Other calves	Calf 83, 84 & 85	Other calves
	%	%	%	%	%	%	%	%
Abomasum								
wall digest	66,7–80,3	61,8–78,9	0	0	0	0,0–0,1	0	0
ingesta filtrate	19,7–31,9	20,3–37,7	0,0–3,2	0,0–4,2	0	0	10,2–21,5	0
ingesta residue	1,1–3,3	0,1–1,2	0,0–0,1	0,0–0,3	0,0–0,06	0	9,2–46,6	0,0–60,2
nylon mesh	0	0,0–1,4	0	0	0	0,0–0,1	0,0–21,4	0
Small intestine								
proximal ingesta filtrate	0	0	75,3–96,8	0,9–96,8	0	0	7,3–23,4	0,0–9,0
distal ingesta filtrate	0	0	2,0–19,3	2,4–99,1	0	0,0–1,3	0,0–0,9	0
proximal ingesta residue	0	0	1,0–2,2	0,0–1,0	0	0,0–0,8	1,1–27,5	0,0–3,0
distal ingesta residue	0	0	0,0–0,03	0,0–2,7	0,0–0,06	0,0–0,9	0,0–3,7	0,0–2,0
nylon mesh	0	0	0	0,0–0,5	0	0	15,3–40,2	0,0–25,2
Caecum & colon								
ingesta filtrate	0	0,0–1,3	0	0	88,7–95,0	0,6–37,4	0	0
ingesta residue	0	0	0	0	4,5–11,1	61,4–98,2	0	0
nylon mesh	0	0	0	0	0,0–1,5	0,2–0,9	0,0–1,8	0
Small intestine, caecum & colon								
wall digest	0	0	0	0	0,0–0,3	0,0–0,7	0,0–1,9	0

TABLE 18 Experiment 5. Anthelmintic efficacy

O. ostertagi		*Cooperia* spp.		*O. radiatum*		*B. phlebotomum*	
Controls	Treated	Controls	Treated	Controls	Treated	Controls	Treated
1 063	79	204	7	423	2	6	0
1 319	197	1 429	10	463	2	6	0
1 449	234	1 588	12	721	4	22	0
1 475	294	1 908	13	876	5	42	0
1 556	367	*2 024*	14	*982*	8	*43*	0
1 779	391	2 191	14	1 021	8	99	0
1 859	530	2 196	18	1 350	9	107	0
2 160	567	2 303	19	1 526	12	109	0
2 236	602	2 388	27	1 581	17	176	0
	682		38		20		0
	805		128		88		0
1 556 × 0,4 = 622,4 2/11 exceed 622,4 Class B		2 024 × 0,25 = 506 0/11 exceed 506 Class A		982 × 0,25 = 245,5 0/11 exceed 245,5 Class A		No worms in 11/11 calves Class A	

The data from Calves 83, 84 and 85 are grouped together in one column while those from the other control calves are given in the adjacent column.

In the former group the coarser nylon, with apertures of 500 microns, was used at autopsy whereas the finer nylon, with apertures of 250 microns, was used for the latter six calves. Neither *O. ostertagi* nor *Cooperia* spp. were trapped in the coarser nylon mesh but in some of the autopsies a small percentage of these species was trapped in the finer mesh. With the caeco-colonic ingesta, however, as many as 1,5% of the worms were trapped in the coarser mesh while in the finer mesh this varied from 0,2 to 0,9% respectively. The coarser mesh retained a relatively large percentage of *B. phlebotomum;* from 0,0 to 21,4% were trapped from the abomasum and from 15,3 to 40,2% from the small intestine. However, this percentage increased to a maximum of 60,2% with the finer mesh from the abomasum and only reached 25,2% in the small intestine respectively. Microscopic examination showed that the males usually managed to pass into the filtrate while the females got trapped in the mesh. This was confirmed by looking at the residue, in which there were very few males but numerous females. It is possible that the females would be able to negotiate an aperture of 700 microns.

When the coarse mesh was used fifth and even adult *O. radiatum* were predominant in the caeco-colonic filtrate and ranged from 88,7 to 95,0%. They never exceeded 37,4% when the fine mesh was used. In the residue from the latter, however, the numbers of larger worms (fifth stage and adults) varied from 61,4 to 98,2%. This finding is important because it is difficult to see the worms in the large mass of ingesta residue, whereas they are much more easily observed in the filtrate.

Cooperia spp. were again predominant in the proximal 10 m of the jejunum, and largely confined to the filtrate of the ingesta. While the digested intestinal wall yielded disappointingly small numbers of *O. radiatum* (0,7% or less) from 60 to over 80% of all stages of *O. ostertagi* present were recovered from the digested abomasal wall.

Anthelmintic efficacy: The anthelmintic efficacy of levamisole met the requirements of Class A for *Cooperia* spp., *O. radiatum* and *B. phlebotomum* but only Class B for *O. ostertagi* (Table 18). In *O. ostertagi* the median worm count and that immediately below it, i.e.: 1 556 and 1 475 respectively, were recounted. In the treated calves the numbers 602 and 567 worms respectively were also obtained by careful recounting and Class B is therefore an accurate estimate of anthelmintic efficacy.

EXPERIMENT 6. COMBINED TEST

This experiment was an attempt to treat different groups of calves, each containing 11 animals, when the

worms were at different stages of development. Only one group of controls was used. A departure was made from the infestation procedures which result in a combination of fourth stage larvae, fifth and adult stages, as in Experiment 3. The disadvantage of this experimental design is that unless the compound is either highly effective or completely ineffective, analysis of the results can be confusing and differ markedly from the analysis of the results derived from a trial aimed at the fourth stage only, e.g. Experiment 2.

Materials and methods

Thirty-three weaned calves of mixed dairy breeds were dosed with levamisole at 15 mg/kg live mass on Day—26. On Day—21 Calf 103 died but no worms were recovered *post mortem* from this animal. The design of this trial is summarized in Table 19.

TABLE 19 Experiment 6. Experimental design

Day	No. of infective larvae dosed to each calf			
	O. ostertagi	*Cooperia* spp.	*O. radiatum*	*B. phlebotomum*
—23	337	—	—	—
—22	431	—	—	—
—21	341	—	—	—
—21	Calf 103 died; no worms recovered at autopsy			
—20	287	693	170	—
—19	329	582	242	—
—18	334	658	212	—
—17	338	626	209	3 114
—16	549	1 296	185	—
—15	459	469	362	—
—14	362	433	190	—
—13	—	493	254	—
—12	—	399	218	—
—11	—	451	287	—
Total	3 767	6 100	2 329	3 114
—10	Calf 114 to 124 inclusive, dosed with levamisole at 5 mg/kg			
0	Calf 104 killed Day 0 control Calf 125 to 135 inclusive dosed with levamisole at 5 mg/kg			
+14	Calf 105 to 113 inclusive killed, Day 14 controls			
+15	11 Calves treated on Day —10 killed			
+16	11 Calves treated on Day 0 killed			

The remaining 32 calves were dosed percutaneously with 3114 infective larvae of *B. phlebotomum* on Day—17. (Method - Experiment 1).

At autopsy the lungs of Calf 104 were examined as described in Experiment 1. The lungs from the other calves were not examined.

At autopsy the abomasum was incised at the pylorus (i.e. the duodenum was not included with it); the entire small intestine was divided into two equal halves; the caecum plus colon was again treated as a unit.

The nylon mesh with apertures of 225 micron was used throughout.

Results

The results are summarized in Tables 20 to 22. Calf 104, the Day 0 control, was infested with fourth stage larvae of *B. phlebotomum,* predominantly fourth stage larvae of *O. radiatum,* fifth stage and adult *O. ostertagi* (more fifth stage than adults) and in the case of the *Cooperia* spp., more adult than fifth stage. For the first time in this series of experiments *B. phlebotomum* was nearly always present in large numbers; one calf only had six worms (Calf 113) but the others had from 109 to 1 290 worms. The worm burdens of the other species were eminently satisfactory.

Worm distribution: This is summarized in Table 21, in which Calf 104 (the Day 0 control) is compared with the controls killed on Day +14. On Day 0, 93% *O. ostertagi* were present in the abomasal wall, but 14 days later 54,0 to 74,4% were in the abomasal ingesta filtrate. From 2,9 to 17,2% were trapped in the nylon mesh. In Calf 104 a surprisingly large number of *Cooperia* spp. (26,1%) were present in the abomasal ingesta filtrate and only 48,3% were in the ingesta filtrate of the proximal half of the small intestine. In the other controls, however, 50,3% to 79,9% were recovered from the ingesta of the proximal filtrate and 3,3 to 22,4% from the proximal residue. A high percentage, varying from 10,4 to 29,8% was present in the nylon mesh from the small intestine.

In the Day 0 control 95,3% of the *O. radiatum* were in the filtrate of the caecum and colon but in calves killed 14 days later from 4,1 to 64,3% were in this filtrate while 34,8 to 93,7% were in its residue. In the case of *B. phlebotomum* in Calf 104 (the Day 0 control), where all the worms were still fourth stage larvae, 100% were present in the filtrate of the ingesta from the proximal small intestine. Once they had developed to the fifth stage in the other controls the majority (57,8 to 99,4%) were recovered from the residue of the proximal part of the small intestine. In one animal (Calf 110), however, 40,6% had migrated through into the filtrate. Very few if any (0 to 0,3%) were trapped in the nylon mesh.

As in the previous experiments digestion of the intestinal wall was disappointing; only 0,8%, if that, *O. radiatum* were present there. In one calf 5,0% *Cooperia* spp. were present in the digested wall.

Anthelmintic efficacy (Table 22): In the controls the median and the numbers above and below it were recounted for each species; in *B. phlebotomum all* the numbers below the median were recounted. In the treated groups the reduced median and the value below it were recounted wherever the compound was effective. It was not done with *Cooperia* spp. because the reduction was so marked that checking was pointless.

The results can be summarized as follows:– Levamisole was ineffective against *O. ostertagi* in the fourth stage but attained Class C against the fifth stage and adults; with *Cooperia* spp. against both third and fourth stage larvae in the one group and fifth stage and adult worms in the other it easily attained Class A; for *O. radiatum* the compound was ineffective against third stage larvae but achieved Class A against fourth stage larvae; in *B. phlebotomum* it achieved Class C for third stage larvae and Class A for fourth stage larvae.

DISCUSSION

The experiments described in this paper have shown that it is feasible to carry out controlled anthelmintic tests with worm-free calves. Suitable experimental groups for these tests can be created by repeated oral dosage with infective larvae of *O. ostertagi, H. placei, Cooperia* spp. and *O. radiatum* and a single percutaneous infestation with infective larvae of *B. phlebotomum.* Calves can be infested in such a way that at treatment worms are either present as third stage larvae, fourth stage larvae or fifth and adult stages. Moreover, enough calves can be infested to enable interpretation of the data by the modified NPM.

TABLE 20 Experiment 6. Worms recovered at autopsy

Group	Calf No.	*O. ostertagi*				*Cooperia* spp.				*O. radiatum*				*B. phlebotomum*	
		Stage of development			Total	Stage of development			Total	Stage of development			Total	Stage of development	Total
		L_4	5	A		L_4	5	A		L_3	L_4	5		5	
Controls															
Killed on Day 0	104	17	1 289	423	1 729	59	1 301	2 477	3 837	7	1 189	0	1 196	51*	51
Killed on Day +14	105	7	38	2 646	2 691	0	0	4 774	4 774	4	442	578	1 024	109	109
Killed on Day +14	106	2	14	1 889	1 905	0	100	5 887	5 987	0	148	1 042	1 190	1 031	1 031
Killed on Day +14	107	18	0	1 909	1 927	0	1	1 694	1 695	0	58	1 247	1 305	816	816
Killed on Day +14	108	36	29	2 460	2 525	0	0	5 023	5 023	0	12	1 546	1 558	1290	1 290
Killed on Day +14	109	10	18	2 079	2 107	0	0	5 023	5 023	0	172	813	985	341	341
Killed on Day +14	110	0	0	2 066	2 066	0	0	3 425	3 425	1	31	697	729	763	763
Killed on Day +14	111	0	0	1 703	1 703	0	0	3 582	3 582	1	201	1 134	1 336	344	344
Killed on Day +14	112	0	0	2 772	2 772	0	0	3 856	3 856	0	137	1 405	1 542	472	472
Killed on Day +14	113	4	0	1 394	1 398	0	1	3 343	3 344	0	1	1 072	1 073	6	6
Treated on Day —10 with levamisole at 5 mg/kg															
Killed on Day +15	114	9	14	1 637	1 660	0	0	7	7	1	37	660	698	1	1
Killed on Day +15	115	1	8	1 447	1 456	0	0	29	29	1	54	881	936	38	38
Killed on Day +15	116	0	0	1 472	1 472	0	0	10	10	0	51	1 032	1 083	293	293
Killed on Day +15	117	1	0	1 299	1 300	0	0	42	42	0	70	600	670	92	92
Killed on Day +15	118	0	0	1 907	1 907	0	0	15	15	0	36	954	990	298	298
Killed on Day +15	119	5	14	1 388	1 407	0	0	10	10	0	35	257	292	332	332
Killed on Day +15	120	9	9	2 054	2 072	0	0	3	3	0	36	839	875	98	98
Killed on Day +15	121	10	2	1 847	1 859	0	0	31	31	0	41	882	923	417**	417
Killed on Day +15	122	4	0	2 114	2 118	0	0	61	61	0	57	1 233	1 290	6	6
Killed on Day +15	123	1	0	1 515	1 516	0	0	0	0	0	28	748	776	28**	28
Killed on Day +15	124	1	0	1 720	1 721	0	0	32	32	0	68	753	821	214	214
Treated on Day 0 with levamisole at 5 mg/kg															
Killed on Day +16	125	9	7	1 016	1 032	0	0	0	0	0	60	204	264	1**	1
Killed on Day +16	126	4	11	949	964	0	0	0	0	1	27	107	135	1**	1
Killed on Day +16	127	11	2	1 294	1 307	0	0	5	5	0	8	210	218	3**	3
Killed on Day +16	128	0	0	404	404	0	0	0	0	0	16	112	128	0	0
Killed on Day +16	129	9	5	1 174	1 188	0	0	2	2	0	13	19	32	1**	1
Killed on Day +16	130	4	8	333	345	0	0	0	0	0	46	15	61	0	0
Killed on Day +16	131	2	5	477	484	0	0	0	0	0	9	64	73	0	0
Killed on Day +16	132	6	6	647	659	0	0	0	0	0	10	39	49	0	0
Killed on Day +16	133	10	12	973	995	0	0	2	2	0	22	142	164	2	2
Killed on Day +16	134	2	4	839	845	0	0	1	1	0	8	57	65	0	0
Killed on Day +16	135	15	3	978	996	0	0	0	0	0	39	104	143	0	0

*Fourth stage larvae **Including one fourth moult

TABLE 21 Experiment 6. Worm distribution in the control calves

Site of recovery	*O. ostertagi*		*Cooperia* spp.		*O. radiatum*		*B. phlebotomum*	
	Calf 104	Other calves	Calf 104	Other calves	Calf 104	Other calves	Calf 104	Other calves
	%	%	%	%	%	%	%	%
Abomasum								
wall digest	93,0	7,1–19,7	3,1	0,0–5,0	0	0	0	0
ingesta filtrate	5,7	54,0–74,4	26,1	0,0–1,8	0	0	0	0
ingesta residue	1,2	5,2–16,8	5,0	0,0–0,3	0	0,0–0,4	0	0,0–0,3
nylon mesh	0	2,9–17,2	7,6	0,0–1,4	0	0	0	0
Small intestine								
proximal ingesta filtrate	0	0,0–1,3	48,3	50,3–79,9	0	0	100,0	0,0–40,6
distal ingesta filtrate	0	0,0–0,01	0,6	0,0–0,6	3,5	0,0–3,5	0	0,0–4,9
proximal ingesta residue	0	0	2,7	3,3–22,4	0	0,0–0,5	0	57,8–99,4
distal ingesta residue	0	0	0,01	0,0–0,3	0	0,0–0,5	0	0,0–1,7
nylon mesh	0	0	6,4	10,4–29,8	0	0,0–0,02	0	0,0–0,3
Caecum and colon								
ingesta filtrate	0	0	0	0	95,3	4,1–64,3	0	0,0–0,1
ingesta residue	0	0	0	0–0,01	0,6	34,8–93,7	0	0,0–0,3
nylon mesh	0	0	0	0	0	0,0–1,5	0	0,0–0,4
Small intestine, caecum & colon								
wall digest	0	0	0,4	0,01–5,0	0,6	0,0–0,8	0	0,0–1,4

TABLE 22 Experiment 6. Anthelmintic efficacy

O. ostertagi			*Cooperia* spp.			*O. radiatum*			*B. phlebotomum*		
Controls	L_4	5th & A	Controls	L_3 & L_4	5th & A	Controls	L_3	L_4	Controls	L_3	L_4
1 398	1 300	345	1 695	0	0	729	292	32	6	1	0
1 703	1 407	404	3 344	3	0	985	670	49	109	6	0
1 905	1 456	484	3 425	7	0	1 024	698	61	341	28	0
1 927	1 472	659	3 582	10	0	1 073	776	65	344	38	0
2 066	1 516	845	*3 856*	10	0	*1 190*	821	73	*472*	92	0
2 107	1 660	964	4 774	15	0	1 305	875	128	763	98	0
2 525	1 721	995	5 023	29	0	1 336	923	135	816	214	1
2 691	1 859	996	5 023	31	1	1 542	936	143	1 031	293	1
2 772	1 907	1 032	5 987	32	2	1 558	990	164	1 290	298	1
	2 072	1 188		42	2		1 083	218		332	2
	2 118	1 307		61	5		1 290	264		417	3
2 066 × 0,5 = 1 033	11/11 exceed 1 033	2/11 exceed 1 033	3 856 × 0,25 = 964	0/11 exceed 964	0/11 exceed 964	1 190 × 0,25 = 297,5 1 190 × 0,5 = 595,0	10/11 exceed 595	0/11 exceed 297,5	472 × 0,25 = 118 472 × 0,5 = 236	4/11 exceed 236	0/11 exceed 118
	Class X	Class C		Class A	Class A		Class X	Class A		Class C	Class A

The methods of carrying out these tests have been described in detail in previous pages. In this discussion attention is only drawn to the more important points that may not have been sufficiently emphasised.

(i) *Worm-free calves:* The best hosts are weaned dairy calves that have been reared for most of their lives in batteries or stables where the chances of worm infestation are minimal. They can adapt to different rations and are reasonably resistant to most of the diseases of neonatal calves. None the less they should be housed in hygienic stables and regarded as potential sources of infectious or contagious diseases for at least 2 weeks before they are used in anthelmintic tests. It is essential to dose them with anthelmintics at two to three times the usual therapeutic dose before they are used in experiments. It is a wise precaution to include two extra calves in every experiment in case unexpected deaths occur.

(ii) *Infective larvae:* Pure strains of infective larvae of each species used in the experiment are desirable. They are not necessarily essential in every trial, e.g. a mixture of *O. ostertagi* and *C. oncophora* was successfully used in Experiment 4. This, however, can only be done when the contaminant does not interfere with the main object of the experiment. Wherever possible newly harvested larvae should be used. In trials with cattle parasites, however, this is frequently impractical (if not impossible) because the donor calves only excrete large numbers of worm eggs in their faeces for a very short period. Three practical ways of solving this problem are:–

1. Collection of the entire faecal output of a calf with a "negative" egg count (50 c.p.g. or less) and the preparation of hundreds of faecal cultures from it. This had to be done in Experiments 5 and 6 to collect enough infective larvae of *O. ostertagi,* when up to 60 cultures were harvested three times a day. When the faeces are completely negative this approach cannot be used.
2. Bulk collection of faeces while calves have positive egg counts is essential. Cortisone and its derivatives can be used as immunosuppressants to boost egg production. Faeces are stored at 4°C in the refrigerator and can be kept for at least 2 months before viable cultures of *H. placei, O. ostertagi, Cooperia* spp. are made. This method of egg storage was satisfactory for at least 4 weeks for *B. phlebotomum* but additional research is essential to prove the long-

evity of eggs of the different species stored in this way.

3. Storage of infective larvae in de-ionised water in flat-sided medicine bottles is highly recommended. Larvae should be stored at concentrations not exceeding 5 000 larvae per ml water in bottles laid on their sides in which the water level must not exceed 5 mm. Here these are stored in cupboards at room temperature but in Weybridge storage in a refrigerator at 4°C is advocated (Anon., 1971). With the exception of *B. phlebotomum* most species, if they are ensheathed and fully motile, remain infective for as long as 4 months if stored in this way.

(iii) *Experimental design:* In Tables 23, 24, 25 and 26 optimal patterns of experimental infestation, treatment and slaughter are summarized. There is no doubt that experiments designed to test the efficacy of a compound against a specific stage of development are the best (see Tables 23, 24 and 25) but a combined test can be equally good if it is limited to 3 species and does not include the entire range given in Table 26. The combined test may be used in experiments with mebendazole against *H. placei* and with levamisole against *Cooperia* spp. because these compounds are examples of excellent anthelmintics for these species. If preliminary tests show that the compound is only moderately effective against any particular species this combined test must be avoided.

If a combined test is used the design advocated in Table 26, (Experiment 6) is better than that of Experiment 3. In the latter, fourth stage larvae, fifth stage and adult worms are all present on the

TABLE 23 Third stage larvae. Experimental design

Day	Infestation procedure (L = infective larvae)						
	H. placei	*O. ostertagi*	*Cooperia* spp.	*B. phlebotomum*	*O. radiatum*	*T. axei*	*N. helvetianus*
—10	—	—	—	—	L	—	—
— 9	—	—	—	—	L	—	—
— 8	—	—	—	—	L	—	—
— 7	—	—	—	L	L	—	—
— 6	—	—	—	—	L	—	—
— 5	—	—	—	—	L	—	L
— 4	—	—	—	—	L	—	L
— 3	—	L	L	—	L	L	L
— 2	L	L	L	—	L	L	L
— 1	L	L	L	—	L	L	L
0	Treat. Slaughter Indicator control						
+35	Slaughter controls and treated calves						

Total larval doses

H. placei	5 000L
O. ostertagi	4 000L
Cooperia spp.	6 000L
B. phlebotomum	3 000L
O. radiatum	2 500L
T. axei	5 000L
N. helvetianus	5 000L

Total No. of calves

Indicator control	1
Other controls	9
Minimum	5
Treated calves	11

TABLE 24 Fourth stage larvae. Experimental design

Day	Infestation procedure (L = infective larvae)						
	H. placei	*O. ostertagi*	*Cooperia* spp.	*B. phlebotomum*	*O. radiatum*	*T. axei*	*N. helvetianus*
—20	—	—	—	Max — 20	L	—	—
—19	—	—	—	↑	L	—	—
—18	—	—	—	L	L	—	—
—17	—	—	—	↓	L	—	—
—16	—	—	—		L	—	L
—15	—	—	—	Min — 15	L	—	L
—14	L	—	—	—	L	—	L
—13	L	—	—	—	L	—	L
—12	L	—	—	—	L	—	L
—11	L	L	—	—	L	L	L
—10	L	L	—	—	—	L	L
— 9	L	L	—	—	—	L	L
— 8	L	L	L	—	—	L	L
— 7	L	L	L	—	—	L	L
— 6	L	L	L	—	—	L	L
— 5	L	L	L	—	—	L	—
— 4	L	L	L	—	—	L	—
— 3	L	—	—	—	—	—	—
0	Treat. Slaughter indicator control						
+28	Slaughter controls and treated calves						

TABLE 25 Fifth Stage and adults. Experimental design

Day	Infestation procedure (L = infective larvae)						
	H. placei	*O. ostertagi*	*Cooperia** spp.	*B. phlebotomum*	*O. radiatum*	*T. axei*	*N. helvetianus*
—60	—	—	—	L (Adult)	—	—	
—40	—	—	—	or L (5th)	L	—	
—39	—	—	—	—	L	—	
—38	—	—	—	—	L	—	—
—37	—	—	—	—	L	—	—
—36	—	—	—	—	L	—	—
—35	—	—	—	—	*L	—	—
—34	—	—	—	—	L	—	—
—33	—	—	—	—	L	—	—
—32	—	—	—	—	L	—	—
—31	—	—	—	—	L	—	—
—30	L	—	—	—	L	—	—
—29	L	—	—	—	L	—	—
—28	*L	L	—	—	L	L	L
—27	L	L	—	—	L	L	L
—26	L	L	—	—	L	L	*L
—25	L	L	—	—	L	L	L
—24	L	L	—	—	L	L	L
—23	L	*L	—	—	L	L	L
—22	L	L	—	—	L	L	L
—21	L	L	—	—	L	*L	L
—20	L	L	L	—	—	L	L
—19	L	L	L	—	—	L	L
—18	L	L	L	—	—	L	L
—17	L	L	L	—	—	L	L
—16	L	L	L	—	—	L	—
—15	L	L	L	—	—	L	—
—14	—	L	*L	—	—	L	—
—13	—	L	L	—	—	L	—
—12	—	L	L	—	—	L	—
—11	—	—	L	—	—	—	—
—10	—	—	L	—	—	—	—
— 9	—	—	L	—	—	—	—
0	Treat. Slaughter indicator control						
+14	Slaughter controls and treated calves						

*The oldest worms should exceed the prepatent period but the number of infestations in the patent period are optional

TABLE 26 Combined trial Experimental design

Day	Infestation procedure (L = infective larvae)						
	H. placei	*O. ostertagi*	*Cooperia** spp.	*B. phlebotomum*	*O. radiatum*	*T. axei*	*N. helvetianus*
—26	—	—	—	—	—	—	L
—25	—	—	—	—	—	—	L
—24	L	—	—	—	—	—	L
—23	L	L	—	—	—	L	L
—22	L	L	—	—	—	L	L
—21	L	L	—	—	—	L	L
—20	L	L	L	—	L	L	L
—19	L	L	L	—	L	L	L
—18	L	L	L	—	L	L	L
—17	L	L	L	L	L	L	L
—16	L	L	L	—	L	L	L
—15	L	L	L	—	L	L	—
—14	L	L	L	—	L	L	—
—13	L	—	L	—	L	—	—
—12	—	—	L	—	L	—	—
—11	—	—	L	—	L	—	—
—10	Treat one group of 11 calves. Slaughter indicator control						
0	Treat the other group of 11 calves. Slaughter indicator control						
+24	Slaughter control and treated calves						

The minimum number of calves is 33 but 35 are advocated in case of unforeseen deaths.
*Unless the compound is known to be highly effective it is not advisable to follow this design for *Cooperia* spp. but separate it into three stages as suggested in Tables 23, 24 and 25.

day of treatment. On that day or within a day or two thereafter the controls are killed and the stages of development determined microscopically. This depends on individual interpretation and is therefore subject to error. From these data the median of the fourth stage larvae on the one hand and the fifth stage and adult stages on the other must be determined. The treated animals are killed a few days later when normal development to the next stages has taken place. Despite this the same analysis is applied to the worms recovered from the treated animals. Another unknown factor is introduced and the validity of the data once more becomes problematical.

These experiments have shown that the number of larval stages never (or very rarely) exceeds the number of worms that are present in the same animal a few weeks later. It must be stressed, however, *that the host must be entirely susceptible otherwise the opposite applies.* If the host is resistant fewer worms will develop, which probably accounts for the difficulty experienced in attempts to establish *Cooperia* spp. in Calf 87 in Experiment 5 (Table 16). It must be admitted that the resistance of the host to the establishment of infestation has ruled much of our thinking on anthelmintic tests in sheep, where resistance in a group of Merino wethers to *H. contortus* was proved by Reinecke, Snijders & Horak (1962). They stated categorically that, because of the sharp reduction in the number of worms they found in their animals over a period of a few weeks, slaughter should take place while the larvae are still present. The present experiments show that this does not apply to tests using fully susceptible hosts and a delay in slaughter is desirable because worm counts are more accurate.

(iv) *Worm recovery post-mortem:* It has been proved in Experiments 4, 5 and 6 respectively that the best results are achieved as follows:–

1. Slaughter should not take place until the worms have developed to the adult or at the very least to advanced fifth stage. This means that, for optimal results, after the last dose of infective larvae slaughter must be delayed for 35 days for *O. radiatum:* 30 for *H. placei,* 28 for *O. ostertagi* and 25 for *Cooperia* spp. The highest worm recoveries *post-mortem* after a single infestation with infective larvae of *B. phlebotomum* were made when the worms were 32 days old (Experiment 6, Table 20), but the number decreased markedly over the next 30 days when the worms at slaughter were 62 days old (Experiment, 5 Table 16). This is probably due to poor technique at infestation. In other experiments in this laboratory it has been shown that this species is not recovered 10 to 15 days after infestation. Even at 17 days (Calf 104, Table 20) the numbers recovered are much lower than they probably would have been if a further 15 days had elapsed before slaughter.

   The greatest advantage of allowing the worms to develop into adults is that it increases the ease with which they can be seen, the accuracy of the worm counts and the subsequent microscopic identification.

2. In those species which migrate into the gut wall, the digestive processes destroy some of the worms, and many others are partially digested, so that they are difficult to recover and identify. In partly digested worms either heads or tails are identified and counted. For this reason the accuracy of the worm counts decreases as the number in the gut wall rises. As many as 93% of the *O. ostertagi* were recovered from the abomasal digest from Calf 104 when this species was 14 to 23 days old. In the abomasal digests of the other controls this varied from 7,1 to 19,7% when the worms were 29 to 38 days old respectively (Table 21).

   As many as 99% of *O. radiatum* occur in the gut wall when the worms are 1 to 9 days old. Seventeen to 19 days later this percentage varies from 0,02 to 6,9% (Table 14). When the youngest worm of this species is 26 days old the percentage varies from 0,0 to 0,8% for those recovered from the digested gut wall (Table 21).

   The decreasing numbers of worms released from the digested abomasal and intestinal gut wall when slaughter is delayed is another advantage of allowing them to grow to the adult stage before slaughter.

   Digestion of the intestinal wall is unnecessary where *O. radiatum* has reached the fifth or adult stage at the time of slaughter. The gut wall, however, must be thoroughly washed to remove any adherent *Cooperia* spp. and can then be discarded if the experiment is planned as suggested above.

3. *Nylon cloths:* Numerous worms are trapped in the nylon mesh, particularly the finer mesh with apertures of 225 microns. This has been solved for *Cooperia* spp. and *O. ostertagi* by using coarse grit-gauze with apertures of 500 microns. (Calves 83, 84 and 85 in Experiment 5, Table 17). In this experiment most of the adult *O. radiatum* and the adult males of *B. phlebotomum* (but not the females of the latter) migrated through this cloth. However, 4,5 to 11,1% of *O. radiatum* remained in the residue of the ingesta of the caecum and colon. Further trials with nylon mesh with apertures of 700 microns should be carried out to facilitate free migration of both female *B. phlebotomum* and the entire adult population of *O. radiatum* through the mesh into the filtrate.

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