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CRYOPRESERVATION OF THE INFECTIVE LARVAE OF THE COMMON NEMATODES OF RUMINANTS

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ABSTRACT

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Exsheathed infective larvae (L 3) of 19 species of nematodes were tested for infectivity in either sheep or cattle after they had been frozen in 0,9% NaCl solution, stored for a relatively short time in the gas phase of liquid nitrogen and subsequently thawed. In addition, 13 of these species were tested after similar storage for up to 18 months.

In sheep, *Haemonchus contortus, Ostertagia circumcincta, Trichostrongylus axei, Trichostrongylus colubriformis, Nematodirus spathiger* and *Oesophagostomum columbianum* were viable after 2 years of cryopreservation, a mean of >90% of the L 3 being alive when thawed after this period. Similar results were obtained with *Chabertia ovina* L 3 after 18 months and with *Marshallagia marshalli, Trichostrongylus falculatus* and *Dictyocaulus filaria,* after a short period of freezing. On the other hand, *Gaigeria pachyscelis* and *Strongyloides papillosus* survived freezing for up to 7 months but neither was viable at the end of this period, nor was exsheathed *G. pachyscelis* viable without freezing.

Most of these infestations were established by inoculating the infective larvae into the abomasum and/or duodenum. *M. marshalli, T. falculatus* and *C. ovina* also proved infective after oral dosing. *D. filaria,* the only other species tested by this route, was not infective when dosed *per os* after thawing.

The infective larvae of the bovine nematodes, *Haemonchus placei, Ostertagia ostertagi, Nematodirus helvetianus, Oesophagostomum radiatum, Cooperia pectinata* and *Cooperia punctata* survived freezing for a mean of 26 months, >90% being alive on thawing, but infectivity was generally lower than with the same genera in sheep. Even when not frozen, exsheathed *Bunostomum phlebotomum* was non-infective. When *Cooperia* spp. after thawing were tested for infectivity by the oral route, more worms developed in one calf infested orally than in another infested by inoculation into the duodenum.

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Ova of *H. contortus, M. marshalli, O. circumcincta, T. colubriformis, T. falculatus, N. spathiger, C. ovina, H. placei, O. ostertagi, Cooperia* spp. and *N. helvetianus* were recovered from the faeces of animals infested with cryopreserved L 3. No ova of *O. columbianum* or *O. radiatum* were recovered from faeces, because differential larval counts were performed before they were patent. Nevertheless, gravid females were obtained post-mortem. Frozen L 3 of *N. helvetianus* were used to re-establish a pure strain in calves, 2,3 million ova being recovered from infestations with 10 670 L 3 frozen for 26 months.

The infectivity of the progeny of frozen L 3 was tested with *M. marshalli* and *C. ovina*. In both instances infectivity was high and the worms which developed also produced ova, thus completing the cycle.

This appears to be the first report of infective larvae of parasitic nematodes retaining their infectivity after being frozen in liquid nitrogen (gas phase) for longer than 2 years. This is also apparently the first time that *M. marshalli, T. colubriformis, T. falculatus, T. axei, N. spathiger, C. ovina, D. filaria, H. placei, O. ostertagi, Cooperia* spp., *N. helvetianus* and *O. radiatum* have been shown to be infective after freezing in liquid nitrogen (gas phase), and that *G. pachyscelis, S. papillosus* and *B. phlebotomum* have been found to survive similar freezing and thawing, even though they do not appear to be infective thereafter.

Résumé

LA CRYO-PRÉSERVATION DES LARVES INFECTIEUSES DE NÉMATODES COMMUNS CHEZ LES RUMINANTS

Des larves infectieuses (L 3) dépouillées de leur cuticule, appartenant à 19 espèces de nématodes, ont été testées pour leur infectiosité aux moutons ou aux gros bétail après avoir été congelées dans une solution de NaCl à 0,9%, conservées pendant un temps relativement court dans la phase gazeuse de l'azote liquide et décongelées ensuite. En outre, 13 de ces espèces ont été testées après une conservation similaire durant jusqu'à 18 mois.

Chez le mouton, Haemonchus contortus, Ostertagia circumcincta, Trichostrongylus axei, Trichostrongylus colubriformis, Nematodirus spathiger *et* Oesophagostomum columbianum *étaient viables après 2 ans de cryo-préservation, une moyenne de plus de 90% des L 3 étant vivantes lorsque décongelées après ce laps de temps. Des résultats similaires ont été obtenus avec* Chabertia ovina *(L 3) après 18 mois et avec* Marshallagia marshalli, Trichostrongylus falculatus *et* Dictyocaulus filaria *après une courte période de congélation. D'autre part,* Gaigeria pachyscelis *et* Strongyloides papillosus *ont survécu jusqu'à 7 mois à la congélation mais ni l'un ni l'autre n'était viable à la fin de cette période et* G. pachyscelis *dépouillé de sa cuticule n'était pas non plus viable sans congélation. La plupart de ces infestations ont été opérées en inoculant les larves infectieuses dans la caillette et/ou dans le duodénum.* M. marshalli, T. falculatus *et* C. ovina *se sont aussi avérés infectieux après administration par voie buccale.* D. filaria, *la seule autre espèce testée de cette façon, n'était pas infectieuse lorsque administrée* per os *après décongelation.*

Les larves infectieuses des nématodes du boeuf, Haemonchus placei, Ostertagia ostertagi, Nematodirus helvetianus, Oesophagostomum radiatum, Cooperia pectinata *et* Cooperia punctata *ont survécu à la congélation pendant 26 mois en moyenne, plus de 90% d'entre elles étant vivantes lorsque décongelées, mais l'infectiosité était généralement inférieur à celui qu'ont ces mêmes genres chez le mouton.* Bunostomum phlebotomum *dépouillé de sa cuticule était non-infectieux même s'il n'avait pas été congelé. Lorsque des espèces de* Cooperia, *après avoir été dégelées, ont été testées pour leur infectiosité par voie buccale, davantage de vers s'est développé chez un veau infesté* per os *que chez un autre infesté par inoculation intra-duodénale.*

Des oeufs de H. contortus, M. marshalli, O. circumcincta, T. colubriformis, T. falculatus, N. spathiger, C. ovina, H. placei, O. ostertagi, Cooperia *spp. et* N. helvetianus *ont été retrouvés dans les matières fécales d'animaux infestés avec des L 3 cryo-préservées. On n'y a pas retrouvé d'oeufs de* O. columbianum *ni de* O. radiatum *parce que la numération différentielle des larves a été faite avant qu'ils ne fussent manifestes. Néanmoins l'on a obtenu post-mortem des femelles pleines. Des L 3 congelées de* N. helvetianus *ont été utilisées pour réétablir une lignée pure chez les veaux: à partir de 10 670 L 3 congelées pendant 26 mois et utilisées pour l'infestation, on a récupéré 2,3 millions d'oeufs.*

L'infectiosité de la descendance des L 3 congelées a été testé avec M. marshalli *et* C. ovina. *Dans les deux cas elle était élevée et les vers qui se sont développés ont également produit des oeufs, complétant ainsi le cycle.*

Ceci semble être la première mention de larves infectieuses de nématodes parasites conservant leur infectiosité après avoir été congelées la phase gazeuse de l'azote liquide pendant plus de deux ans. C'est aussi la première fois, semble-t-il, qu'on a montré que M. marshalli, T. colubriformis, T. falculatus, T. axei, N. spathiger, C. ovina, D. filaria, H. placei, O. ostertagi, Cooperia *spp.,* N. helvetianus *et* O. radiatum *restaient infectieux après avoir été congelés dans la phase gazeuse de l'azote liquide, et que* G. pachyscelis, S. papillosus *et* B. phlebotomum *peuvent survivre à une manipulation similaire (congélation—décongelation), même s'ils semblent ne plus être infectieux par la suite.*

INTRODUCTION

Since, with certain notable exceptions, nematode larvae or ova do not survive very well in the laboratory by the usual methods of storage (Herlich, 1966; Persson, 1974), and as pure strains of nematodes must be readily available to the helminthologist, particularly for work with anthelmintics, the pure strains are generally maintained in animals.

The donor animals must be replaced frequently owing to the development of resistance to worm infestation or cross infestation with other species of nematodes. Furthermore, as much effort is required at frequent intervals to ensure that the strains are uncontaminated and to monitor the levels of infestation of the hosts, this method of maintenance is very expensive and the number of strains that can be maintained is usually strictly limited.

Because certain pure strains (for instance, those showing resistance to certain anthelmintics which are usually effective against the species concerned) are either irreplaceable or extremely difficult to replace, they are often maintained even though they are used infrequently in experiments.

The need for improving the storage of larvae has been realised for a long time and freezing has been considered on numerous occasions. Weinman & McAllister (1947), who were apparently the first to report that nematode larvae survived freezing and thawing, found that the microfilariae of *Wuchereria bancrofti, Dirofilaria immitis* and *Litomosoides carinii* survived freezing well. They found that only a small number of *Ancylostoma caninum* infective larvae (L 3) survived freezing for 37 days, but a few of these survivors retained their infectivity after thawing.

Subsequently it was found that exsheathing nematode infective larvae before freezing considerably enhanced their infectivity and numerous species of nematodes have been tested for survival and/or infectivity after having been frozen for up to 44 weeks (Campbell, Blair & Egerton, 1972; Campbell & Thomson, 1973; Campbell, Blair & Egerton, 1973 and Kelly, Campbell & Whitlock, 1976). On the other hand very few ensheathed larvae survived (Isenstein & Herlich, 1972; Campbell & Thomson, 1973).

It was decided to investigate all the pure strains of nematodes available in this laboratory for viability after short term storage in the gas phase of liquid nitrogen. Thereafter, most of the species that gave encouraging results were retested after longer periods of storage.

MATERIALS AND METHODS

1. *The following species were used in these investigations in sheep:*

Haemonchus contortus (2 strains)
Marshallagia marshalli
Ostertagia circumcincta
Trichostrongylus axei
Trichostrongylus colubriformis
Trichostrongylus falculatus
Gaigeria pachyscelis
Nematodirus spathiger
Strongyloides papillosus
Chabertia ovina
Oesophagostomum columbianum
Dictyocaulus filaria.

In calves:

Haemonchus placei
Ostertagia ostertagi
Bunostomum phlebotomum
Cooperia pectinata and *Cooperia punctata* (mixed)
Nematodirus helvetianus
Oesophagostomum radiatum

2. *Isolation of the pure strains of parasites*

With the exception of *T. falculatus, M. marshalli* and one strain of *H. contortus*, the worms were isolated by Reinecke (1973).

The remaining strain of *H. contortus* was benzimidazole-resistant and was obtained in pure culture after sheep were treated with thiabendazole*.

T. falculatus was obtained from a farm in the Orange Free State where regular treatment with rafoxanide† suppressed the ubiquitous *H. contortus* without affecting the *T. falculatus*. For unknown reasons the latter was not mixed with other species.

M. marshalli was obtained from a mixed culture in sheep from Barkly East. A pure culture was obtained by passing the faeces through a nylon sieve‡ with 100 μm apertures onto a 64 μm aperture sieve. Nematode ova in the residue were further concentrated by flotation in 40% sucrose solution and resieved on a 64 μm sieve to remove any remaining contaminating ova. A few *N. spathiger* ova were present but were separated by collecting hatched *M. marshalli* before the *N. spathiger* started hatching.

* Thibenzole, M.S.D.
† Ranide, M.S.D.
‡ Swiss Bolt' Cloth Co.

3. *Exsheathing the infective larvae*

Preliminary investigations agreed with the findings of Campbell *et al.* (1972) that all the species of ensheathed larvae survived poorly after thawing. Furthermore, freezing in 0,9% NaCl apparently gave better results than freezing in water.

In most experiments, freshly collected infective larvae (L 3) were exsheathed in freshly prepared 0,16% sodium hypochlorite (NaOCl) solution*. When more than 90% of the larvae were exsheathed (usually after about 20 minutes), they were washed with 0,9% saline (NaCl) on wet-strengthened filter paper† in a Baermann funnel attached to a water pump, collected in 0,9% NaCl and sealed in thin-walled glass 10 ml ampoules.

Most species exsheathed after about 20 min, but larvae in cold water took longer to exsheath than those in warmer water and air bubbled through the suspension appeared to stimulate exsheathment when this was slow.

Rose (1973) reported that the larvae he tested (among others *O. circumcincta, O. ostertagi* and *N. helvetianus*) became exsheathed after 20 minutes in 0,01% NaOCl solution. In our investigation, however, the larvae did not exsheath in this concentration of NaOCl even after a few hours. Possibly Rose (1973) referred to the percentage of available chlorine, in which case our results are similar. Because of the unstable nature of the chemical, it is perhaps preferable to refer to the available chlorine rather than to the concentration of the NaOCl solution.

4. *Freezing and thawing the infective larvae*

The ampoules of larvae were frozen by submerging them in the gas phase of liquid nitrogen (Campbell *et al.*, 1973) in a semen storage tank of 40 l capacity‡ and were thawed in hot water at 50–55 °C (Campbell *et al.*, 1973) until only a small piece of ice remained. They were then transferred to water at room temperature. The larvae were diluted in 0,9% saline (at room temperature).

5. *Collection of larvae*

Sheep faecal pellets were moistened and homogenized in an electrical food mincer§, using a cutting plate with holes 10 mm in diameter. They were then mixed with vermiculite to obtain a crumbly, moist consistency for culturing the larvae (Reinecke, 1973).

Faeces were incubated in a Perspex hatching chamber (Fig. 1 & 2), a modification of a model which is commercially available||.

This chamber, developed and built at Onderstepoort, has a capacity of 6 kg of faeces mixed with vermiculite, and has a plastic tube at each end instead of a single burette for collecting the larvae. The faeces are placed in 4 separate narrow rectangular containers. Up to 7,5 million *H. placei* L 3 have been collected from this apparatus loaded only once with faeces.

* B.D.H.
† Whatmans No. 111
‡ M.V.E. Model A—1 000
§ Hobart Model A—200
|| R.N. Powell Scientific Instruments, Australia

FIG. 1 The hatching chamber (oblique view). The dimensions are: height, 37 cm; breadth, 34 cm; length, 59 cm

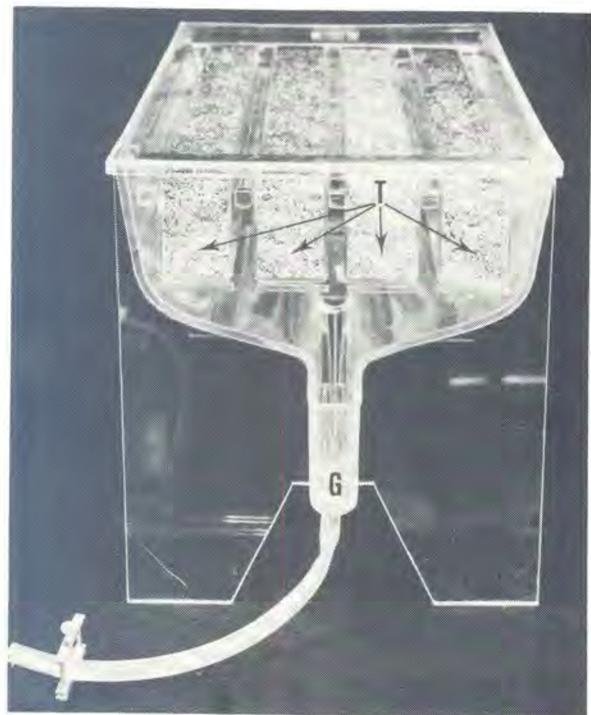

FIG. 2 End view of the hatching chamber. The faecal trays, T are 57 cm long, 5 cm wide and 7,5 cm deep. The collecting groove, G, has a maximum depth of 12,5 cm and is 2,5 cm wide (i.d.)

6. *The sheep*

Dorper sheep, raised worm-free, were used in the experiments. They received sterilized lucerne hay during the experiment and were kept under worm-free conditions. Before infestation, faecal examinations were done to confirm the absence of nematode ova but they were also treated with levamizole* or parbendazole† or mebendazole‡ at double the therapeutic dose.

If more than one group of sheep was used in an experiment, the sheep were ranked by mass or age and thereafter randomly allocated to the various groups.

7. *Infestation*

Larval doses were determined by counting aliquots of larval suspension (Reinecke, 1973).

* Ripercol, 15 mg/kg (Ethnor)
† Helmatac, 60 mg/kg (Coopers)
‡ Multispec, 40 mg/kg (Ethnor)

In most of the experiments larvae were injected into the abomasum and/or the duodenum which had been exposed by laparotomy (local anaesthesia* after sedation with acepromazine maleate†).

After-care consisted of therapy with long-acting penicillin preparations‡ in addition to the usual nursing procedures.

8. *Definitions*

For the purposes of this publication the following definitions apply unless otherwise stated:

a. *Storage, cryopreservation* or *freezing* of larvae denotes larvae frozen and stored in the gas phase of liquid nitrogen without being submerged in the fluid (Campbell & Thomson, 1973). The larvae were always thawed before being used for infestation. "Fresh larvae" denotes freshly-hatched larvae which are used without having been frozen or exsheathed.

b. *Eggs per gram of faeces* (e.p.g.) refers to counts performed by the modified McMaster procedure (Reinecke, 1973). An e.p.g. count by flotation means total examination of 5 g of faeces by a concentration technique (Whitlock, 1959) so that all the ova present may be counted and the concentration per gram calculated.

c. *Percentage survival* and *percentage alive* refer to larvae which were motile when examined under the microscope. If the larvae appeared lethargic, formalin was added to a final concentration of between about 1 % and 3 % to stimulate motility. In this case motility was usually assessed before and after addition of the formalin and the best result recorded. Some larvae, for example, *S. papillosus* and *G. pachyscelis,* usually reacted unfavourably to the addition of formalin. Other larvae which did not move may of course have been alive.

d. *Viability* refers to the ability of the surviving (live) larvae to develop in the host.

9. *Worm recovery*

The worms and parasitic larvae were recovered, killed and fixed as described by Reinecke (1973), and at least 40 % of each ingestum was examined under a stereo-microscope. In each sample, the first 100 worms recovered were identified, but if fewer than 100 were recovered, they were all identified (Reinecke, 1973).

10. *Statistical evaluation*

The frozen and fresh larvae used in Experiment 2 were tested for significant differences in viability by means of the Mann-Whitney U Test (Siegel, 1956). No other statistical comparisons were done.

11. *Comparative tables*

For the purpose of cross reference, the viability of the larvae used in more than one experiment is listed in Tables 12 and 26 for sheep and cattle respectively. Please note, however, that the experiments from which these results are obtained are not strictly comparable.

* Planocaine (M & B)
† Acetylpromazine (Boots)
‡ Dipenilente (Novo Industries) or Compropen (Glaxo-Allenbury)

I. Sheep Nematodes

The experiments carried out are reported in the following sequence: firstly, preliminary investigations and subsequent medium- and long-term storage of a group of nematode species; secondly, the results with species in which only preliminary investigations were done, the storage of naturally exsheathed *C. ovina* and the infectivity of the (unfrozen) progeny of cryopreserved *C. ovina* and *M. marshalli*; and, lastly, unsuccessful storage of 2 species and an unfavourable result with *H. contortus*.

(A) Satisfactory cryopreservation

Experiments 1 to 8

EXPERIMENT 1

H. contortus, O. circumcincta, T. colubriformis, N. spathiger *and* O. columbianum — *Preliminary investigations*

Method

Small numbers of larvae (at a concentration varying from about 300 per ml saline for *N. spathiger*—900 per ml saline for *O. circumcincta*) of the above 5 species of nematodes were frozen separately on the same day and thawed 17 days later, after which small numbers were examined to determine the percentage of survivors and to estimate the numbers used for infestation. The remaining larvae were divided into 2 doses (Table 1) and injected into the abomasum of Sheep 1 (7 months old at infestation) and the duodenum of Sheep 2 (8 months old at infestation). The duodenum of Sheep 2 was partially compressed by adjacent organs and some of the larval suspension appeared to enter the abomasum.

Faecal examinations were done 24 and 31 days after infestation and worms were recovered after 42 days.

Results (Table 1)

The surviving larvae of all species were very active when thawed and diluted with 0,9% saline. The *O. columbianum* larvae contained vesicles in the intestinal cells when thawed (Van Wyk, 1977).

Nematode ova counts in Sheep 1 and 2 were respectively 200 and 500, 24 days after infestation and 0 and 300, 31 days after infestation.

Worm development varied between 2,4% for *N. spathiger* and 14,2% for *H. contortus* (mean of the 2 routes of infestation, Table 1).

Most of the female worms of all species were gravid.

Comment

There appeared to be very little difference between the 2 routes of infestation tested in this experiment, but this may be due to the overflow of larval suspension from the duodenum into the abomasum in Sheep 2. This surmise is supported by the results of Experiment 5 in which *H. contortus* larvae from a mixed culture stored in liquid nitrogen developed in one sheep when infested *per os*, but not in another, in which the larvae were deposited in the duodenum.

The survival and viability of the *H. contortus* in our experiment was lower than that reported by Campbell *et al.* (1973) after oral infestation with similar frozen larvae. However, the results obtained in Experiments 2 and 3 (below) were similar to those of Campbell *et al.* (1973).

Campbell & Thomson (1973) were unable to infest sheep dosed *per os* with either frozen or unfrozen exsheathed *T. colubriformis*, but this experiment confirms their suggestion that infestation in the duodenum may give positive results. The percentage development was low, but better results were obtained in Experiments 2 and 3.

For investigations of the medium and long-term storage of the following 8 species of nematode larvae, sufficient larvae of each species were frozen for the survival of the same batches of larvae to be tested after 2 or more periods of storage.

EXPERIMENT 2

H. contortus, O. circumcincta, T. axei, T. colubriformis, N. spathiger, O. columbianum *and* D. filaria *frozen for a mean of* 227 *days and* C. ovina *for* 26 *days* (*Medium-term storage*)

Method

For this experiment, 4–12 ampoules of infective larvae of the above 8 species of nematodes were frozen on different days and some of each species thawed on the same day. The times of cryopreservation (in days) are shown in Table 2.

TABLE 1 Experiment 1: Sheep infested with larvae frozen for 17 days

Worm species	Larvae		Sheep No.	Route of infestation	Worm recovery	
	Alive (%)	No. (alive) dosed			No.†	Development (%)
H. contortus	52,9	276	1	Abomasum	35	12,7
			2	Duodenum	43	15,6
O. circumcincta	96,6	612	1	Abomasum	91	14,9
			2	Duodenum	63	10,3
T. colubriformis	95,9	385	1	Abomasum	41	10,6
			2	Duodenum	34	8,8
N. spathiger	93,0	424	1	Abomasum	2	0,5
			2	Duodenum	18	4,2
O. columbianum	58,1	?*	1	Abomasum	46	—
			2	Duodenum	235	—

* The number used for infestation was not determined

† All adult worms, except 2 *O. columbianum* 4th stage larvae recovered from Sheep 2. Gravid females of all species were present

TABLE 2 Experiment 2: Viability of larvae stored for a mean of 227 days (26 days in the case of *C. ovina*)

Worm species	Concentration§	Frozen larvae*				Fresh larvae†		
		Days frozen	No. examined	Alive (%)	No. (alive) dosed per sheep	No. examined	Alive (%)	No. (alive) dosed per sheep
H. contortus	38	**223**	164	**85,9**	1 059	64	**100,0**	1 075
O. circumcincta	12	**229**	88	**96,6**	981	135	**96,3**	948
T. axei	130	**232**	203	**98,0**	1 021	50	**100,0**	845
T. colubriformis	2	**229**	63	**100,0**	712	146	**99,3**	803
N. spathiger	10	**220‡**	177	**87,0**	1 518	146	**93,8**	1 484
O. columbianum	240	**232**	163	**83,3**	1 611	124	**98,4**	1 476
D. filaria	5	**232**	165	**65,5**	1 424	55	**92,7**	1 414
C. ovina	8	**26**	132	**87,9**	917	82	**100,0**	960
S. papillosus	9	**183**	98	**83,7**	1 345	—	—	—

* Exsheathed, frozen and stored in liquid nitrogen and thawed for the experiment
† Freshly collected larvae (not frozen)
‡ Mean time. Two batches, frozen for 217 and 222 days, were mixed
§ Concentration in thousands per ml saline during storage

TABLE 3 Experiment 2: Faecal egg counts (e.p.g.)

Sheep No. \ Days after infestation	20	22	23	26	30	33
Frozen larvae*						
3	1 500	3 400	2 100	4 900	3 600	10 500
4	1 000	400	1 100	1 100†	3 000	1 600
5	0	2 900	1 000	800	200	600
6	0	0	0	100	0	0
7	0	100	200	800	100	900
Mean e.p.g.	500	1 360	880	1 540	1 380	2 720
Fresh larvae*						
8	2 600	1 400	1 600	2 400	1 200	2 800
9	1 600	1 400	1 900	800	2 700	1 100
10	200	900	1 200	1 500	1 200	800†
11	600	900	1 300	700	—‡	1 100
12	900†	500	900	1 400†	1 300†	900
Mean e.p.g.	1 180	1 020	1 380	1 360	1 600	1 340

* See Table 2
† Including 100 *Nematodirus* e.p.g.
‡ Not determined

TABLE 4 Experiment 2: Differential larval counts 29 days after infestation

Sheep No.	Differential larval count (%)		
	Haemonchus	*Trichostrongylus/Ostertagia**	*Strongyloides*
Frozen† larvae:			
3	100	0	0
4	83	16	1
5	78	22	0
6	0	80	20
7	92	8	0
Fresh† larvae:			
8	96	4	0
9	100	0	0
10	97	3	0
11	87	13	0
12	100	0	0

* These genera were not differentiated
† See Table 2

Sheep 3–7 were infested with *H. contortus, O. circumcincta* and *T. axei* by injection into the abomasum, and *T. colubriformis, N. spathiger, O. columbianum, D. filaria* and *C. ovina* by injection into the duodenum. Each sheep was infested with all species except *D. filaria* which was dosed only to Sheep 3 and 4. Larval doses are listed in Table 2.

An additional 5 sheep (8–12) were infested *per os* with freshly collected, unfrozen infective larvae of the same 8 species, except that *D. filaria* was dosed to only Sheep 8 and 9. These sheep served to control the susceptibility of the hosts to this combination of species.

The sheep were killed for worm recovery either 36 days after receiving frozen larvae or 35 days after receiving fresh larvae.

Results (Tables 2, 3, 4, 5 & 6)

The frozen larvae were very active when thawed. Survival of the larvae varied from 65,5% for *D. filaria* to 100% for *T. colubriformis* (Table 2).

In the case of *O. columbianum* and *C. ovina*, most of the larvae had prominent vesicles in the intestinal cells as described by Van Wyk (1977). A few of the *T. colubriformis* and *N. spathiger* larvae also had some vesicles in the intestinal cells.

The mean faecal egg counts (e.p.g.) varied from 500–2 720 for the cryopreserved larvae and from 1 020–1 600 for the fresh larvae. On 5 occasions (1 with frozen larvae and 4 with fresh larvae) *Nematodirus* ova were present (Table 3).

TABLE 5 Experiment 2: Number of worms recovered from sheep infested with larvae frozen for 26–232 days

Worm species \ Sheep (age at infestation)	Infested with cryopreserved‡ larvae						Infested with fresh‡ larvae (controls)					
	3 (12 m)	4 (6 m)	5 (6 m)	6 (16 m)	7 (14 m)	Mean for the group	8 (6 m)	9 (6 m)	10 (6 m)	11 (15 m)	12 (6 m)	Mean for the group
H. contortus												
Total (adults)	757	310	179	0	397	336*	704	140	133	333	279	318
Development (%)						**31,7%***						**29,6%**
O. circumcincta												
L4	7	0	0	0	0	2*	0	29	0	27	3	12
L5	0	0	0	0	0	0*	0	13	0	0	0	3
Adult	251	274	50	0	147	180*	279	142	94	82	109	141
Total	258	274	50	0	147	182*	279	184	94	109	112	156
Development (%)						**18,6%***						**16,5%**
T. axei												
L4	0	0	0	0	0	0*	11	12	0	0	0	5
L5	0	39	0	0	0	10*	0	6	0	11	0	3
Adult	57	214	20	0	55	87*	619	326	261	209	138	311
Total	57	253	20	0	55	96*	630	344	261	220	138	519
Development (%)						**9,4%***						**61,4%**
T. colubriformis												
Total (adults)	358	507	234	86	437	324	390	336	629	394	347	419
Development (%)						**45,5%**						**52,2%**
N. spathiger												
L4	22	1	13	66	21	25	20	13	6	38	0	15
L5	7	0	9	137	48	40	45	89	85	70	0	58
Adult	127	717	122	69	737	354	65	93	440	271	164	207
Total	156	718	144	272	806	419	130	195	531	379	164	280
Development (%)						**27,6%**						**18,9%**
O. columbianum												
L4	12	97	18	71	17	43	87	39	65	195	27	83
L5	293	420	531	0	0	249	536	381	125	26	775	368
Total	305	517	549	71	17	292	623	420	190	221	802	451
Development (%)						**18,1%**						**30,6%**
C. ovina												
L4	12	1	23	23	19	16	38	189	0	227	124	116
L5	156	100	79	0	0	67	206	14	9	0	59	58
Total	168	101	102	23	19	83	244	203	9	227	183	174
Development (%)						**8,9%**						**18,0%**
D. filaria												
L5	0	0	—†	—†	—†	0	174	157	—†	—†	—†	166
Adult	0	1	—	—	—	1	74	0	—	—	—	37
Total	0	1	—	—	—	1	248	157	—	—	—	203
Development (%)						**0,1%**						**14,4%**

* Excluding Sheep 6

† was not infested with *D. filaria*

‡ See Table 2

TABLE 6 Experiment 2: The immature worms as a percentage of the total worm burdens

Worm species	Immature worms (%)	
	Sheep 3–7 (frozen larvae)	Sheep 8–12 (fresh larvae)
H. contortus	0,0	0,0
O. circumcincta	1,1	9,6
T. axei	10,4	1,5
T. colubriformis	0,0	0,0
N. spathiger	15,5	26,1
*O. columbianum**	14,7	18,4
*C. ovina**	100,0	66,7
D. filaria	0,0†	81,8

* Only 4th and 5th stage larvae recovered; 4th stage larvae expressed as a percentage of the total worm recovery
† Only 1 worm recovered (an adult—Table 5)

On Day+29, when differential larval counts were done (Table 4), *Haemonchus* was the predominant species in all the sheep except Sheep 25. Some *Trichostrongylus*/*Ostertagia* were also present in most of the animals. *Strongyloides* larvae were recovered from the faeces of Sheep 4 and 6 which had received frozen larvae, but none from the sheep that received fresh, unfrozen larvae.

The mean percentages of development of frozen and fresh larvae are shown in Table 5. With the exception of *D. filaria*, of which only 0,1% of the frozen larvae developed, the development varied from 8,9% (*C. ovina*)—45,5% (*T. colubriformis*) for the frozen L 3, and 14,4% (*D. filaria*)—61,4% (*T. axei*) for the fresh larvae (Table 5).

The immature worms recovered, including early 5th stage worms (Reinecke, 1973), are expressed as a percentage of the total worm burden for both the frozen and fresh larval groups as shown in Table 6. In the case of *O. columbianum* and *C. ovina* the sheep were killed before the worms could become adult and only 4th stage larvae and 5th stage worms were recovered. The 4th stage larvae of these 2 species are expressed as a percentage of the total worm burden.

Comment

The larvae appeared as active when thawed after 7 months as other batches when thawed after 17 days (Experiment 1). The survival rate of these larvae was similar to that of those stored for 17 days (Table 1) as well as to that of fresh unfrozen larvae (Table 2).

The faecal egg counts of the 2 groups of sheep were also similar (Table 3). If the negative counts of Sheep 6 (in which 3 worm species did not develop) are excluded, the mean egg counts of the group receiving frozen larvae were lower than those of the controls on only 2 occasions out of 6.

No *Haemonchus* larvae were recovered from the faeces of Sheep 6, which had a consistent negative e.p.g. count (except on Day 26 when a low count was obtained), nor were *H. contortus, O. circumcincta* or *T. axei* found post-mortem. Probably the larvae intended for injection into the abomasum (all 3 species mentioned) were injected into the wall of the abomasum or the needle may have traversed the abomasum, depositing the larvae in the abdominal cavity. Similarly, the very low numbers of these worms recovered from Sheep 5 could be ascribed to a partial failure of larval inoculation.

The percentage development of the worms was disappointing in both experimental groups (Table 5). The development of *H. contortus* (P>0,5), *O. circumcincta* (P>0,5), *T. colubriformis* (P>0,5), *N. spathiger* (P>0,55), *O. columbianum* (P>0,2) and *C. ovina* (P>0,07) was similar for both groups. Only with *T. axei* (P<0,02) did significant differences occur. The development of the *H. contortus*, while slightly inferior to the results obtained by Campbell *et al.* (1973), was much better than that in Experiment 1.

It is interesting that *Strongyloides* larvae were encountered among the larvae hatched from the faeces of Sheep 4 and 6 (frozen larvae) while no adult *S. papillosus* was recovered post-mortem, nor were *Strongyloides* ova detected in the faecal examinations. Contamination of the cultures may have been responsible for the larvae in the faeces of the 2 sheep, but it is pertinent that none were detected in the faeces of the control sheep (fresh larvae). It is possible, though improbable, that *S. papillosus*, like *B. phlebotomum* (Experiment 15), may have developed. More work on this aspect of the investigation is indicated.

Cryopreservation does not seem to have stimulated hypobiosis in the larvae (Table 6). In the cases of *T. axei* and *C. ovina*, the sheep receiving frozen larvae contained higher percentages of immature worms, while the reverse was true with *O. circumcincta, N. spathiger* and *O. columbianum*.

EXPERIMENT 3

H. contortus, O. circumcincta, T. axei, T. colubriformis, N. spathiger *and* O. columbianum *frozen for a mean of* 24 *months and* C. ovina *frozen for* 17 *months* (*long-term storage*)

Method

Some of the remaining batches of frozen larvae used in Experiment 2 were used for this experiment. All the larvae were thawed on the same day (Table 7). The concentrations of larvae per ml of saline corresponded to those for Experiment 2 (Table 2).

To determine the percentage of surviving larvae (Table 7), those that had burst from freezing and thawing were disregarded because their proportion appeared constant irrespective of the time of storage in liquid nitrogen. We were interested in determining the effect of prolonged storage on larval mortality as distinct from a constant loss attributed to freezing and thawing.

Eleven sheep were divided into 3 groups and infested as shown in Table 8. The larval doses are listed in Table 7.

Worms were recovered from Groups A, B and C on Days 27, 28 and 29, respectively, after infestation. The first 2 groups (A and B) received frozen larvae, the 3rd (Group C) fresh larvae.

Results (Tables 7, 8 & 9)

Table 12 gives a comparison between the development of the various ovine nematodes in different experiments.

The mean percentage of survival of the frozen larvae (excluding *C. ovina*) after a mean storage time of 727 days was 93,5% (range 87,3%–96,7%, Table 7) while that of fresh larvae was 91,1% (range 88,3%–94,2%).

As in previous experiments, the frozen larvae were very active when thawed. Most of the *O. columbianum* and *C. ovina* larvae and some *T. colubriformis* and *N. spathiger* contained vesicles in the intestinal cells.

TABLE 7 Experiment 3: The infective larvae

Worm species	Frozen larvae*				Fresh larvae*		
	Days frozen	No. examined	Alive (%)	No. (alive) dosed per sheep‡	No. examined	Alive (%)	No. (alive) dosed per sheep
H. contortus	**722**	119	**96,6**	8 414	120	**90,8**	2 856
O. circumcincta	**728**	183	**95,6**	1 773	—	—	—
T. axei	**731**	212	**96,7**	4 970	—	—	—
T. colubriformis	**728**	169	**93,5**	1 355	120	**94,2**	1 991
N. spathiger	**719†**	156	**91,0**	4 404	—	—	—
O. columbianum	**731**	173	**87,3**	4 932	120	**88,3**	1 823
C. ovina	**525**	189	**86,8**	3 272	—	—	—

* See Table 2
† Mean time. Two batches, frozen for 716 and 721 days respectively
‡ Three worm species were dosed to each of 4 sheep and the other 4 species to a second group of 4 sheep

TABLE 8 Experiment 3: Routes of infestation of Sheep 13–23

Group	Type of larvae	Sheep	Route of infestation		
			Abomasum	Duodenum	*Per os*
A	Frozen*	13–16	*H. contortus*	*T. colubriformis* *O. columbianum*	—
B	Frozen*	17–20	*O. circumcincta* *T. axei*	*N. spathiger* *C. ovina*	—
C	Fresh*	21–23	—	—	*H. contortus* *T. colubriformis* *O. columbianum*

* See Table 2

TABLE 9 Experiment 3: Faecal egg counts (e.p.g.)

Group*	Sheep No.	Type of larvae	Days after infestation			
			20	21	22	25
A	13	Frozen§	300	100	400	1 000
	14		2 300	1 600	3 100	10 600
	15		600	1 100	1 300	800
	16		7 400	— †	— †	8 600
	Mean e.p.g.		2 650	933	1 600	5 250
B	17	Frozen§	100	300	200	500‡
	18		200	300	300‡	200
	19		200	0	0	100
	20		0	500‡	200‡	100
	Mean e.p.g.		125	275	175	225
C	21	Fresh§	0	500	400	500
	22		300	200	700	300
	23		0	0	100	100
	Mean e.p.g.		100	233	400	300

* See Table 8 for details of the worm species with which the sheep were infested
† No faeces obtained for an egg count
‡ Including 100 *Nematodirus* e.p.g.
§ See Table 2

TABLE 10 Experiment 3: Number of worms recovered from sheep infested with larvae frozen for 525–731 days

Worm species \ Sheep (age at infestation)	Group A—infested with frozen* larvae					Group B—infested with frozen* larvae					Group C—infested with fresh* larvae			
	13 (5 m)	14 (8 m)	15 (8 m)	16 (6 m)	Mean for the group	17 (8 m)	18 (9 m)	19 (9 m)	20 (6 m)	Mean for the group	21 (8 m)	22 (5 m)	23 (8 m)	Mean for the group
H. contortus														
L4	0	0	0	33	8	0	0	0	0	0	0	0	0	0
Adult	1 951	5 037	126	6 264	3 345	3	0	2	1	2	114	63	28	68
Total	1 951	5 037	126	6 297	3 353	3	0	2	1	2	114	63	28	68
Development (%)	23,2	59,9	1,5	74,8	**39,9%**	n†	n	n	n	n	4,0	2,2	1,0	**2,4**
T. colubriformis														
Total (Adults)	939	553	976	926	849	578	871	758	597	651	301	119	78	166
Development (%)	69,3	40,8	72,0	68,3	**62,7%**	n	n	n	n	n	15,1	6,0	3,9	**8,3%**
O. columbianum														
L4	630	979	257	1 563	857	0	0	0	0	0	210	168	28	135
L5	1 489	0	0	3	373	5	6	65	3	20	86	367	431	295
Total	2 119	979	257	1 566	1 230	5	6	65	3	20	296	535	459	430
Development (%)	43,0	19,8	5,2	31,8	**24,9%**	n	n	n	n	n	16,2	29,3	25,2	**23,6%**
O. circumcincta														
L4	‡—	—	—	—		0	0	0	27	7	—	—	0	0
L5	—	—	—	—		18	30	10	0	15	—	—	0	0
Adult	—	—	—	—		970	1 216	1 150	658	999	—	—	48	16
Total	—	—	—	—		988	1 246	1 160	685	1 020	—	—	48	16
Development (%)	—					55,7	70,3	65,4	38,6	**57,5%**			n	n
T. axei														
L4	—	—	—	—		0	45	0	3	12	—	—	—	
L5	—	—	—	—		0	68	0	0	17	—	—	—	
Adult	—	—	—	—		1 404	589	2 019	1 376	1 347	—	—	—	
Total	—	—	—	—		1 404	702	2 019	1 379	1 376	—	—	—	
Development (%)						28,2	14,1	40,6	27,7	**27,7%**				
N. spathiger														
L4	—	—	—	—		41	119	292	0	113	—	—	—	
L5	—	—	—	—		0	533	0	0	133	—	—	—	
Adult	—	—	—	—		3 583	1 756	1 555	3 363	2 564	—	—	—	
Total	—	—	—	—		3 624	2 408	1 847	3 363	2 811	—	—	—	
Development (%)	—					82,3	54,7	41,9	76,4	**63,8%**				
C. ovina														
L4	—	—	—	—		36	273	114	112	134	—	0	—	0
L5	—	—	—	—		1 222	902	1 418	986	1 132	—	3	—	1
Total	—	—	—	—		1 258	1 175	1 532	1 098	1 268	—	3	—	1
Development (%)	—	—	—	—		38,4	35,9	46,8	33,6	**38,8%**		n		n

* See Table 2

† No pure cultures of this species dosed—these worms originated from mixed cultures (see Comment, Experiment 3)

‡ No larvae dosed or worms recovered

The mean faecal egg counts (e.p.g.) varied from 933–5 250 per day for Group A, 125–275 for Group B and 100–400 for Group C (Table 9). *Nematodirus* ova were encountered on 4 occasions in Group B.

The mean percentages of frozen larvae that actually developed varied from 24,9% for *O. columbianum* to 63,8% for *N. spathiger*, while from 2,4% (*H. contortus*) to 23,6% (*O. columbianum*) of the fresh larvae developed (Table 10).

The immature worms recovered post-mortem varied from 0% (*T. colubriformis*)–8,8% (*N. spathiger*) of the total burdens in the sheep infested with frozen larvae (Table 11). As in Experiment 2, no adult *O. columbianum* or *C. ovina* were recovered and the 4th stage larvae constituted 69,7% and 10,7% respectively, of the total worm burdens (Table 11).

TABLE 11 Experiment 3: The immature worms as a percentage of the totals recovered

Worm species	Immature worms (%)	
	Sheep 13–20 (frozen larvae)	Sheep 21–23 (fresh larvae)
H. contortus	0,2	0,0
T. colubriformis	0,0	0,0
O. columbianum	69,7*	31,4
O. circumcincta	2,2	—
T. axei	2,1	—
N. spathiger	8,8	—
C. ovina	10,7*	—

* No adult worms recovered; 4th stage larvae expressed as a percentage of the total worms recovered

No immature *H. contortus* and *T. colubriformis* were found in the animals receiving fresh larvae, and the 4th stage *O. columbianum* constituted 31,4% of the total recovered.

While no *H. contortus, T. colubriformis* or *O. columbianum* L 3 were dosed to our knowledge to the sheep in Group B, and no *O. circumcincta* or *C. ovina* L 3 to Group C, small numbers of these species were nevertheless recovered from them (Table 10).

Comment

In this experiment frozen and fresh larvae of each species were not compared as in Experiment 2. The fresh larvae used in this experiment controlled the method of infestation.

The larvae survived storage surprisingly well for approximately 2 years. In Table 13 a comparison is made of the survival rate of the various species after 7,5 months and 2 years storage. It appears that more larvae of *H. contortus, N. spathiger* and *O. columbianum* survived 2 years of storage than 7,5 months. However, the differences are probably due to the fact that larvae that had burst, while being included in the percentage of dead larvae in Experiment 2, were ignored in this experiment. From this it can be deduced that very few of the *O. circumcincta, T. axei, T. colubriformis* and *C. ovina* burst.

In every instance it appeared that a higher percentage of worms had developed from the frozen larvae after 2 years storage than after 7,5 months (Table 13). No explanation can be given for this, except that the lambs in Experiment 3 were younger than those in Experiment 2, were infested with larger numbers of larvae, and were killed sooner after infestation. Exclusion of the burst larvae in the present experiment could not have played a role as the larval doses listed were of live larvae; dead larvae (including those that had burst), were ignored in both trials.

TABLE 12 Comparative development of sheep nematodes in different experiments

Sheep	Larvae	Short-term storage		Medium-term storage	Long-term storage
		I	II		
H. contortus	Days L3 frozen	17	17	223	722
	Development (%)	**12,7**	**15,6**	**31,7**	**39,9**
	Exp. No.	1	1	2	3
O. circumcincta	Days L3 frozen	17	17	229	728
	Mean development (%)	**14,9**	**10,3**	**18,6**	**57,5**
	Exp. No.	1	1	2	3
N. spathiger	Days L3 frozen	17	17	220	719
	Mean development (%)	**0,5**	**4,2**	**27,6**	**63,8**
	Exp. No.	1	1	2	3
O. columbianum	Days L3 frozen	17	17	232	731
	Mean development (%)	**?**	**?**	**18,1**	**24,9**
	Exp. No.	1	1	2	3
T. colubriformis	Days L3 frozen	17	17	229	728
	Mean development (%)	**10,6**	**8,8**	**45,5**	**62,7**
	Exp. No.	1	1	2	3
T. axei	Days L3 frozen	—	—	232	731
	Mean development (%)	—	—	**9,4**	**27,7**
	Exp. No.			2	3
T. falculatus	Days L3 frozen	12	—	—	—
	Development (%)	**4,9**	—	—	—
	Exp. No.	5			
C. ovina	Days L3 frozen	26	—	—	525
	Mean development (%)	**8,9**	—	—	**38,8**
	Exp. No.	2			3
D. filaria	Days L3 frozen	28	44	232	—
	Mean development (%)	**0,2**	**4,3***	**0,1**	—
	Exp. No.	4	5	2	
M. marshalli	Days L3 frozen	12	—	—	—
	Mean development (%)	**9,4**	—	—	—
	Exp. No.	6			

* These *D. filaria* L3 exsheathed but not those in the other 2 experiments

TABLE 13 Experiments 2 & 3: A comparison of the survival of larvae stored for 7,5 months and 2 years

Worm species	Survival (%)	
	After 7,5 months	After 2 years
H. contortus	85,9	96,6
O. circumcincta	96,6	95,6
T. axei	98,0	96,7
T. colubriformis	100,0	93,5
N. spathiger	87,0	91,0
O. columbianum	83,3	87,3
*C. ovina**	87,9	86,8

* Larvae stored for only 1 month and 17 months

Worms that developed from frozen larvae laid numerous eggs and gave high faecal counts (Table 9).

We cannot explain why the fresh larvae were very poorly viable in the present experiment (Table 10), since, judging from the development of the frozen larvae, the sheep were fully susceptible to infestation. Only with *O. columbianum* did comparable percentages of larvae develop in Groups A and C; 2,4% *H. contortus* fresh larvae developed, compared to 39,9% frozen larvae, while with *T. colubriformis* 8,3% and 62,7% larvae respectively developed.

Prolonged freezing did not appear to have had any effect on the rate of development of the larvae as very few immature worms were found in either the frozen or fresh larval groups (Table 11). The difference was appreciable only with *O. columbianum*.

This trial confirms what was predicted by Weinman & McAllister (1947) that: "If an organism withstands the freezing and thawing processes which are the destructive ones, it will usually survive prolonged storage". They concluded: "Although the period of storage (of hookworm infective larvae) was relatively short (44 days), there is an excellent probability that similar results might be obtained after storage of 1–2 years or longer".

It is obvious that some of the stored, so-called, pure larvae, were contaminated with other species of larvae. In Group B, for instance, as a mean of 651 *T. colubriformis* developed (Table 10) while the sheep ostensibly received only *T. axei*, it is most probable that the culture was contaminated with *T. colubriformis*. In Experiment 2 this was not realised because both species of worms were administered to every sheep. It is interesting, though, that the percentage development of *T. colubriformis* was by far the highest of the worm species in Experiment 2, probably because the sheep received more larvae than calculated from the pure *T. colubriformis* culture. The low level of contamination with *H. contortus* and *O. columbianum* detected in Group B probably had little influence on the calculated worm development in Experiment 2.

EXPERIMENT 4

D. filaria

Method

D. filaria larvae collected from sheep faeces as described by Reinecke (1973), were aerated in evaporating dishes until infective. Thereafter they were treated for 20 min with NaOCl solution but were not observed to shed their sheaths. The concentration of larvae frozen was about 1 500 per ml of saline.

After being frozen for 28 days, 757 live larvae were injected into the abomasum of Sheep 24 and 520 into the duodenum of Sheep 25.

The sheep were killed for worm recovery 60 days later.

Results

The percentage of larvae that survived cryopreservation was 79,1%. Two gravid female worms were recovered from Sheep 25 infested via the duodenum but no worms from Sheep 24.

Comment

The low worm development from apparently viable larvae in this trial and in Experiment 2 (p. 177) may be because the larvae failed to exsheath before freezing. In Experiment 5, more worms developed when exsheathed larvae were used.

EXPERIMENT 5

T. falculatus *and* D. filaria

Method

After 40 min of exposure to NaOCl, the *D. filaria* larvae exsheathed. The larvae were thawed when the *T. falculatus* had been frozen for 12 days and the *D. filaria* for 44 days (Table 14).

Sheep 26 (8 months old at infestation) was infested by inoculation into the duodenum, while Sheep 27 (14 months at infestation) was dosed *per os*, in both cases with the same cultures of larvae. Details of infestation and worm recovery are summarized in Table 14.

Sheep 26 was killed for worm recovery 30 days after infestation but Sheep 27 was not killed.

TABLE 14 Experiment 5: *T. falculatus* and *D. filaria* in 2 sheep

Worm species	Frozen larvae						Worm recovery (Sheep 26)	
	Concentration*	Days frozen	No. examined	Alive (%)	No. (alive) dosed			
					Sheep 26 (in duo.)	Sheep 27 (*per os*)	Number	Development (%)
T. falculatus	25	12	276	91,3	24 446	48 891	1 197	4,9
D. filaria	17	44	277	77,3	10 420	10 420	452	4,3

* Concentration in thousands/ml saline during storage

TABLE 15 Experiment 5: *T. falculatus* e.p.g. of faeces and examination for *D. filaria* larvae

Days after infestation	Sheep 26		Sheep 27	
	E.p.g.	*D. filaria* examination for larvae	E.p.g.	*D. filaria* examination for larvae
20	negative	—*	negative	—
21	38†	—	positive	—
22	300	—	negative	—
26	400	—	100‡	—
28	500	negative	100	negative
29	400	negative	100	negative
30	200	negative	100	negative

* No examination done
† E.p.g. determined by total flotation
‡ Only 1,3% *Trichostrongylus* eggs; the remainder were *Haemonchus* (see text)

Results (Tables 14 & 15)

When thawed after 12 and 44 days of storage, 91,3% *T. falculatus* and 77,3% *D. filaria* respectively were alive (Table 14).

The infestations of *T. falculatus* in both sheep became patent after 21 days (Table 15); no eggs were found in the faeces on Days 15, 19 and 20 after infestation.

Two months after infestation, a differential larval count (Sheep 27) showed 1,3% *Trichostrongylus* and 98,7% *Haemonchus* larvae, the latter obviously being "contaminants". The fact that in Sheep 26 no *Haemonchus* developed was possibly because the larvae (presumably a mixed culture of *T. falculatus* and *H. contortus*) were deposited in the duodenum, which is unfavourable for *H. contortus*.

No larvae of *D. filaria* were found in the faeces of either sheep (Table 15) but gravid females were recovered from the sheep slaughtered on the 30th day. In addition 20 000 ova in all stages of development were recovered from the lung washings.

The worm recovery from Sheep 26 (Table 14) was 1 197 *T. falculatus* (4,9% development) and 452 *D. filaria* (4,3% development).

Comment

Apparently the larval cultures (most probably those of *T. falculatus*) were contaminated with *H. contortus*, since *Haemonchus* L 3 was recorded in a differential larval count for Sheep 27. Nevertheless, no *Haemonchus* developed in Sheep 26 in which the larvae were inoculated into the duodenum.

As with *T. colubriformis*, *T. falculatus* survived freezing and thawing very well without entirely losing viability, but the percentage development, comparable with that of *T. colubriformis* in Experiment 1, was poor.

It is interesting that an apparently light infestation of *T. falculatus* developed in Sheep 27, infested *per os*, while Campbell *et al.* (1973) failed to infest sheep *per os* with either frozen or unfrozen exsheathed *T. colubriformis*. This possibly reflects a difference in the infectivity of the 2 species.

It is difficult to interpret the results obtained with the larvae of *D. filaria* in this experiment. In Experiments 2 and 4, only 3 of 4 125 (0,1%) L 3 developed in 4 sheep, while in the present experiment 452 of 10 420 L 3 (4,3%) developed in one sheep. This batch of larvae, which exsheathed before being frozen, was subsequently retested in 2 sheep after 44 days of storage and again 4,3% and 2,9% of the larvae developed to adult worms (Van Wyk, unpublished data, 1976). The period of storage probably did not play a role as the 30 days of storage in this experiment is intermediate between the 28 days and 232 days in the other experiments. The intervals between infestation and necropsy were also similar.

The only obvious difference between the experiments is that the larvae in this experiment exsheathed, while no exsheathment was noticed in Experiments 2 and 4.

According to results obtained by other workers (Campbell *et al.*, 1972; Campbell & Thomson, 1973; Campbell *et al.*, 1973; Kelly *et al.*, 1976) with different species of larvae and from experiments in the present series, it seems unlikely that exsheathment has any beneficial effect other than to protect larvae from the effects of freezing and thawing. It is notable that in the present experiment exsheathment does not seem to have played a major role in the survival of *D. filaria* larvae, as 77,3% of the exsheathed L 3 survived freezing and thawing, while 79,1% and 65,5% of the apparently ensheathed L 3 survived in Experiments 2 and 4 respectively.

Thus, although the 3 experiments are not strictly comparable because different larval batches were used in small numbers of animals, exsheathment may have enhanced the infectivity of the *D. filaria* larvae which survived freezing.

It is probable that the *D. filaria* infestation would have become patent, because both gravid females and ova containing fully developed larvae were recovered from the bronchi.

EXPERIMENT 6

M. marshalli

Method

The larvae were concentrated to about 20 000 per ml saline, frozen, and thawed 12 days later.

Sheep 28 was infested *per os* and Sheep 29 by injection into the abomasum (Table 16). Both sheep were 6 months old at infestation. Worms were recovered 39 days after infestation.

TABLE 16 Experiment 6: *M. marshalli* larvae frozen for 12 days

Sheep No.	Age at infestation	Larvae		Route of infestation	Worms recovered			Development (%)
		Survival (%)	No. (alive) dosed		5th stage	Adult	Total	
28	6 months	89,9	16 824	*per os*	340	661	1 001	6,0
29	6 months	89,9	12 785	abomasum	295	1 343	1 638	12,8

Results (Table 16)

When thawed, 89,9% of the larvae were alive. They were very active and some contained vesicles of various sizes in the intestinal cells.

The faecal egg counts were negative, except in Sheep 28 on Day 26 (100 e.p.g.) and Day 36 (20) and in Sheep 29 on Day 25 (3) and Day 36 (100).

After 39 days, immature 5th stage worms constituted 34% and 18% of the total worm burden in Sheep 28 and Sheep 29 respectively; the remainder were mature adults.

The percentage development was 6,0% and 12,8% in Sheep 28 and Sheep 29 respectively.

Comment

Although a high percentage of larvae survived freezing, the percentage development was low in both sheep.

EXPERIMENT 7

Naturally exsheathed C. ovina

Method

A batch of *C. ovina* which had been stored at 4 °C had exsheathed when examined after 6 months (177 days).

Some were frozen in 0,9% NaCl without prior treatment with NaOCl, were thawed 2 days later and tested for infectivity in Sheep 30 (12 months old at infestation) which received 10 000 L 3 *per os*.

The rest of the larvae were maintained at 4 °C for a further 5 months (163 days), when 10 000 of the remaining larvae were frozen and their infectivity tested 2 days later by infesting Sheep 31 (9 months old at infestation). At the same time Sheep 32 (10 months old at infestation) was infested *per os* with 5 000 unfrozen larvae from the same batch to serve as a control. These sheep were not killed for worm recovery.

Similarly, some of the remaining larvae were retested after 742 days of storage at 4 °C. Sheep 33 was infested *per os* with 6 200 live L 3, frozen, and thawed 15 minutes later, and Sheep 34 was infested *per os* with 6 160 live refrigerated larvae which had not been frozen. These 2 sheep were killed for worm recovery 58 days after infestation.

Results

Fourteen per cent of the *C. ovina* L 3 survived storage at 4 °C for 742 days.

Faecal examination and larval identification showed that Sheep 30, 31 and 32 were heavily infested with *C. ovina* (Table 17).

In Sheep 33 (frozen larvae) and Sheep 34 (refrigerated larvae), the maximum faecal egg counts were 200 e.p.g. and 1 500 e.p.g. respectively, and 84 (1,4% development) and 498 (8,1% development) worms were recovered.

Comment

Even after storage at 4 °C for 24,5 months, the larvae that became exsheathed spontaneously were successfully frozen and thawed without treatment with NaOCl. Previously such exsheathed larvae were regarded as uninfective in this laboratory.

Campbell *et al.* (1973) suspected that the low percentage of larvae (usually about 5–10% as shown by Isenstein & Herlich, 1972) that usually survive freezing and thawing when not exsheathed beforehand, may be naturally exsheathed larvae. Our results confirm that such larvae can retain infectivity after freezing in liquid nitrogen.

TABLE 17 Experiment 7: *C. ovina* naturally exsheathed during storage at 4 °C

Days after infestation	E.p.g.		
	Sheep 30* Larvae frozen	Sheep 31† Larvae frozen	Sheep 32† Larvae not frozen
54	200	200	700
55	—	100	400
56	100	—	—
59	—	100	400
61	—	300	600
62	200	—	—
63	—	300	500
64	200	—	—
70	1 200	—	—
82	1 400	—	—

* Larvae stored for 6 months at 4 °C before use
† Larvae stored for 11 months at 4 °C

The excellent development of exsheathed larvae after oral dosing indicates that more of the species reported on in this paper should be tested for infectivity by this route after freezing and thawing to reduce laparotomies to the minimum.

EXPERIMENT 8

The infectivity of the progeny of cryopreserved larvae of C. ovina *and* M. marshalli

Previously only the infectivity of larvae that had been frozen and thawed was tested, and not that of the progeny of these larvae (Campbell *et al.*, 1972; Campbell & Thomson, 1973; Campbell *et al.*, 1973 and Kelly *et al.*, 1976). Furthermore, no one seems to have tested the viability of ova produced by worms that developed from cryopreserved larvae. If the offspring of such larvae are not infective, then naturally this technique will be of very little practical significance.

Method

C. ovina and *M. marshalli* L 3 were obtained from the ova passed in the faeces of Sheep 31 (Experiment 7) and Sheep 29 (Experiment 6), both of which had been infested with cryopreserved larvae only.

The larvae harvested were used to infest Sheep 35, which received 1 000 *C. ovina* and 9 000 *M. marshalli* L 3 *per os* at different times. These progeny were not exsheathed or frozen before their infectivity was tested.

Sheep 35 was killed for worm recovery 54 days after infestation with *C. ovina* and 19 days after receiving *M. marshalli*.

Results

The number of worms recovered post-mortem were as follows: 704 *C. ovina* (all adult) and 8 936 *M. marshalli* (8 813 5th stage worms and 123 adults). The percentages of development were 70,4% for *C. ovina* and 99,3% for *M. marshalli*.

C. ovina ova were recovered from the sheep's faeces as follows:

Day 53 after infestation: 100 e.p.g.

Day 54 after infestation: 4 000 e.p.g.

The viability was not tested.

Although no *M. marshalli* ova were recovered from the faeces on these 2 days, a few of the females recovered post-mortem were gravid; the sheep was probably killed too soon for this infestation to be patent.

Comment

The offspring of cryopreserved larvae were infective in the case of these 2 species, and, by producing ova in their turn, they completed the cycle.

It is probably unlikely that the development of *M. marshalli* was as high as that indicated above; presumably the aliquot method used for predicting the numbers of larvae administered was inaccurate.

The increase in egg count between Day 53 and Day 54 after infestation is probably due to an increase in the number of worms which became patent at this time.

(B) Unsatisfactory cryopreservation

Experiments 9 and 10

EXPERIMENT 9

G. pachyscelis *and* S. papillosus

Method

Each of Sheep 36–44 was infested with exsheathed *G. pachyscelis* larvae by one of the following routes: subcutaneous (injection), percutaneous (intact skin), intravenous (injection), intra-abomasal, intraduodenal and via the conjunctiva (ocular). Three of these sheep were also exposed simultaneously to *S. papillosus* larvae which had been frozen and then thawed after 8 days.

Despite the fact that *S. papillosus* L 3 were not ensheathed, these larvae were exposed to NaOCl and washed and frozen as described for ensheathed larvae.

Results (Table 18)

When thawed 8 days after freezing, 66,7% *S. papillosus* were alive. The survival rate of *G. pachyscelis* was 68,0% after 17 days, 75,0% after 22 days, and 65,0% after 40 days.

No worm ova were detected in the faeces, nor were worms recovered from any of these sheep.

Comment

Even though a high percentage of larvae of both worm species survived freezing and thawing, and although some *G. pachyscelis* were only exsheathed and not frozen, they were apparently not infective. Weinman & McAllister (1947) reported good survival of *Strongyloides simiae* L 3 after freezing for 49 days at —70 °C, but did not test the viability of the larvae.

Both *S. papillosus* and *G. pachyscelis* usually infest the sheep percutaneously, and possibly freezing and/or exsheathment affected "enzymes" necessary for tissue penetration, making them unviable. However, on one hand various routes of inoculation, regarded as obviating the need for tissue-penetrating substances, were equally unsuccessful, and on the other hand 2 similar nematode genera have been frozen successfully without total loss of infectivity. Weinman & McAllister (1947) froze *Ancylostoma caninum* larvae in water without exsheathment and dosed about 80 surviving L 3, thawed after 37 days, *per os* to a dog. The worms apparently did develop and copulate, as the infestation became patent. Kelly *et al.* (1976) repeated this with exsheathed L 3 *A. caninum* frozen in the gas phase of liquid nitrogen for 90 days and inoculated them subcutaneously into dogs. Exsheathment alone (without freezing) caused only a 45–59% reduction of infectivity compared to that of intact larvae, but not total loss of viability as in our trial with *G. pachyscelis*. Furthermore, Kelly & Campbell (1974) obtained similar results with *Nippostrongylus brasiliensis* in rats.

Possibly a physiological method of exsheathing *G. pachyscelis* L 3, for example by skin penetration, would give better results. The feasibility of freezing hookworm ova should also be investigated.

TABLE 18 Experiment 9: Infestation of sheep with *G. pachyscelis* and *S. papillosus*

Sheep No.	Age at infestation (months)	Worm species	Larvae			Route of infestation§	Result
			Time stored* (days)	Survival (%)	No. (alive) dosed		
36	5	*G. pachyscelis*	17	68,0	308	s/c	negative
		S. papillosus	8	66,7	746	percut.	negative
37	5	*G. pachyscelis*	17	68,0	308	percut.	negative
		S. papillosus	8	66,7	746	s/c	negative
38	17	*G. pachyscelis*	40	65,0	292	abo. & duo.	negative
		S. papillosus	8	66,7	1 492	abo. & duo.	negative
39	8	*G. pachyscelis*	22	75,0	431	s/c	negative
40	7	*G. pachyscelis*	22	75,0	431	i.v.	negative
41	5	*G. pachyscelis*	22	75,0	431	percut.	negative
42	8	*G. pachyscelis*	unfrozen†	n.d.‡	575	s/c	negative
43	8	*G. pachyscelis*	unfrozen†	n.d.	575	i.v.	negative
44	14	*G. pachyscelis*	unfrozen†	n.d.	21 600	conjunctiva	negative

* If frozen

† Unfrozen but exsheathed

‡ Not determined. Larvae freshly collected and very active

§ s/c: Subcutaneous; percut: percutaneous; abo: in abomasum; duo.: in duodenum; i.v.: intravenous; conjunctiva: in ocular conjunctival sac

Another possibility is to freeze the larvae immediately after penetration into a piece of host tissue. McCall, Jun & Thompson (1975) were successful in preserving *Dipetalonema viteae* L 3 in ticks frozen in dimethyl sulfoxide (DMSO), but not when frozen without the ticks. These larvae retained infectivity after they had been preserved for 595 days.

Perhaps the solution is to enhance the preservation, not by freezing but by disinfecting the larvae as for hookworm larval vaccines (Miller, personal communication, 1976).

EXPERIMENT 10

An unsatisfactory result with H. contortus *stored for 1 year*

Method

A high concentration (±230 000 L 3/ml saline) of a strain of *H. contortus* relatively resistant to thiabendazole was frozen.

After 403 days an ampoule was thawed, diluted with 0,9% NaCl solution and 22 000 live L 3 inoculated into the abomasum of Sheep 45.

Eleven faecal egg counts were done between 21 and 53 days after infestation.

Results

About 37% of the larvae survived storage. The live larvae were sluggish and had a curious, granular, semi-opaque appearance.

Although the maximum faecal egg count was 200 at 41 days after infestation, only 3 of 11 egg counts were positive between 21 and 53 days after infestation (Days 22, 41 and 53).

Comment

Considering the large number of larvae dosed, the faecal egg count which developed was very low. One sheep only was used in this experiment, but previous observations (Van Wyk, unpublished data, 1975) in 2 other sheep in which smaller numbers of these larvae were used for infestation confirmed the poor viability of these larvae.

The difference in the viability of these larvae compared with that of larvae in other experiments may be due to differences in strains or in the concentrations of larvae during storage. Unpublished data (Van Wyk, 1977) support the supposition that the differences may be due to differences in strains. In other experiments where the concentrations of the larvae differed, variable results were obtained as in Experiments 2 and 3 (Tables 2, 5 and 10).

Sufficient larvae were obtained from Sheep 43 to re-establish the strain in another donor from which large numbers of infective larvae were cryopreserved. These have not yet been tested for survival and infectivity.

II Bovine nematodes

(*A*) *Satisfactory cryopreservation*

EXPERIMENT 11

H. placei, O. Ostertagi, Cooperia *spp*., N. helvetianus *and* O. radiatum *stored for* 42 *days* (*preliminary investigations*)

Method

Small numbers of the above species were frozen separately on the same day and thawed 42 days later. The concentration of larvae during storage was very low, varying from 80 per ml (*O. ostertagi*) to 1 000 per ml (*N. helvetianus*). Two ampoules of larvae of each species were available, one of which was used for infesting Calf 1 (12 months old at infestation) by injection into the abomasum and the other by injection into the duodenum of the same calf.

The percentage survivors of each species was determined separately on small aliquots of the contents of each ampoule.

The calf was not killed for worm recovery as a very low faecal egg count was obtained on only one occasion.

Results (Table 19)

With the possible exception of the *H. placei*, there was little difference in the percentage live larvae at thawing between the 2 ampoules of larvae of each species (Table 19). The mean percentage of larvae that survived in 2 ampoules varied from 54,1% for *H. placei* to 96,6% for *O. radiatum*.

Twenty-eight days after infestation the egg count was 50 e.p.g. On a few occasions no ova were detected in the faeces.

Comment

Experiment 11 is included for comparison of the percentage survival of duplicate ampoules of larvae of each species.

It is interesting that the percentage survival of the pairs of ampoules thawed was similar. However, too few repetitions were examined to make these results comparable with the more thorough investigations of Kelly & Campbell (1974). These workers found that the survival and infectivity of exsheathed frozen larvae both between different batches and between different vials of the same batch, varied to such an extent that they concluded that "certain unknown experimental variables need to be brought under control before the cryogenic preservation of these species can be introduced as a standard laboratory procedure."

TABLE 19 Experiment 11: Larvae in each of 2 ampoules per worm species

Worm species	Ampoule 1		Ampoule 2		Total larvae dosed*
	No. examined	Survivors %	No. examined	Survivors %	
H. placei	93	44,9	76	63,2	800
O. ostertagi	10	90,0	7	85,7	140
Cooperia spp.	23	91,3	38	92,1	500
N. helvetianus	127	94,5	88	88,6	1 800
O. radiatum	8	100,0	15	93,3	200

* Not accurately determined in some cases as very few larvae were available

TABLE 20 Experiment 12: The infective larvae

Worm species	Concentration†	The larvae			
		Days frozen	No. examined	Alive (%)	No. (alive) dosed
H. placei	16	**60**	89	**43,8**	6 000*
O. ostertagi	6	**35**	49	**90,0**	5 000*
Cooperia spp.	14	**161**	74	**89,2**	11 843
N. helvetianus	3	**112**	121	**99,2**	9 000*
O. radiatum	8	**101**	117	**90,6**	6 507

* These larval cultures contained a few small clusters of larvae which hampered accurate dose determination
† Concentration in thousands per ml saline during storage

EXPERIMENT 12

H. placei, O. ostertagi, C. pectinata, C. punctata, N. helvetianus *and* O. radiatum *frozen for* 35–161 *days (short- and medium-term storage)*

Method

As for the experiments on medium- and long-term storage of the sheep nematodes (Experiments 2 and 3), sufficient larvae of each of these species were frozen so that the survival of the same batches of larvae could be compared (in this experiment and Experiment 13) after 2 periods of storage.

Six ampoules of *Cooperia* spp. and 9 ampoules of the other 4 species of larvae were frozen on different occasions as they became available, and some of each species were thawed on the same day immediately before being used to infest Calf 2. No control calf (receiving fresh, unfrozen larvae) was used.

Calf 2 (3 months old at infestation) was infested by the injection of the *H. placei, O. ostertagi* and 47% of the *Cooperia* spp., *N. helvetianus* and *O. radiatum* L 3 into the abomasum, while the remaining larvae of the latter 3 genera were inoculated into the duodenum.

Larval doses are listed in Table 20.

Faecal egg counts were done on 7 occasions between 21 and 49 days after infestation and a differential larval count was performed of larvae hatched from faeces passed 36 days after infestation.

Results (Tables 20 & 21)

The infective larvae were very active when thawed. Percentages of surviving larvae varied from 43,8% (*H. placei*) to 99,2% (*N. helvetianus*) as shown in Table 20.

TABLE 21 Experiment 12: Worm recovery data

Worm species	No. of worms recovered*	Development (%)
H. placei	940	14,5
O. ostertagi	1 059	19,8
Cooperia spp.	2 741	23,1
N. helvetianus	320	3,5
O. radiatum	11	0,2

* Gravid females of all species were recovered

The faecal egg count was 300 per g on Day 21 after infestation and 200, 500, 1 000, 600 and 600 on Days 22, 29, 36, 43, 44 and 49 after infestation respectively. A differential count of larvae hatched from the faeces at the time of the maximum e.p.g. showed: *Haemonchus,* 31%, *Ostertagia,* 23%; and *Cooperia,* 46%.

Worms of all 5 species developed, the percentage of larvae recovered as adult worms ranging from 0,2% for *O. radiatum* to 23,1% for *Cooperia* spp. (Table 21). Unfortunately, a few small larval clumps occurred in the exsheathed larval suspension and hence these percentages of development are not as accurate as in other experiments where the larval doses could be determined more exactly.

Comment

Larval survival rates were similar to those in Experiment 11 (Table 22).

TABLE 22 Experiments 11 & 12: A comparison of larvae that survived freezing and thawing

Worm species	Surviving L 3 (%)	
	Experiment 11	Experiment 12
H. placei	54,1	43,8
O. ostertagi	87,9	90,0
Cooperia spp.	91,7	89,2
N. helvetianus	91,6	99,2
O. radiatum	96,6	90,6

A high faecal egg count developed. All species except *O. radiatum* were found on larval culture, but gravid female *O. radiatum* were recovered post-mortem.

The percentage development of worms was low, with a maximum of 23,1% (*Cooperia* spp.) and minimum of 0,2% (*O. radiatum*). It is not clear why the *O. radiatum* and *N. helvetianus* were so poorly viable when high percentages of the larvae survived freezing and thawing and appeared very active before infestation. The development in sheep of unfrozen, intact *N. spathiger* L 3 is known to be very erratic (Groeneveld & Reinecke, 1969).

EXPERIMENT 13

H. placei, O. ostertagi, C. pectinata, C. punctata, N. helvetianus *and* O. radiatum *frozen for* 24–28 *months (long-term storage)*

Method

The concentrations of larvae per ml saline were the same as in Experiment 12 (Table 20). The times of storage of the larvae are listed in Table 23.

TABLE 23 Experiment 13: Viability of infective larvae stored for 24–28 months

Worm species	Calf No.	Larvae (frozen)			
		No. days frozen	No. examined	Alive (%)	No. (alive) dosed per calf
H. placei	3	**742**	130	**86,2**	11 939
O. ostertagi	4	**716**	82	**96,3**	12 567
Cooperia spp	3	**843**	141*	**97,2**	14 434
Cooperia spp	4	**843**	141*	**97,2**	7 217
N. helvetianus	4	**794**	182	**98,4**	2 470
N. helvetianus	5	**794**	85	**98,8**	8 200
O. radiatum	3	**783**	81	**95,1**	7 831

* A total of 141 *Cooperia* L3 was examined

As in Experiment 3, burst larvae were disregarded when the percentages of live larvae were determined. Larvae dosed are listed in Table 23 and the routes of infestation are shown in Table 24. Calf 3 was 7 weeks old at the time of infestation while Calves 4 and 5 were 3 weeks old and 1 week old, respectively.

TABLE 24 Experiment 13: Routes of infestation

Calf		Route of infestation		
Number	Age (weeks)*	Abomasum	Duodenum	*Per os*
3	7	*H. placei*	*Cooperia* spp. *O. radiatum*	—
4	3	*O. ostertagi*	*N. helvetianus*	*Cooperia* spp.
5	1	—	*N. helvetianus*	—

* Age at the time of infestation

Faecal egg counts were done on Days 18, 19, 20, 22 and 25 after infestation.

Worms were recovered from Calves 3 and 4, 28 days after infestation. Calf 5 was retained for recovery of *Nematodirus* ova, and killed only 102 days after infestation.

TABLE 25 Experiment 13: Number of worms recovered from calves infested with larvae frozen for 716–843 days

Worm species \ Calf (age at infestation)	3 (7w)	4 (3w)	Immature worms as a % of the total
H. placei			
Total (Adult)	1 261	—*	
Development (%)	10,6%	—	0,0
Cooperia spp.			
Total (adult)	51	565	
Development (%)	0,4%	7,8%	0,0
O. radiatum			
L4	79	—	
5th	313	—	
Total	392	—	
Development (%)	5,0%	—	20,2†
O. ostertagi			
Total (adult)	—	4 536	
Development (%)	—	36,1%	0,0
N. helvetianus			
L4	—	5	
5th	—	3	
Adult	—	289	
Total	—	297	
Development (%)	—	12,0%	2,7

* No larvae dosed or worms recovered
† L4 as a percentage of the total worms (no adult worms recovered)

TABLE 26 Comparative development of bovine nematodes in different experiments

Worm species	Larvae	Short-term storage (Exp. 12)	Long-term storage (Exp. 13)
H. placei	Days L3 frozen	60	742
	Development (%)	14,5	10,6
O. ostertagi	Days L3 frozen	35	716
	Development (%)	19,8	36,1
N. helvetianus	Days L3 frozen	112	794
	Development (%)	3,5	12,0
O. radiatum	Days L3 frozen	101	783
	Development (%)	0,2	5,0
Cooperia spp.	Days L3 frozen	161	843
	Development (%)	23,1	0,4*/7,8†

* Infested in duodenum
† Infested *per os*

Results (Tables 23, 24 & 25)

The infectivity of the various nematode larvae of bovines used in Experiments 12 and 13 is given in Table 26.

The mean percentage survival of the larvae after a mean storage time of 776 days was 94,7% (Table 23). The larvae were very active when thawed.

The infestation in all 3 calves was patent 22 days after infestation. The following maximum e.p.g. counts were obtained: 150 each in Calf 3 and Calf 5 and 200 in Calf 4. No differential larval counts were performed.

Approximately 2,39 million *Nematodirus* ova were recovered from the faeces of Calf 4 and Calf 5 by sieving, flotation and sedimentation procedures. From these ova, approximately 1,8 million (75,6%) larvae were hatched. The infectivity of these larvae was not determined.

The percentage of larvae that developed after infestation (considering only the best results obtained per genus) varied from 5,0% for *O. radiatum* to 36,1% for *O. ostertagi* (Table 25).

The immature worms recovered post-mortem from Calf 3 and Calf 4 varied from nil for *H. placei, O. ostertagi,* and *Cooperia* spp., to 2,7% for *N. helvetianus,* and 20,2% for *O. radiatum* (Table 25).

Comment

This experiment has demonstrated effectively that frozen larvae can be used for re-establishing *N. helvetianus* in the laboratory. Large numbers of larvae were obtained after infestation of 2 calves with smaller numbers of frozen infective larvae of *N. helvetianus* than fresh larvae used to infest *Nematodirus* donors

in this laboratory (Reinecke, 1973). The fact that very young calves were used for infestation may have had an important bearing on these favourable results (Table 24).

TABLE 27 Experiments 12 & 13: A comparison of the survival of the frozen larvae

Worm species	Survival (%)	
	After 35–161 days*	After 2 years†
H. placei	43,8	86,2
O. ostertagi	90,0	96,3
Cooperia spp.	89,2	97,2
N. helvetianus	99,2	98,6
O. radiatum	90,6	95,1

* Experiment 12
† Experiment 13

The survival of the larvae used for Experiment 12 (after 65-161 days of storage in the gas phase of liquid nitrogen) and that of the larvae in the present experiment are compared in Table 27. In the present experiment 42,4% more larvae of *H. placei* and 4,5%–6,3% of the other species were alive than after a short period of storage (Experiment 12). Except for *H. placei*, where no explanation is suggested, the differences were probably due to larvae which had burst and were recorded as dead in Experiment 12, while being ignored in the present experiment.

The faecal egg counts seemed disappointingly low considering the larger numbers of larvae used in this experiment. Possibly larger counts would have been obtained had faecal examinations been continued for a longer period.

The viability was similar in larvae stored for more than 2 years and those stored for short periods; with *H. placei* and *Cooperia* spp. the development was lower after 2 years and vice versa in *O. ostertagi*, *N. helvetianus* and *O. radiatum* (Table 26).

Further experiments are intended with the larvae that remained from the batches frozen for Experiments 2 & 3 and 12 & 13.

(B) *Unsatisfactory Cryopreservation*

Experiments 14 *and* 15

EXPERIMENT 14

Exsheathed (unfrozen) B. phlebotomum

G. pachyscelis L 3 which, like *B. phlebotomum*, infest the host percutaneously, and which were exsheathed in NaOCl, but not subsequently frozen, were not infective to sheep (Experiment 9). It was decided to test the infectivity of *B. phlebotomum* L 3 exsheathed in bovine serum heated to 40 °C and, similarly, not frozen.

Method

Freshly collected *B. phlebotomum* L 3 were exsheathed by placing them for 50 min in bovine serum heated to 40 °C.

Thereafter 344 000 of these larvae were injected intravenously into Calf 6.

The calf was killed 23 days later for worm recovery.

Results

No worm eggs were found in the faeces and no worms were recovered from the calf post-mortem.

Comment

Although the larvae were not frozen, this method of exsheathing was as unsuccessful as the NaOCl method with *G. pachyscelis*.

EXPERIMENT 15

Exsheathed (frozen) B. phlebotomum

Method

The skin of the shank of one leg of a bovine foetus was stripped to resemble a sausage casing. This "tube" was everted, closed at one end with a screw clamp and received approximately 300 000 L 3 *B. phlebotomum* pipetted into the lumen of the "tube". The open end was clamped after the air had been expelled and the tube itself submerged in bovine serum maintained at 37 °C.

On the following day the larvae that had traversed the skin and those remaining in the lumen of the "tube" were frozen separately (each in 1 ml saline) and stored for a few hours before being thawed and injected intravenously into Calf 7 (2 weeks old at infestation).

The calf was killed for worm recovery 139 days after infestation.

Results

Approximately 5 000 L 3 traversed the skin and appeared in the serum. Most of the remainder became exsheathed, but remained in the lumen of the skin preparation.

Whereas the 5 000 L 3 were very active upon thawing, the remainder of the 300 000 appeared dead, only a few larvae moving very sluggishly.

Fifty-four days after infestation 4 nematode ova were obtained by flotation from 5 g faeces and on Day 55 after infestation another 3 were recovered. Thereafter all examinations for ova were negative. Furthermore, cultures of faeces yielded no larvae.

No worms were recovered post-mortem from the calf.

Comment

Only 1 of the 7 ova recovered from the faeces of this calf resembled that of *B. phlebotomum*.

The fact that the calf was killed long after infestation may account for the failure to detect any *B. phlebotomum* post-mortem.

Hence, even though it seems highly unlikely that any *B. phlebotomum* developed, experiments with larger numbers of highly active larvae, different routes of infestation and a shorter period of infestation might produce different results.

The exsheathment of *B. phlebotomum* L 3 by this method was retested on numerous occasions subsequently with extremely variable results (Van Wyk, unpublished data, 1975). In one case at least 30–50% of a large number of larvae penetrated the skin and emerged in the serum, but in most other cases very small numbers of larvae were successful. The age of the foetus from which the skin was obtained may have played a role in the ability of the larvae to traverse it. Unfortunately no record was kept of the approximate age of the foetus from which each preparation

was obtained, but the fact that most had sparsely developed hair on the skin of the shank was some indication of the age of the foetus. Older foetuses should be tested because their skin resembles more closely that usually penetrated by the *B. phlebotomum* L 3 in nature (i.e. the hirsute skin of calves and older cattle).

Discussion and Conclusions

As far as can be ascertained, this is the first time that the following species of nematodes have been tested for infectivity after freezing in liquid nitrogen: *H. placei, O. circumcincta, O. ostertagi, T. axei, T. falculatus, M. marshalli, N. spathiger, N. helvetianus, S. papillosus, G. pachyscelis, C. ovina, O. radiatum, D. filaria, Cooperia* spp. and *B. phlebotomum*. Furthermore this appears to be the first time that nematode larvae have been tested for infectivity after storage in the gas phase of liquid nitrogen for as long as 2 years.

All attempts to freeze *S. papillosus, G. pachyscelis* and *B. phlebotomum* were unsuccessful. The other species were satisfactorily stored in liquid nitrogen, some retaining viability even after being frozen for longer than 2 years.

The viability of the larvae of sheep and bovine nematodes after various periods of storage is listed, respectively, in Tables 12 and 26. Although it appears that viability increased with longer storage, this conclusion is probably erroneous because the various experiments were not strictly comparable. It is possible, for instance, that the handling of the larvae during thawing and counting improved with experience.

While most of the larvae were inoculated into the abomasum and/or duodenum, a few were administered *per os* and found infective, namely, *C. ovina, M. marshalli, C. pectinata* and *C. punctata*. More of the remaining species should be tested by this simple route.

The results were reasonably predictable from the work of Campbell *et al.* (1972); Campbell & Thomson (1973); Campbell *et al.* (1973); Kelly *et al.* (1976) and McCall *et al.* (1975). These workers showed that larvae of 2 species (*D. viteae* & *H. contortus*) were viable after prolonged storage (Campbell *et al.*, 1973; McCall *et al.*, 1975). Furthermore, the suggestion by Campbell & Thomson (1973) that some species found uninfective after oral administration may be infective after duodenal inoculation, has been confirmed.

There seems little doubt that the preservation of larvae in liquid nitrogen is vastly superior to storage in the refrigerator at 4 °C or at room temperature. Under the latter conditions most larvae lose their viability after relatively short periods (Herlich, 1966; Persson, 1974; Todd, Levine & Boatman, 1976) although there are some notable exceptions, for example, *Cooperia* which survived storage at 4 °C for at least 3 years (Herlich, personal communication, 1974).

Because of the work and costs involved in laparotomies, we considered that cryopreservation would be unpractical unless larvae remained viable for at least 2 years. The results obtained after this period of storage are encouraging.

Storage of larvae in liquid nitrogen presents new possibilities not previously available to the helminthologist. Because of the expense of maintaining nematodes in pure strain in the laboratory in the past, the numbers of strains had to be strictly limited. It is very difficult to maintain strains which are resistant to certain anthelmintics, numerous species of, for example, *Trichostrongylus*, etc. Cryopreservation in liquid nitrogen after exsheathment may enable the helminthologist to store the nematodes with much the same ease as the microbiologist stores different viruses and bacteria.

We concur with Kelly & Campbell (1974) that survival and infectivity of different batches and vials of frozen larvae may be variable and that ". . . certain unknown experimental variables need to be brought under control". However, we do not agree that this technique cannot at present "be introduced as a standard laboratory procedure." This technique makes it possible to safeguard against the loss of valuable material; for example, if not only various batches of a certain strain of nematode are frozen but also numerous vials of each batch. In exceptional cases it may also be advisable to retain fresh larvae or a donor animal until representative vials of at least one batch of the larvae have been tested for viability after freezing for a relatively short time.

In the past, worms or larvae could not be stored for long periods and this led to frequent passages in animals, often in abnormal hosts, for example, *T. colubriformis* in guinea-pigs (Gordon, Mulligan & Reinecke, 1960). These passages in animals tend to change the character of a strain by unintentional selection, as, for example, against hypobiosis, since faecal cultures are generally made at the time of peak egg excretion shortly after patency and before hypobiotic worms have become adult (Michel, personal communication, 1974).

The high percentages of larvae which survive storage for long periods in liquid nitrogen combined with a low frequency of passages through animals, suggest that this method may cause a smaller selection pressure on the strain of nematode than other existing methods of maintenance.

More work should be done with those species of infective larvae that were not stored successfully in liquid nitrogen (*S. papillosus, G. pachyscelis* and *B. phlebotomum*) and the work should be extended to other species that have not yet been tested.

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