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ORIGINAL PAPER

# Storage of gastrointestinal nematode infective larvae for species preservation and experimental infections

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**Abstract** Techniques to preserve the infective third-stage larvae (L3) of gastrointestinal nematodes are of considerable interest to preserve rare species and to maintain a stable source for routine experimental infections. This study compares the relative pros and cons of the two most common techniques, cryopreservation and refrigeration by comparing how they influence consequent infection outcome parameters in terms of life-history traits and fitness as a function of time using the gastrointestinal nematode of sheep *Haemonchus contortus* as a study species. Establishment capacity was found to be significantly reduced in cryopreserved stocks of L3 compared to refrigerated stocks, but this was followed by significant increases in their fecundity. Refrigeration did not affect L3 stocks consequent fitness by 16 months (the maximum examined) although they did incur a significant reduction in establishment, followed once again by an augmentation in fecundity. The study highlights potential areas for bias in comparing studies using L3 larvae maintained for different periods of time under different techniques.

**Keywords** Cryopreservation · Fitness · *Haemonchus contortus* · Life-history traits · Refrigeration

## Introduction

Techniques to preserve the infective third-stage larvae (L3) of gastrointestinal nematodes (GIN) are of great experimental interest, both to preserve rare species selected for particular aptitudes, i.e. anthelmintic resistance, morphology etc. and as a source for routine experimental infections. Effective preservation techniques reduce labour and financial costs associated with maintaining differing isolates in constant cycle through their obligate hosts and further minimize the potential for genetic drift or unexpected selection that may result from repeated passage (Chehresa et al. 1997; Gasnier et al. 1992). Two techniques are commonly used in the preservation of GIN L3 stages. The first is cryopreservation whereby the L3 are ex-sheathed and maintained in vials of physiological serum in liquid nitrogen (VanWyk et al. 1977). Studies using the sheep GIN *Haemonchus contortus* have found that not only are the worms recoverable after 10 years of storage (Rew and Campbell 1983), they also remain infective to sheep (Campbell et al. 1973). The second technique is to maintain stocks of L3 refrigerated at 4 °C (MAFF 1986). This technique takes advantage of GIN L3 physiology. The L3 possess an external protective sheath to provide greater resistance against environmental variables; however, it also prevents the L3 from feeding (O'Connor et al. 2006). The L3 therefore exist on a limited energy budget. While elevated temperatures can cause a rapid decline in their energy reserves by increasing their metabolic rate (Vlassoff et al. 2001), maintaining L3 stocks at cooler temperatures can lower their metabolism to sustain their energy reserves. The refrigeration technique was first outlined in the Ministry of Agriculture, Fisheries and Food (MAFF) manuals (1977, 1986); however, the sources on which this information was based are not documented. While there is some information regarding the efficacy of the cryopreserved techniques (Van Wyk et al. 1977, 2000; Rew and Cambell 1983; Van Wyk and Gerber 2000), no studies have compared this technique against refrigeration.

Cryopreservation has a clear duration advantage over refrigeration. Studies by Van Wyk et al. (2000) found that *H. contortus*, among other nematode species, could survive

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and establish in their host following more than 15 years in cryopreservation (Van Wyk and Gerber 2000). However, this produced highly variable results for establishment ranging from only a few percent up to 40 % based on infections introduced directly into the abomasum or rumen by means of laparotomy (Van Wyk et al. 2000) rather than traditional infections per os. On the other end of the spectrum, stocks of GIN L3 maintained at room temperature are thought to lose their infectivity in a matter of weeks (Kerboeuf 1978a; Mallet and Kerboeuf 1984, 1985). No studies could be found exploring the effect of refrigeration on GIN L3 establishment.

The relative pros and cons of cryopreservation and refrigeration techniques on consequent GIN infection outcome have never been compared. This study thereby compares the effects of cryopreservation and refrigeration on the establishment capacity and fecundity of GIN using *H. contortus* as a model species. Furthermore, the effect of various periods of refrigeration (3–16 months) on the viability of the L3 and consequent infection is explored by comparing their performance at different life-history traits and determining their absolute fitness. Given GIN are well documented to alter their life-history traits to compensate for any challenges (Chehresa et al. 1997; Poulin 1998), these measures provide a more complete picture on the effects of refrigeration age and enable direct comparisons between the stocks. To ensure there were no *H. contortus* isolate-related effects on these measures, two different isolates were compared. Similar studies were carried out on the effect of cryopreserved stocks of GIN maintained for up to 15 years, but the results are limited to information on establishment and fecundity (Van Wyk et al. 2000).

## Materials and methods

### Sheep

The trial comparing cryopreserved vs. refrigerated stocks was tested on Ile-de-France male lambs, 3 months of age. The study comparing refrigerated stocks at various time intervals tested on 15 Romane rams, 9 months of age. Both breeds are considered susceptible to infection by *H. contortus*. All sheep were maintained in worm-free conditions prior to the experiment and their worm-free status verified with negative faecal egg counts (FEC) prior to the experiment. This experiment was approved (no 2012-06-10) by the regional (Val de Loire) ethical committee.

### Cryopreservation technique

This technique was derived from that described by Van Wyk et al. (1977) with some small alterations made. The main differences used here include (1) following ex-sheathment of infective larvae, they were washed four times with 10× phosphate buffered saline (PBS) solution at pH 7.2; (2) the larvae were cryogenized in 1× PBS; (3) freezing was done gradually, decreasing by 1.4 °C every minute for 30 min in gas nitrogen before being introduced to liquid nitrogen where they remained; (4) prior to use, the stocks were thawed at 30 °C and 1× PBS was subsequently added. The number of mobile L3 was counted under a microscope to determine the percentage surviving and calculate the infective dose. Stocks were left at room temperature for 24 h prior to infection in sheep.

### Refrigeration technique

Following extraction of the L3 larvae from a faecal sample using the Baermann funnel technique (Baermann 1917), the stocks were placed in Roux flasks in water and maintained at 4 °C with approximately 2500 L3 per ml. Generally, stocks are not used within the first 30 days as a delay of maturation seems needed for attaining full infective capacity (Kerboeuf et al. 1978a, b). Prior to infection, the stocks are removed from the fridge and left for 24 h at room temperature to revive fully. The living L3 were separated from the dead larvae by placing them once again through a Baermann funnel. The stock densities were then calculated and then diluted to obtain the desired infective dose.

### Infective dose

All sheep received an infective dose of 10,000 L3 from their respective isolate per os.

### *Haemonchus contortus isolates*

The study comparing the establishment and fecundity of cryopreserved vs. refrigerated stocks of *H. contortus* compared two different isolates: ISE, an isolate that is susceptible to the three main anthelmintic groups (see Roos et al. 2004 for line history) and the same line after passage in highly resistant Martinik blackbelly sheep ISE Bb and Kokstad (KOK), an isolate obtained from a farm in South Africa with resistance against the three main anthelmintic drugs, i.e. levamisole, benzimizadole and macrocyclic lactones, kindly donated by J. Van Wyk. The length of time the isolates had been preserved and the number of sheep the respective isolate was tested on are presented in Table 1.

The study comparing the fitness of refrigerated *H. contortus* stocks after various periods of time compared two isolates. The ISE (described above) and ISE-Bb, an isolate which originated from ISE but had undergone a strong divergent selection process in Martinik Blackbelly sheep, a breed known for its resistance against *H. contortus* infection (Terefe et al. 2007). This resulted in a strong bottleneck for the ISE-Bb isolate. The length of time the isolates had been preserved and the number of sheep the respective isolate was tested on are presented in Table 2.

**Table 1** Cryopreserved vs. refrigerated *H. contortus* infective larvae stocks. Details of isolates, duration of preservation and number of sheep tested

| Isolate | Method preservation | Duration of preservation | Number of sheep |
|---|---|---|---|
| ISE | Refrigeration 4 °C | 15 months | 1 |
| | | 10 months | 2 |
| | | 9 months | 2 |
| | | 3 months | 2 |
| | Cryopreservation | 2 years | 2 |
| KOK | Refrigeration 4 °C | 4 months | 1 |
| | | 6 months | 1 |
| | Cryopreservation | 2 years | 5 |

## Faecal egg counts

Faecal samples were collected to carry out FEC using a modified McMaster technique (Raynaud 1970) in a sodium chloride flotation solution, accurate to 50 eggs per gram (EPG) of faeces. For the cryopreservation vs. refrigeration trial, FEC were carried out on 28 to 35 days postinfection (dpi). For the trial comparing fitness of refrigerated stocks after various periods of time, FEC were carried out on 0, 21, 24, 28, 32 and 35 dpi.

## Establishment

The capacity of the L3 to establish in their sheep host was determined by counting the number of adult worms found in the abomasum at necropsy following the procedure described by Gaba et al. (2006). The fourth-stage larvae (L4) were also counted after having extracted them from the abomasal mucosa by incubating them in water at 37 °C for 4 hours, washing them thoroughly in water which was collected and put through a 30-μm-mesh sieve to collect any L4. The total number of adults and L4 counted for each sheep was divided by the number of L3 in the infective dose (i.e. 10,000) to see how many were able to establish, and the percentage was calculated.

For the trial comparing cryopreserved vs. refrigerated stocks, necropsy took place at 35–40 dpi. For the trial comparing fitness of refrigerated stocks after various periods of time, necropsy took place at 35 dpi.

**Table 2** Refrigerated *H. contortus* stock fitness after various periods of time: isolates, length of preservation and number of sheep tested

| Isolate | Duration of preservation (months) | Number of sheep |
|---|---|---|
| ISE | 4 | 4 |
| | 7 | 3 |
| ISE Bb | 4 | 4 |
| | 16 | 4 |

## Fecundity

For the cryopreservation vs. refrigeration trial, fecundity was determined by dividing the last FEC (when the excretion of eggs is stabilized) by the number of female worms counted.

For the trial comparing refrigerated stocks after various periods of time, the fecundity of the adult female worms was evaluated as described in Cabaret and Ouhelli (1984). In brief, the total daily egg output per sheep was determined. Given the FEC only provide a measure for 1 g of faeces, the FEC on 35 dpi (just prior to necropsy) was multiplied by the total quantity of faecal matter (QFM) in kilograms produced in a day. The QFM was calculated using the following formula (back-transformed from logarithm) developed specifically to the conditions of this study, based on a linear regression between the logarithm of the weight of faecal excretion collected over a 24-h period and the logarithm of the metabolic weight ($W^{0.75}$) of Romane rams:

$$\mathrm{QFM} = 0.041\mathrm{W}^{0.75}\ p = 0.00;\ r = 0.95$$

where W is the weight of the individual sheep (kg).

The total daily egg output (EPG × QFM) was divided by the number of female adult worms found at necropsy to give the number of eggs produced per female, per day.

## Egg to L3 larvae development ratio

The faeces collected on 28, 32 and 35 dpi were also used to test the capacity of the eggs to develop into infective L3. This was done by culturing 5 × 5 g faecal samples for each sheep in conditions favourable to *H. contortus* development, i.e. 23 °C, 70% H, 10 days (Rossanigo 1992). The L3 were then extracted from each 5-g sample of faecal matter using the Baermann funnel technique over a 24-h period at room temperature (Baermann 1917) and counted under a microscope to obtain the number of L3 developed per 5 g of faeces. Only living L3 were included in the count. The number of L3 counted for each 5-g sample was then divided by 5 to obtain the number of L3 per 1 g of faeces then divided again by the individual sheep's respective FEC for that day. This provided the ratio of larvae developing and surviving from the *H. contortus* eggs excreted.

## Absolute fitness

Absolute fitness (W) for the four isolate/age groups was determined to reflect their capacity to survive and reproduce. Absolute fitness is the number of offspring (L3) produced by an individual L3, i.e. one complete life cycle (Maynard-Smith 1989). This was calculated by multiplying the egg to L3 larvae development ratios (i.e. number of L3 per gram of faeces) by the QFM to provide the total number of living L3 produced in a day. This was done for three different days for each sheep,

**Table 3** General linear model analyses comparing effect of cryopreserved vs. refrigerated stocks of *H. contortus* on establishment and fecundity including effect of isolate and length of stored stock

| | Source | F | Significance |
|---|---|---|---|
| Establishment | | | |
| (log transformed) | Corrected model | 2.511 | 0.108 |
| | Intercept | 615.016 | 0.000 |
| | Cryopreserved vs. refrigerated stocks | 7.230 | 0.020 |
| | Isolate | 0.736 | 0.408 |
| | Age of stored stock | 0.101 | 0.756 |
| Fecundity | Corrected model | 7.350 | 0.005 |
| | Intercept | 457.449 | 0.000 |
| | Cryopreserved vs. refrigerated stocks | 4.844 | 0.048 |
| | Isolate | 6.413 | 0.026 |
| | Age of stored stock | 0.014 | 0.907 |

i.e. 28, 32 and 35. The number of L3 produced in a day was then divided by the number of L3 in the infective dose, i.e. 10,000 to provide the daily absolute fitness value (W) for the *H. contortus* in each sheep. The mean absolute fitness value was based on three sample days then calculated for each of the four isolate/age groups to provide the mean daily fitness.

### Statistical analyses

A general linear model—GLM with (SPSS software version 11.5) was used on raw or transformed data when Gaussian distribution was not present. It allows greater flexibility than standard analysis of variance by freely combining quantitative (age of L3) and categorical (preservation) factors.

## Results

### Cryopreservation vs. refrigeration

Using linear model (LM) analyses, we found that the refrigerated stocks established significantly ($p$=0.02) better than the cryopreserved stocks. The mean establishment rate for the cryopreserved stocks was 19 %±5 SE—standard error and 35 %±4 SE for the refrigerated stocks (both isolates and all ages included). Neither the isolate nor the age of the stored stock interacted with the establishment (Table 3).

The isolate ($p$=0.026) and the cryopreservation of the refrigerated stock ($p$=0.048) acted significantly on the fecundity. Fecundity was greater in the KOK (5.6 eggs per female ±0.0.4 SE) than in ISE (4.7 eggs per female ±0.3 SE). The mean fecundity for all cryopreserved stocks was greater (5.9 eggs per female ±0.4 SD) than for the refrigerated stocks (4.8 eggs per female ±0.3 SE).

### *Fitness of refrigerated stocks compared after various periods of time in storage*

The results show there were no significant differences in fitness between the isolates, irrespective of the length of time the L3 were stored (Table 4). There were however significant differences between the life-history traits of the ISE Bb 16 months old compared to the others. This group had a significantly diminished capacity to establish in their host and to develop egg into larvae. This was not reflected in the fitness as the same group also had a significantly increased fecundity.

## Discussion

The cryopreserved stocks demonstrated a significantly reduced capacity (19 %) to establish compared to the refrigerated stocks (35 %). In this case, the cryopreserved stock was only 2 years old but it attained a lower establishment capacity than that of the 13.8-year-old cryopreserved stock (29 %) observed by Van Wyk et al. (2000). Two procedural differences may account for the differences between these studies: (i) the present study froze the L3 in increments of gradually decreasing temperatures whereas Van Wyk et al. (2000) froze the L3 directly in liquid nitrogen, and (ii) the present study infected the sheep per os whereas Van Wyk et al. (2000) infected the sheep parentally. Although studies have

**Table 4** The daily absolute fitness value and mean life-history trait±standard deviation of different of *H. contortus* isolates stored at 4 °C for different periods. Significant differences between isolates denoted with different letters in superscript. Significant differences (S), $p$=0.05; not significant (NS)

| | ISE 4 months ($N$=4) | ISE 7 months ($N$=3) | ISE Bb 4 months ($N$=4) | ISE Bb 16 months ($N$=4) | Significance |
|---|---|---|---|---|---|
| Fitness | 46.39±9.6 | 35.7±8.97 | 38.53±7.83 | 55.89±0.72 | NS |
| Establishment (no. adults) | 4341±1826$^{a}$ | 4341±1826$^{a}$ | 5351±603$^{a}$ | 2398±500$^{b}$ | S |
| FEC (EPG) | 6918±169 | 5527±1262 | 5535±1004 | 4523±1024 | NS |
| Fecundity (eggs/female) | 3599±2429 | 3148±1585 | 2836±783 | 4572±1400 | NS |
| Egg-larvae development | 0.06±0.01$^{a}$ | 0.06±0.02$^{a}$ | 0.06±0.02$^{a}$ | 0.1±0.02$^{b}$ | S |

suggested these different routes of administration should result in little difference in GIN success (Van Wyk and Gerber 1980), the possibility cannot be excluded entirely. The two studies did however share one thing in common, a high variability in the establishment capacity of cryopreserved stocks. This is likely due in part to the differing protective responses of the hosts as the lambs used were all genetically unrelated. Studies exploring *H. contortus* in the extreme conditions of desiccation found that only about 35 % of the population were able to survive exposure and revive following hydration thereafter (Chylinski et al. 2014). The present study verified that the infective doses of cryopreserved stocks consisted of only living L3, yet it is possible that the physiological and metabolic demands of reviving from a frozen state negatively impacted the lipid reserves of the L3 and their consequent capacity to establish. A reduced establishment capacity owing to reduced lipid reserves was observed in desiccated *H. contortus* L3 (Siamba et al. 2011, 2012).

Given the refrigerated *H. contortus* stocks were not subjected to such extreme conditions, this likely contributed to their better establishment capacity observed in this study. The refrigerated stocks had another advantage over the cryopreserved stocks in that they still maintained their external protective sheaths which were otherwise removed prior to cryopreservation. In natural infections, the L3 do not ex-sheath until arrival at the rumen, this likely affords them some degree of protection against the hostile host environment en route. The lack of sheath in the cryopreserved L3 would have made survival to establishment for the cryopreserved stocks more challenging when infected per os.

Interestingly, the cryopreserved stocks appeared to respond to the challenge of their preservation conditions by augmenting their reproductive output, an effect which was not triggered in the less extreme conditions of refrigeration. Fecundity also varied significantly as a function of *H. contortus* isolate in cryopreserved stocks. Previous studies have noted that the performance of *H. contortus* isolates at different life-history traits can vary substantially, but sometimes with little overall effect on fitness (Chylinski et al. under review).

The fitness results from refrigerated stocks suggest that *H. contortus* L3 may be maintained at 4 °C for up to 7 months without any change to their respective fitness and life-history traits. This is in sharp contrast to what was observed for the GIN *Trichostrongylus retortaeformis* maintained at 24 °C which showed decreases in establishment as early as 9 weeks (Kerboeuf 1978a; Mallet and Kerboeuf 1985). This highlights the important role a cooler temperature has to play in extending the viability of the L3.

Despite the significant reduction in establishment of the 16-month-old ISE Bb isolate, they did not incur any costs to their absolute fitness. Instead, the isolate appeared to compensate for this reduced establishment by significantly augmenting their fecundity. This echoes what was observed for the cryopreserved stocks of L3 above. Similar patterns of decreased establishment followed by increased fecundities in older compared to younger stocks of GIN have previously been observed in the Humeau isolate of *H. contortus* (3 vs. 11 months old maintained at 4 °C) (G. Sallé personal communication 2014), *Trichostrongylus colubriformis* (fresh vs. 9 weeks old maintained at 24 °C) (Mallet and Kerboeuf 1985), *Heligimosoides polygyrus* (fresh vs. 9 weeks old maintained at 22 °C) (Kerboeuf 1978b). As reductions in establishment capacity as a function of L3 age have been observed across species, i.e. *H. contortus*, *T. colubriformis* (Mallet and Kerboeuf, 1985) and *H. polygyrus* (Kerboeuf, 1978a), it may occur in other GIN species as well. However, the rapidity the infectivity is affected may be specific to the species and certainly, as a function of the temperature in which they are maintained (Mallet and Kerboeuf 1985). Similar studies for other common GIN species maintained experimentally may be useful.

While the cryopreserved stocks experienced a reduced capacity to establish relative to the refrigerated stocks, we cannot conclude that their establishment was altogether poor. Indeed, we maintain that there is a place for cryopreservation as we performed, especially for the long-term maintenance of the isolates or rare GIN species. The relative aim of the experiment at hand will also influence whether refrigerated or cryopreserved stocks should be used. For example, where *H. contortus* establishment is of prime relevance to a study, refrigerated stocks less than 7 months of age would clearly be more useful. However, where offspring production is of interest such as for multiplication and culturing of stocks, cryopreserved stocks would be perfectly adequate. Importantly, in the interest of comparing and contrasting different experimental studies in the literature, we suggest it would substantially reduce bias if refrigerated stocks less than 7 months of age were used in laboratory experiments using *H. contortus*.

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