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ORIGINAL PAPER

Adaptations to the Arctic: low-temperature development and cold tolerance in the free-living stages of a parasitic nematode from Svalbard

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Abstract For nematodes with a direct life cycle, transmission is highly dependent on temperature-related development and survival of the free-living stages. Therefore, in the Arctic, where the winter lasts from October to May, nematode transmission is expected to be focused in the short summer season, yet there is strong evidence that as well as focussing egg output during winter months, the nematode parasite, *Marshallagia marshalli*, infects Svalbard reindeer during the Arctic winter when temperatures are persistently below freezing. To investigate the potential for development and survival of eggs and infective third-stage larvae in winter and therefore the possibility of for winter transmission, we ran a series of low-temperature laboratory experiments. These provide five key insights into the transmission and survival of the free-living stages of *M. marshalli*: (1) eggs hatched at temperatures as low as 2 °C, but not below 0 °C, (2) eggs were viable and developed after being exposed to sub-zero temperatures for up to 28 months, (3) infective-stage larvae survived for up to 80 days at 5 °C, (4) infective-stage larvae could survive rapid exposure to temperatures below −30 °C, and (5) desiccation resistance may be important for long-term larval survival at low temperatures. Together, these results indicate that eggs deposited during the winter are highly tolerant of prevailing environmental conditions and have the potential for rapid development with the onset of spring. It is therefore likely that the parasite remains in the egg stage in the faeces during the winter of deposition, hatch and develop into the infective larval stage in the summer, remaining viable on the tundra until the reindeer host returns to the winter feeding grounds the following winter.

Keywords Svalbard reindeer · Parasite · Over-winter survival · Egg development · Larvae survival

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Introduction

The importance of parasites in wild animal systems is becoming increasingly recognised because of the role they play in influencing their host's population dynamics (Anderson and May 1978; Dobson and Hudson 1992; Hudson et al. 1992; Albon et al. 2002; Tompkins et al. 2002). However, in the majority of wild systems, knowledge of the basic ecology of the parasites themselves is lacking. For directly transmitted nematodes, eggs are typically passed in host faeces and development through three larval stages (L1, L2 and L3) occurs on the pasture. Transmission occurs when a suitable host ingests infective-stage larvae (L3) during grazing. Thus, the success of infection and persistence of the parasite population in the host depends to a large extent on the survival and development of the free-living stages, as well as the temporal and spatial overlap of hosts and parasites (Stromberg 1998; O′Connor et al. 2006; Crossan et al. 2007). The development and survival of the free-living stages is known to be sensitive to temperature, moisture and humidity and

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because of this, transmission in temperate livestock systems exhibits strong seasonal dynamics with peak egg output and transmission typically occurring during the main grazing period and little or no egg output and low worm burdens during the winter (Anderson 1992; Stromberg 1998; O′Connor et al. 2006), presumably because transmission is poor during this period. Various life-history strategies have evolved to deal with this. For example, some species of nematodes have the propensity to arrest development as larvae in the host to only resume development to adults (and subsequent egg production) when the conditions outside the host are favourable to the development and survival of free-living stages. This is thought to have evolved to limit exposure of the free-living stages to adverse conditions (Sommerville and Davey 2002). However, other nematode species survive adverse periods as free-living stages on the pasture (Troell et al. 2005; Hrabok et al. 2006; Makovcova et al. 2009; Hoar et al. 2012a), waiting until hosts become available again. These adaptations have the potential to introduce time delays in parasite recruitment to the host which can affect the dynamics of the parasite–host relationship and are therefore important to identify as part of understanding the ecology of these parasites (May and Anderson 1978; Dobson and Hudson 1992; Albon et al. 2002).

The high Arctic archipelago of Svalbard (75°–81°N, 9°–33°E) experiences extreme seasonality. For example, winter, defined as the period when temperatures drops below 0 °C for at least 48 h until the first rise above zero in the following spring, can last for up to 79 % of the year (Coulson et al. 1995b), and the ground is generally frozen and snow covered from October until May. The polar night, when the sun remains continuously below the horizon, lasts from the end of October until mid-February (Halvorsen and Bye 1999), and warmer temperatures, suitable for plant growth, occur only during a short period of time from June to August (Coulson et al. 1993, 1995b). In winter, reindeer graze in exposed wind-blown areas, often snow-free ridge habitats, which resemble a polar desert zone compromising a sparse plant community (Punsvik et al. 1979; Coulson et al. 1995b; Jonsdottir 2005). These environmental conditions would be expected to select for nematode transmission and egg output during the short summer, when conditions are more favourable for the development of the free-living stages (Halvorsen et al. 1999).

Svalbard reindeer (*Rangifer tarandus platyrhynchus*) are infected with two directly transmitted nematodes, *Ostertagia gruehneri* and *Marshallagia marshalli*. As is typical of a directly transmitted nematode in a temperate environment, peak egg output and transmission occur during the summer for *O. gruehneri* (Irvine et al. 2000; Stien et al. 2002) and studies have indicated that this parasite has the propensity to arrest development, protecting it from the harsh conditions during the Arctic winter (Hoar et al. 2012b). However, *M. marshalli* has a counter-intuitive life history because during the summer the within-host parasite population size is small, egg output is low (Irvine 2001) and there is no propensity for arrested development within the host (Irvine 2001; Carlsson et al. 2012). Instead, there is high transmission of infective larvae as well as a large within-host parasite population size and high egg output during the winter (Irvine et al. 2000, 2001; Irvine 2001; Carlsson et al. 2012). To understand how the relationship between *M. marshalli* and Svalbard reindeer persists, we need to understand the survival and development of the parasite's free-living stages.

During the summer, Svalbard reindeer graze in the valley floors, whilst in winter they congregate and graze on exposed wind-blown ridges with little snow accumulation. Therefore, larvae hatched from eggs deposited in winter are unlikely to be ingested by hosts in summer, due to the mismatch in spatial overlap of hosts and parasites. There are thus two hypotheses to explain the successful transmission to the host of the free-living stages of *M. marshalli*. First, eggs deposited in winter may develop into L3 and infect reindeer during the same winter they are deposited (October–May). Second, the free-living stages may adopt an over-wintering strategy that requires them to survive sub-zero temperatures and the potential lethal risks of freezing and desiccation for prolonged periods of time; that is, eggs deposited in areas where reindeer graze in winter (October–May) remain undeveloped in the faeces until the following summer (June–July) when temperatures increase and then develop into L3, which remain on the pasture until reindeer return to their winter grazing grounds (October–November). This implies harsh conditions for the free-living stages, because eggs deposited on wind-blown ridges will not benefit from the protective layer of insulation offered by snow (Coulson et al. 1995b; Ávila-Jiménez et al. 2010) and may experience soil surface temperatures below −20 °C (Coulson et al. 1995b). Larvae hatched from these eggs in summer would lie exposed on the tundra for several months until reindeer return to these ridges in winter. Moisture input to the ridge communities is low after the snow melt and can occasionally drop below zero even during August (Coulson et al. 1995a). Larvae are unlikely to burrow into the soil as they need to maximise transmission potential (O′Connor et al. 2006; van Dijk and Morgan 2011) and will therefore be exposed to soil surface temperatures that average 8 °C in July and August (Coulson et al. 1993). Here, we test these two hypotheses to improve our understanding of the ecology of the free-living stages of *M. marshalli* in order to gain new insights into the survival and transmission of this parasite during the long Arctic winter. Specifically, we aimed to determine patterns

of egg development at low temperatures, cold tolerance of eggs and the survival of L3 at low temperatures and after desiccation. We discuss the implications of these results in light of the over-wintering survival strategies in other invertebrates.

Materials and methods

Thermal conditions on Svalbard

Soil surface temperatures were recorded between August 2010 to July 2012 using TGP-4020 Tinytag dataloggers (Gemini, Chichester, West Sussex, UK) deployed at the Nordlysstasjon (the Northern Light station) in Adventdalen adjacent to Longyearbyen. The site is approximately 8 km from the Norwegian Meteorological Institute weather station at Svalbard airport and is in the same valley. The dataloggers were fitted with external thermister probes (PB-5009, Tinytag) which were located in the surface layers of the organic soil at a depth of approximately 3 mm. Care was taken to avoid direct sunlight impinging on the thermistor. Loggers recorded temperature every, hour and daily means were subsequently calculated from these data. Air temperature data for the same period were acquired from www.eklima.met.no based on records from the Norwegian Meteorological Institute weather station at Svalbard airport.

Isolation of *M. marshalli* eggs

Female adult Svalbard reindeer were caught (Albon et al. 2002) in April 2009, 2010 and 2011 in the Reindalen-Semmeldalen-Colesdalen valley system (Fig. 1), and fresh dung was collected during defecation. None of the reindeer from which dung was collected had been treated with anthelmintics in the previous 12 months. Dung was frozen upon collection at ambient outdoor temperature, and mean temperatures during April of 2009, 2010 and 2011 were −15.9, −7.7 and −5.8 °C. Although temperature fluctuated during the collection period, they did not rise above 0 °C and no thawing of the dung was observed. Dung was transferred to a −20 °C freezer within 10 days of collection. Unless otherwise specified, eggs were extracted from the 2011 samples, 2–4 weeks post-collection. For egg extraction, faeces was thawed at 5 °C for approximately 6 h; 200 g of faeces was then homogenised in tap water (not chemically treated) until all faecal pellets were dissolved. The liquid was passed through a 250 µm sieve and allowed to sediment at 2 °C overnight. The watery supernatant was removed and the left-over roughage centrifuged at 1,500 rpm for 10 min. The resulting supernatant was discarded and the remaining material mixed with saturated salt solution and centrifuged at 1,500 rpm. The mixture was then filtered through a 200 µm sieve and eggs collected in a 63 µm sieve. Eggs were repeatedly washed in tap water to remove any salt residue and collected in a Petri dish. Individual *M. marshalli* eggs were identified (as outline in Irvine et al. 2000) and isolated using a 2–20-µL pipette under a binocular microscope.

Egg hatching rates

Egg hatching rates of *M. marshalli* were monitored in two experiments to determine (i) hatching rates at low temperatures (ii) the vital rates of egg development close to the lower-developmental threshold. For (i), six temperature treatments were used to establish the rate of hatching at different temperatures over 62 days in climate chambers. The mean temperatures experienced by the eggs were

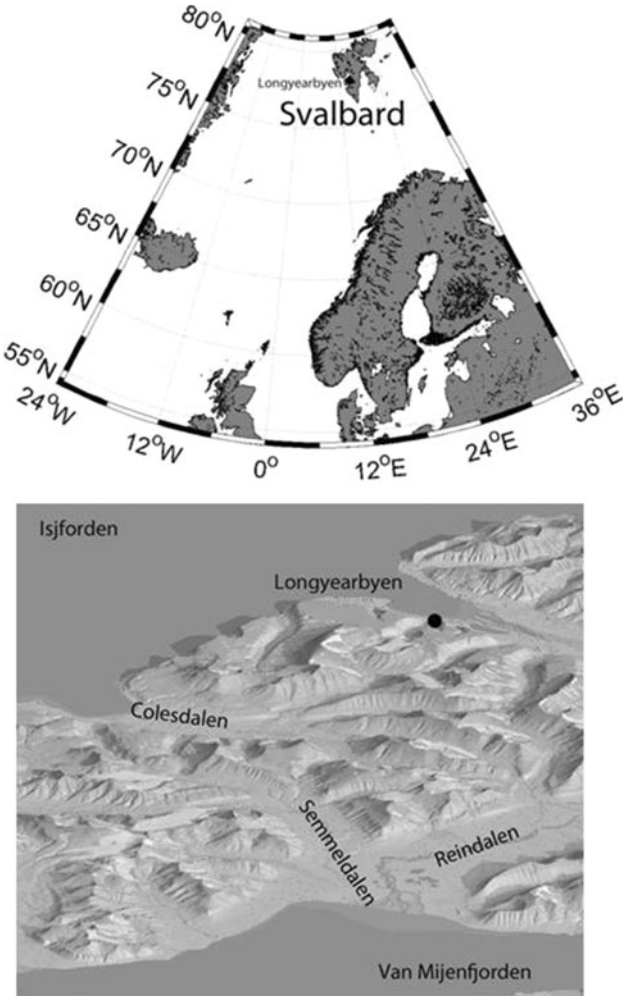

Fig. 1 Overview of the study area. The study area encompasses the Reindalen-Semmeldalen-Colesdalen valley system (*bottom*) on Spitsbergen, Svalbard (*top*). Maps adapted from http://toposvalbard.npolar.no/ with permission from the Norwegian Polar Institute

13 °C (24 h cycle; min ~6, max ~23); 8 °C (min 7, max 9); 0 °C (min −4, max 5); −2 °C (min −6, max 8); −3 °C (min −6, max 0) and −6 °C (min −8, max 0). Thirty replicate Petri dishes, each containing *c*. 30 *M. marshalli* eggs suspended in untreated tap water, were placed in each temperature treatment. At days 3, 6, 12, 21 and 62, six replicates from each treatment were examined and the proportion of eggs hatching in relation to Time ($\log_{10}$ (day +1)) and Treatment was analysed using generalised linear models with binomial error and logit link function using the *lmer* function in *R* (R core development team). Although the temperature treatments were a mixture between constant temperatures and temperatures cycling above and below zero, no development was observed at treatments with a mean temperature below zero. Therefore, all below-zero treatments were combined into one group leaving Treatment as a three-level factor (mean temperature = 13, 8 or <0 °C).

For experiment (ii), 10 Petri dishes, each containing 10 *M. marshalli* eggs, were incubated at a constant 2 °C. All eggs were examined for development every 5 days. *Marshallagia marshalli* follows the traditional life cycle of a gastrointestinal nematode, with the exception that larvae develop inside the egg and undergo only one moult to the infective L3 after hatching. Although moulting of larvae inside the egg has currently not been confirmed, the presence of a double cuticle on the L3 suggests that this does occur and that larvae emerge as L2 (Igrashev 1973). Development was therefore divided into three categories and scored as: egg, larvae inside egg or hatched larvae (i.e. L2). Development rates were analysed using Cox proportional hazard survival model from the *survival package* in *R*.

Based on development rates at the lower temperature threshold, accumulated day-degrees (i.e. the total number of heat units occurring above the lower temperature threshold for development in a 24-h period) were used to predict the timing of egg hatching and availability of infective-stage larvae on the tundra, assuming constant development of egg hatching to L3. Average daily ground temperatures, recorded at the Nordlysstasjon, Svalbard weather station from August 2010 to July 2012 (Fig. 1), were used to predict when eggs would hatch.

Egg cold tolerance

Marshallagia marshalli eggs isolated from dung collected during April 2009, 2010 and 2011 were used to establish the effect of long-term freezing on egg development. Faeces collected in each of the above sampling years were stored at −20 °C until eggs were extracted in June 2011 and had thus been frozen for 28, 14 and 2 months, respectively. For each sampling year, 10 Petri dishes, each containing 10 *M. marshalli* eggs, were placed in an incubator set at a constant 10 °C. All eggs were examined for development every 3 days. Eggs were scored according to their stage of development, as outlined above. Egg hatching rates were compared for the different periods of freezing by using Cox's proportional hazard model. Here, the period of freezing was coded as a three-level factor. However, due to the synchronised development of eggs, the model fit was sub-optimal and so to test the robustness of these findings, the survival data were also analysed using generalised linear models with binomial errors, coding period of freezing as a numerical variable (2, 14 or 28 months). The hatching rate of eggs that had developed into larvae inside the egg was also analysed using generalised linear models.

Larval desiccation and survival

To assess long-term survival of L3 and the effects of desiccation, all larvae that hatched from the 2011 samples from the above experiments were kept in an incubator at 10 °C. Water was allowed to evaporate from the samples. Sixty days post-hatching all samples had been desiccated for 2 days. Samples were then rehydrated for 48 h and larval development stage and survival were determined by examining all samples and recording how many larvae were motile.

In a separate experiment, 10 centrifuge tubes were filled with 100 µL of water and 10 L3, cultured at room temperature, were added. Five replicates were placed in a temperature chamber set at 5 °C, and the remaining five placed in a −20 °C freezer. After 80 days, larvae were examined after recovering at room temperature for 24 h.

Larval cold tolerance

To assess survival rates of *M. marshalli* L3 at temperatures below zero, eggs were cultured at room temperature to produce infective-stage larvae. Three centrifuge tubes, each containing 10 L3, were placed in a plastic box with a picolog temperature recorder (Pico Technology, UK) and cooled to approximately 5, −5, −25, −35, −40, −45, −50, −60 and −80 °C in a −80 °C freezer. There were three replicate boxes (each containing 3 × 10 larvae) for each treatment. Each box was monitored individually and removed when cooled to the pre-determined temperature. Samples were then placed in a polystyrene box with ice clamps to control and slow down the warming rate. Cooling and warming rates were monitored for each individual replicate. Larvae were considered to be alive if they were motile after a 24-h recovery period at room temperature. Larval survival was analysed in relation to temperature and cooling and warming rates using generalised linear mixed

effects models with binomial errors using the *lmer* package in *R*, with replicate as a random effect.

Results

Thermal conditions on Svalbard

As illustrated in Fig. 2, the ground temperature tracks that of air temperatures for most of the year in snow-free areas. The data presented represent a relatively mild winter.

Egg hatching rates

In the experiment testing hatching rates at low temperatures no eggs developed or hatched in the three below-zero treatments or in the variable temperature treatment with a mean of around 0 °C ($z_{2,354} = 0.007$, $p = 0.99$). Eggs kept at circa (*c.*) 13 °C hatched quicker than eggs kept at *c.* 8 °C ($z_{2,354} = -3.821$, $p < 0.001$), and the time taken for 50 % of the eggs to hatch was 11 and 22 days, respectively (Fig. 3). The hatching success rate was over 90 % for both of these treatments.

In the second experiment exploring development at 2 °C, eggs did develop but only 46 % (±5 % se) of eggs developed into larvae inside the egg by the time monitoring was terminated at 120 days. Development to this stage took on average 90 days for those eggs where any development occurred (Fig. 4a). Only 18 % (±4 % se) of eggs had hatched when the experiment was terminated. Hatching took an average of 113 days for those eggs that hatched (Fig. 4b). Although development had decreased by the end of the experimental period, it remains unclear if the remaining undeveloped eggs, and eggs containing larvae, would have hatched if monitoring had continued.

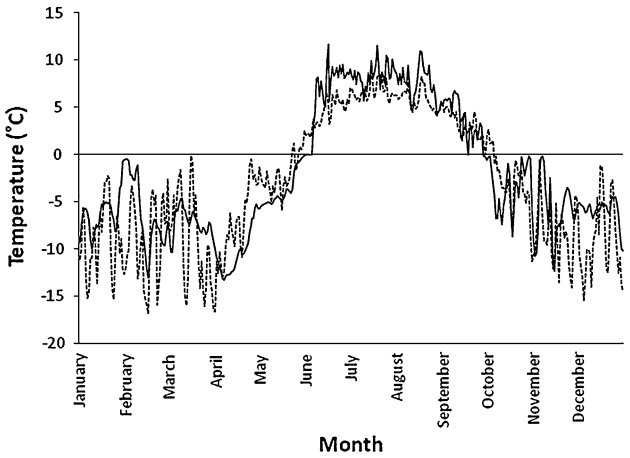

Fig. 2 Mean daily air temperature (*dotted line*) and ground temperatures (*solid line*) on Svalbard. Mean ground temperatures were calculated from hourly recordings 3 mm soil depth at the Nordlysstasjon (Northern Lights Station), Svalbard, as measured from August 2010 to July 2012. Air temperatures are based on daily means measured at the Svalbard airport weather station, obtained from www.eklima.no

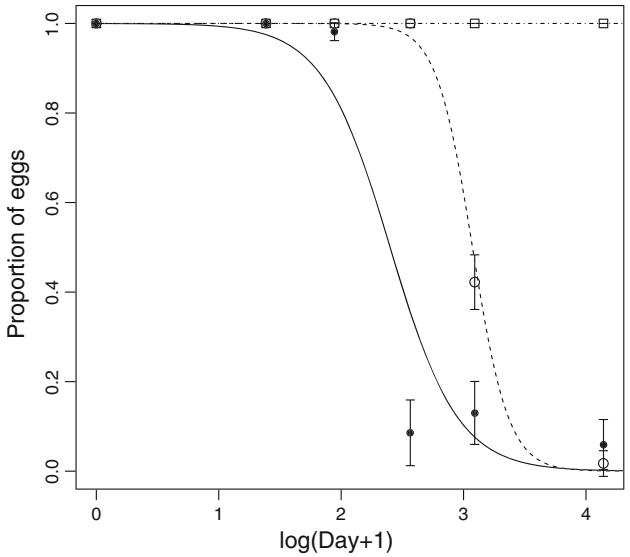

Fig. 3 Hatching of *M. marshalli* eggs at *c.* 13 °C (*solid line*, *filled circles*), *c.* 8 °C (*dashed line*, *open circles*) and below 0 °C (*dashed* and *dotted line*, *open squares*). The mean proportion of parasites remaining as eggs, with standard errors, is plotted for each sampling day (on a natural log scale). The *lines* give a continuous time prediction of development as estimated from the minimal generalised linear model

Using 2 °C as a lower temperature threshold for development and assuming that the development rates observed at this temperature were constant throughout the parasite's life cycle, accumulated day-degrees (based on ground temperature data observed between 2010 and 2012) indicated that eggs would hatch in early- to mid-July, and infective-stage larvae would be available on pasture in late July or early August. The maximum estimated day-degrees required to develop to L3 was 270, and in total, 368 day-degrees were accumulated between August 2010 and July 2012.

Egg cold tolerance

Survival analysis revealed that there was a significant effect of period of freezing at −20 °C on egg development ($\chi^2_2 = 167.26$, $p < 0.001$; Fig. 5). Eggs that had been frozen for 28 months were the least likely to develop and, for eggs frozen for 14 or 2 months, the daily likelihood that eggs would hatch increased by 8 (95 % CI 4–17) and 26 (95 % CI 13–52) times that of eggs frozen for 28 months, respectively. These results were corroborated when the data were analysed using generalised linear models, which showed that the proportion of hatched larvae increased

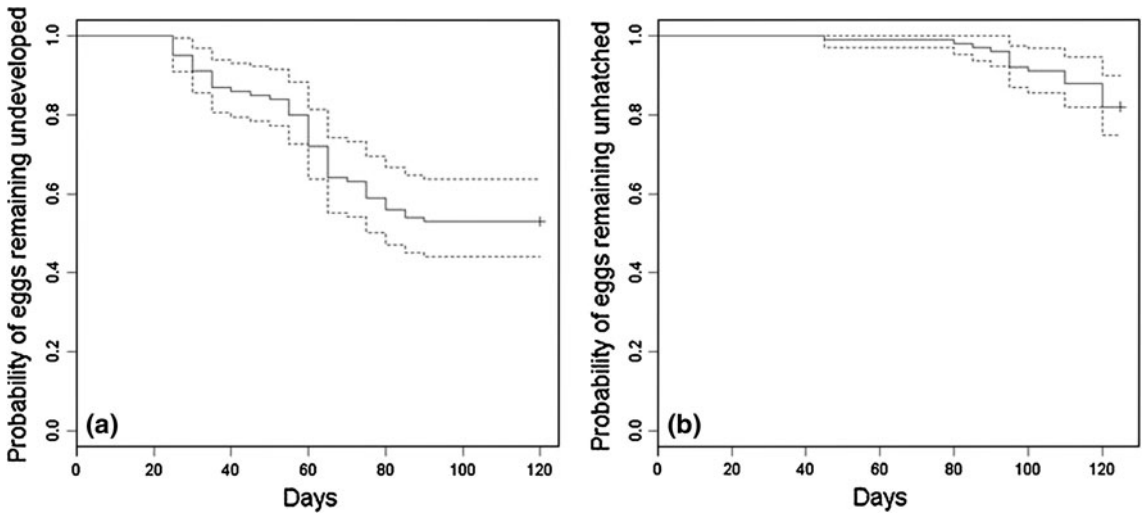

Fig. 4 Development of *M. marshalli* eggs at 2 °C represented as **a** proportion of eggs with larvae developed inside and **b** proportion of hatched eggs. *Lines* show fitted values from Cox proportional hazard model with 95 % CI

significantly as the period of freezing was reduced ($\chi^2_{1,298} = 253.7$, $p < 0.001$). Only 9 % of eggs frozen for 28 months hatched, whereas 56 % of eggs frozen for 14 months and 91 % of eggs frozen for 2 months hatched. Hatching appeared to be synchronised for viable eggs in each treatment group, with more than 50 % of the total hatching that eventually took place occurring by day 14 post-warming in each case (Fig. 5). Some eggs developed a larva inside but did not hatch (between 2 and 7 %); however, the proportion of eggs reaching this stage did not vary with period of freezing ($\chi^2_1 = 113.4$, $p = 0.11$).

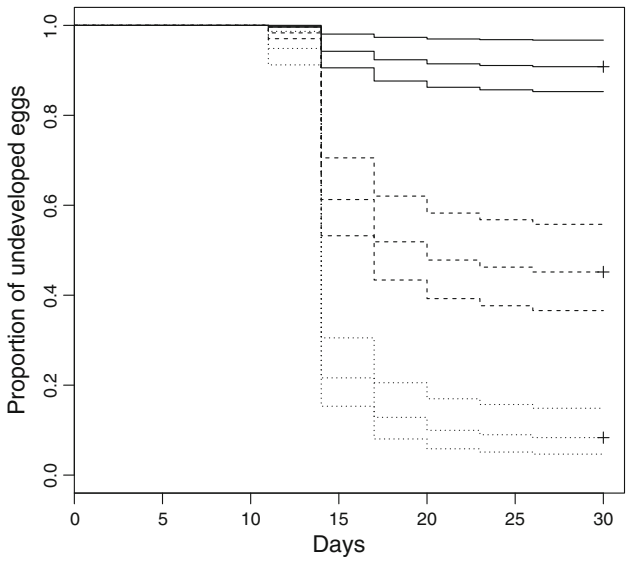

Fig. 5 Hatching rate of eggs frozen for 28 months (*solid lines*), 14 months (*dashed lines*) and 2 months (*dotted lines*). *Lines* show fitted values from Cox proportional hazard model with 95 % CI

Larval desiccation and survival

In total, 92 out of 100 larvae hatched from the 2011 samples cultured for the egg cold tolerance experiment. After being stored at 10 °C for up to 60 days post-hatching and allowed to naturally desiccate, all larvae had developed to L3 and 88 % (±14 % se) of larvae were still motile after rehydration. All larvae stored in water at 5 °C were alive and motile after 80 days. However, none of the larvae stored for the same period at −20 °C were observed to be motile after a 48-h recovery period.

Larval cold tolerance

L3 survival decreased significantly as temperature decreased ($z_{1,25} = 6.14$, $p < 0.008$), with lower lethal temperature estimated at −48 °C (Fig. 6), although even at −80 °C, 4 % of the larvae were motile after a 24-h recovery period. Cooling and warming rates varied between replicates and treatments (mean cooling rate = 1 °C/23 s (±2 se, min 11, max 55); mean warming rate = 1 °C/40 s (±2 se, min 20, max 83), but this had no significant effect on larval survival ($z_{1,25} = 0.95$, $p = 0.34$; $z_{1,25} = -1.31$, $p = 0.19$, respectively).

Discussion

Here, we have shown that *M. marshalli* free-living stages have the ability to rapidly develop once temperatures rise above zero and that they can survive for long periods at low temperatures as well as periods of desiccation. This life history is consistent with our hypothesis that eggs

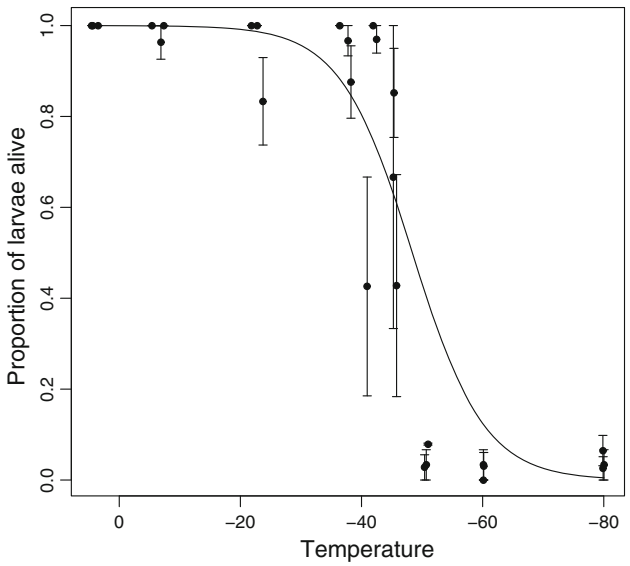

Fig. 6 Mean proportion and standard errors of *M. marshalli* third-stage larvae survival at low temperatures. *Line* gives predicted survival according to minimal generalised linear mixed effects model

deposited on winter grazed habitats develop rapidly in summer and can survive as infective larvae until reindeer return the following year. This accounts for the observed winter transmission in this species (Carlsson et al. 2012). The development and survival of the free-living stages of *M. marshalli* in the Arctic has not previously been investigated, and these results demonstrate that the basic ecology and transmission dynamics of parasites can be very different from what would be predicted from the literature. Although laboratory experiments cannot precisely replicate field conditions and interactions with other environmental factors, they can give a good indication of development rates of the free-living stages. Here, we have identified five important features of the free-living stages of *M. marshalli* that have implications for our understanding of the transmission and survival of this parasite.

Our results clearly demonstrate that *M. marshalli* eggs do not develop at sub-zero temperatures. This means that they are unlikely to develop into the infective-stage in winter and so cannot account for increases in host worm burdens during the winter in which they were deposited, thus introducing significant time delays into the transmission process. However, successful hatching of *M. marshalli* eggs was observed at temperatures as low as 2 °C, which is, to our knowledge, the lowest hatching temperature recorded for a species of the Trichostrongyloidea (Anderson 1992). In line with previous research on the free-living stages of gastrointestinal nematodes, development rates also increased with increasing temperature (Anderson 1992; O′Connor et al. 2006), with 50 % of eggs hatching within 22 days when incubated at *c.* 8 °C, within 14 days for those at *c.* 10 °C and within 11 days for those at *c.* 13 °C. Based on ground temperatures recorded in 2010–2012 (Fig. 1), the day-degrees accumulated during summer were sufficient for eggs to complete development and hatch in one summer season. It should, however, be noted that this estimation assumes constant development and survival from hatching to development to L3. We did not monitor stage-specific development post-hatching, and for many nematode species, pre-infective stages are more sensitive to fluctuations in temperature, which could impact upon development and survival rates (reviewed in Anderson 1992). However, the day-degree prediction is in line with field studies where infective-stage *M. marshalli* were recovered in July from eggs placed out on ridges on Svalbard in April (Stien et al., unpublished).

Eggs were viable and developed even after prolonged periods of freezing. Most eggs of common nematodes of vertebrates do not tolerate freezing well (reviewed in Anderson 1992), but 9 % of *M. marshalli* eggs stored at −20 °C for 28 months were still viable and hatched, suggesting that this species is better adapted to sub-zero temperatures than other species. As expected, decreasing the freeze period radically increased hatching rate, with 56 % of eggs successfully hatching after 14 months and 91 % after 2 months. Thus, if soil temperatures do not increase above the developmental threshold in summer, viable eggs will still be available to develop and infect reindeer in the subsequent year. *M. marshalli* is mainly a parasite of cold deserts and is therefore expected to have adapted to the environment in which the host resides (William et al. 2001). Another nematode remarkably tolerant to cold environments is *Nematodirus battus*, a pathogen of sheep that has been suggested to have an Arctic origin (van Dijk and Morgan 2008). This parasite completes development to L3 within the egg, an adaption which may serve to buffer the free-living stages against adverse temperatures (Anderson 1992; van Dijk and Morgan 2008). Similarly, in *M. marshalli* eggs, which are clearly also highly tolerant of sub-zero temperatures, hatching occurs after development to L2 (Igrashev 1973), which could also be an adaptation to the harsh Arctic environment.

Experiments demonstrated that *M. marshalli* L3 survive well for prolonged periods at 5 and 10 °C, indicating that *M. marshalli* could survive the summer as infective larvae, which could partly be achieved through the protection afforded by the retention of the L2 sheath. In line with these results, ensheathed infective-stage nematode larvae of other species are also known to have high survival rates and successful transmission after prolonged exposure to adverse conditions (Slocombe 1974; Troell et al. 2005; Hrabok et al. 2006; Makovcova et al. 2009).

The experiments demonstrate that *M. marshalli* L3 larvae can survive exposure to rapid freezing at very low

temperatures, where 50 % of larvae survived −48 °C and even at −80 °C, 4 % of larvae survived. However, no larvae survived prolonged exposure to −20 °C. This apparent contradiction could potentially be explained by the rate and length of the freeze events. Studies on insect cold hardiness have shown that time spent at sub-zero temperatures (Sinclair et al. 2003; Bale and Hayward 2010; Lee 2010), and rate of cooling (Wang and Kang 2005) can greatly influence survival. Although we did not find any effect of cooling rate on survival, cooling rates were very rapid in all experiments and are probably not optimal for long-term survival. Slower rates of cooling, better approximating conditions in the field, may allow larvae to acclimatise better.

Finally, the present study also revealed that *M. marshalli* larvae survived desiccation at 10 °C. In line with these results, laboratory experiments from the Russian literature indicate that L3 were vulnerable to prolonged freezing but highly resistant to desiccation (Igrashev 1973). Desiccation is an important mechanism of survival at sub-zero temperatures. For example, by slowly losing water from their bodies, the Antarctic free-living nematode, *Panagrolaimus davidi*, and the collembolan *Megaphorura arctica* from Svalbard can survive for long periods of time in a state of anhydrobiosis (Sinclair et al. 2003; Wharton 2003; Sorensen and Holmstrup 2011). Experiments with L3 of *Trichostrongylus colubriformis* also showed that desiccation enhanced the ability of L3 to survive when exposed to low temperatures (Andersen and Levine 1968; Wharton and Allan 1989). In reference to these studies, our results suggest that long-term survival at sub-zero temperatures of *M. marshalli* may be linked to cooling rates and desiccation. However, further laboratory and field experiments are needed to determine the exact mechanism of cold tolerance of *M. marshalli*.

Winter transmission of *M. marshalli* has been experimentally demonstrated to occur on Svalbard (Carlsson et al. 2012) and suggested elsewhere (Kutz et al. 2012; Morgan et al. 2007). Perhaps surprisingly, there is no evidence that *M. marshalli* undergo arrested development in the host (Irvine 2001; Carlsson et al. 2012) as a mechanism to survive adverse periods for transmission, opting instead for a survival strategy based on hardiness of the free-living stages. Our results support our second survival and transmission hypothesis in that *M. marshalli* does not develop at sub-zero temperatures, but eggs deposited in one winter can survive until spring and then undergo rapid development, allowing them to develop into infective larvae in the summer. These larvae then survive on the tundra until the following winter when they are available to infect reindeer as they return to their winter grazing habitats. This provides strong evidence to support the interpretation that *M. marshalli* has a 1 year lag in its life cycle, with possible consequences for the stability of the parasite–host interaction (May and Anderson 1978; Dobson and Hudson 1992). In contrast, evidence suggests that the main overwinter survival strategy of the other dominant nematode species of Svalbard reindeer (*O. gruehneri*) is arrested development within the host (Irvine 2001; Hoar et al. 2012b). Although larvae of *O. gruehneri* have been demonstrated to survive the winter on the Canadian tundra (Hoar et al. 2012a), it has been reported that eggs are intolerant of sub-zero temperatures (Kutz et al. 2012). The remarkable difference in these life-history strategies allows the co-existence of two potentially competing species on Svalbard, with differing implications for their relationship with the host.

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