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Phenotypic plasticity and local adaptation in freeze tolerance: Implications for parasite dynamics in a changing world

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A R T I C L E I N F O

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A B S T R A C T

Marshallagia marshalli is a multi-host gastrointestinal nematode that infects a variety of artiodactyl species from temperate to Arctic latitudes. Eggs of *Marshallagia* are passed in host faeces and develop through three larval stages (L1, L2, and L3) in the environment. Although eggs normally hatch as L1s, they can also hatch as L3s. We hypothesised that this phenotypic plasticity in hatching behaviour may improve fitness in subzero and highly variable environments, and this may constitute an evolutionary advantage under current climate change scenarios. To test this, we first determined if the freeze tolerance of different free-living stages varied at different temperatures (−9 °C, −20 °C and −35 °C). We then investigated if there were differences in freeze tolerance of *M. marshalli* eggs sourced from three discrete, semi-isolated, populations of wild bighorn and thinhorn sheep living in western North America (latitudes: 40°N, 50°N, 64°N). The survival rates of eggs and L3s were significantly higher than L1s at −9 °C and −20 °C, and survival of all three stages decreased significantly with increasing freeze duration and decreasing temperature. The survival of unhatched L1s was significantly higher than the survival of hatched L1s. There was no evidence of local thermal adaptation in freeze tolerance among eggs from different locations. We conclude that developing to the L3 in the egg may result in a fitness advantage for *M. marshalli*, with the egg protecting the more vulnerable L1 under freezing conditions. This phenotypic plasticity in life-history traits of *M. marshalli* might be an important capacity, a potential exaptation capable of enhancing parasite fitness under temperature extremes.

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1. Introduction

Parasites are a major component of biological diversity and a direct determinant in many ecological processes (Dobson et al., 2008). For parasites with stages that develop in the external environment, survival and development are intrinsically tied to environmental factors such as temperature and humidity. As a consequence, these types of parasites are very sensitive to climatic conditions, and thus climate change (Altizer et al., 2013). To persist in adverse or extreme environments, parasites have evolved a variety of behavioural and physiological traits (Perry and Moens, 2011). While some nematodes have capacities to avoid exposure to unsuitable conditions (e.g. seasonal arrested development inside the host to avoid cold conditions in *Ostertagia gruehneri* (Hoar et al., 2012a)) or are capable of resisting environmental extremes (e.g. the marked freeze tolerance of the arctic lungworms *Umingmakstrongylus pallikuukensis* and *Varestrongylus eleguneniensis* (Kafle et al., 2018)), other species have evolved plastic phenotypic responses, switching developmental pathways depending on short-term environmental cues. For instance, when the nematode of wild rats, *Strongyloides ratti*, is outside the host it has two different developmental trajectories depending on environmental cues such as temperature: (i) a heterogonic pathway, as a free-living adult with sexual reproduction in the environment, and (ii) a homogonic pathway, as an infective larval stage that must be ingested in order to reproduce, asexually, in the gut of the final host (Viney, 1996). Phenotypic plasticity can thus allow short-term modifications in development patterns to cope with unexpected environmental events. Additionally, plasticity facilitates fast and flexible evolutionary responses, when they act as adaptive pathways enhancing parasite fitness under changing environments and circumstances (Viney and Diaz, 2011; Forsman, 2015). The study of phenotypic plasticity is key to understanding and

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predicting the adaptive and non-adaptive responses of biological systems to current rapid environmental changes (Reed et al., 2011; Burggren, 2018).

The nematode *Marshallagia marshalli* infects the abomasum of wild and domestic ungulates from the Holarctic region (Hoberg et al., 2001; Meradi et al., 2011; Kutz et al., 2012). It has negative consequences for small-scale farming of small ruminants in the Middle East (Meradi et al., 2011), and is associated with poor condition and lower pregnancy rates in Dall's sheep (*Ovis dalli dalli*) in North America (Neilsen and Neiland, 1974; Aleuy et al., 2018), and poor body condition in female juvenile saiga antelope (*Saiga tatarica*) in Kazakhstan (Morgan et al., 2005). *Marshallagia marshalli* originated in dry and cold mountain-steppe habitats in Eurasia and expanded to North America across Beringia through a series of episodic expansion events linked to climatic extremes, and periods of climatic suitability (Hoberg, 2005; Hoberg et al., 2012a). Today, its range of distribution in North America is latitudinally broad, and soil temperature profiles in single locations may range up to 70 °C, from −40 °C to ≥30 °C (Hoar et al., 2012b).

Given the evolutionary history of *M. marshalli* and the current environments in which it persists, it is likely that adaptive traits have emerged in response to extremes in temperatures and the different sources of temperature variations (e.g. high seasonal and inter-annual temperature variability) (Aleuy et al., 2019). This nematode has a direct life cycle in which eggs are passed through the faeces to the environment where they develop to the infective stage (L3) and are ingested by a new host. Our previous work demonstrated that, although hatching as an L1 is the primary developmental pathway in *M. marshalli*, hatching as an L3 can occur at higher incubation temperatures. We hypothesised that development to L3 in the egg enhances the capacity of early larval stages of *M. marshalli* to cope with a wide variety of unfavourable environmental conditions including subzero temperatures (Aleuy et al., 2019). Understanding such specific adaptations is a key step to inform predictive models to anticipate future consequences of climate change on parasite dynamics and, ultimately, host populations.

Here, we used the parasitic nematode *M. marshalli* and its phenotypic plasticity in hatching behaviour as a model to explore the hypothesis that phenotypic plasticity is a key attribute for coping with extreme and unpredictable environmental conditions in host/parasite systems. We predict that the egg and L3 of *M. marshalli* have greater survival at subzero temperatures than the hatched L1, similar to what is observed in other nematode species (Perry and Moens, 2011). Thus, developing to L3 in the egg confers a fitness advantage for *M. marshalli*, enhancing the survival of the L1 at subzero temperatures. In order to test this, we first compared the differences in freeze survival among free-living stages (eggs, L1s and L3s), second, we tested if the survival of the L1 in the egg differed from that of the L1 outside the egg at subzero temperatures and, finally, we compared the freeze tolerance of eggs sourced from locations with different winter temperature regimes to determine if there was evidence of local thermal adaptation in the egg. Local adaptation was used as a proxy to understand the role of evolutionary history in freezing survival of *M. marshalli*. We hypothesised that the capacity of free-living stages of *M. marshalli* to cope with subzero temperatures was the consequence of the deep evolutionary history of this species in Eurasia and its expansion to North America rather than an adaptation to local conditions in the current range of distribution of *M. marshalli*. Understanding the role of evolutionary history in the capacities of parasites to survive extreme or unpredictable conditions can play an important role in assessing and predicting the effect of climate change on parasite dynamics (Hoberg et al., 2017).

2. Material and methods

2.1. Collection and extraction of M. marshalli eggs

Marshallagia marshalli eggs were extracted from faecal samples of wild sheep collected from different populations in North America. Faecal samples were collected from Rocky Mountain bighorn sheep (*Ovis canadensis*) populations located in Sheep River Provincial Park (50°40′N 114°40′W), Alberta, Canada (hereafter referred as Alberta), and Rocky Mountain National Park (40°43′N, 105°81′W), Colorado, USA (hereafter referred as Colorado). For Dall's sheep (*Ovis dalli dalli*), faecal samples were collected at Wildhorse Mountain (64°48′N, 139°25′W) in central Yukon, Canada (hereafter referred as Yukon) (Fig. 1). For all the collections, sheep were observed from a distance and individual faecal samples were collected into individual Ziploc bags within 60 min of defecation when sheep voluntarily moved to a different location. Samples were immediately placed in a portable cooler, maintained at ~2 °C, and transported to the University of Calgary, Canada, within 36 h, where the samples were refrigerated at ~1 °C until used in the experiments within 2–3 days post collection. For all the experiments, eggs of *M. marshalli* were extracted from faeces following the protocol described by Aleuy et al. (2019). *Marshallagia marshalli* eggs were identified based on the keys by Monnig, (1940) and Jacobs et al. (2015). These eggs are easy to identify morphologically under a 30-40x power microscope, due to their unique morphology and large size.

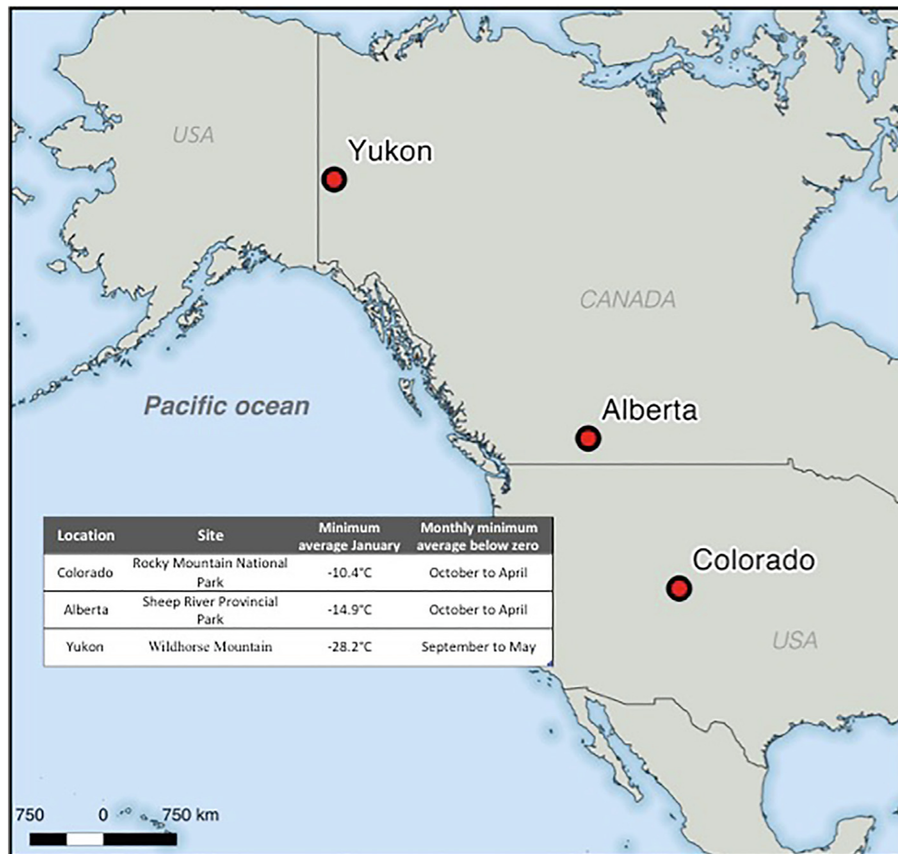
2.2. Freeze survival of eggs, hatched L1s and L3s

To compare the effect of subzero temperatures on the survival of free-living stages of *M. marshalli,* we exposed eggs, L1s, and L3s to −9 °C, −20 °C, and −35 °C for 3, 7, 15, 30, 60, and 90 days in a full factorial experimental design.

2.2.1. Egg and larval preparation

Approximately 4000 eggs of *M. marshalli* were extracted from bighorn sheep faeces collected in Alberta (see Fig. 1). The eggs were allocated to two petri dishes containing distilled water. Petri dish N °1, containing ~1000 eggs, was used as an immediate source of eggs, while the petri dish N°2, containing ~3000 eggs, was used as a source of L1s and L3s. Petri dish N°2 was placed in a 20 °C incubator to allow parasite development. Eggs were cultivated in distilled water with no extra nutrient media as we had previously determined that *M. marshalli* does not feed during larval development (Aleuy et al., 2019).

Immediately after extraction, 20 eggs were collected from petri dish N°1 and placed, with 150 μL of distilled water, in 20 individual wells in 96-well culture plates (96 Well Assay Plates, Corning Incorporated, NY, USA). Thirty-two plates in total were prepared and then placed in a 5 °C incubator for 24 h to acclimate the eggs to a cooler temperature. After 24 h, 30 plates were evenly distributed among three temperature treatments (−9 °C, −20 °C, and −35 °C), while two plates were retained in a 20 °C incubator as controls for all the stages in this experiment (eggs, L1s and L3s) (Fig. 2A). Control plates were not exposed to freezing temperatures in order to assess the quality/survival of eggs used in the experiments. The temperature in each freezer was monitored every ~30 min using LogTag recorders (LogTag TRIX-8, MicroDAQ, Contoocook, NH, USA) (Supplementary Table S1; Table 1 and Fig. 1). At 3, 7, 14, 30, and 90 days, two plates from each temperature treatment were moved to a 5 °C incubator for 24 h to allow gradual thawing conditions and then placed into a 20 °C incubator for the remainder of the experiment. Once in the 20 °C incubator, survival and development to L3 of each egg were monitored every



Location	Site	Minimum average January	Monthly minimum average below zero
Colorado	Rocky Mountain National Park	-10.4°C	October to April
Alberta	Sheep River Provincial Park	-14.9°C	October to April
Yukon	Wildhorse Mountain	-28.2°C	September to May

Fig. 1. Locations of collection sites for *Marshallagia marshalli* eggs used in experiments investigating local thermal adaptation to freezing. Also included is information on the minimum average January temperature in each location and the months in which the monthly average minimum temperature is lower than 0 °C.

3 days under 200× magnification with an inverted microscope until the individual was either an L3 (as described by Aleuy et al. (2019)) or dead. Eggs with no development, no hatching, and abnormally dark in colour for two consecutive observation periods were recorded as dead. Similarly, early larval stages with no development or movement over three observation periods were recorded as dead. Control plates were monitored and assessed in the same way (Supplementary Table S2). The design allowed us to assess the survival of 40 individual parasites per stage/temperature/day of exposure.

Petri dish N°2 was monitored daily for L1s and L3s as defined by Aleuy et al. (2019). L1s and L3s were extracted from the dish on the day that they first appeared and allocated to separate wells in 96-well plates as described above, acclimated at 5 °C for 24 h, and then assigned to one of the three temperature treatments (Fig. 2A). The sampling and data collection procedures followed the same protocols as described for petri dish N°1.

2.3. Freeze survival of hatched L1s versus L1s in eggs

To determine if remaining in the egg has a protective effect for L1s exposed to subzero temperatures, we compared the survival of L1s in the egg with hatched L1s after exposure to −9 °C, −20 °C, and −35 °C for 3, 7, 14, and 60 days.

2.3.1. Egg and larval preparation

Approximately 2000 *M. marshalli* eggs were extracted from bighorn sheep faeces collected in Alberta, placed in a petri dish containing distilled water, and incubated at 20 °C. Egg development was monitored daily using an inverted microscope at 400× magnification to look for two specific developmental stages: (i) L1 inside the egg in an advanced stage of development and close to hatching, determined by block-shaped intestinal cells in the abdomen, decreased density of granules in the cranial area, and continuous larval movement with consequent egg elongation (Aleuy et al., 2019) (hereafter referred to as 'eggs'), and (ii) free-living L1 within 24 h of hatching.

Immediately upon identification, hatched L1 or eggs were pipetted into individual petri dishes and stored in a 1 °C incubator to delay their development until at least 640 individuals in each stage were obtained. This process extended over 48 h. Thirty-six 96-well culture plates were then prepared with 20 larvae and 20 eggs placed individually in wells containing 150 μL of distilled water. Eggs and larvae were placed in alternating wells to control for the location within the plate. Plates were evenly allocated to one of the three freezing treatments, −9 °C, −20 °C, and −35 °C. The temperature in each freezer was monitored every ~30 min using LogTag recorders (Supplementary Table S1 and Supplementary Fig. S1). At each exposure time (i.e. 3, 7, 14, and 60 days) two plates per temperature treatment were moved to a 1 °C incubator for 24 h and then to a 5 °C for another 24 h to allow gradual thawing conditions, and finally to a 20 °C incubator to assess survival. Survival of individual eggs and larvae was monitored on a weekly basis using an inverted microscope. Only eggs and larvae that subsequently developed to L3s were considered alive. Monitoring continued until the individual was either dead or reached the mature L3 stage.

2.4. Local thermal adaptation in freeze tolerance

We assessed differences in freeze tolerance among *M. marshalli* eggs collected in Colorado, Alberta, and Yukon. These locations

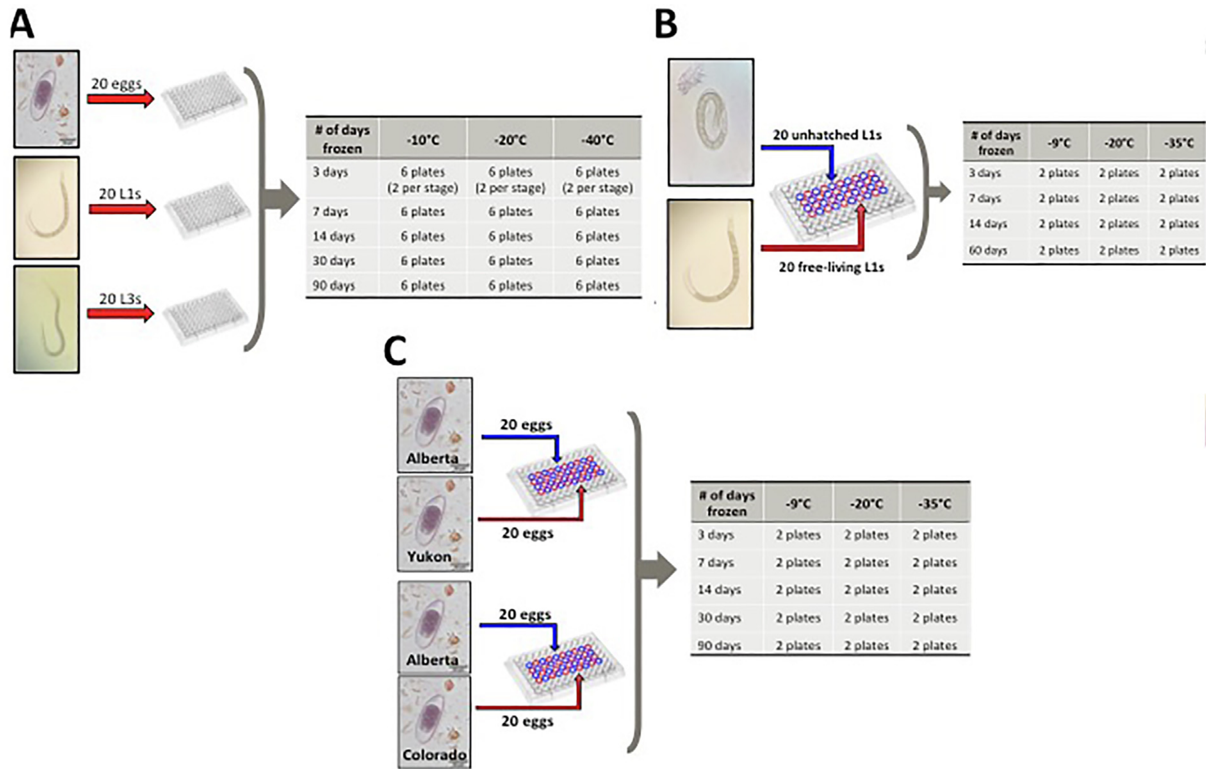


Fig. 2. Experimental design of the three experiments investigating survival of free-living stages of *Marshallagia marshalli* at subzero temperatures. Experiment A tested differences in survival among free-living stages, experiment B tested differences in survival between hatched and unhatched L1s, and experiment C tested differences among *M. marshalli* eggs sourced from different locations in North America. In each experiment, the free-living stages were allocated in individual wells of 96-well culture plates containing ~150 µl of distilled water.

Table 1
Final models (binomial Generalised Linear Model, logit link) for three experiments exploring the survival of free-living stages of the nematode *M. marshalli* after exposure to subzero temperatures for different time periods.

Models for subzero survival of:	Independent variables	Estimates	S.E.	*P* value	95% CI
Free-living stages (eggs, L1 and L3)					
	Day	−0.023	0.006	<0.001	−0.039–0.011[a]
	Stages (Ref: L1)				
	Egg	5.288	0.361	<0.001	4.602 6.018[a]
	L3	3.719	0.295	<0.001	3.149 4.306[a]
	Temperature	0.259	0.014	<0.001	0.234 0.287[a]
	Day: Stage Egg	−0.012	0.008	0.159	−0.027 0.005
	Day: Stage L3	0.012	0.007	0.128	−0.002 0.029
L1 in the egg and free-living L1					
	Day	−0.006	0.003	0.094	−0.014 0.001
	Temperature	0.146	0.011	<0.001	0.125 0.168[a]
	Stage (Ref: Free-living L1)				
	L1 in the egg	1.612	0.181	<0.001	1.262 1.975[a]
Eggs sourced from different locations					
	Day	−0.044	0.006	<0.001	−0.063–0.037[a]
	Temperature	0.346	0.024	<0.001	0.301 0.396[a]
	Site (Ref: Colorado, USA)				
	Sheep River (Colorado)	−2.468	0.729	<0.001	−3.942–1.072[a]
	Yukon, Canada	−2.117	0.742	0.004	−3.613–0.691[a]
	Sheep River (Yukon)	−2.992	0.713	<0.001	−4.437–1.632[a]
	Day: Temperature	−0.001	0.002	<0.001	−0.001–0.000[a]
	Day: Site Sheep River (Colorado)	0.009	0.005	0.102	−0.001 0.021
	Day: Site Yukon	−0.002	0.006	0.759	−0.013 0.009
	Day: Site Sheep River (Yukon)	0.009	0.006	0.129	−0.002 0.019
	Temp: Site Sheep River (Colorado)	−0.079	0.028	0.003	−0.136–0.026[a]
	Temp: Site Yukon	−0.094	0.027	<0.001	−0.142–0.034[a]
	Temp: Site Sheep River (Yukon)	−0.078	0.024	0.001	−0.149–0.042[a]

CI, confidence interval.
[a] Statistically significant.

were chosen to represent the wide range of freezing temperatures that *M. marshalli* experience during winter in North America (Fig. 1). Due to the logistical challenge of obtaining fresh samples from three distant geographic locations at the same time, we performed two experiments, simultaneously comparing Yukon versus Alberta and then Colorado versus Alberta. This experimental design allowed us to use fresh faecal samples in both experiments (i.e. collected within 2–3 days) and control for differences among locations and season of collection using Alberta as a control location (Fig. 2C). The collection of faecal samples, maintenance/transport to the laboratory, and egg extraction procedures were performed using the same protocols described above.

2.4.1. Experimental design and data collection

The experimental design was similar to previous experiments (Fig. 2). After 24 h acclimation at 5 °C, 36 plates, with 20 eggs per location placed in alternating wells in each plate, were allocated in one of the temperature treatments at −9 °C, −20 °C, and −35 °C (12 plates per temperature). Egg survival was monitored by sampling two plates with no replacement from each temperature treatment at 3, 7, 14, 30, 60 or 90 days of exposure. On the day of sampling, the plates were held at 1 °C for 24 h, then at 5 °C for another 24 h, and maintained at 20 °C for the duration of monitoring. Individual egg survival was checked once each week under 400x magnification using an inverted microscope until the individual was either dead or reached the L3 stage.

2.5. Data analysis

The three experiments were analysed separately using binomial generalised linear models (GLM; logit link), where the binomial response variable was the fate of each individual L2 within each experiment (0 = dead, 1 = alive). The models were fitted directly on the raw data, and temperature treatment and day of exposure to sub-zero temperatures were included as fixed effects in the three analyses. The temperature and days of exposure were fitted as continuous variables. Additionally, the stage of development was included as a fixed effect in the analyses of the first and second experiments, and the collection site was added as a fixed effect in the analysis of the third experiment. Initially, the plate was included as a random effect but the inclusion of the random effect did not change model outcomes and was excluded in the final results for simplicity. Interactions between the above fixed effects were included and tested when they represented biologically meaningful hypotheses (e.g., the interaction between temperature and location in experiment 3) within each experiment, models that included different combinations of fixed effects and interactions were compared using the Akaike Information Criterion (AIC).

3. Result

3.1. Freeze survival of free-living stages

In the best model (i.e. lowest AIC), the survival of eggs, L1s and L3s significantly differed among stages and decreased with temperature and days of exposure (Fig. 3, Table 1). The stage of development was a significant fixed effect in the two top models, with the survival of L1s at subzero temperatures being significantly lower than that of eggs and L3s (Supplementary Table S3). The second-best model (delta AIC < 2) further included the interaction between temperature and stage of development, but the effect of this interaction was small. In addition, there was a significant interaction between days of exposure and stage of development, where egg survival decreased significantly faster with days of exposure than that of the L3 (GLM; L3: $b = 0.023$, SE = 0.005, $z = 4.053$, $P < 0.001$).

3.2. Freeze survival of hatched L1 versus L1 in the egg

The total survival of the L1 in the egg was significantly higher than that of hatched L1 (odds ratio = 5.01, 95% CI = 3.53–7.21). Temperature and L1 stage (hatched/unhatched) were included as fixed effects in all the top five models (Supplementary Table S4). The survival of both stages decreased significantly as temperature decreased, approaching zero at −35 °C. Survival of both hatched and unhatched L1s appeared to decrease with days of exposure, although not significantly over the duration of our experiment (maximum 60 days) (Table 1 and Fig. 4).

3.3. Local thermal adaptation in freeze tolerance

The overall survival of eggs significantly decreased with decreasing temperature, and increased days of exposure, in all of the 10 top fitted models (Supplementary Table S5). After running the model with the lowest AIC using different reference levels for the fixed effect site, the survival of eggs from Colorado was significantly higher than eggs from Sheep River and Yukon (Fig. 5). However, this trend was significant only in the first two top models. The survival of eggs from Yukon and Sheep River (in both experiments) did not significantly differ in any of the models (Supplementary Table S6). The interaction between temperature and collection site was significant, indicating that the difference in survival among sites decreased with increasing temperatures (Table 1).

4. Discussion

Marshallagia marshalli is a common parasite in arid environments, experiencing wide annual temperature fluctuations including soil temperature extremes that can range from < − 40 °C to >30 °C (Morgan et al., 2007; Hoar et al., 2012b). Our work has demonstrated that high freeze tolerance of eggs, L1s in eggs, and L3s may be an important life-history trait that allows persistence in these frigid environments. Altogether, these results support the general hypothesis that phenotypic plasticity in hatching behaviour of *M. marshalli* might increase parasite fitness by protecting the more vulnerable L1 under freezing conditions. Despite the wide latitudinal distribution of *M. marshalli*, and the wide range of temperatures associated with its distribution, we observed weak evidence of local adaptation in freeze tolerance of eggs from discrete populations from different locations. Here we discuss these findings in the context of how specific developmental traits, phenotypic plasticity, and the insights from evolutionary history are important determinants of parasite dynamics under highly variable environmental conditions. Further, we discuss how they may constitute a critical piece of information to determine and predict the impact of climate change on host–parasite interactions.

Marshallagia marshalli is unique among most temperate and arctic trichostrongylids in that its egg production is characterised by a seasonal increase during winter, and winter transmission is common (Irvine et al., 2000; Morgan et al., 2007; Carlsson et al., 2012; Kutz et al., 2012). These pathways for transmission require that both eggs and L3s are able to resist freezing. Under selection, nematodes have evolved two major trajectories to survive exposure to sub-zero temperatures: freeze tolerance and freeze avoidance (Wharton, 1999). Freeze-tolerant species survive ice formation in at least some of their tissues. In contrast, freeze avoidance results from manipulation of the substances that initiate the formation of ice crystals (ice nucleators) allowing body fluids to be liquid at temperatures below their melting point

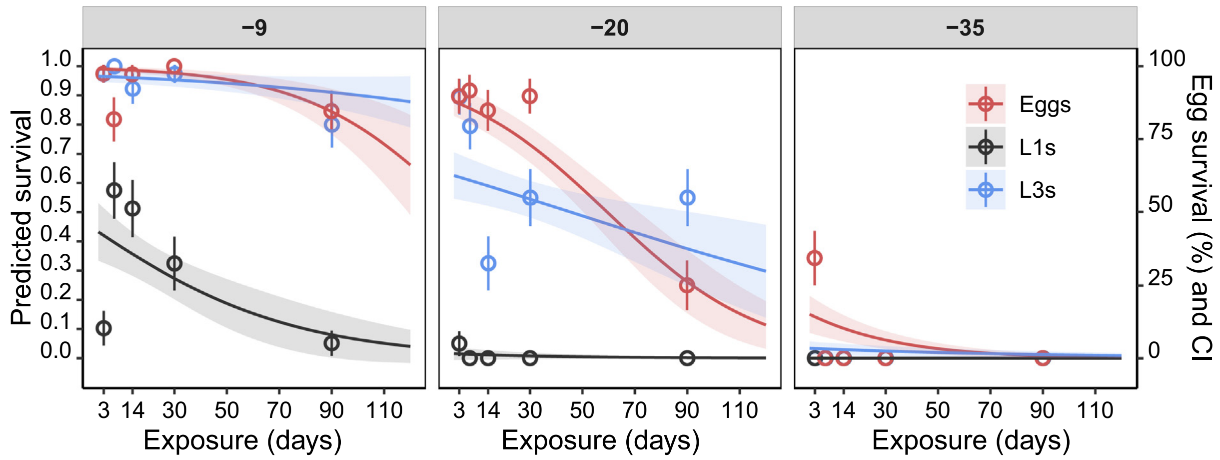


Fig. 3. Survival of eggs, L1s and L3s of *Marshallagia marshalli* after exposure to subzero temperatures. The circles represent the proportion of live eggs/L1s/L3s after exposure to −9 °C, −20 °C and −40 °C for 3, 7, 14, 30 and 90 days. The bars indicate 95% confidence intervals. The lines and shaded areas represent the prediction and 95% confidence interval of the binomial model (Generalised Linear Model, logit link) with lower Akaike Information Criterion (AIC) explaining survival of eggs, L1s and L3s. Proportions and confidence intervals of survival at each level were calculated directly from the raw data. CI, confidence interval.

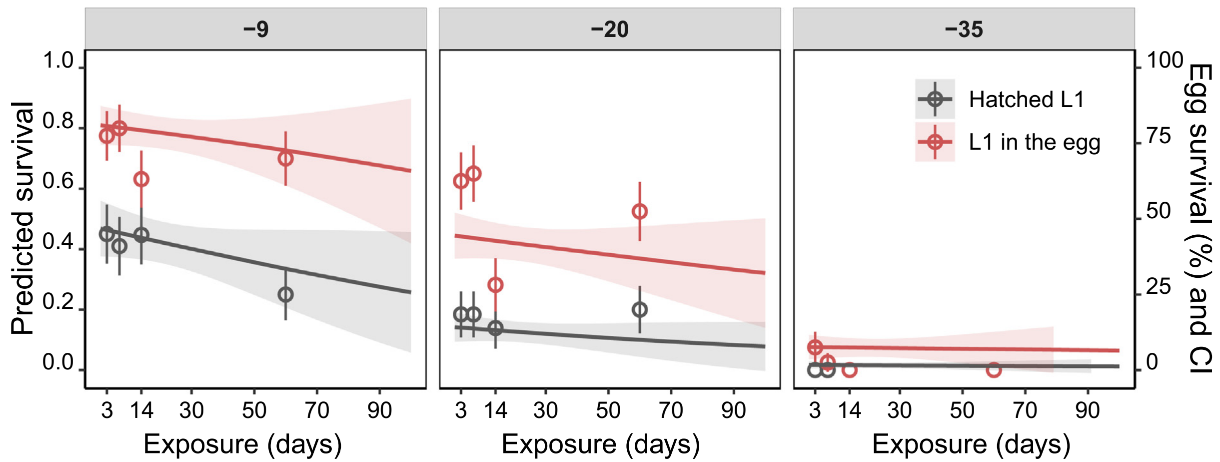


Fig. 4. Survival of free-living L1s and unhatched L1s of *Marshallagia marshalli marshalli* after exposure to subzero temperatures. The circles represent the proportion of live L1s and L1s in the eggs after exposure to −10 °C, −20 °C and −40 °C for 3, 7, 14, and 60 days. The bars indicate 95% confidence intervals. The lines and shaded areas represent the prediction and 95% confidence interval of the binomial model (Generalised Linear Model, logit link) with lowest Akaike Information Criterion (AIC) explaining survival of free-living L1s and unhatched L1s. Proportions and confidence intervals of survival at each level were calculated directly from the raw data. CI, confidence interval.

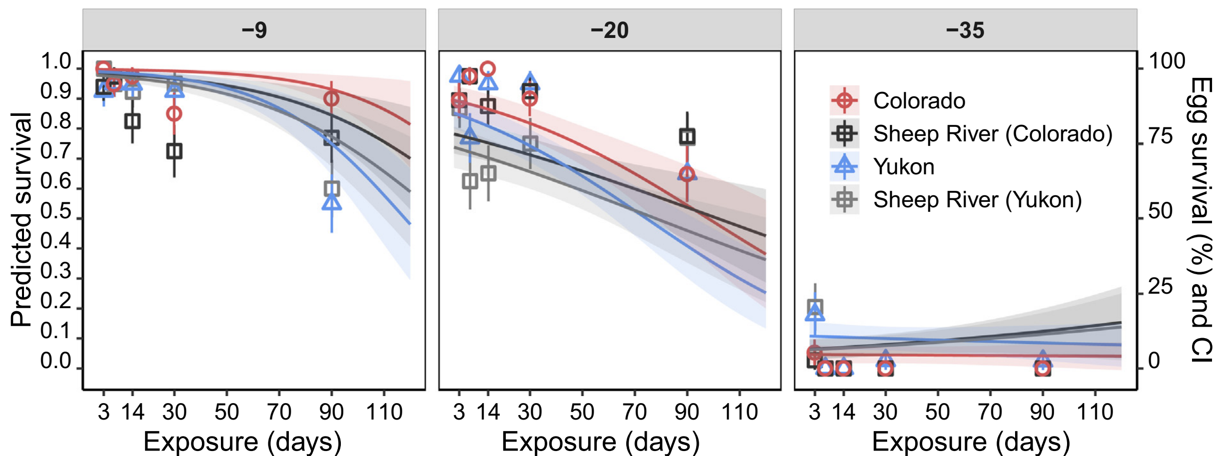


Fig. 5. Survival of eggs of *Marshallagia marshalli* collected from Colorado (USA), Alberta and Yukon (Canada) after exposure to subzero temperatures. The circles represent the proportion of live eggs from different locations after exposure to −10 °C, −20 °C and −40 °C for 3, 7, 14, 30, and 60 days. The bars indicate 95% confidence intervals. The lines and shaded areas represent the prediction and 95% confidence interval of the binomial model (Generalised Linear Model, logit link) with the lowest AIC explaining survival of eggs sourced from different locations. Proportions and confidence intervals of survival at each level were calculated directly from the raw data. CI, confidence interval.

(i.e. supercooled state) (Perry and Moens, 2011; Wharton, 2011). In the case of *M. marshalli*, the multilayered eggshell likely allows freeze avoidance, as occurs in other nematodes with similar survival capacities against below-zero temperatures. The eggshell can prevent inoculative freezing in the egg by lowering the freezing point and by mobilising and accumulating different carbohydrates (e.g. trehalose, glycogen) that can reduce water loss and act as cryoprotectants (Ash and Atkinson, 1983; Wharton, 1994). In the case of larvated eggs of nematodes, structural proteins in the chitinous layer of the eggshell, and to a lesser extent the egg fluid surrounding the larvae, are important in preventing exogenous ice nucleation, and in allowing supercooling (Wharton et al., 1993). For the L3 of *M. marshalli*, the prominent sheath may contribute to freeze-avoidance by promoting supercooling in the presence of external ice and in delivering mechanical protection against the advancing ice crystal front, as occurs in the related species, *Trichostrongylus colubriformis* (Wharton and Allan, 1989).

Freeze tolerance of eggs and L3s may be intimately linked, and adaptive, to the seasonal migration patterns of the host population. Dall's sheep typically undergo seasonal migrations determined by regional climate dynamics and local habitat features (Geist, 1971; Dertien et al., 2017). Migration patterns can vary among sheep groups, for instance, with female bands moving to higher elevations for lambing, however, sheep groups reunite in fall for the rut and to overwinter (Bunnell, 1982). The high number of *M. marshalli* eggs produced during winter are capable of surviving subzero temperatures and developing to L3s during the following summer when the more vulnerable L1s are less likely to be exposed to subzero temperatures. Winter transmission will be favoured in this case, as the freeze-resistant L3s will be temporally and spatially synchronised with the return of sheep to their winter range. Similar transmission mechanisms of *M. marshalli* have been suggested for the migratory reindeer in the Arctic archipelago of Svalbard (Carlsson et al., 2012), and of saiga antelope (Morgan et al., 2006). Additionally, sheep habitat during winter is highly restricted by snow depth, and as a consequence, habitat use is reduced to wind-swept slopes where forage is more accessible (Seip and Bunnell, 1985; Dertien et al., 2017). This may lead to overcrowding, increasing host density and local faecal concentration, enhancing parasite transmission during the following winter. This close association between life-history traits of parasites and seasonal migration of their host is also seen in other related nematodes. For example, the eggs of *O. gruehneri* in migratory caribou are not freeze tolerant and although adult parasites may present year round, parasite production ceases in the winter on winter range and resumes the following spring on the calving grounds. L3s, and perhaps L2s of this parasite are freeze tolerant, thus contamination on the spring/summer range may carry over to the following summer, synchronising with the annual caribou migration (Hoar et al., 2012a).

Our previous finding of plasticity in the hatching capacity for *M. marshalli*, where some larvae remained in the egg throughout their development to L3, made us question whether this was an adaptive behaviour related to selection for larval survival under unsuitable environmental conditions. While limited availability of L3s in the eggs meant we could not compare the survival of L3 in eggs versus free-living L3s, we were able to demonstrate a cryoprotective effect of eggs on L1s and presume a similar effect for L2s and L3s. Our results suggest that the phenotypic plasticity of *M. marshalli* in their hatching behaviour may play an important role in its capacity to persist in extreme environments. Although egg production by *M. marshalli* peaks in winter, eggs are produced throughout the year (Irvine et al., 2000; Kutz et al., 2012). Development of eggs to L3s takes a minimum of 10 days at a constant temperature of ~20 °C (Aleuy et al., 2019). Thus, for those eggs shed late in the summer, full development to L3 may not be possible prior to winter. Our data demonstrate that hatched L1s are highly susceptible to subzero temperatures and would be unlikely to survive throughout winter. Conversely, high freeze tolerance of the L1 in the egg suggests that overwintering in the egg and resuming development the following summer would increase transmission potential. The eggshell can also protect against desiccation, however, we were unable to investigate this phenomenon. The lipidic layer of nematode eggshells can control and significantly decrease water loss, directly impacting parasite survival in dry and warm conditions, but also perhaps moderating the interaction of desiccation with subzero temperatures (Perry and Moens, 2011; Wharton, 2011). Thus, remaining in the egg for extended periods of time, including through to L3, may increase the survival of *M. marshalli* in the highly seasonal environments, which are typically arid and range from frigid to hot, that characterise its endemic distribution. Delayed hatching may also provide a phenotypic advantage under different climate change scenarios and, in particular, with unpredictable and/or extreme weather events. In addition, considering that extreme and variable environments are a generality in the distribution range among *Marshallagia* spp. (Morgan et al., 2007; Hoberg et al., 2012a), future research should determine the nature of these adaptations in the genus and whether these are general phenomena or just limited exclusively to *M. marshalli*.

We had only weak evidence of local adaption of *M. marshalli* across our three study sites. This is initial support for the hypothesis that the evolutionary forces shaping the adaptations of *M. marshalli* to freezing conditions occurred prior to the establishment of these populations in their current locations (Hoberg, 2005; Hoberg et al., 2012b). *Marshallagia* originated in Eurasia, associated mostly with Caprini and Rupricaprini inhabiting mountain-steppe habitats in western China, Tibet, Mongolia, Kazakhstan and Azerbaijan (Hoberg et al., 2012a). Between 2.5 million years to 11.7 thousand years ago (i.e., late Pliocene and Pleistocene), northern systems experienced episodic extreme climatic events characterised by glaciations, fluctuations in humidity, and habitat disruption. Changing environments during the late Pliocene lead to biotic philtres for species expansions out from Eurasia into North America through Beringia prior to the Pleistocene. Then later in the Pleistocene, glacial-interglacial cycles created the physical and biological conditions (e.g. critical ice-free zones or refugia) for expansion and isolation that structured the northern fauna, and to a great degree the fauna in the Nearctic (Fernández and Vrba, 2005; Hoberg et al., 2012b, 2017; Cook et al., 2017). The freezing tolerance of *M. marshalli* seems to be evenly distributed across its distribution in North America, and thus may constitute a conserved capacity arising prior to the Pleistocene in the original distribution of *Marshallagia*.

Our study sought to investigate the general hypothesis that phenotypic plasticity in hatching behaviour of *M. marshalli* might increase its persistence under extreme and changing environments. For instance, plasticity in essential traits can allow short-term modifications in developmental pathways that may serve to buffer the immediate impact of climate change and thus extend the potential window for selection of evolutionary adaptations (Reed et al., 2011; Forsman, 2015). Maybe even more importantly, there is also the potential for a shortcut to rapid evolutionary adaptation if the context of capacities defined by plasticity (e.g., hatching as L3 in the case of *M. marshalli*) fits well with new environmental conditions and boundaries. Natural selection would thus promote such plastic traits, as they can be expressed in many individuals within just a few generations (Pfennig et al., 2010; Merilä and Hendry, 2014; Forsman, 2015). Although this hypothesis is very intuitive and has strong theoretical support (see Pfennig et al., 2010), its study in natural settings is particularly challenging (however, for more cases see Furness et al., 2015; Rutherford et al., 2017; Kingsolver and Buckley, 2018). Issues such as quantifying

the extent to which plasticity in developmental traits varies among natural populations, and the degree of association between these specific traits and parasite fitness, are crucial in our ability to predict the effects of climate change on the geographic diversity and distribution of parasites.

We have characterised the survival at subzero temperatures of the free-living stages of the nematode *M. marshalli*. Our results provide mechanisms that can explain overwinter transmission of this parasite in reindeer, muskoxen, Dall's sheep, and saiga antelope. Also, our results in part corroborate that the nature of plasticity for developmental pathways in *M. marshalli* may be outcomes from historical climate extremes during the radiation of the genus in Eurasia. Developmental plasticity as manifested in these nematodes may facilitate short term (i.e. phenotypic response) and long term (i.e. evolutionary response) patterns of persistence under the current regime of accelerating climate and environmental perturbation. Our ability to anticipate the effects of climate change on parasitic diseases relies on our capacity to gather knowledge about specific life-history traits of parasite species, their geographic distribution and host ranges and relationships (Molnár et al., 2013; Brooks et al., 2014, 2019).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2019.12.004.

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