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Adaptations and phenotypic plasticity in developmental traits of *Marshallagia marshalli*

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ABSTRACT

Despite the economic, social and ecological importance of the ostertagiine abomasal nematode *Marshallagia marshalli*, little is known about its life history traits and its adaptations to cope with environmental extremes. Conserved species-specific traits can act as exaptations that may enhance parasite fitness in changing environments. Using a series of experiments, we revealed several unique adaptations of the free-living stages of *M. marshalli* that differ from other ostertagiines. Eggs were isolated from the feces of bighorn sheep (*Ovis canadensis*) from the Canadian Rocky Mountains and were cultured at different temperatures and with different media. Hatching occurred primarily as L1s in an advanced stage of development, morphologically very similar to a L2. When cultured at 20 °C, however, 2.86% of eggs hatched as L3, with this phenomenon being significantly more common at higher temperatures, peaking at 30 °C with 28.95% of eggs hatching as L3s. After hatching, free-living larvae of *M. marshalli* did not feed nor grow as they matured from L1 to infective L3. These life history traits seem to be adaptations to cope with the extreme environmental conditions that *Marshallagia* faces across its extensive latitudinal distribution in North America and Eurasia. In order to refine the predictions of parasite dynamics under scenarios of a changing climate, basic life history traits and temperature-dependent phenotypic behaviour should be incorporated into models for parasite biology.

1. Introduction

The importance of understanding parasite ecology has gained renewed scientific interest over the last few decades as a direct consequence of large scale environmental changes (i.e., climate change) that were/are altering host-parasite interactions (e.g. van Dijk et al., 2008; Altizer et al., 2013; Gallana et al., 2013). Many parasite species have stages that develop in the environment prior to infecting their final host. The development and survival of those stages are intrinsically tied to environmental factors such as temperature and humidity. As a consequence, parasite fitness is determined by complex interactions, and adaptive traits that together either benefit or limit the development and survival of each free-living stage of the parasite (Molnár et al., 2013; Raffel et al., 2013). In nematodes for example, it has been suggested that larvated eggs and infective L3s are, in general, more resistant to temperature and desiccation extremes than eggs, and L1s or L2s (Perry and Moens, 2011). Similarly, while warmer temperatures can increase the development rate of eggs and free-living larvae, they can simultaneously decrease survival time (Molnár et al., 2013). This stage-specific interaction with the environment becomes even more complex when species-specific adaptations to fluctuating or extreme environments are considered. For instance, van Dijk and Morgan (2010) described a geographic polymorphism in the larval behaviour of *Nematodirus battus*, manifested as differing chilling requirements for egg hatching. They hypothesized this behaviour could be an adaptation to cope with environmental unpredictability, particularly under cooler climates, playing a key role in population fitness of the species (van Dijk and Morgan, 2010). Further, these capabilities interact to determine host geographic range in the context of ecological fitting; representing the outcomes of evolutionary conservative capacities of parasites in the arena of environmental opportunity (Araujo et al., 2015; Hoberg and Brooks, 2015).

Marshallagia marshalli (Ransom, 1907) is an ostertagiine nematode infecting the abomasum of wild and domestic ungulates from

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the Holarctic region (Hoberg et al., 2001, 2012a; Meradi et al., 2011; Kutz et al., 2012). This species is one of the major causes of parasitic gastroenteritis in domestic sheep and goats in Middle East regions (Eslami et al., 1979; Altaş et al., 2009). In Canada, it is associated with reduced body condition and pregnancy rates in wild Dall's sheep (*Ovis dalli dalli*) ewes from Canada (Aleuy et al., 2018) and reduced body condition of female juvenile saiga antelope (*Saiga tatarica*) in Kazakhstan (Morgan et al., 2005). It is believed that *M. marshalli* originated in Eurasia, in dry and cold mountain-steppe habitats with large temperature fluctuations. *Marshallagia,* together with an assemblage of diverse nematodes in ungulates, later expanded from Eurasia into North America in the Pliocene-Pleistocene during episodic extremes in climate, and changing environments, that defined glacial-interglacial stages over the past 3.0–2.5 million years ago (Mya) (Hoberg et al. 2012b). This evolutionary scenario might have generated developmental traits adapted to extreme conditions. These traits could have persisted over time and may influence the parasite's ecology under the current conditions of environmental change.

The literature on the developmental strategies of the free-living stages of *M. marshalli* is limited and contradictory. For instance, Durette-Desset (1985) suggested that the development of *M. marshalli* to infective L3 relied only on vitelline reserves. However, other authors have included different food sources in incubation experiments, inferring the need for external food sources to complete development in *M. marshalli* (Carlsson et al., 2013). Regarding hatching, there is anecdotal evidence suggesting that hatching occurs as L2 with the first moult occurring inside the egg (Taylor et al., 2007). In contrast, our preliminary research on *M. marshalli* suggested that it has a differential hatching strategy depending on temperature, and that hatching as L2 is the least probable developmental pathway. Understanding the basic life history traits that *M. marshalli* uses to interact with its environment is fundamental to studying parasite dynamics and assessing/predicting the impacts of large scale environmental disturbances such as climate change (Molnár et al., 2013; Verschave et al., 2016).

The objective of this study was to describe several phenotypic attributes of the free-living stages of *M. marshalli* in order to elucidate key life history traits relevant to parasite transmission dynamics. Obtaining nutrition from the environment is a key feature for free-living stages of most species among the Trichostrongyloidea, thus, the first step was to determine the effect of food supplementation on development, survival and morphometrics of *M. marshalli* during development from L1 to L3. This also allowed the optimization of in vivo culture methodologies for subsequent experiments. Then, the development from egg to L3 was characterized in order to provide morphological and morphometric definitions for each larval stage. In early trials, it was possible to detect some differences in the degree of development of the larval stages emerging from the eggs. This phenomenon was then further explored to determine if the temperature of incubation had an effect on larval development stage at the time of hatching. It was hypothesized that due to the evolutionary history of *M. marshalli*, developmental plasticity related to temperature would be a critical determinant of patterns associated with hatching under differing environmental regimes.

2. Materials and methods

2.1. Collection and extraction of M. marshalli eggs

Fresh fecal samples were collected from a bighorn sheep (*Ovis canadensis*) population located at Sheep River Provincial Park (50°40′N 114°40′W), Alberta, Canada. To ensure that only fresh samples were collected, sheep were located and observed from a distance until defecation occurred. Fecal samples were collected within an hour of defecation, stored in a portable cooler (∼2°C) and transported to the University of Calgary (Canada) within 3 h of collection. Samples were refrigerated at 1 °C for one to 3 days until they were used in the experiments.

Eggs of *M. marshalli* were extracted from feces using a modified version of the technique described in Hubert and Kerboeuf (1984). In short, to obtain an egg sample representative of the host population, 10 g of feces from at least five different animals (50 g total) were mixed in a plastic container. A sub-sample of 30 g of feces was removed and placed in a plastic bag with some tap water. The bag was then sealed and massaged by hand until fecal pellets were homogenized. The material was then passed through a 300 μm sieve and the sieve was washed with abundant tap water. Sediment passing through the sieve was collected in several 3000 ml beakers and then passed through a 63 μm sieve. The material collected in the 63 μm sieve was washed into 250 ml plastic bottles using tap water and centrifuged at 400*g* for 10 min. The resulting supernatant was discarded, the pellet obtained was vortexed and a 13% salt solution was added, then the slurry was centrifuged again at 400*g* for 10 min. The resultant supernatant was then filtered through the top of a fluke finder (Flukefinder®, Soda Springs, ID, USA) and the eggs collected in a 63 μm sieve. The eggs were washed off the sieve with a gentle jet of distilled water and collected in a plastic petri dish. *Marshallagia marshalli* eggs were identified at 30–40× power using a dissecting microscope and then isolated into another dish. The eggs of *M. marshalli* (Fig. 1A) are easy to distinguish as they are significantly larger than other strongyles eggs, except for *Nematodirus* spp., and have a characteristic

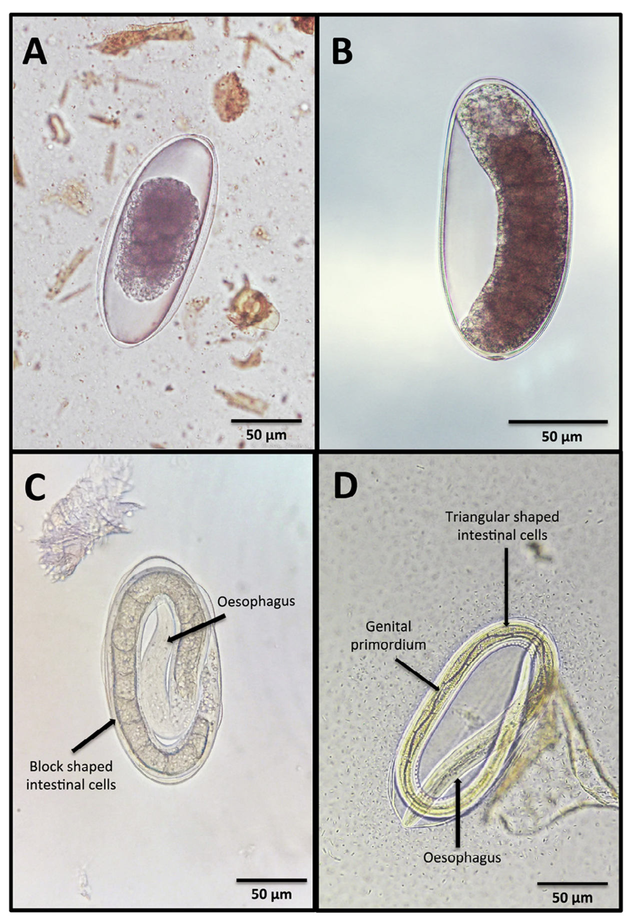

Fig. 1. Different *Marshallagia marshalli* stages during development inside the egg. (A) Egg of *M. marshalli*; (B) elongated embryo; (C) L1; (D) Infective L3.

elongated shape, and a well-developed morula (Monnig, 1940; Jacobs et al., 2015).

2.2. External nutrition requirements during the development of free-living stages

Experiment 1 was performed to determine whether the development and survival rates of free-living stages of *M. marshalli* were influenced by the presence of external nutrients. Extracted eggs were incubated in three different nutritive mediums (bacteria, yeast and distilled water) for 2 months. Nutritive mediums were prepared according to Sutherland and Scott (2010) for the bacteria medium, and Hubert and Kerboeuf (1984) for the yeast medium. For culturing, we used 96-well cell culture plates, allocating one egg per well that was filled with 100 µl of nutritive medium or distilled water. To control for the location of the egg in the plate, the treatments were placed in alternating wells. Two plates with 32 eggs per treatment in each plate were prepared, covered with their lids, and placed in replicate incubators at the same temperature (~20 °C). The survival of each egg together with hatching and time of larval development to L3 were recorded by examining each well using an inverted microscope at 400× magnification every 2 days, for a period of 60 days. When the volume of diluted nutritive medium or distilled water in the wells decreased by more than 50%, the wells were refilled with their respective mediums. Survival rates among treatments were analyzed using survival analysis with the Cox's proportional hazard model. The days to hatching and L3 appearance were compared among treatments using Generalised linear mixed models (GLMMs) with the variable 'incubator' as a random effect.

Experiment 2 was performed to determine if the size of *M. marshalli* L3 was associated with food availability during development. Extracted eggs were cultured in only two of the three different media (yeast medium and distilled water) due to the low availability of eggs for this experiment and because no difference in survival and development rates were observed in experiment 1 comparing the different nutrient treatments. Two culture plates were prepared containing 10 eggs per food treatment, allocated in alternating wells and each plate was placed in a separate incubator at ~20 °C. After 25 days of incubation, five L3s per treatment were randomly selected from each plate and measured. Larvae were heat-fixed by placing them in 50 µl of tap water on a glass slide and passing the slide six times quickly over a Bunsen burner flame (Kutz et al., 2001). Fixed L3s were examined under 400× magnification using a microscope and digital camera (Olympus BX53 fitted with a digital camera, Olympus DP73, Olympus®) and the measurements were made using the CellSens software (CellSens; Olympus Soft Imaging Solutions GmbH). The features measured were done according to Van Wyk and Mayhew (2013). The morphometrics were compared between treatments using a t-test or permutation test when the assumption of normality was not met.

2.3. Embryonic and post embryonic development

The development of free-living stages of *M. marshalli* was analyzed using two experimental approaches in order to describe the morphological and morphometric changes from egg to L3.

In the first experiment, approximately 200 eggs were placed in distilled water in a petri dish at 20 °C to monitor their development to L3. The developmental stages and morphological changes in the eggs and/or larvae were monitored after 12 and 24 h of incubation and then every 24 h until all the eggs either developed to L3 or were considered dead (determined by the absence of movement and a change into a darker coloration). At each observation period, a sub-sample of 20 eggs in the dish of 200 (i.e. non-probability sampling with replacement) were observed at 400× using an inverted microscope and morphology was described. The sample size was calculated using the resource equation method (Festing, 2006). The criteria used to describe the stages of development were adapted from Cruz et al. (2012) (Supplementary Table S1) and the percentage of eggs in each stage was calculated using the subsample of 20 eggs as the denominator. For describing larval morphology, 40 newly hatched larvae from the same sample were selected and allocated to a different petri dish immediately after hatching. Their developmental stage was recorded on a daily basis until all of the larvae were either L3 or determined dead.

In the second experiment, the post-hatching morphometric changes of *M. marshalli* larvae cultured at a constant temperature were monitored. Approximately 200 eggs were allocated to individual wells in 96-well culture plates containing 100 µl of distilled water and then incubated at 20 °C. Egg development was monitored on a daily basis until hatching. A subset of 10 larvae at both one and 2 days post-hatching (PH) and then every second day after that until 18 days PH, were randomly collected, heat-fixed, examined and measured under the microscope. Data were analyzed by using a permutation test to find differences in larval size among sampling days.

2.4. Temperature dependency of the larval stage at hatching

To test if the larval stage at hatching was different at different temperatures, *M. marshalli* eggs were cultured at increments of 5 °C, from 5 to 40 °C. Twenty eggs were placed individually in 96-well culture plates containing 100 µl of distilled water in each well. A total of 16 plates were prepared and then allocated to the different temperature treatments in duplicate incubators at each temperature. The survival and larval stage at hatching were recorded every 24 h until all the eggs/larvae were dead or had developed to free-living L3. The results are presented using descriptive statistics for hatching times and survival rates. The proportion of eggs hatching in different larval stages were analyzed in relation to temperature using a chi-squared test, with Monte-Carlo significance test procedures (2000 replicates), to account for low cell counts (<5) in the contingency tables. GLMMs with binomial error and logit link function including replicate incubator as a random effect were used. All the analyses were performed using R (Version 3.5.2) (R Core Team, 2013. R: A language and environment for statistical computing. R foundation for statistical computing Vienna, Austria) and using the lme4 (Bates et al., 2015), coin (Hothorn et al., 2006), and survival (Therneau, T.M., 2017. A package for survival analysis in S. 2015. R package version 2) packages.

3. Results

3.1. External nutrition requirements during the development of free-living stages

The day of hatching (median = 6, range = 5–14) did not differ among the three food treatments (GLMM; yeast food: b = 0.0516, S.E. = 0.07, z = 0.73, P = 0.46; distilled water: b = −0.0139, S.E. = 0.07, z = −0.20, P = 0.84), nor did the development rate to L3 (median = 14, range = 10–21) (GMM; fungal food: b = −0.0073, S.E. = 0.05, z = −0.14, P = 0.890; distilled water: b = 0.0168, S.E. = 0.05, z = 0.32, P = 0.748), or the survival rate of *M. marshalli* to 60 days (Chi-squared; χ^2 = 0.4, df = 2, P = 0.825, Supplementary Fig. S1). L3 size did not differ between larvae maintained with the yeast solution compared with those in distilled water (Supplementary Fig. S2).

3.2. Embryonic and post embryonic development

After 12 h of incubation, 10% of the eggs had a morula with a kidney shaped invagination. The remaining 90% of the eggs were in a more advanced stage of development with arched and elongated embryos (Fig. 1B). At 24 h of incubation, 40% of the viable eggs were in a pre-larval stage with elongated embryos. The cephalic and caudal ends of embryos were easily distinguishable at this time. Movement occurred in all the embryos after light stimulation. Well-formed early L1s were observed in 30% of the eggs by 48 h of incubation. In the third day of incubation (72 h), 30% of the eggs were in late L1 stage with a differentiable esophagus, intestinal cells and their length several times longer than the egg (Fig. 1C). Hatching was first observed by 4 days (96 h) of incubation (median = 6 days, range = 4–14 days). The newly hatched L1s were very motile, with well defined block shaped intestinal cells and no distinguishable loose cuticle (Fig. 2A). No moulting, as defined by a visible cuticle sheath around the larva, was observed inside the egg before hatching.

The newly hatched L1s were in an advanced stage of development and morphologically similar to the L2s. At 24 h post-hatching, 10% of larvae were surrounded by a loose L1 cuticle and by 48 h post-hatching this increased to 70% (Fig. 2B). By 72 h post-hatching, some of the L2s entered a state of lethargus with a straight and rigid body and were not responsive to light stimulation. In all the cases this was observed only during one recording event before the larvae recovered their movement and responsiveness to light. The larvae were classified as L3s when the development of intestinal cells was completed or when a loose L2 cuticle sheath was observed around the larvae. The fully developed intestinal cells consisted of 16 well-defined elongated triangular cells ($n = 20$). In 40% of the L3s, the L1 cuticle was maintained and two sheaths were observed on the L3s (Fig. 2C). The L3 body was slender and cylindrical, tapering in the posterior end more prominently than in the anterior end.

The dimensions of *M. marshalli* larvae did not differ between the newly hatched L1s and fully developed L3s. Most of the morphometrics followed a general pattern with larvae decreasing in size 2 days PH with a subsequent progressive increase and recovery to their initial size before attaining the L3 stage. Once the L3 stage was attained, larval dimensions remained more or less constant until the end of the experiment (Table 1). For more details see Fig. 3.

3.3. Influence of temperature on stage of larvae at hatching

The hatching of *M. marshalli* was associated with the temperature of incubation. Hatching as L3s occurred only at 20, 25, 30 and 35 °C (Fig. 1D). The proportion of eggs hatching as L3s significantly increased from 2.86% (95% confidence interval (CI): −0.41–6.12) at 20 °C to 28.95% (95% CI: 20.1–37.8) at 30 °C (Chi-squared; $\chi^2 = 9.029$, $P = 0.003$) (Fig. 4). A similar pattern was observed when analyzing the data using a regression approach. In order to avoid an error in the Hessian matrix by including temperatures with no event of interest, the model was fitted using only 20, 25, 30 and 35 °C (Supplementary Table S2). In most cases, the fully developed L3 stayed inside the egg for several days before hatching. As a consequence, the time until the L3 was available free in the environment, was significantly longer when they hatched as L3 compared with if they hatched as L1 at 25, 30 and 35 °C (Permutation tests, 25 °C: Z = −3.09, $P = 0.006$, 30 °C: Z = −2.11$P = 0.027$, 35 °C: Z = −2.35, $P = 0.042$) (Fig. 5).

Table 1
Comparative measurements between L1s and L3s of *Marshallagia marshalli* cultivated in vivo at 20 °C. There are no significant differences in any measurement among each larval stage (t-test, $P < 0.01$).

Measurements (unit)	L1 Range (mean ± S.E.), $n = 20$	L3 Range (mean ± S.E.), $n = 20$
Total body length (μm)	587.6–696.4 (640.9 ± 23.1)	564.4–711.0 (641.9 ± 36.6)
Length of esophagus (μm)	118.7–167.3 (147.6 ± 11.4)	121.8–175.1 (153.6 ± 11.1)
Esophagus as a percentage of total body length (%)	19.2–25.9% (23.0 ± 1.6%)	21.7–27.13% (23.97 ± 1.3%)
Width at base of esophagus (μm)	22.8–28.2 (24.73 ± 1.3)	21.7–27.45 (25.1 ± 1.3)
Cephalic extremity to genital primordium (μm)	312.2–405.0 (362.8 ± 19.0)	313.4–415.2 (353.1 ± 28.1)
Length intestine (μm)	337.0–434.4 (397.2 ± 25.9)	343.3–447.6 (385.9 ± 28.4)
Intestine as a percentage of total body length (%)	57.1–67.4% (61.9 ± 2.8)	56.0–63.6% (60.14 ± 1.7)
Sheath tail extension (STE) (μm)		5.67–18.9 (13.85 ± 2.7)
Tail width (μm)		3.8–8.7 (6.4 ± 1.3)

4. Discussion

Several of the life history traits of *M. marshalli* observed in our experiments differed from those documented among closely related genera and species of the Trichostrongyloidea (e.g.,

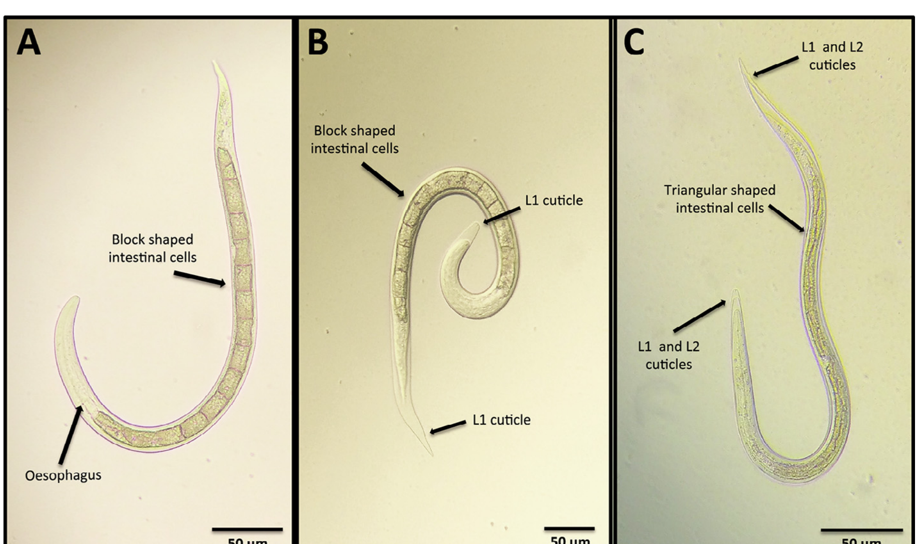

Fig. 2. Different *Marshallagia marshalli* larval stages during their development from L1 to L3 outside of the egg. (A) First stage larvae (L1); (B) second stage larvae (L2) retaining a loose L1 cuticle; (C), infective larvae (L3) retaining L1 and L2 loose cuticles.

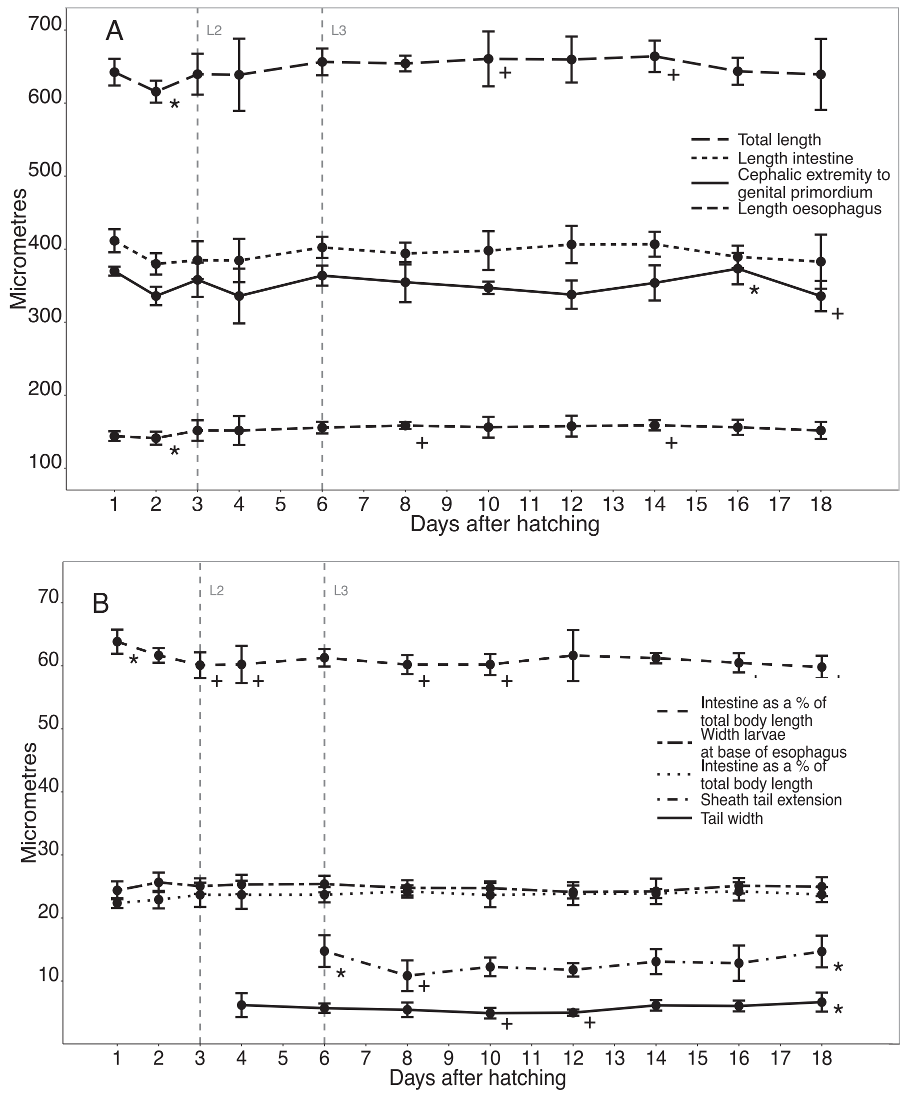

Fig. 3. Mean measurements of larvae of *Marshallagia marshalli* cultivated in vivo (20 °C) at various days post hatching. A) Measurements higher than 100 micrometres. B) Measurements lower than 100 micrometres. All measurements in micrometers except for esophagus as a percentage of total body length (% esophagus) and intestine as a percentage of total body length (% intestine). Error bars represent standard errors (S.E.). The vertical dashed lines indicate the average time for L2 and L3 development.

Ostertagia spp., *Haemonchus* spp., *Cooperia* spp., *Teladorsagia* spp., and *Trichostrongylus* spp.) (Anderson, 2000).

Our results demonstrate that the free-living stages of *M. marshalli* do not depend on nutrition from the external environment for their development and survival. The development from egg to L3 in distilled water was quantitatively (i.e. development and survival rate) and qualitatively (i.e. morphology) equal to that in two different types of nutrient-rich media. As a general rule in Trichostrongyloidea, external nutrients (e.g. microorganisms) are essential for the survival and development of the free-living L1s and L2s, whereas the L3s may remain ensheathed, and do not feed (Durette-Desset, 1985; Durette-Desset et al., 1994). Interestingly, the pattern of development of *M. marshalli*, where nutrition is independent from the external environment, is shared with species from the sister superfamily Molineoidea (e.g., *Nematodirus* and *Nematodirella* spp.) (Durette-Desset et al., 1994; Chilton et al., 2001), where the development to L3 occurs within the egg and depends entirely on vitelline reserves (Boulenger, 1915). The absence of feeding of the free-living parasite stages has direct implications for strategies used to control parasite infections in livestock production systems. For instance, the use of different plant species as non-chemical methods to control the availability of infective stages in the pasture appears as an alternative to reduce the use of antiparasitic drugs and the presentation of drug resistance (Robertson et al., 1995). Among the anthelmintic effects suggested for these plant species is the damage to the gastrointestinal mucosa and to the pharyngeal muscles of the parasite larvae caused by the ingestion of these herbages (Molan and Faraj, 2010). Given that L1 and L2 of *M. marshalli* and *Nematodirus* spp. do not ingest nutrients from the external environment during their development to L3, it could provide them with specific resistance against this type of control method.

Morphometrics of newly hatched L1s of *M. marshalli* remained constant during development to L3s. Generalized growth curves for nematodes suggest that juvenile stages follow a continuous growth rate after hatching that may be specific for each instar (i.e. developmental stage between each moult) (Yeates and Boag, 2003). Although this holds true in the most studied trichostrongyloid nematode species (Anderson, 2000), this is not the case for *M. marshalli*. Despite the absence of growth after hatching, the larvae of *M. marshalli* overlap in length ($\bar{x}$=641.9 ± 36.6 μm) with L3s of some other trichostrongyloids with a continuous growth pattern

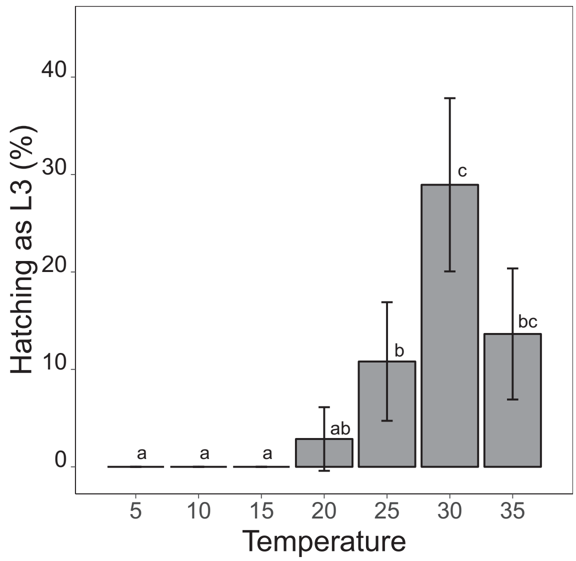

Fig. 4. Percentage of eggs hatching as L3 of *Marshallagia marshalli* cultivated in vivo under a range of temperatures (5, 10, 15, 20, 25, 30, 35 °C). Forty eggs per temperature treatment were cultivated and divided into duplicate incubators. The percentage is calculated excluding eggs that died before hatching. The error bars represents 95% confidence intervals. Different letters represents statistically significant differences (Chi-square, $P < 0.05$).

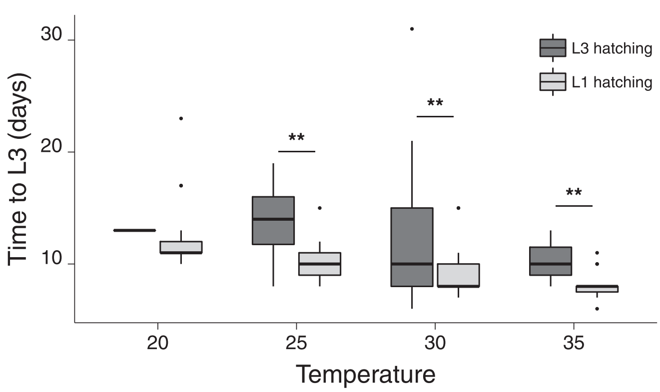

Fig. 5. Time to *Marshallagia marshalli* free-living L3s among eggs that hatched as L1s and L3s at a range of temperatures. The lines and ** represent statistically significant differences among hatching strategies within each temperature treatment (Permutation test, $P < 0.05$).

such as *Trichostrongylus colubriformis* ($\bar{x}$=710 ± 33 μm), *Trichostrongylus axei* ($\bar{x}$=685 ± 33 μm), and *Haemonchus contortus* ($\bar{x}$=730 ± 75 μm). In contrast, larval *M. marshalli* are considerably shorter than the L3s of others such as *Ostertagia ostertagi* ($\bar{x}$=850 ± 65 μm), *Ostertagia gruehneri* ($\bar{x}$=978 ± 78), *Cooperia oncophora* ($\bar{x}$=890 ± 95 μm), *Nematodirus helvetianus* ($\bar{x}$=1107 ± 62 μm) (Herlich, 1954; Hoar et al., 2012; Van Wyk and Mayhew, 2013).

Marshallagia marshalli presented two pathways related to hatching. In the first pathway, hatching occurred as L1s in an advanced stage of development. This was the most common pathway observed in *M. marshalli* and is consistent with trichostrongyloid nematodes in general (Anderson, 2000). The second and less common pathway was that larvae developed to L3s inside the egg prior to hatching, and the likelihood of this happening increased at higher temperatures. Development to L3 in the egg is an unprecedented finding, not only for an ostertagiine nematode but also for the remaining subfamilies in the family Trichostrongylidae (e.g. Libyostrongylinae, Cooperiinae, Graphidiinae, Trichostrongylinae, and Haemonchinae) (Anderson, 2000). In contrast, development to L3 in the egg is a conserved trait for Nematodirines, and is thought to improve survival of the parasites in extreme or variable environments by decreasing dependence on the external environment (e.g., for food) together with taking advantage of the protective properties of the eggshell (Durette-Desset, 1985).

Although the debate about hatching and larval development in *M. marshalli* is longstanding, it has always been restricted to whether or not larvae hatch as L1s or L2s (Durette-Desset, 1985; Taylor et al., 2007; Carlsson et al., 2013). The possibility of hatching as L3s has not been raised in the literature. Development to the L3 in the egg might confer to *M. marshalli* the capacity to cope with a wide variety of unfavourable environmental conditions, including extremes in temperature and humidity (Perry and Moens, 2011). The endemic range of *M. marshalli* includes dry temperate to Arctic and alpine environments characterized by high summer temperatures typically associated with decreased relative humidity and extreme subzero temperatures in winter with also very low relative humidity (Meradi et al., 2011; Hoberg et al., 2012a). Phenotypic characteristics that protect the parasite from these extremes are likely to be conserved (Cook et al., 2017). Remaining in the egg through to the L3 stage could offer several advantages under these conditions. The eggshell in *N. battus*, for instance, plays a role protecting the unhatched larvae against inoculative freezing (i.e. induced internal ice formation) by participating in the mobilization and accumulation of carbohydrates (e.g. trehalose, glycogen), that act as major cryoprotectants and water loss retardants under freezing conditions (Ash and Atkinson, 1983). Our preliminary work supports a protective role of the egg at subzero temperatures, wherein newly hatched L1s have lower survival rates than L1s in the egg (O.A. Aleuy, unpublished observations). Similarly, protection from desiccation at high temperatures is likely to enhance survival of these parasites that reside in arid to semi-arid environments or in the case of eggs shed during unusually warm events. The lipidic layer of the eggshell is the main permeability barrier to water loss during embryonic development and significantly enhances the survival of larval stages in the egg of several groups of parasitic nematodes (Wharton, 1980; Perry and Moens, 2011). Further research is needed testing the hypothesis that the eggshell could have the capacity to confer a protective effect for *M. marshalli* larvae during their development to L3s under unfavourable conditions.

We also observed a delay in the hatching of L3s of *M. marshalli*, even after they were fully developed in the egg. Developing to L3 in the egg with a subsequent delay in hatching also occurs with *Nematodirus* spp. For some species, e.g. *Nematodirus filicollis*, a fully developed L3 in the egg may enter arrested development, with hatching only occurring when environmental conditions are favourable (Viljoen, 1972; Sommerville and Davey, 2002). This hatching behaviour is thought to improve parasite fitness in at least two ways: (i) eggs membranes provide protection to cope with unfavourable winter conditions (Thomas and Stevens, 1960), and (ii) reproductive success of the parasite is enhanced by synchronizing the appearance of L3s with the seasonal abundance of susceptible hosts (i.e. for *N. battus* hatching in spring when lambs are more numerous and the possibility of being ingested is higher) (Perry, 1989). The occurrence, drivers, and potential epidemiological significance of delayed hatching for L3s of *M. marshalli*, across its different host species with their different life histories, and across different geographic ranges, deserves further consideration.

We have elucidated unique features of *M. marshalli* that distinguish it from its most closely related genera. Larvae do not require external nutrition to develop to the infective stage, thus, there can be plasticity in development and hatching behaviour, such as developing to L3 in the egg and remaining there until conditions are suitable for transmission. This ecological fitting and phenotypic plasticity may help to explain the contemporary epidemiological patterns that we observe for this parasite and its potential for persistence and/or expansion in changing environmental settings (Agosta et al., 2010; Araujo et al., 2015; Hoberg and Brooks, 2015). These features are likely interrelated and contribute to its survival under highly variable temperature conditions and extremes in humidity. Developing in the egg to L3 and the plasticity observed in hatching behaviour traits may become increasingly important under the current regime of climate change, particularly where warming and the occurrence of extreme weather events are accentuated across much of the Arctic and subarctic range of *Marshallagia*. While we identified an association between high temperatures and hatching as L3s, behavioural and physiological responses in nematodes may be triggered by a set of environmental stimuli or by complex interactions linking parasite biology and the ambient environment (McSorley, 2003) (see, for instance, *O. ostertagi* hypobiosis, Lützelschwab et al., 2005). Other stimuli such as light regime, relative humidity, and genetics likely influence both the occurrence of development of L3 in the egg and timing of hatching of the L3. Refining our understanding of the mechanisms triggering development to L3 in the egg, and the genetic component of developmental plasticity and its variation among parasites from different populations, are fundamental steps in defining the limits for faunal assembly in this system.

Currently, the described diversity of *Marshallagia* in North America is limited to only two species. This is very low compared with what it is seen in Eurasia, where at least 11 species of *Marshallagia* are recognized (including *M. marshalli*) (Hoberg et al., 2012a; Hoberg et al., 2012b). Such diversity gradients are consistent with the hypothesis that *Marshallagia* originated in Eurasia and then dispersed into North America during repeated invasions during the Pliocene and Pleistocene (Hoberg, 2005). Groups of herbivores with high sympatry, density and diversity populated Beringia during the Pleistocene, linking the Palearctic and Nearctic, representing the ideal host substrate for these repeated parasite colonization events (Fernández and Vrba, 2005; Hoberg et al., 2012b, 2017; Cook et al., 2017). During the late Pliocene and Pleistocene, Beringia experienced extreme climatic events including intermittent glaciations, climate fluctuations, different degrees of desiccation, and habitat perturbation (Hoberg et al., 2012b; Cook et al., 2017). These conditions likely acted as a strong selective bottleneck for parasite species that could not cope with or rapidly adapt to a changing environment, and for specific developmental traits in parasitic nematodes adapted to transmission in extreme environments in Eurasia, such as *M. marshalli* (Hoberg, 2005). For instance, there are remarkable similarities in the developmental patterns of *M. marshalli* and *Nematodirus* spp., which are phylogenetically disparate nematodes partitioned in different families. Such life history similarities suggest common phylogenetic and historical constraints, shaped by similar evolutionary forces selecting for resilience in extreme conditions. A similar effect of Beringia and the extreme climatic events during the Plio-Pleistocene can be seen in the adaptations to extreme environments of different groups of arctic parasitic fauna such as the family Protostrongylidae (e.g. *Umingmakstrongylus pallikuukensis*, Hoberg et al., 1995; Kutz et al., 2005; Hoberg and Brooks, 2015; Kafle et al., 2018), and Ostertagiinae (e.g. *Teladorsagia boreoarcticus*, (Hoberg et al., 1999; Cook et al., 2017)).

Several of the life history traits of *M. marshalli* observed in our experiments differed from those documented among closely related genera and species of the Trichostrongyloidea (e.g., *Ostertagia* spp., *Haemonchus* spp., *Cooperia* spp., *Teladorsagia* spp., and *Trichostrongylus* spp.) (Anderson, 2000), and are consistently similar to specific traits observed in Nematodirines. Similar historical selection processes among these groups, particularly in the ecosystems where these nematodes originated (e.g. Eurasia and high altitude systems), together with, in some cases, similar historical and geographical patterns of radiation from Eurasia, might have acted as a common selectivity filter for these traits. Under the current conditions of climate change, these historically conserved traits may represent exaptations that are enhancing parasite fitness (Gould and Vrba, 1982). Phenotypic plasticity in development traits among these nematodes may constitute conserved capacities manifested in potential responses to environmental change, and thus are at the core of understanding faunal dynamics (expansion, extinction and mosaic faunal assemblages) under a regime of accelerating perturbation (Agosta et al., 2010; Araujo et al., 2015; Hoberg et al., 2017). Gathering knowledge about species-specific life history traits is a fundamental step in refining our ability to anticipate and predict the effects of climate change on parasitic diseases (e.g., Brooks et al., 2014). Such insights gain particular importance when a parasite such as *M. marshalli* has negative economic, social or conservation impacts.

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Appendix A. Supplementary material

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