View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Open Archive Toulouse Archive Ouverte

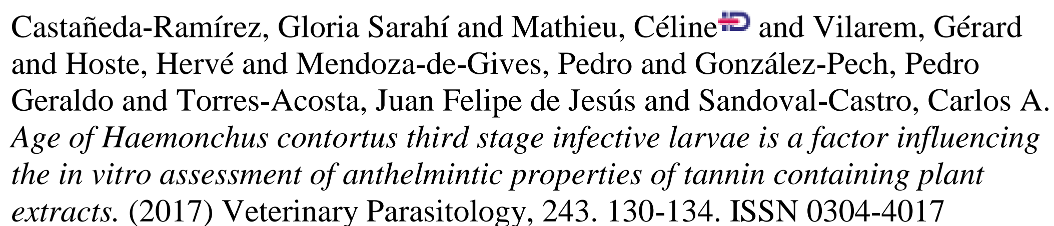
Open Archive Toulouse Archive Ouverte (OATAO)

OATAO is an open access repository that collects the work of Toulouse researchers and makes it freely available over the web where possible

This is an author's version published in: http://oatao.univ-toulouse.fr/25140

Official URL: https://doi.org/10.1016/j.vetpar.2017.06.019

To cite this version:



Castañeda-Ramírez, Gloria Sarahí and Mathieu, Céline and Vilarem, Gérard and Hoste, Hervé and Mendoza-de-Gives, Pedro and González-Pech, Pedro Geraldo and Torres-Acosta, Juan Felipe de Jesús and Sandoval-Castro, Carlos A. *Age of Haemonchus contortus third stage infective larvae is a factor influencing the in vitro assessment of anthelmintic properties of tannin containing plant extracts.* (2017) Veterinary Parasitology, 243. 130-134. ISSN 0304-4017

Any correspondence concerning this service should be sent
to the repository administrator: tech-oatao@listes-diff.inp-toulouse.fr

Age of *Haemonchus contortus* third stage infective larvae is a factor influencing the *in vitro* assessment of anthelmintic properties of tannin containing plant extracts

G.S. Castañeda-Ramírez[a], C. Mathieu[c], G. Vilarem[c], H. Hoste[b], P. Mendoza-de-Gives[d], P.G. González-Pech[a], J.F.J. Torres-Acosta[a,*], C.A. Sandoval-Castro[a]

[a] *Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma de Yucatán, Km 15.5 Carretera Mérida-Xmatkuil, C.P. 97100, Mérida, Yucatán, Mexico*
[b] *INRA UMR 1225 Interactions Hôte Agents Pathogènes, 23 Chemin des Capelles, F31076, Toulouse, France*
[c] *Université de Toulouse, INP-ENSIACET, LCA (Laboratoire de Chimie Agro industrielle), F31030 Toulouse, France*
[d] *Centro Nacional de Investigación Disciplinaria en Parasitología Veterinaria, INIFAP, Carretera Federal Cuernavaca-Cuautla No. 8534, Col. Progreso, Municipio de Jiutepec, Estado de Morelos, CP 62550, Mexico*

A R T I C L E   I N F O

Keywords:
Haemonchus contortus
Age of larvae
Anthelmintic effect
Exsheathment inhibition
Larval motility

A B S T R A C T

The larval exsheathment inhibition assay (LEIA) of infective larvae (L_3) is an *in vitro* method used to evaluate the anthelmintic (AH) activity of tannin-containing plant extracts against different species of gastrointestinal nematodes, including *Haemonchus contortus*. Some conditions remain to be defined in order to standardize the LEIA, i.e. the optimal age of larvae produced from donor animals to use in the assays. Therefore, this study aimed at identifying the effect of age and age-related vitality of *H. contortus* infective larvae produced under tropical conditions, on the *in vitro* AH activity measured with the LEIA. The same acetone:water (70:30) extract from *Acacia pennatula* leaves was used to perform respective LEIA tests with *H. contortus* L_3 of different ages (1–7 weeks). Each week, the L_3 were tested against different concentrations of extract (1200, 600, 400, 200, 100, 40 μg/mL of extract) plus a PBS control. Bioassays were performed with a benzimidazole (Bz) resistant *H. contortus* (Paraíso) strain. In order to identify changes in L_3 vitality on different weeks (1–7), two assays testing larval motility were included only with PBS: the larval migration assay (LMA) and the larval motility observation assay (LMOA). Mean effective concentrations causing 50% and 90% exsheathment inhibition (EC_{50}, EC_{90}) were obtained for every week using respective Probit analyses. On the first week, the larvae had lowest EC_{50} and EC_{90} (39.4 and 65.6 μg/mL) compared to older larvae ($P < 0.05$). The EC_{50} and EC_{90} for weeks 2–5 were similar ($P > 0.05$), while older larvae tended to show higher EC_{50} and EC_{90} ($P < 0.05$). Motility showed strong negative correlations with age of larvae ($r \geq 0.83$; $P < 0.05$) and EC_{50} ($r \geq 0.80$; $P < 0.05$), suggesting that the lower extract efficacy could be associated with decaying vitality of larvae associated with age. More stable efficacy results were found between two to five weeks of age.

1. Introduction

The identification of plants with non conventional anthelmintic (AH) effect against gastrointestinal nematodes (GIN) requires *in vitro* screening methods to enable a quick assessment of plant materials at relatively low cost (Hoste et al., 2008, 2015). Some *in vitro* methodologies have been first developed to test conventional AH drugs against parasite eggs (Coles et al., 1992). They were later adapted to examine the AH effects of crude plant extracts, extract partitions or fractions and even pure metabolites (Vargas Magaña et al., 2014; Von Son de Fernex et al., 2015; Chan Perez et al., 2016). Other *in vitro* methodologies were proposed to screen specifically the AH activity of plant extracts (Bahuaud et al., 2006; Jackson and Hoste, 2010). Whatever the stages of GIN used, and the objective of the *in vitro* assay, the different proposed protocols need to explore ways to improve their standardization in order to better define the conditions of use in each laboratory. The latter is particularly relevant when considering that parasites are biological materials that may show phenotypic variation (Chan Perez et al., 2016).

The larval exsheathment inhibition assay (LEIA) as described by Bahuaud et al. (2006), needs to be adjusted to take into account ambient temperature inside the laboratory, namely at 22 23 °C, to achieve

* Corresponding author.
E-mail addresses: tacosta@correo.uady.mx, jfj.torresacosta@gmail.com (J.F.J. Torres-Acosta).

http://dx.doi.org/10.1016/j.vetpar.2017.06.019

an exsheathment process that lasts 60 min in > 95% of L_3 in the control groups, with the least variation possible. Similarly, other adjustments might be needed, for example the quantity of sodium hypochloride required for every batch of tests performed (Jackson and Hoste, 2010).

The need to adapt experimental protocols to the conditions of dif ferent laboratories is also evident when comparing the time to produce *H. contortus* L_3 from faecal cultures under different environmental conditions. In hot tropical areas, the L_3 of *H. contortus* may be harvested from faecal cultures after four to five days of incubation, while it may take seven to ten days of incubation under the conditions of temperate laboratories. Variability in L_3 development has been suggested for outdoor environmental conditions, where a cooler macro and micro climate slows the development of eggs in the faeces, while warm and humid conditions speed up L_3 development (Liebano, 2011). Such dif ference in speed of L_3 development has been observed even for *H. contortus* eggs cultured with incubators that control humidity and temperature. If speed of larvae development differs, then it is possible that the age at which the L_3 die or loose vitality under hot conditions could also be reached earlier than under temperate conditions, even when maintained under refrigeration (4 5 °C). If that was the case, the LEIA in the tropical conditions would need to be performed with larvae younger than the three month recommendation by Bahuaud et al. (2006). Thus, there is a need for a planned systematical attempt to test the effect of age of larvae on the AH effect recorded for plant extracts measured with LEIA under laboratory conditions. Such evaluation must include changes in L_3 vitality with age, to confirm whether the AH ef fect is associated with vitality. Therefore, this study explored the effect of age and age related vitality of *H. contortus* infective larvae produced under tropical conditions, on the *in vitro* anthelmintic activity measured with the larval exsheathment inhibition assay.

2. Materials and methods

2.1. Production of Haemonchus contortus infective larvae

Two donor lambs (25 ± 1 kg) were raised free of GIN infections, and were kept in individual pens with raised slatted floors before and during the experiment. Prior to their artificial infection with *H. con tortus*, donor lambs were confirmed free of GIN infection using the centrifuge flotation technique and the McMaster technique on faecal samples obtained on three consecutive days. Then, the four month old donor lambs were orally inoculated with 4000 *H. contortus* infective L_3 to obtain a mono specific infection. The *H. contortus* isolate used for this study was “Paraiso”, which is a benzimidazole resistant isolate showing low susceptibility to polyphenol rich plant extracts (Chan Perez et al., 2016). Samples of faeces were obtained 28 days after infection to confirm the presence of *H. contortus* eggs. Animals were fed with a balanced diet based on grass hay and a commercial concentrate feed with *ad libitum* access to water. All the procedures performed on donor animals complied with the ethical standards of the Bioethics Committee (licence No. CB CCBA D 2014 003).

Faecal pellets were collected from donor sheep to harvest eggs. Faecal pellets were washed with tap water to remove grass and other debris. Faecal material was then placed in Petri dishes and were maintained inside an incubator at 28 °C for 5 days. Faecal cultures were moistured every second day using a water sprayer. Infective larvae were harvested using a Baermann apparatus and were stored at 4 °C. The *H. contortus* L_3 were produced once every week during eight consecutive weeks. The larvae were identified by microscopic observation at 100 × magnification according to the keys for identification proposed by Bowman and Lynn (1999). The L_3 were kept in culture flasks for each respective week.

2.2. Production of acetone:water extract from Acacia pennatula leaves

Fresh *A. pennatula* leaves were collected on April 2015 in Xmatkuil, Yucatan, Mexico. The herbarium staff at the Faculty of Veterinary Medicine botanically identified the plant material and a respective voucher number was assigned (No. 20101). The acetone:water extract was produced using 75 g of fresh leaves. Fodder material was grinded and placed inside acetone:water (70:30) containing ascorbic acid (1 g/L) to avoid oxidation. The mix was left to rest for 24 h; subsequently the extract was filtered (paper filter No 50). Acetone was evaporated using a rotovapor (IKA®, Germany). Thereafter, the water fraction was rinsed with methylene chloride in a portion volume 1:1 (two washes) to re move chlorophyll and lipids. The extract was again roto evaporated to eliminate solvent residues. Then extract was lyophilized and stored at 4 °C until bioassays (Alonso Díaz et al., 2008).

2.3. Infective larvae vitality

Two motility tests were used to identify the age related vitality of L_3 from weeks 1 7. In both tests the motile larvae were considered viable, while the non motile larvae were considered non viable. The purpose of these two tests was to use the motility of the larvae as an aging in dicator to interpret any variation in susceptibility to plant extracts.

- Larval migration assay (LMA). This technique evaluated the *H. contortus* L_3 migration without *A. pennatula* extract. The metho dology was based on the procedure described by Wagland et al. (1992), modified by Rabel et al. (1994). The LMA was performed on seven consecutive weeks using larvae of different ages (from weeks 1 7). The methodology was performed as follows. Seven vials were added with 4 mL of the larvae suspension (1000 L_3/mL phosphate buffered saline solution (PBS)), each corresponding to the different ages of L_3 (weeks 1 7). Subsequently, the larvae in the vials were left incubating for 1 h at 23 °C. A 24 multi well plate was used to perform the assay on each week. Three non consecutive rows of 4 wells were added with 2350 µL of PBS for each well. The remaining three non consecutive rows were maintained empty for the process of insert washing. Inserts were placed inside the wells containing the PBS. After incubation with PBS, larvae suspension was homogenized with a vortex. A 150 µL aliquot of larvae suspension from each tube was placed on top of the inserts’ mesh (20 µm) (four repetitions). Multi well plates were placed in an incubator at 28 °C. After 3 h of incubation, inserts were removed from the respective wells. The insert was rinsed with 500 µL PBS and the contents left inside the insert were placed on the respective empty well for every insert. The larvae that migrated through the mesh were recorded as viable, while the larvae failing migration through the mesh were recorded as non viable.
- Larvae motility observation assay (LMOA). The LMOA was per formed once a week with L_3 of different ages (weeks 1 7). For each test, seven vials corresponding to the different L_3 age were prepared with 4 mL of larvae suspension (1000 L_3/mL PBS). Subsequently, the vials were incubated without *A. pennatula* extract for 1 h at 23 °C. After incubation, the contents of the tubes were homogenized with a vortex to obtain two aliquots of 50 µL from each tube (larvae suspension in PBS). The aliquots were placed on slides and larvae were observed at microscope with the 10 x objective (100 × mag nification). The same procedure was repeated in six replicates for each respective week. This process was repeated for 7 weeks. Larvae were classified as motile depending whether they moved (with or without progression) or not within a 3 min observation period. Larvae showing no movement in that period of time were con sidered non motile. For every tube, the number of motile and non motile larvae was determined and the motility percentage was cal culated. A total of 500 ± 100 L_3 were observed per tube to estab lish the percentage.

2.4. Larvae exsheathment inhibition assay (LEIA)

The assays were performed once every week for seven consecutive weeks. Moisture, temperature and general conditions in the laboratory were kept homogenous along the entire experimental period. The only condition that varied was the age of larvae (from 1 to 7 weeks). The LEIA were conducted following the procedure described by Jackson and Hoste (2010). The negative controls used were larvae not treated with extract and only exposed to the PBS. The different concentrations applied to evaluate the AH effect of *A. pennatula* acetone:water extract were 1200, 600, 400, 200, 100, 40 μg/mL.

Stock solution (5000 μg/mL) of acetone:water extract were made in PBS prepared with purified water. One tube was used as negative control containing 1000 μL of PBS without extract. Finally, 1000 μL of infective larvae solution (L_3 ~ 1000/mL) were added to each tube to obtain the final extract concentrations (1200, 600, 400, 200, 100, 40, 0 μg/mL PBS). Infective larvae were incubated with the plant extract for 3 h at 24 °C. After incubation, larvae were centrifuged for 3 min at 168*g* and washed 3 times with PBS solution. Then, aliquots of each larvae solution were placed in eppendorf vials (200 μL each). Four repetitions were performed for each concentration and PBS control. The process of exsheathment was artificially induced by contact with Milton® (Laboratoire Rivadis, France), which is a solution of sodium hypochlorite (2.0%) and sodium chloride (16.5%) diluted in PBS. The quantity of Milton® solution to use for each assay was determined every week by testing different concentrations (25, 30, 35 and 40 μL/6 mL PBS). During the first two weeks the concentration used for the bioassays was 30 μL/6 mL PBS and it was changed to 25 μL/6 mL PBS for the following weeks. The exsheathment kinetic was observed with a microscope using the 10× objective and recorded at 0, 20, 40 and 60 min.

2.5. Calculation of data for the different in vitro assays

The percentage of exsheathment inhibition (EI%) obtained from results of the Larvae Exsheathment Inhibition Assays (LEIA), was calculated using the following two formulae:

$$Exsheathment\ \% = \frac{\text{L3 without sheath}}{\text{L3 with sheath} + \text{L3 without sheath}} \times 100$$

$$EI\% = 100 - \text{Exsheathment}\%$$

For the larval migration assay (LMA), the proportion of migration was determined according to the following formulae:

$$\text{Migration\%} = \frac{\text{Number of migrated L3}}{\text{Number of migrated L3} + \text{Number of L3 that remains on the inserts}} \times 100$$

Similarly, the percentage of larval motility assay (LMOA) was calculated using the formulae:

$$\text{Motility\%} = \frac{\text{L3 active motile}}{\text{L3 active motile} + \text{L3 not motile}} \times 100$$

2.6. Statistical analyses

Exsheathment% inhibitions were analyzed with respective GLM to assess differences between the results of the control PBS and the different concentrations of the *A. pennatula* extract tested within each week.

The results of the respective LEIA performed on different weeks were used to determine the effective concentrations 50% (EC_{50}) and 90% (EC_{90}), with respective 95% confidence intervals (95%CI), using the probit analysis of the Polo Plus 1.0 software (LeOra Software, 2004). The resulting EC_{50} and EC_{90} and their respective 95% CI obtained for each week were compared to test differences depending on the age of larvae. When the respective 95%CI did not overlap then the EC_{50} or the EC_{90} were considered significantly different.

For the LMA and LMOA, percentages of motility and migration, a respective post hoc analysis was performed using the Fisher's least significant difference (LSD) to assess differences between ages (weeks).

Spearman correlation tests were performed between age (week) of larvae and the respective LMA or LMOA weekly averages. Moreover, a Spearman correlation between the different values of the two motility tests (LMA and LMOA) was also determined. Lastly, respective correlations between EC_{50} values of LEIA and motility (LMA or LMOA) values were also assessed. The correlation analyses were performed using Statgraphics Centurion XV (Statpoint Technologies Inc., 2005).

3. Results

3.1. Effect of L_3 age on exsheathment inhibition percentage

Table 1 shows the influence of L_3 age on the EI% at different concentrations of the *A. pennatula* acetone:water extracts. When larvae were between 1 4 weeks, the *A. pennatula* extract caused significant exsheathment inhibition ($P < 0.05$) from the lowest concentration tested (40 μg/mL). Also, on weeks 1 4 the EI% reached 100% at a concentration of 400 μg/mL. Meanwhile, the EI% of older larvae, from five to seven weeks of age, did not reach more than 77.7% at a concentration of 400 μg/mL. Furthermore, the EI% of seven week old larvae only reached 93.5% at the highest extract concentration tested (1200 μg/mL), with an increased proportion of L_3 exsheathing spontaneously (4.9 ± 2.4%).

3.2. Effect of L_3 age on the effective concentrations of *Acacia pennatula* extract

The mean EC_{50} and EC_{90} of the *A. pennatula* extract used to induce the exsheathment inhibition of *H. contortus* L_3 on different weeks of age are shown in Table 1. The EC_{50} and EC_{90} against one week old larvae were significantly lower than any other found for older larvae. The EC_{50} and EC_{90} tended to remain similar for larvae between 2 and 5 weeks of age. From the sixth week of age, the EC_{50} and EC_{90} became significantly higher than for previous weeks.

3.3. Effect of L_3 age on larval migration and larval motility

The motility observed for L_3 from 1 to 7 weeks of age using two different procedures, the LMA and LMOA, is presented in Table 2. Both procedures showed that L_3 were significantly more motile on week one (93.37% for the LMA and 99.87% for the LMOA; $P < 0.05$). Similarly, motility of larvae on week 2 was again significantly higher than L_3 of older ages ($P < 0.05$). The motility of L_3 was lower than 70% on week 4 for the LMA and that same threshold was reached for larvae on week 6 for the LMOA. Finally on the seventh week of age, the larvae motility reached 56.6% and 52.66% for the LMA and LMOA respectively ($P < 0.05$).

3.4. Correlation between larvae motility and age of L_3 or extract EC_{50} values

Negative correlations were found between age of larvae and their motility measured either with the LMA ($r = -0.83$; $n=52$; $P \leq 0.05$) or the LMOA ($r = -0.92$; $n = 52$; $P \leq 0.05$). Also, a negative correlation was found between the extract's EC_{50} values and the L_3 motility measured either with the LMA ($r = -0.80$; $n = 7$; $P < 0.05$), or the LMOA ($r = -0.85$; $n = 7$; $P < 0.05$). Finally, a positive correlation was observed between the motility values obtained with the LMA and the motility values measured with the LMOA ($r = 0.88$; $n = 52$; $P < 0.05$).

Table 1
Exsheathment inhibition percentage (EI%) (mean ± SEM) caused by different concentrations of the *Acacia pennatula* acetone:water (70:30) extract (leaves) on *Haemonchus contortus* L_3 at different weeks of age (one to seven weeks) and the resulting mean effective concentrations (EC_{50} and EC_{90}) with 95% confidence intervals (95%CI) needed to induce exsheathment inhibition.

Concentration [µg/mL]	Proportion of L_3 showing exsheathment inhibition on different weeks of age						
	1	2	3	4	5	6	7
PBS	2.9	5.6	4.6	8.7	6.8	7.8	6.6
40	86.8*	26.7*	13.9*	48.1*	9.2	5.7	6.1
100	100*	27.8*	13.5*	52.2*	18.6	15.4	14.7
200	100*	60*	26.7*	67.7*	37.3*	15.4	29.4*
400	100*	100*	100*	100*	77.7*	39.5*	75.4*
600	100*	100*	100*	100*	99.1*	95.2*	85.6*
1200	100*	100*	100*	100*	100*	99.1	93.5*
SEM	1.7	1.1	1.2	6.2	5.6	3.9	4.3
EC_{50} and EC_{90} resulting from exposure of L_3 to the plant extract on different weeks of age							
	1	2	3	4	5	6	7
EC_{50}	39.4 a	207.5 b	258.1 b	219.8 b	263.1 b	418.1 c	283.2b, c
95%CI	30.5–43.2	176.1–232.9	231.6–277.8	172.9–251.8	187.1–308.5	314.8–496.4	197.3–357.3
EC_{90}	52.1 a	304.8 b	344.9b	328 b	454.3b	815.3 c	804.8 c
95%CI	48.9–57.0	264.5–433.6	319.8–386.6	282.3–473.4	404.2–541.3	683.8–1099.8	619.3–1278.6
R^2	93.59	92.78	90.54	90.06	99.48	91.29	83.44

Different letters between effective concentrations (EC_{50} and EC_{90}) indicate significant differences ($P < 0.05$).
SEM: Standard Error of Mean.
R^2: Coefficient of determination.
* Indicates significant differences within column compared to respective PBS controls on each week ($P < 0.05$).

4. Discussion

The present study aimed at identifying the effect of the age of L_3 larvae and the age related vitality on the anthelmintic effect recorded for a plant extracts measured with the LEIA under laboratory conditions in the tropics. This study used a single acetone:water (70:30) plant extract of *A. pennatula* and included two tests to evaluate larvae motility as indicators of the L_3 vitality.

Results presented in Table 1 showed that *H. contortus* L_3 were more sensitive to the *A. pennatula* extract in its first week of age, when the lowest extract concentration (100 µg/mL) caused a 100% inhibition of exsheathment. Meanwhile, the larvae of 2 4 weeks reached 100% in hibition from 400 µg/mL and the 5 week larvae needed 1200 µg/mL to reach 100% exsheathment inhibition. After that age, the larvae needed to be exposed to higher concentrations of extracts to show a total ex sheathment inhibition effect. Thus, the evidence shown in Table 1 suggests that the inhibition of exsheathment caused by the plant extract differs with the L_3 age. To the best of our knowledge, no other studies have explored the effect of L_3 age on the outcome of an *in vitro* test. Other factors are known to affect the inhibition of exsheathment ob tained with the LEIA *in vitro* tests besides the bioactive plant secondary metabolites of plant extracts. For example, recent studies using LEIA showed that *H. contortus* susceptibility towards tannin rich extracts differed between the parasite isolates, with some isolates showing higher EC_{50} than other even when exposed to exactly the same plant extracts (Chan Perez et al., 2017). Also, the required concentrations of sodium hypochloride needed to promote L_3 exsheathment in controls can change with the L_3 age or between parasite isolates. Thus, the quantity of sodium hypochloride must be confirmed before performing LEIA (Jackson and Hoste, 2010).

The EC_{50}% and EC_{90}% of the *A. pennatula* extract were estimated for L_3 at different weeks of age to confirm that the exsheathment inhibition result varied with the age of L_3 (Table 1). It is important to remind that all the larvae were exposed to the same extract to avoid any variation due to extract biological activity. The one week old L_3 showed the lowest EC_{50} and EC_{90} compared to all the other ages tested. Meanwhile, L_3 of ages between 2 and 5 weeks showed similar EC_{50}% and EC_{90}% when exposed to the *A. pennatula* extract ($P > 0.05$). Thus, it seems reasonable to suggest that the exsheathment inhibition test should be performed with 2 to 5 week old L_3. Older larvae (6 or 7 week old) resulted in significant increments either in the EC_{50}% or EC_{90}%. Fur thermore, L_3 older than 5 weeks of age showed a wide 95% CI for the EC_{90}, which is undesirable for the assay.

It is not known why the ability of the extract to inhibit exsheath ment of L_3 changes with larvae age. We propose that one aspect that might be involved in the L_3 exsheathment effect of the extract is related to the vitality of larvae. However, there is no method to evaluate L_3 vitality. Thus, the present study implemented two larvae motility measurements, the LMA or the LMOA, as indirect measurement of L_3 vitality. Similar to what was observed for the extract efficacy, larval motility was reduced for older L_3 (Table 2). To the best of our knowl edge, this constitutes the first report of the effect of L_3 age on its

Table 2
Mean percentages (± SEM) of migration and motility recorded for *Haemonchus contortus* L_3 larvae at different ages (weeks 1–7) using the larval migration assay (LMA) or the larval motility observation assay (LMOA).

Assay	Weeks of age							
	1	2	3	4	5	6	7	SEM
LMA	92.37 a	80.63 b	70.85 b, c	67.24 c	57.46 d	56.99 d	56.62 d	10.00
LMOA	99.86 a	91.36 b	82.08 c	77.77 c, d	70.78 d, e	66.08 e	52.66 f	7.06

Means with different letters in the same row are different at $P < 0.05$.
SEM: Standard error of means.

motility. The highest motility was displayed for the youngest larvae on week 1 ($P < 0.05$), followed closely by L_3 of 2 weeks of age that were also more mobile than older L_3. Motility was lower than 70% on week 4 when using the LMA and on week 6 for the LMOA. The motility on week 7 was lower than 57% for both methods.

A negative correlation was also found between the extract EC_{50} of LEIA recorded for each week of age and values of L_3 motility measured with LMA or LMOA. Such negative correlations represent a salient feature of the present study. It was hypothesized that "older" L_3 with lower vitality might be already primed to exsheath more easily than "younger", more motile L_3. Therefore, higher quantity of extract is re quired to inhibit the exsheathment of older L_3. The infective L_3 must exsheath in order to continue the parasitic life cycle. Hence, older larvae, with lower energy reserves, might have already accomplished some physiological "steps" to facilitate exsheathment either in the rumen or in the abomasum, depending on the anatomical location of the nematode species (Hertzberg et al., 2002). Thus, in spite of the reduced motility/vitality of L_3, a high exsheathment was observed for surviving old larvae. Even more, nearly 5% of L_3 exsheathed sponta neously in the control groups incubated with PBS on weeks 6 or 7. It could be interesting to compare the morphological changes induced by the *A. pennatula* extract on the L_3 of different ages to identify any as sociation between age and damage to the larvae sheath. Furthermore, it could be interesting to confirm whether other plant extracts, containing tannins of different structure and size, may also show variations in exsheathment inhibition efficacy when using L_3 of different age. Fi nally, variation in the exsheathment inhibition due to L_3 age should be compared between different *H. contortus* isolates. Comparison of iso lates should be performed in a single location to avoid variations due to laboratory conditions or conditions of donor animals.

This study represents the first evidence indicating the importance of L_3 age on the evaluation of the AH activity of plant extracts using the LEIA. As a direct consequence, some basic recommendations are pro posed when implementing the LEIA in different laboratories:

- Sheath must be present in > 97% L_3 before starting the LEIA. As it was mentioned above, a proportion of L_3 are already exsheathed on the 7th week of age in the control groups (4.9 ± 2.4%). Thus, larvae of such age may not be used for the LEIA.
- Motility of L_3 should be at least 65% before performing the LEIA. Larvae showing motility above that threshold produced more stable EC_{50}% for LEIA. Motility could be confirmed either with the LMA or the LMOA described in the present study.

Recent evidence indicates that *H. contortus* isolates obtained from different geographical regions may show variations in their sensitivity towards plant extracts (Chan Perez et al., 2016). In the light of that study it is essential to avoid confounding factors such isolate origin and the strong effect of L_3 age when the LEIA is implemented. This will help to make more reliable and reproducible bioassays.

5. Conclusion

The age of *H. contortus* L_3 affects the *in vitro* assessment of anthel mintic properties of plant extracts. Older L_3 required higher extract EC50 and EC90 to inhibit exsheathment. More stable efficacy results were found between 2 5 weeks of age. Using L_3 of such age range may improve repeatability and reduce variability in the bioassay results. The strong negative correlations between EC_{50} EC_{90} data and larvae mo tility suggest that variation in the former might be associated with decaying vitality of the larvae.

Conflict of interest

None.

Ethical standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and in stitutional guides on the care and use of laboratory animals (licence No. CB CCBA D 2014 003).

Acknowledgments

This work was financed by CONACYT Mexico (Project CB 2013 01 221608) and CONACYT PCP No. 229330 between Mexico France. The first authors (G.S. Castañeda Ramírez) acknowledge the scholarship from CONACYT to undergo PhD studies.

References

Alonso-Díaz, M.A., Torres-Acosta, J.F.J., Sandoval-Castro, C.A., Aguilar-Caballero, A.J., Hoste, H., 2008. *In vitro* larval migration and kinetics of exsheathment of *Haemonchus contortus* larvae exposed to four tropical tanniniferous plant extracts. Vet. Parasitol. 153, 313–319.

Bahuaud, D., Martínez-Ortiz de Montellano, C., Chaveau, S., Prevot, F., Torres-Acosta, F., Fouraste, I., Hoste, H., 2006. Effects of four tanniferous plant extracts on the *in vitro* exsheathment of third stage larvae of parasitic nematodes. Parasitology 132, 545–554.

Bowman, D.D., Lynn, R.C., 1999. Georgis' Parasitology for Veterinarians, 7th edition. W.B. Saunders Company, Philadelphia, USA, pp. 320–324.

Chan-Perez, J.I., Torres-Acosta, J.F.J., Sandoval-Castro, A.C., Hoste, H., Castañeda-Ramírez, G.S., Vilarem, G., Mathieu, C., 2016. *In vitro* susceptibility of ten *Haemonchus contortus* isolates from different geographical origins towards acetone:-water extracts of two tannin rich plants. Vet. Parasitol. 217, 53–60.

Coles, G., Bauer, C., Borgsteede, F., Klei, T., Taylor, M., Waller, P., 1992. Methods for the detection of anthelmintic resistance in nematodes of veterinary importance. Vet. Parasitol. 44, 35–44.

Hertzberg, H., Huwyler, U., Kohler, L., Rehbein St Wanner, M., 2002. Kinetics of exsheathment of infective ovine and bovine strongylid larvae *in vivo* and *in vitro*. Parasitology 125, 65–70.

Hoste, H., Torres-Acosta, J.F.J., Alfonso-Díaz, M.A., Brunet, S., Sandoval-Castro, C., Adote, S.H., 2008. Identification and validation of active plants for the gastrointestinal nematode in small ruminants. Trop. Biomed. 25, 56–72.

Hoste, H., Torres-Acosta, J.F.J., Sandoval-Castro, C.A., Mueller-Harvey, I., Sotiraki, S., Louvandini, H., Thamsborg, S.M., Terrill, T.H., 2015. Tannin containing legumes as a model for nutraceuticals against digestive parasites in livestock. Vet. Parasitol. 212, 5–17.

Jackson, F., Hoste, H., 2010. *In vitro* methods for primary screening of plant products for direct activity against ruminant nematodes. In: Vercoe, P.E., Makkar, H.P.S., Schlink, A.C. (Eds.), *In Vitro* Screening of Plant Resources for Extra-nutritional Attributes in Ruminants: Nuclear and Related Methodologies. Springer, pp. 25–45.

LeOra Software, 2004. Polo Plus. Probit and Logit Analysis. LeOra Software, Berkeley, California, U.S.A.

Liebano, H.E., 2011. Ecología de larvas de los nematodos gastrointestinales de bovinos, ovinos y caprinos. In: Quiroz-Romero, H., Figueroa-Castillo, J.A., Ibarra-Velarde, F., López-Arellano, M.E. (Eds.), Epidemiología de enfermedades parasitarias en animales domésticos, pp. 254–272.

Rabel, B., Mcgregor, R., Douch, P.G.C., 1994. Improved bioassay for estimation of inhibitory effects of ovine gastrointestinal mucus and anthelmintics on nematode larval migration. Int. J. Parasitol. 24, 671–676.

Statpoint Technologies Inc, 2005. Statgraphics Centurion XV. Statpoint Technologies Inc., Warranton, Virginia, U.S.A.

Vargas-Magaña, J.J., Torres-Acosta, J.F.J., Aguilar-Caballero, A.J., Sandoval-Castro, C.A., Hoste, H., Chan-Perez, J.I., 2014. Anthelmintic activity of acetone-water extracts against *Haemonchus contortus* eggs: interactions between tannins and other plant secondary compounds. Vet. Parasitol. 206, 322–327.

Von-Son-de-Fernex, E., Alonso-Díaz, M.A., Mendoza-de-Gives, P., Valles-de-la-Mora, B., González-Cortazar, M., Zamilpa, A., Epigmenio-Castillo, G., 2015. Elucidation of Leucaena leucocephala anthelmintic-like phytochemicals and the ultrastructural damage generated to eggs of Cooperia spp. Vet. Parasitol. 214, 89–95.

Wagland, B.M., Jones, W.O., Hribar, L., Bendixsen, T., Emery, D.L., 1992. A new simplified assay for larval migration inhibition. Int. J. Parasitol. 22, 1183–1185.