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Survival, physical and physiological changes of *Taenia hydatigena* eggs under different conditions of water stress

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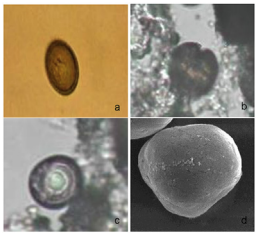
HIGHLIGHTS

- The physical and physiological changes of *T. hydatigena* eggs exposed at different conditions of water stress are described.
- The formation of clumps and the use of triacylglycerols are key to long-term eggs resistance to these stress conditions.
- The findings shown herein provide bases to better comprehend basic biology and epidemiology of *T. hydatigena*

GRAPHICAL ABSTRACT



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ABSTRACT

Taenia hydatigena eggs were investigated for morphological and physiological changes under water stress conditions. Fresh eggs were exposed at 31%, 47% and 89% of relative humidity (RH), and survival, size and ultrastructural changes were accounted up to 365 days of exposition. The article shows how each RH environment affects the vitality of the eggs. Results of this study suggest that *T. hydatigena* eggs have mechanisms to withstand water stress, indicating that the eggs clustering improves protection against desiccation, and that endogenous metabolism using triacylglycerols play an important role in the maintenance of embryo vitality under low, medium and high relative humidity conditions. This contributes to understanding the water stress resistance mechanism in eggs belonging to Taeniidae family. The findings shown herein have provided a basis to better comprehend basic biology and epidemiology of the cysticercosis caused by *T. hydatigena*.

1. Introduction

Taenia hydatigena is a cosmopolitan and widespread parasite found in of canids that can infect a wide range of mammals when it is in the larval stage, the *Cysticercus tenuicollis.* This parasite has both veterinary and economic consequences since it is a source of economic loss for the meat industry. It has been estimated that the

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total economic loss due to liver lambs condemnation amounts to € 333, 657.7 per year, affecting 14.6% of 30–40 day-old lambs with cysticercosis in Sardinia, Italy (Scala et al., 2015).

T. hydatigena egg is the free-living stage of the cestode. It has been accepted that temperature, relative humidity and sunlight are some of the most important abiotic factors controlling egg survival in Taeniidae (Matsumoto and Yagi, 2008; Wachira et al., 1991; Gemmel et al., 1987; Willis and Herbert, 1984; Colli and Williams, 1972; Sweatman and Williams, 1963). Until now several studies have focused mainly on the temperature deleterious effect to taeniid eggs (Silverman, 1956; Laws, 1968; Coman, 1975; Buttar et al., 2013). Water stress eggs tolerance has been investigated in relatively few species of the Taeniidae family mostly focusing on *Echinococcus* genus (Veit et al., 1995; Gemmel et al., 2001) and some on *T. pisiformis*. (Coman,1975; Coman and Rickard, 1977). Many of these studies have covered either a short period of observation or only extreme relative humidity conditions (0% and >95% of relative humidity).

In Patagonia region, one of the most important environmental stresses facing taeniid eggs in nature is desiccation. Our previous studies revealed that eggs *Echinococcus granulosus* exhibited high persistence under environmental conditions in the inferior arid climate of Chubut Province (Patagonia, Argentina) and that they were still able to infect sheep after 41 months of ageing (Sánchez Thevenet et al., 2003; Thevenet et al., 2005). In this province, *T. hydatigena* has a prevalence of 25% in dogs, and in relative natural humid conditions varied from a minimum of 17% to a maximum of 99% (INTA, 2016).

Desiccation resistance is defined as the capacity of any organism to withstand an arid environment without loss viability. The resistance to desiccation can be related to both physical features and physiological strategies. Numerous researchers have shown that the organism responds to individual morphological changes and or collective structures to water stress. In Culicidae, widespread genus of mosquito eggs, size and structure of eggshell layers plays an important role in egg resistance to desiccation (ERD) (Farnesi et al., 2015). In eggs of the ticks *Boophilus microplus* and in larvae of the Antartic midge *Belgica antarctica* it has been demonstrated that forming aggregations is one of the mechanisms to drought acclimation (Gibbs et al., 1997; Benoit and Denlinger, 2007). In nematodes, coiling and clump formation are evidenced as adaptations to desiccation (Wharton, 1996). There are few studies regarding the ultrastructure of the eggs of cestodes and the majority of these studies focus on other affecting factors rather than the desiccation of free living parasites (Wang et al., 1981; Morseth, 1965; Nieland, 1968; Chomicz et al., 1995).

From a physiological point of view, in organisms such as insects and bacteria, the presence, the amount and the endogenous metabolism of metabolites such as glycerol trehalose, glycogen and triacylglycerol (TAG) were demonstrated to be the key factors with regards to its water stress resistance (Gray and Bradley, 2005; Alvarez et al., 2004). As well in cestodes it has been postulated that TAG plays an important role in the maintenance of embryo viability in *T. hydatigena* eggs (Sánchez Thevenet et al., 2010).

The understanding of the mechanisms of resistance to water stress conditions of free living stages as in the representative example of *Taenia* organisms is of great importance for predicting parasite activities in the environment and for establishing effective prevention and control measures in the field. In this context, our study attempts to contribute further information about whether the eggs have the ability to survive under water stress conditions and to elucidate some of the mechanisms involved in confronting this environmental challenge. The aims of the study were (i) to evaluate the effect of different relative humidity conditions on *T. hydatigena* eggs survival, (ii) to determine and quantify effect of moisture level on the physical features of eggs and, (iii) to evaluated the hypothesis that TAG have a key role in the physiological response to water stress in taeniid eggs.

2. Materials and methods

2.1. Source of Taenia hydatigena eggs

They were obtained from naturally infected dogs which were deparasited by oral administration of arecoline hydrobromide, from the Chubut Province Argentinian patagonic regions of Comodoro Rivadavia (45° 52′ 0″ S, 67° 30′ 0″ W), Esquel (42° 54′ 0″ S, 71° 19′ 0″ W), and Paso de Indios (43° 54′ 0″ S, 69° 4′ 0″ W). The gravid worms were preserved in a sterile 0.85% sodium chloride solution. Eggs were collected from the gravid proglottids washed with sterile 0.85% sodium chloride and centrifuged at 370 g for 10 min twice in a Cavour® (VT 3216D model) centrifuge (Heath and Lawrence, 1981). Then, they were counted by light microscopy in a Fuchs-Rosenthal chamber (Marienfeld-Superior®) and resuspended in sterile 0.85% sodium chloride solution at a final concentration of 203 eggs/μl (initial suspension, Si).

2.2. Experimental conditions

Fresh *T. hydatigena* eggs were exposed to different relative humidity conditions in glass desiccators (Numak®) with humidity adjusted to low (System 1, S1), medium (S2) and high (S3) relative humidity (RH) conditions (Table 1). Humidities environment were established with 200 ml of 42.8% (S1). 34.7% (S2) and 7% (S3) saturated salt solutions of potassium hydroxide in distilled water according to Pandey et al. (1993). Solutions were renewed monthly. 50 μl of the Si suspension per tube was aliquoted into each of the 45 sterile plastic tubes of 1.5 mm of diameter and 10 cm of height. Fifteen tubes were used in each treatment (S1, S2, and S3) and put into the glass desiccators and maintained for 365 days under light protected conditions at 19.5 °C (S.D. 2.37 °C). Temperature and RH was done using a digital hygrometer/thermometer (TFA/Germany).

The eggs were analyzed before and after the exposure by optical and scanning electron microscopy (MEB) and for neutral lipid and vitality studies. Those studies were performed at different time of exposition: initial time (T0), 24 h (T1), 48 h (T2), 72 h (T3), 96 h (T4), 7 days (d, T5), 15 d (T6), 35 d (T7), 49 d (T8), 90 d (T9), 121 d (T10), 185 d (T11). 210 d (T12), and 365 d (T13). In the case of the vitality study a total of 300 eggs were evaluated at each time at each system and of the morphology study by optical microscopy were evaluated 90 eggs at each time at each system a total of. To a particular case of MEB studies observations were made at: 0 h (T0), 35 d (T7). 90 d (T9), and 210 d (T12). And the lipid study was performed before and after 210 days (T12) of ageing.

Table 1
Different values of relative humidity (RH) conditions used to expose *T. hydatigena* eggs. Values of RH are expressed in %.

Statistical descriptor	S1	S2	S3
$\bar{x}$	30.79	46.88	89.20
S.D	2.52	4.59	3.45
Md	31	45	89
Mo	32	44	89
C. V	0.082	0.098	0.037

Mean ($\bar{x}$). Median (Md). Mode (Mo). Standard deviation (S.D). Coefficient of Variation (C.V). S1, system 1. S2, system 2. S3, system 3.

2.3. Evaluation of the vitality of T. hydatigena eggs

The evaluation of the vitality of fresh and of exposed eggs to different RH conditions was tested for their ability to exclude 0.1% aqueous trypan blue as described by Ciarmela et al. (2005) in terms of vitality associated with the ability to prevent the dye staining. In this way we consider that eggs that did not change color remain vital and the dead ones were stained blue because by loss of vitality.

2.4. Egg lipid analysis

To characterize the changes of neutral lipid fractions it has been prepared suspensions of fresh and exposed eggs of *T. hydatigena*, by counting by light microscopy in a Fuchs-Rosenthal chamber (Marienfeld-Superior®) and resuspended in sterile in sterile 0.85% sodium chloride solution at a final concentration of 40 eggs/μl. The eggs were then separated by centrifugation at 500*g* for 3 min and lipids were extracted with chloroform and methanol (2:1. v/v) at 4 °C for 1 h, maintaining a 10:1 extraction solution:sample relationship. The lipids were separated by HPTLC by High Performance Thin Layer Chromatography (HPTLC) as previously described by Sánchez Thevenet et al. (2010).

2.5. Light microscopy

Three 50 μl aliquots of the initial suspension (Is) were placed on perfectly clean and degreased glass slides and the microdroplet water was evaporated at room temperature. Each slide was placed in the chamber under the different RH conditions previously described. The major diameter (MD) and minor diameter (md) of the eggs was measured at each defined time of exposure using an optical microscope at 450x. The observations were photographically documented using a Zeiss-Axioplan microscope with a Yashica camera.

2.6. Scanning electron microscopy

Three sterile plastic tubes each containing 0.25 ml of S1 were placed under each RH condition. Tubes were withdrawn at 35, 90 and 210 days of exposure respectively and the eggs were processed following the phytoplankton net technique for SEM of *T. hydatigena* eggs used by Sarmiento et al. (2006). All the samples were mounted in a double-sided carbon tape and gold metallidingin a Joel Fine Ion Sputter JCF 100. Observations and photomicrographs were obtained with a SEM JMS-T 100 and with JEOL Model 6360 LV SEM.

2.7. Statistical analysis

Determinations were done in triplicate in each different time. A total of 12.000 eggs were studied during the total exposition period of 365 days. The percentage of viable eggs was calculated and expressed as means and standard deviation. For dispersion forecasts around the mean, as a measure of dispersion, quartiles were used. Differences in vitality were studied by Variance Analysis. We carried out 3600 measurements of each diameter (minor and major) on the *T. hydatigena* eggs, 90 measurements on fresh eggs and 3510 measurements on water stress post-exposure eggs. The differences in diameter were analyzed with Student's *t*-test.

Univariate relationships were studied by Pearson correlation. P values < 0.05 were considered as significant. The software used was Instat V2.02 and Sigma Plot 4.0.

3. Results

3.1. Effect of the relative humidity on the vitality of Taenia hydatigena eggs

After 365 days of exposure to all three imposed RH conditions, viable and non-viable *T. hydatigena* eggs were recovered (Fig. 1, Table 2). The regression analysis (Fig. 2) showed the following results: r = - 0.967 for 31%RH ($p < 0.01$), r = - 0.965 for 47%RH ($p < 0.01$) and r = - 0.858 for 89%RH ($p = 0.01$). The variance analysis carried out to compare the vitality values for the eggs across the different RH regimes revealed that the differences observed were statistically significant ($p < 0.01$ S1 31%RH *vs* 47%RH, $p < 0.01$ 31%RH *vs* 89%RH y $p < 0.05$ 47%RH *vs* 89%RH).

3.2. Effect of the relative humidity on the morphology of Taenia hydatigena eggs

There was variation in the major and minor diameters of the eggs after 365 days of exposure to the three RH conditions imposed (Table 3). The intra-regime difference between initial and final values (T0 *vs.* T365 days) in the md and MD of the eggs analyzed was significant in those eggs exposed to 31%RH and 47%RH: p(md. S1) = 9.343×10^{-4}, p(MD. S1) = 4.321×10^{-12}, p(Dm. S2) = 4.622×10^{-6}, p(Dm. S2) = 1.458×10^{-6}. It was not significant for eggs exposed to 89%RH (pmd. = 0.565 and pMD = 0.113). In terms of the differences observed in the size of eggs exposed to 31% RH and 47%RH, we observed a significant difference between minor and major diameters in the case of exposure to 89%RH throughout most of the exposure time (Table 3).

Although intact eggs in good morphological condition were observed under all three RH regimes throughout the period of analysis, eggs exposed to 31%RH and 47%RH started to show alterations on the outer shells consisting of partial or complete cracking and deformation over the exposure time (Fig. 3). At 31%RH and 47%RH eggs were seen to be closer to each other in relation to the distribution observed at 89%RH. Under low and medium RH exposure, the bulk of the viable eggs were grouped and laid on a matrix.

3.3. Ultra-structure of fresh and exposed to water stress Taenia hydatigena eggs

The SEM of the *T. hydatigena* fresh eggs showed round or oval intact eggs and eggs with transverse sections (Fig. 4). In the case of fresh and intact eggs the outer membrane covering the embryo was slightly smoothed and showed numerous holes (Fig. 4). The images of cracked fresh eggs showed that the medium layer of the embryophore is formed by sub-units whose length is superior to

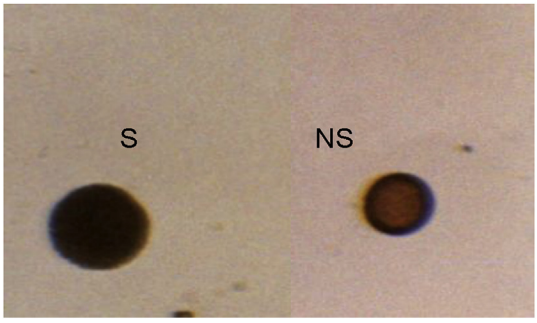

Fig. 1. Damaged and not damaged *T. hydatigena* eggs recovered after 365 days of exposition at 31% of relative humidity. Eggs stained (S) or not (NS) with Trypan Blue.

Table 2
Percentage[a] of vital eggs of *T. hydatigena* exposed at different conditions of relative humidity (RH) during 365 days. N = 12,000.

RH (%)	Days of exposition													
	0	1	2	3	4	7	15	35	49	90	121	185	210	365
31	94 ± 1.531	93 ± 1.15	92 ± 2.89	87 ± 3.51	81 ± 4.51	80 ± 6.35	79 ± 5.69	76 ± 4.00	71 ± 4.16	70 ± 0.58	63 ± 7.52	49 ± 1.73	46 ± 3.06	39 ± 1.00
47	94 ± 1.53	93 ± 3.06	93 ± 4.16	91 ± 4.04	87 ± 2.89	87 ± 4.00	82 ± 5.57	80 ± 1.53	85 ± 2.77	76 ± 2.31	71 ± 1.53	70 ± 1.16	65 ± 2.08	62 ± 2.52
89	94 ± 1.53	94 ± 3.21	94 ± 2.00	92 ± 2.89	86 ± 5.20	87 ± 2.89	87 ± 1.15	84 ± 1.53	82 ± 7.51	86 ± 3.61	87 ± 2.52	86 ± 3.00	82 ± 2.89	81 ± 1.00

[a] All values are expressed as mean ± standard deviation of three replicates.

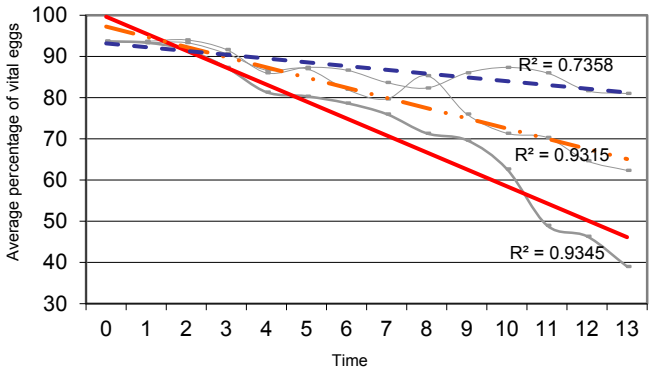

Fig. 2. Decline in the vitality of *T. hydatigena* eggs exposed at three different conditions of relative humidity during 365 days. N = 4000 eggs by system.
—: 31%RH, — · —: 47%RH, - - -: 89%RH. T0: pre-exposed time. T1 and following exposition time: T1: 24 h, T2: 48 h, T3, 72 h, T4: 96 h, T5: 7 days (d), T6: 15 d, T7: 35 d, T8: 49 days, T9: 90 d, T10: 121 days, T11: 185 d, T12: 210 d y T13: 365 d.

Table 3
Minor (Dm) and major (DM) diameter of *T. hydatigena* pre (T0) and post exposed eggs, during 365 days (T365), at three different relative humidity (RH) conditions. N = 360.

	RH		
Diameter[a] (μm)	31%	47%	89%
Dm (T0)	30 (1)[1]	30 (1)	30 (1)
Dm (T365)	27 (2)	28 (2)	30 (2)
DM (T0)	34 (2)	34 (2)	34 (2)
DM (T365)	30 (2)	31 (2)	33 (2)

T: time.
[a] All values are expressed as mean ± standard deviation of three replicates.

their width, their average length being 2.61 μm (Range, R: 2.50–2.71 μm). These sub-units tend to be irregular tetragonal to hexagonal structures (Fig. 5). Numerous pores with an average size of 0.12 μm (R: 0.08–0.15 μm) are observed in the inner part of the sub-units (Fig. 5). The number of pores ranged between 5 and 9 per sub-unit and they were mainly located on the lower two-thirds area of these sub-units. Amorphous content is observed inside some of these pores. The inner part of the embryophore is rough and granular and also has pores connected to the inside part. The oncosphere is surrounded by the afore mentioned structures and in terms of its geometry it is spherical or semi-spherical with a multicellular aspect (Fig. 4).

As to the eggs exposed to the different RH regimes over 35, 90 and 210 days, we observed spherical or oval intact eggs with characteristics similar to those of fresh eggs. At 90 post-exposure days some of the eggs subject to 31%RH and herb47%RH showed irregular polygonal shapes and a reduced size when compared to eggs exposed to 89%RH (Fig. 6). The outer membrane showed cracking and cracked eggs showed a detached inner membrane and deformation of the sub-units of the embryophore (Fig. 7). These alterations were also observed on eggs exposed to low and medium RH after 210 days of exposure. In the case of cracked eggs, starting at 90 days of exposure to the three RH conditions, the pores of the sub-units of the embryophore are seen to be located mainly on the medium and upper third area of these sub-units, which showed an increase in length when compared to their length on fresh eggs. This length reached a maximum average value of 3.10 μm (D. E. 0.34 μm) in the sub-units of the eggs exposed to 89%RH/210 days. The micrographs corresponding to 90 days of exposure to 31%RH and 47%RH showed that the majority of eggs which conserved their morphology and structure are grouped and enclosed by an aggregation substance (Fig. 8). The amount of aggregation substance increases over time (Fig. 9). This matrix adapted to the shape of the eggs and was connected to the external surface of the embryophore (Fig. 10). The outer holes of the eggs embryophore are gradually covered by this substance and become increasingly less visible. The groupings described formed a compact mass and continued to be observed on the micrographs corresponding to 210 days of exposure.

Consistent with the observations on a microscopic scale, the macroscopic aspect of the suspensions prepared to obtain the eggs' micrography differed according to the RH regime to which the eggs to be studied had been exposed to. S1 suspensions looked turbid and featured a great number of whitish lumps. S2 suspensions had an aspect similar to that of S1 suspensions and had a moderate number of whitish lumps. S3 suspensions were translucent and contained fine particles (Fig. 8).

3.4. Neutral lipids in Taenia hydatigena eggs exposed to different conditions of relative humidity

Six fractions of neutral lipids were detected, corresponding to esterified cholesterol (Rf 0,93), free cholesterol (Rf 0,15), triacylglycerides (Rf 0,64), diacylglycerides (Rf 0,09), free fatty acids (Rf 0,35) and an unidentified fraction which remained at the point of sowing. After 210 days of exposure, the TAG fraction had disappeared in eggs aged under the three conditions of RH, a fraction observed in fresh eggs. This disappearance was more obvious in the case of eggs exposed to 31%RH and 47%RH when compared to eggs exposed to 89%RH (Fig. 11).

4. Discussion

The larva, adult and egg stages of *T. hydatigena* are potential targets of cysticercosis control, the egg being the less well-known stage. In this context, this study contributes information in terms of the potential effect on these eggs of a key environmental determinant such as relative humidity.

Taeniidae eggs represent the free life stage of these cestodes and are subject to the demands of the external environment (Herbert et al., 1984). Low or moderate temperatures and high relative humidity are seen as favorable situations for the long-term survival of *E. multilocularis* and *T. pisiformis* eggs (Coman, 1975; Coman and Rickard, 1977; Ilsøe et al., 1990; Veit et al., 1995; Gemmel et al., 2001). At high RH values, viability continued through at least 300

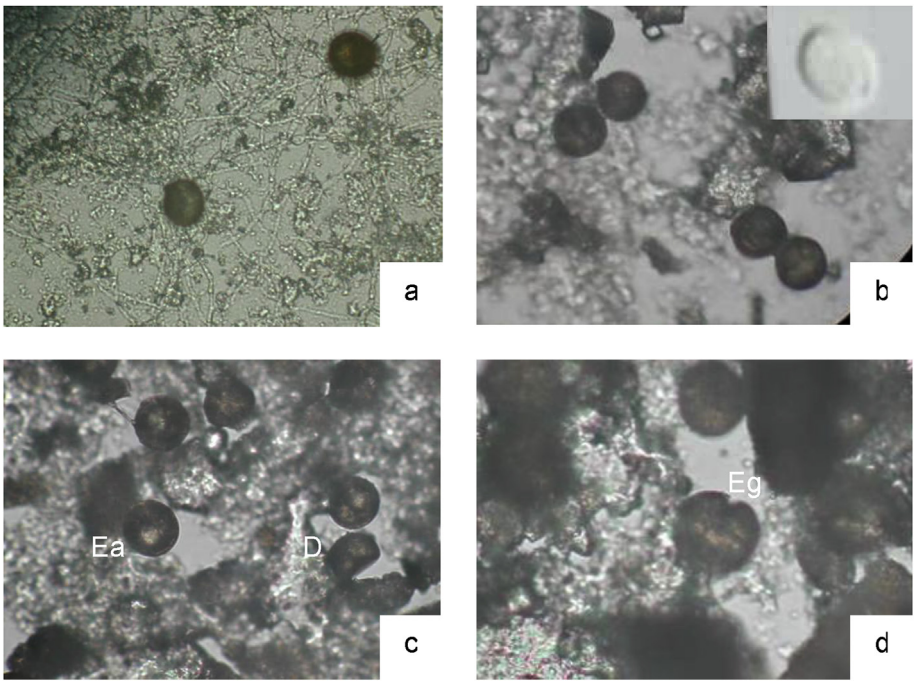

Fig. 3. Fresh and aged *T. hydatigena* eggs. Ageing at 31% of relative humidity during 365 days. a: fresh eggs. b: aged eggs in a good morphologic general condition. c: aged egg showing slimming of the embryophoric layer (Ea) and deformed egg (D). d: aged egg with a break of the embryophoric layer (Eg). 400x. Top right edge: macroscopic view of the dried stamp of eggs after 365 days of exposition at 31% of relative humidity.

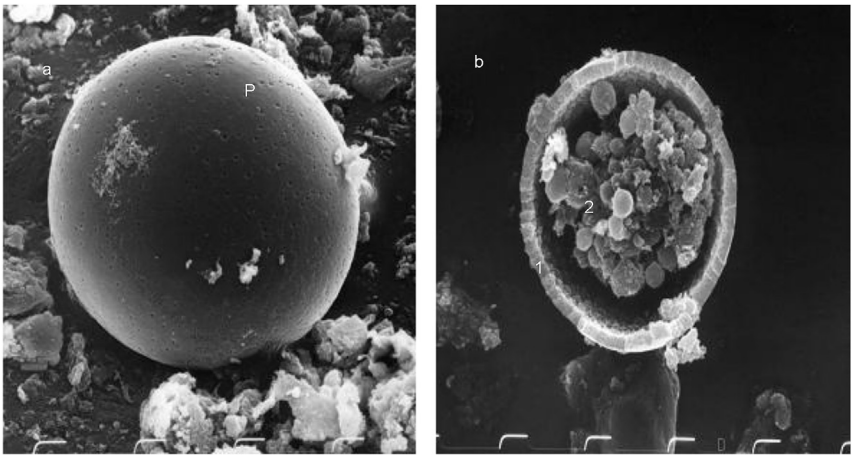

Fig. 4. SEM micrographs of fresh *T. hydatigena* eggs. a: intact egg showing embryophoric pits (P), 2,000x. b: fractured egg, 2,000x. The scale bars correspond to 10 μm. 1: middle embryophoric subunit layer, 2: oncosphere.

days for *T. pisiformis* at 85–95% HR/4–5 °C and 111 days for *E. multilocularis* at 85–95% HR/25 °C, whereas extremely low relative humidity significantly affects the viability of the eggs of these parasites. In the case of *E. multilocularis* exposed to 27%RH/25 °C and *T. pisiformis* eggs exposed to 32–33% HR/3–5 °C, viability was lost at 48 h and 56 days respectively. The experimental evidence reported so far is limited to these two species and does not elaborate on the adaptation mechanisms eggs might possess to tolerate these water stress conditions. Additionally, temperature could be a confusion variable masking the specific effect of relative humidity in these models. Our study shows that *T. hydatigena* eggs have different degrees of tolerance vis à vis water stress conditions. A portion of these eggs survived even after 365 days of exposure to high, medium and low relative humidity conditions and the differences observed in survival values according to the different regimes imposed were significant. The lowest survival values corresponded to eggs exposed to 31%RH.

The results obtained under controlled laboratory conditions are useful when it comes to understanding the epidemiological patterns of parasitic diseases (Pandey et al., 1993). In this sense, the RH regimes imposed in this study were intended to reflect the conditions of the different scenarios among which specimens of *T. hydatigena* were recovered. In these scenarios we recorded average RH minimum and maximum values of 40%RH in summer and 78%RH in winter, absolute extreme minimum and maximum values reaching 20%RH in June and 95%RH in January (Servicio Meteorológico Nacional, 2016). This study showed that after 365 days of exposure median survival values of *T. hydatigena* eggs

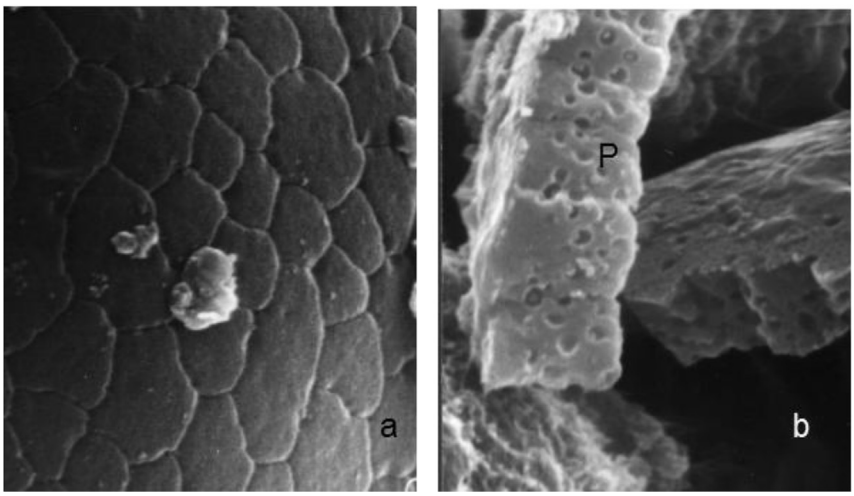

Fig. 5. High magnification detail of the embryophore of fresh *T. hydatigena* eggs by SEM. a: external embryophoric surface with tetra to hexagonal subunits, 10,000x. b: transversal section of embryophoro showing pits (P), 7,500x.

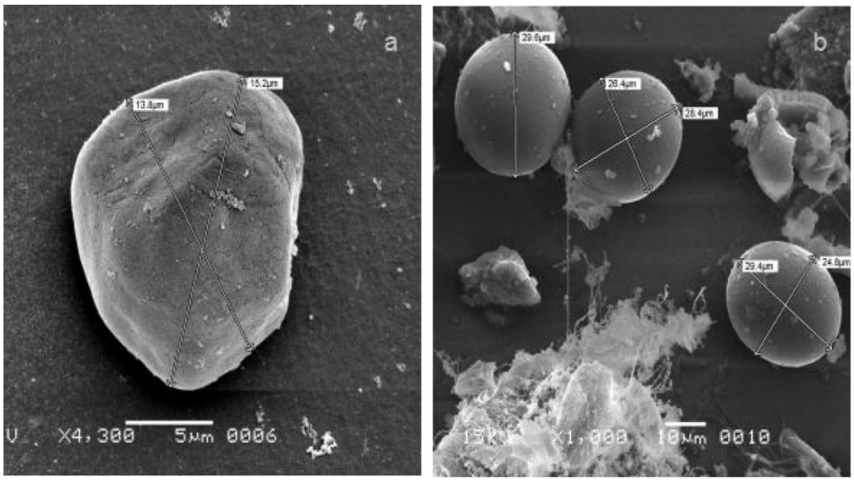

Fig. 6. SEM micrographs of *T. hydatigena* exposed eggs at 47% and 89% of relative humidity during 90 days. a: exposed egg at 47% of RH, deformed and poliedric with a decrease of normal size, 4,300x. b: espheric and subespheric eggs with normal size at 89% of RH, 1,000x.

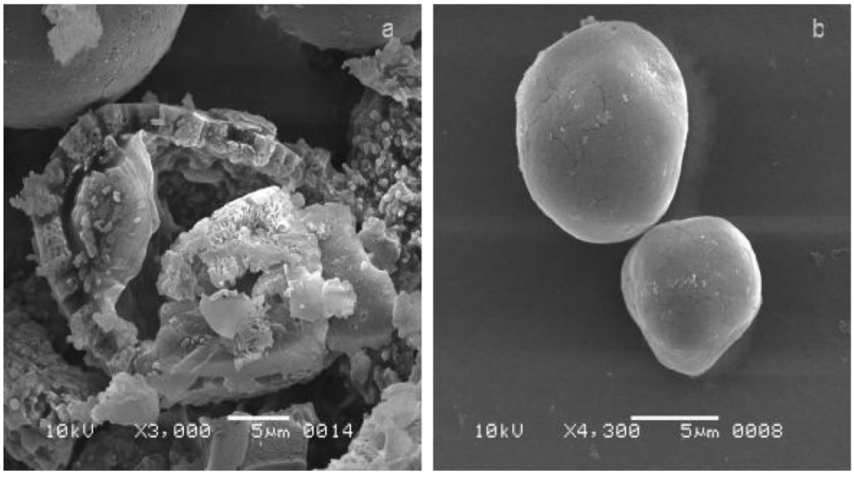

Fig. 7. SEM micrographs of *T. hydatigena* exposed eggs at 31% of relative humidity during 90 days. a: fractured egg with inner membrane detachment and deformed embryophoric subunits, 3,000x. b: deformed eggs with poliedric shape and outer embryophore surface with fissures, 4,300x.

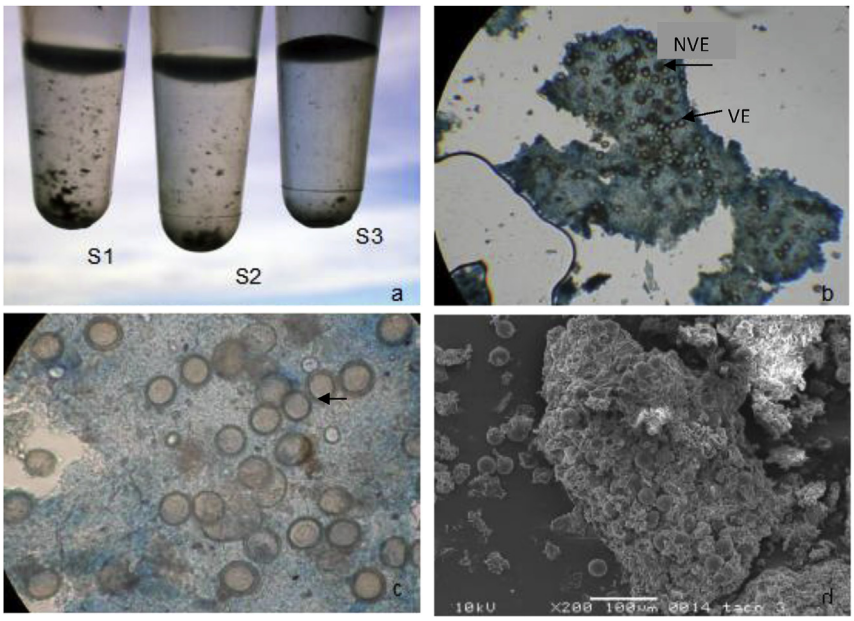

Fig. 8. Views of eggs aggregates forming by the *T. hydatigena* eggs after 210 days of exposition at three different relative humidity conditions. a: views of suspensions showing in increasing in particules-like bodies presence due to each relative humidity (RH) condition, S1 = 31%RH, S2 = 47%RH, S3 = 89%RH. b: clusters of vital eggs formed at 31%RH, 10x. b: vital eggs inside off de aggregate body. Tripan Blue coloration, 45x. c: SEM micrographs off aggregates of eggs exposed to 47% RH 200x. (d). VE: vital egg, HNE: non-vital egg, S1: 31% RH, S2: 47% RH, S3: 89% RH. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ranged between 80% and 82% at 89%RH, between 59.5% and 64.5% at 47%RH, and between 38% and 40% at 31%RH.These results suggest that viable parasite eggs are likely to be found under the different natural prevailing RH conditions in the region. Considering that for *T. hydatigena* the average number of eggs per mature proglottid is 38,000, with 7/100 of eggs becoming viable cysticerci (Gemmel, 1997), under the most unfavorable RH conditions imposed (31% RH/365 days), 39% of these eggs remained viable, which would represent 14,820 eggs of a mature proglottid and this would suppose that some 1000 cysticerci could potentially be generated in the intermediate host.

A relationship was revealed between the level of relative humidity in the environment and egg viability in terms of vitality. Our results showed that as RH decreases, the percentage of viable eggs falls. The regression analysis confirmed that lower RH values reduce the number of viable eggs over the period of exposure. Furthermore, the coefficients of determination (R^2) obtained for each system revealed that 93.45% of reduced vitality was caused by exposure to 31%RH, 93.15% by exposure to 47%RH and 73.58% by exposure to 89%RH. Morseth (1966) described how the blocks making up the embryophore of *T. hydatigena* eggs are constituted by a keratin protein, a water insoluble protein which provides properties such as hardness, hydrophobic nature and resistance to substances containing it. Thus the chemical nature of the sub-units of the embryophore of *T. hydatigena* eggs could partly account for the fact that these eggs have a high level of tolerance to low and medium relative humidity conditions.

The MEB of *T. hydatigena* fresh eggs allowed us to distinguish the following structures: external membrane, medium layer of embryophore sub-units, internal membrane and oncosphere. The embryophore of *T. hydatigena* eggs was the main lining around the oncosphere, it looked consistent and was formed by elongated sub-units; it constitutes a thick spherical or sub-spherical structure which physically isolates the embryo from the external environment. Some of these structures have also been reported in studies using transmission electron microscopy (TEM) on T. *ovis, T. pisiformis, T. solium, T. saginata, T. hydatigena* and *E. granulosus* eggs (Morseth, 1965; Swiderski, 1983; Holcman et al., 1994). Our study provides a description of the superficial topography of the embryophore and adds further information on the species studied to that generated by TEM.

Wang et al. (1981) proposed that the sub-unit pores are part of a network system whose functions can be related to the breathing of embryos, to humidity control through the egg external shell and to the provision of nutritional material for the development of the oncosphere. Furthermore, Morseth (1965) argued that these pores are related to the replacement of the embryophore material, the latter elongating from the base at the expense of the deposit of substances coming from the internal membrane. Thus the sub-units would gradually add constituent material and increase in length from the internal and lower area of the embryophore causing the displacement of the pores to the upper area. Throughout the period of study the analysis of the embryophore sub-units showed differences in terms of the length of these sub-units and the localization of the pores between fresh eggs and eggs exposed to the different RH conditions. We observed an increase in the length of these sub-units over time of exposure as well as displacement in the location of pores from the middle area to the upper third area of the sub-unit. Furthermore, the predominantly hexagonal geometry observed in the embryophore sub-units coincided with observations carried out on *T. pisiformis* and *T. solium* eggs by Morseth (1965) and Wang et al. (1981) respectively. Wang et al. (1981) pointed out that in the case of *T. solium* immature eggs hexagonal sub-units are not well developed whereas Ilsøe et al. (1990) argued that some Taeniidae eggs reach maturity outside their definitive host. Our observations reveal that some of the eggs are likely to have continued their process of maturing and generating the embryophore outside the host regardless of their RH condition.

Throughout the 365 day period analyzed under the three RH conditions imposed we observed individual and group changes in intact eggs. At the individual level the most remarkable alterations

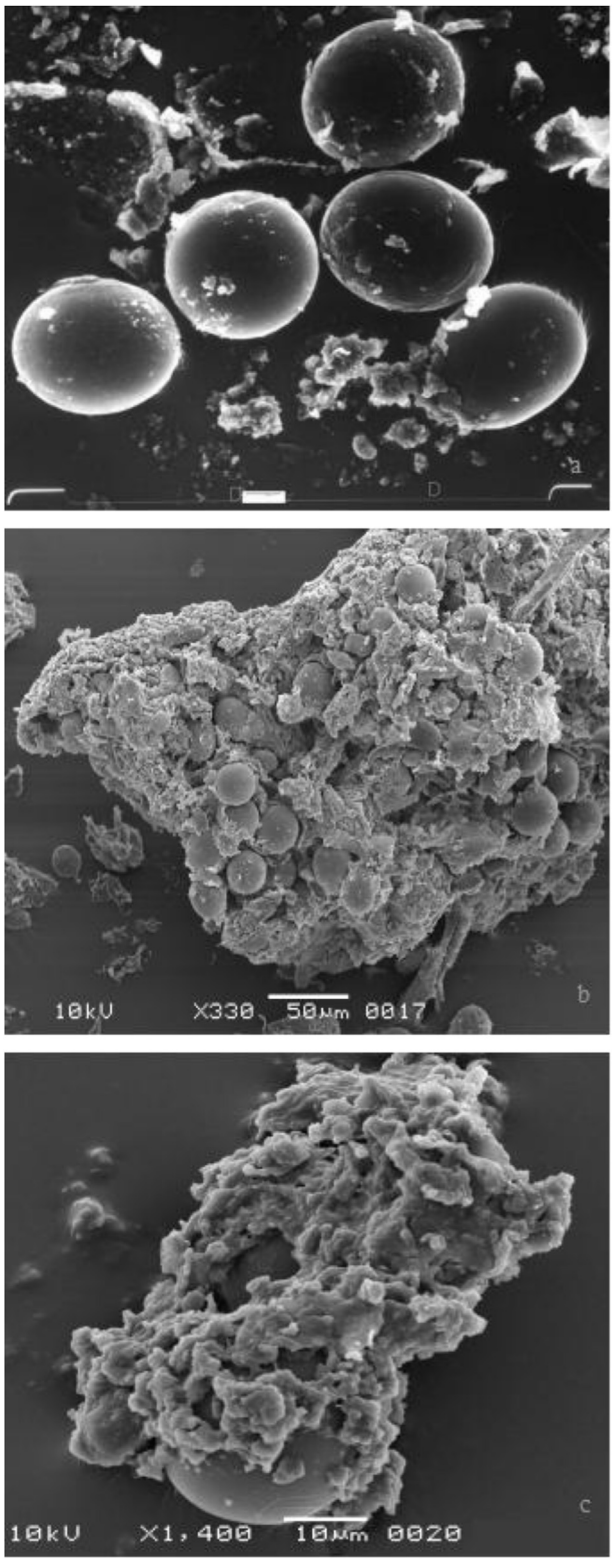

Fig. 9. a: SEM micrographs of fresh *T. hydatigena* eggs, b: clusters formed after 90 days under 31% of relative humidity, 330x and c: views of eggs surrounded by a substance of aggregation after 210 days under a 31%RH, 1400x (c).

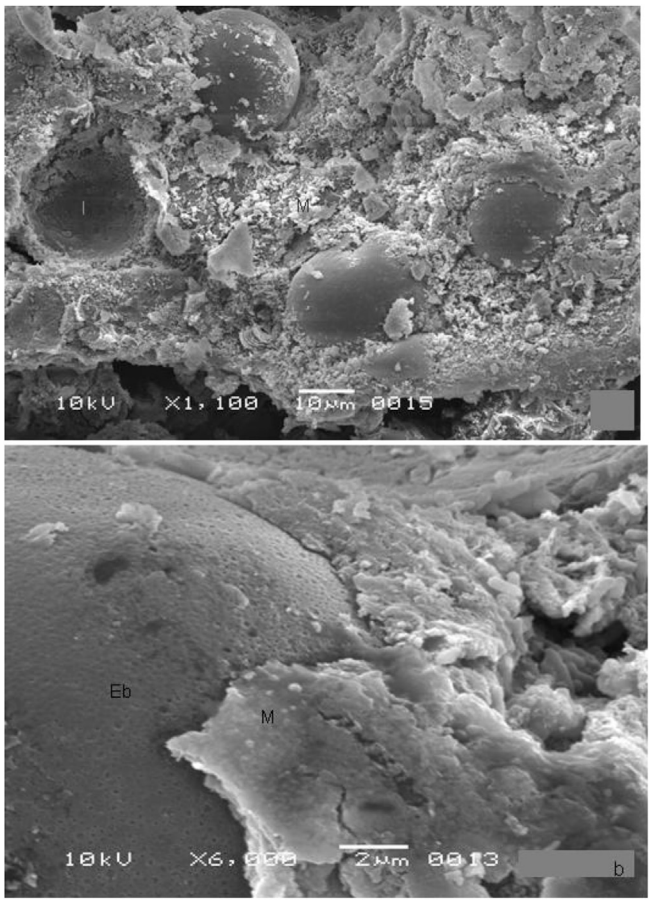

Fig. 10. a: substance of aggregation containing *T. hydatigena* eggs after 90 days of exposition at 47% of relative humidity, views of eggs and a fingerprint of the egg (I), 1100x. b: detail of the substance of aggregation (M) related to outer surface of the embryophore (Eb), after 210 days of exposition at 47%RH, 6000 x.

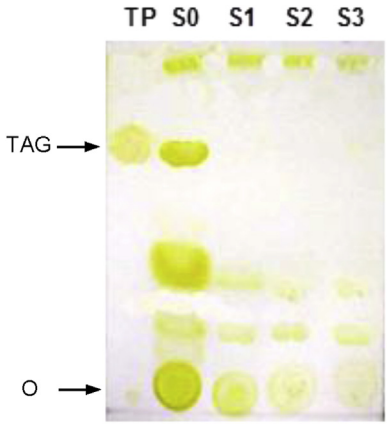

Fig. 11. Triacylglycerol content of fresh and aged *Taenia hydatigena* eggs determined by HPTLC analysis. Ageing time: 210 days. S0: fresh eggs. S1: aged eggs at 31% of relative humidity (RH). S2: aged eggs at 47%RH. S3: aged eggs at 47%RH. TP: tripalmitin. TAG: triacylglycerols. O: origin. Solvent system: hexane: ethyl ether: acetic acid (80:20:1, v/v/v). Lipid fractions were visualized by exposure to iodine vapours.

were an alteration of the egg's general morphology, cracks in the embryophore and detachment of the internal membrane. The change in both major and minor diameter of the embryophore is significantly related to a fall in RH. Furthermore, the damage observed in aged eggs was seen mainly on those eggs exposed to 31%RH and 47%RH, and corresponded to lower vitality values in the population of eggs exposed to these conditions. The structures and membranes surrounding the embryo in taeniid eggs make up their

immediate micro-environment and allow for their survival outside the definitive host (Morseth, 1965). The revealed correspondence highlights the importance of the conservation of morphological integrity of the egg shells for embryo survival.

At the collective level, the main change observed was egg grouping, especially at medium and low RH values. Willis and Herbert (1984) observed that *T. multiceps* eggs are clumped when found on canine faeces outside the proglottids originally containing them and argued that clumping could be a survival strategy for these eggs in the face of adverse environmental conditions. In the case of the plant pathogenic nematode *Ditylenchus dipsaci*, its capacity to form clumps is an adaptation response in order to resist desiccation. These clumps result in a reduction of the water loss rate, and while the worms located on the periphery of the clump die by dehydration, those at the center of this clump survive (Crowe and Madin, 1975; Wharton, 1996). In *B. microplus* (tick) and *B. antartica* (midge), clumping was shown to provide a minimum exposure surface to the outside for their eggs and larvae respectively, which results in a decrease in the water loss rate (Benoit and Denlinger, 2007; Kurup et al., 2008) and observations on bacteria of the genus *Rhodococcus*, which produce an amorphous matrix of extracellular polymeric substances (EPS) when exposed to water stress conditions revealed that the EPS covers the bacterial biomass and retains water in the micro-environment around the bacteria, acting as a protective sponge regulating water loss in cells and buffering external changes in the aqueous potential (Alvarez et al., 2004). In line with this record, our study shows that eggs exposed to 31%RH and 47%RH conditions are mostly clumped and that these clumps were constituted from a binding substance and form a compact mass. Furthermore, the bulk of the eggs included in the clumps were viable whereas those located in the periphery displayed some kind of damage. *T. hydatigena* eggs possibly clump as a response to a decrease in humidity in the surrounding environment and this clumping is at the expense of a substance which binds them together. The chemical nature and origin of this binding substance has not been defined in this study and it could be the object of future research.

As mentioned above, *T. hydatigena* eggs represent the free life stage of the parasite, they remain in the environment until they are ingested by an intermediate host and depend of the embryo and its deposit of reserve material to derive energy throughout that external period (Vinakayan, 1982). Fat reserves play an important role in maintaining long-term viability of higher animals and bacteria (Bequer Urbano et al., 2013). Specifically, TAG reserves can temporally and spatially liberate organisms from the need for an immediate source of energy and provide endogenous reserves which can be used in the absence of external nutrient sources (Coleman and Douglas, 2004; Daniel et al., 2014). Furthermore, they provide protection against toxic metabolites, they are a source of precursors for the synthesis of other lipids and represent an endogenous reserve of metabolic water (Alvarez and Steinbüchel, 2002; Alvarez, 2016). As to the *T. hydatigena* eggs analyzed, we observed the six previously described fractions of neutral lipids for these forms of the parasite (Sánchez Thevenet et al., 2010). The TAG fraction disappeared in eggs aged over 210 days in relation to the fraction observed on fresh eggs; this fraction was mobilized at 89% RH, 47%RH and 31%RH. It is thus proved that the degradation route of these compounds remains active even under different conditions of water stress in these biological forms of the cestode. For these forms, having TAG and being capable of using them under low RH conditions involves possessing energy-efficient reserve compounds with ample physiological versatility which constitute a reserve of metabolic water.

In conclusion our study indicates that although change in RH affects the vitality of *T. hydatigena* eggs, this biological parasite form has responded to desiccation by the formation of clumps and by the endogenous metabolism in the use of TAG. These compounds are key to the long-term resistance under hydrological stress conditions. Thus, the long life of the eggs may require a set of responses to counter the wide variety of hydric conditions which they encounter in the whole Patagonian environment. This work not only deepens but develops the existing knowledge on the adaptation of taeniid eggs to desiccation.

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