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A descriptive study of the occurrence and significance of lipids in *Taenia hydatigena* eggs

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ABSTRACT

The aim of this work was to investigate the lipid content of *Taenia hydatigena* eggs and to evaluate the role of lipids in the maintenance of embryo viability. The total lipid content of the egg was 4.5% (w/w). Five classes of neutral lipids were identified: esterified cholesterol, free cholesterol, triacylglycerols, diacylglycerols and free fatty acids. Our results suggest that triacylglycerols play a key role in the maintenance of embryo viability. In addition, we found that *T. hydatigena* eggs remain metabolically active by mobilisation of stored triacylglycerols. This study contributes to the understanding the survival strategies of a member of the Taeniidae family in the environment outside the host.

1. Introduction

The family Taeniidae includes tapeworms of great importance to human health and veterinary sanitation (Lawson and Gemmel, 1990). The Taeniid egg, which contains an embryo, is the free-living stage of the parasite and can infect intermediate hosts.

Once the infectious egg is passed out of the definitive host, the embryo within must depend on stored material for energy until it enters the intermediate host for further development (Vinakayan, 1982). It has been reported that *Taenia* spp. and *Echinococcus granulosus* eggs can survive outside of their hosts in soil for up to 250 days and 41 months, respectively (Ilsoe et al., 1990; Cabrera et al., 1995; Sánchez Thevenet et al., 2005).

Lipids, and particularly triacylglycerols, are concentrated depots of metabolic energy for the egg (Alvarez and Steinbüchel, 2002; Berg et al., 2003). Studies of the lipid content of taeniid tapeworms have been mainly confined to the larval and adult stages (Frayha et al., 1980; Sánchez Acedo et al., 2000). For example, the presence of free and bound fatty acids, mono-, di- and triacylglycerols, free cholesterol and cholesterol esters has been demonstrated in the cysticerci of *Taenia hydatigena* (Frayha et al., 1980).

Thin layer chromatography (TLC) has been frequently used for lipid analyses of Taeniidae organisms, particularly for larval stages of *E. granulosus* and *T. hydatigena* (Digenis et al., 1970; Frayha et al., 1980; Abidi et al., 1989) and adults of *E. granulosus*, *T. saginata* and *T. taeniformis* (Ciccini et al., 1976; von Brand et al., 1965). In addition, Dennis et al. (1993) characterised glycolipids from hydatid cysts, whereas Bandstra et al. (2006) and Massa et al. (2008) analysed neutral lipid profiles in Trematodes using high-performance thin layer chromatography (HPTLC). TLC is still the simplest and most widely employed technique in lipid analysis, providing rapid and complete separation of most neutral and phospholipid classes (Myher and Kuksis, 1995). Modern HPTLC is an efficient, instrumentalised, quantitative method that is carried out on layers composed of small particles with a diameter of 5 mm, as compared to

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12–20 mm for conventional TLC (Sherma, 2000). The particle size distribution is narrower, the layers are thinner and the development distance is shorter for HPTLC, leading to greater separation efficiency, faster separations and improved detection limits (Sherma, 2008).

To date, no studies have examined stored lipids in Taeniidae eggs and whether they function as an energy source or are otherwise metabolised. Thus, we used traditional validated chemical methodologies to study neutral lipids in *T. hydatigena* eggs, with the goal of improving our knowledge about lipids in parasites and comparing our results on neutral lipids with the available data for other cestodes. In this context, the aim of this work was to characterise the lipid content of *T. hydatigena* eggs and to evaluate the role of lipids in the maintenance of embryo viability.

2. Materials and methods

2.1. Source of Taenia hydatigena eggs

Fresh *T. hydatigena* eggs were obtained from 40 adult worms that came from naturally infected dogs deparasited by oral administration of arecolina hydrobromide. Gravid proglottids were separated, washed with sterile 0.85% sodium chloride solution (SSe) and sectioned to liberate the eggs. The eggs obtained were resuspended in SSe and filtered by Universal® filter system to eliminate parasite remains. The concentration of the egg suspension was determined by triplicate counts in a Neubauer chamber (Marienfeld, Germany).

2.2. Total lipid content determination

The total lipid extraction was performed using lyophilised *T. hydatigena* eggs according to the methods of Folch et al. (1957), using the Free Zone Freeze Dry System (Labconco, Kansas City, MO, USA) with a mixture of chloroform and methanol (2:1, v/v) at 4 °C for 1 h. The lipid extract was dried under a N_2 atmosphere. The total lipid content was determined by gravimetry with an analytical Sartorious scale (GMBH 2842). Determinations were done in triplicate.

2.3. Characterisation of neutral lipids

To characterise the neutral lipid fractions, we first prepared suspensions of fresh eggs of *T. hydatigena* at 40 eggs/μl in SSe. The eggs were then separated by centrifugation at $500 \times g$ 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 (Ciccini et al., 1976). The lipids were separated by HPTLC on 60F254 silica gel plates (Merck, Darmstadt, Germany) using hexane:ethyl ether:acetic acid (80:20:1, v/v/v) as the solvent system (Alvarez et al., 1996). Lipid fractions were visualised by incubation in iodine vapour or by spraying with vanillin sulphuric acid (Wagner and Bladh, 2000). The following reference substances were used: dipalmitin (DP) (Sigma), tripalmitin (TP) (Fluka-Chemika), palmitic acid (PA) (Merk), oleanolic acid (OA) (ICN Biomedicals), cetyl-palmitate (CP) (Merck) and cholesterol (CHE) (ICN Biomedicals). The sample volume used for the HPTLC was 25 μl, and 5 μl of each reference substance was used, except for AP, of which 2 μl was used.

To determine the occurrence of esterified cholesterol in eggs, we performed a saponification step with a mixture of sodium hydroxide and 10% methanol at 80 °C for 1 h, followed by hydrochloric acid treatment in single-egg suspensions before the HPTLC analysis.

2.4. Determination of metabolic activity

Tetrazolium chloride (TTC) (Sigma, St. Louis, MO, USA) reduction was used to measure respiration activity in *T. hydatigena* eggs (Alvarez et al., 2004). Tetrazolium chloride is an artificial electron acceptor that is reduced to a water-insoluble red formazan, which can be dissolved by hexane and measured by absorbance at 546 nm. Fresh *T. hydatigena* eggs were distributed in 12 tubes containing in 2.5 ml of sterile TTC solution (0.3% tetrazolium chloride in 100 mM Tris–HCl buffer, pH 7.4). The tubes were incubated at 27 °C for 120 days (d) in the dark. At 0 h, 24 h, 8 d and 120 d, four drops of the egg/TTC suspension were observed by light microscopy to examine the presence of the red colouration due to formazan production. Next, 500 μl of the egg/TTC suspension was centrifuged at $500 \times g$ for 3 min and resuspended in 2 ml of hexane. After 2 h of incubation in the dark at 20 °C, the eggs were separated by centrifugation ($500 \times g$/3 min) and the absorbance of the supernatant was measured at 546 nm. Determinations were done in triplicate. For the experiments, fresh *T. hydatigena* eggs in 2.5 ml of SSe were used as a negative control.

2.5. β-Oxidation inhibition assay

The effect of the inhibition of the β-oxidation pathway of the eggs was evaluated by incubation of the eggs with acrylic acid. Acrylic acid (Merck, Darmstadt, Germany) was used to inhibit the β-oxidation pathway (Alvarez et al., 2004) to determine the role of this pathway in the survival of *T. hydatigena* eggs. A 5-ml suspension of fresh *T. hydatigena* eggs (68.75 eggs/ml) in 0.85% sodium chloride, containing 5 mg/l of acrylic acid (AA) and a sterile antibiotic solution (SSa) (Heath and Smith, 1970) of 1000 UI/ml penicillin G sodium (Richet S.A., Argentina), 1000 μg/ml streptomycin sulphate (Richet S.A., Argentina), 1000 U/ml nistatin (Bristol-Myers, Argentina) and NaCl 0.85 g/100 ml (Dorwill, Argentina), was incubated at 27 °C the dark. A 5-ml suspension of an equal concentration of fresh eggs in SSa without acrylic acid was used as a negative control.

Triacylglycerol analysis, metabolic activity analysis and viability examinations were performed to evaluate the inhibitory effects of AA. The triacylglycerol analyses were performed by HPTLC according to the method described in Section 2.3. Metabolic activity was measured according to Section 2.4 and the viability study was performed according to Section 2.7. The measurements were made after 0 h, 24 h and 8 d and 120 d of incubation of the eggs in each condition.

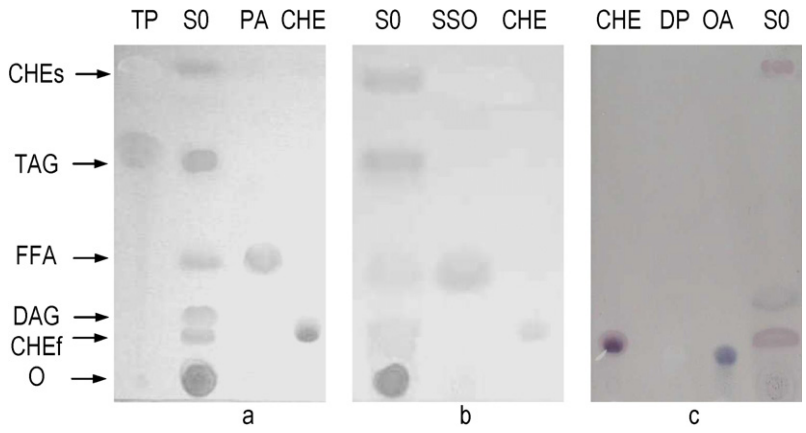

Fig. 1. Characterisation of neutral lipids in fresh *T. hydatigena* eggs by HPTLC. S0: fresh eggs, SS0: saponified fresh eggs. TP: tripalmitin, PA: palmitic acid, CHE: cholesterol, OA: oleanolic acid, DP: dipalmitin. Solvent system: hexane:ethyl ether:acetic acid (80:20:1, v/v/v). Lipid fractions were visualised by exposure to iodine vapours (a and b) or to vanillin-sulphuric acid (c). CHEs, esterified cholesterol; TAG, triacylglycerols; DAG, diacylglycerols; FFA, free fatty acids; CHEf, free cholesterol; O, origin.

2.6. Ageing assay

Fresh *T. hydatigena* eggs were subjected to an ageing process by incubation in an incubator. For this purpose, a 203 eggs/ml suspension of fresh eggs in SSe was prepared, and 50 μl per tube was aliquoted into 15 sterile plastic tubes of 1.5 mm of diameter and 10 cm of height. The tubes were put into the incubation cabinet and maintained for 210 days under light protected conditions at 19.5 °C (S.D. 2.37 °C) and 89.2% (S.D. 3.45) relative humidity (r.h.). Temperature and humidity control was done using a digital hygrometer/thermometer (TFA/Germany). In the incubation cabinet, the r.h. atmosphere was produced using 200 ml of a 34.7% KOH solution in distilled water (Pandey et al., 1993). After the ageing time (210 days), eggs were analysed by neutral lipid and viability studies according to Sections 2.3 and 2.7, respectively.

2.7. Viability evaluation

The evaluation of the viability of fresh *T. hydatigena* eggs and the eggs exposed to the conditions described in Sections 2.4, 2.5 and 2.6 were tested for their ability to exclude 0.1% aqueous trypan blue. The eggs stained with trypan blue were counted as nonviable, and the unstained eggs (clear) were counted as viable (Wang et al., 1997). Determinations were done in triplicate, and the percentage of viable eggs was calculated.

2.8. Statistical analysis

To determine the nature of the distribution of the studied variables, the Armitage principle was used. The viability results were expressed as means and standard deviation. The Dumm's test was used as a post-hoc test (Dawson-Saunders and Trapp, 1994). 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

The average weight of the total lipid content of fresh *T. hydatigena* eggs was 45 mg/g (dry weight) or 4.5% (w/w). Five classes of neutral lipids were detected by HPTLC analysis of lipids extracted from the fresh eggs and were identified by comparing their R_f to those of reference compounds. The neutral lipid fractions detected were: cholesterol esters (R_f 0.93), free cholesterol (R_f 0.15), triacylglycerols (R_f 0.64), diacylglycerols (R_f 0.09) and free fatty acids (R_f 0.35) (Fig. 1). One fraction that was retained at the starting point could not be identified.

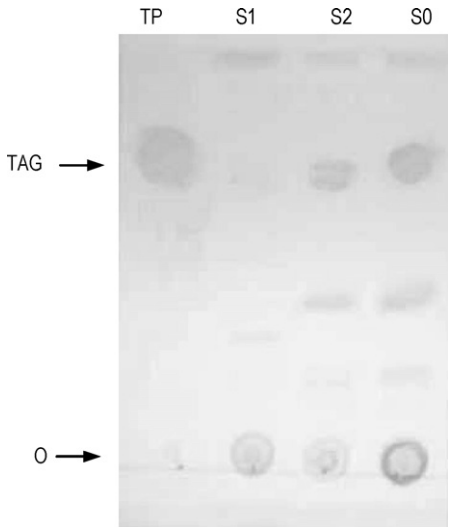

Fig. 2. Variation of the triacylglycerol fraction from *T. hydatigena* eggs incubated in the presence or absence of acrylic acid (AA), analysed by HPTLC. S0: fresh eggs. S1: control eggs incubated without AA for 8 days. S2: eggs incubated in the presence of 5 mg/ml of AA for 8 days. TP: tripalmitin. TAG: triacylglycerols. O: origin. Solvent system: hexane:ethyl ether:acetic acid (80:20:1, v/v/v). Lipid fractions were visualised by exposure to iodine vapours.

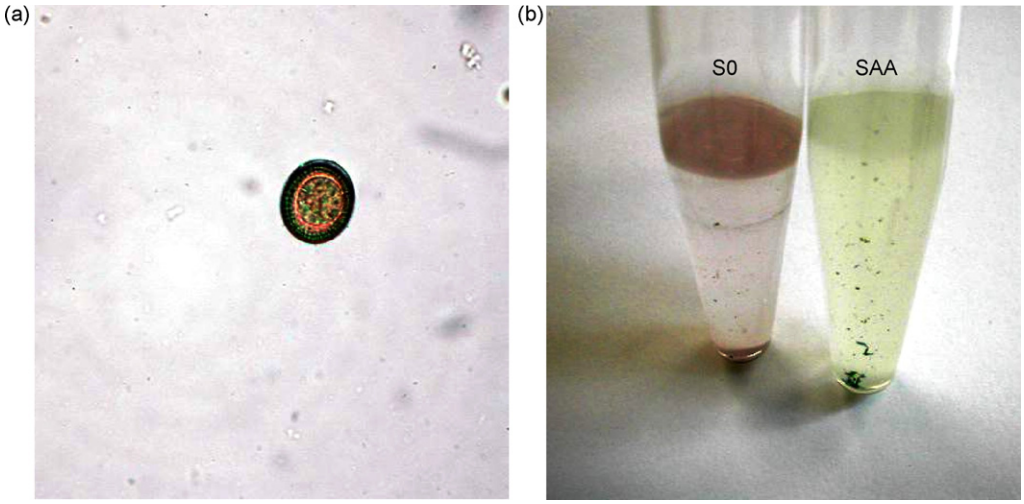

Fig. 3. Metabolic activity of *T. hydatigena* eggs. (a) Fresh eggs with metabolic activity (notice the red colouration of the embryo); (b) egg suspension in tetrazolium chloride solution in the absence (S0) or presence (SSA) of acrylic acid (AA). The metabolic activity of eggs was determined using tetrazolium chloride as a colourimetric indicator of the respiratory chain activity, as described in Section 2.4. Eggs were incubated with 5 mg/ml acrylic acid at 27 °C in the dark for 8 days. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

The survival experiments were performed to elucidate the role of the TAG in viability maintenance. The HPTLC analyses of the eggs incubated in the presence of 5 mg/ml of acrylic acid showed that the neutral lipid profile of the studied eggs changed in comparison with those incubated without the inhibitor (control eggs). In the control system, we observed a relative decrease of TAG fraction and a simultaneous relative increase of the free fatty acid fraction in the eggs during the incubation time. In contrast, the most dramatic change observed in eggs incubated with acrylic acid was an apparent inhibition of mobilisation of the TAG fraction (Fig. 2).

Metabolic activity was investigated using tetrazolium chloride as a colourimetric indicator of respiratory activity. Fresh *T. hydatigena* eggs reduced the TTC to red formazan, producing a red colour in the egg and surrounding solution (Fig. 3). In contrast, the eggs incubated in the presence of AA did not show the red colouration. Moreover, a drastic decrease of metabolic activity in the eggs incubated with acrylic acid was observed in comparison with control eggs (Table 1).

Table 1
Variation of the metabolic activity of *T. hydatigena* eggs incubated at different times in the absence or the presence of 5 mg/ml of acrylic acid (AA).

Incubation conditions	Time of incubation			
	0 h	24 h	8 d	120 d
Without AA	0.003[a]	0.103	0.212	1.039
With AA	0.003	0.058	0.040	0.187

Metabolic activity of the eggs was determined using tretrazolium chloride as colorimetric indicator of the respiratory chain activity, as described in Section 2.4.

[a] The values are expressed as absorbance at 546 nm.

The viability study showed that viable *T. hydatigena* eggs were recovered after 210 days of ageing. The average viability value of the fresh eggs was 94% (S.D. 2) and the average viability value of aged eggs was 82% (S.D. 2.89). A regression study of the viability values produced an R value of -0.858 ($P = 0.01$). In addition, HPTLC of the aged *T. hydatigena* eggs showed that the TAG fraction disappeared after 210 days, suggesting the mobilisation of these lipids (Fig. 4).

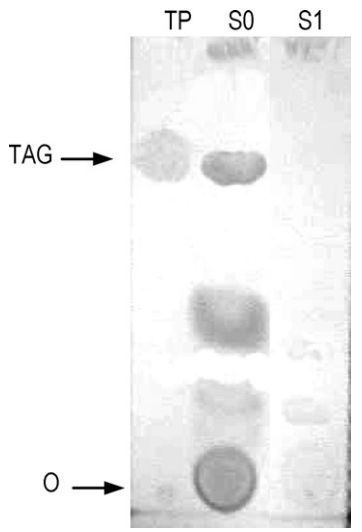

Fig. 4. Triacylglycerol content of fresh and aged *T. hydatigena* eggs determined by HPTLC analysis. S0: fresh eggs. S1: aged eggs. Ageing conditions: 89% r.h./19.5 °C/210 days of incubation. TP: tripalmitin. TAG: triacylglycerols. O: origin. Solvent system: hexane:ethyl ether:acetic acid (80:20:1, v/v/v). Lipid fractions were visualised by exposure to iodine vapours.

4. Discussion

Very little is known about the lipid constituents of Cestoda eggs. This study is the first on the presence and possible role of neutral lipids in Taeniidae eggs.

A previous study of *Cysticercus tenuicollis* (the larval stage of *T. hydatigena*) found total lipid contents of 2.1% and 1.4% (w/w of the dry tissue) from goat and pork cysticerci, respectively (Abidi et al., 1989). However, no data exist on the lipid content of the egg stages of the parasite. In this study, the total lipid content of the egg was found to be 4.5% (w/w of the dry tissue), which is two times higher than the amounts found in larvae. The higher content of stored lipids in the egg may be due to an adaptation to different habitats and nutritional situations. The adult and larval stages, which live in a low oxygen or anaerobic environment in the hosts, are completely dependent on their hosts for lipids because they have lost the capacity for *de novo* cholesterol and fatty acid synthesis (Tielens, 1994). Thus, carbohydrate fermentation is the major source of energy for larvae and adults (Vinakayan, 1982; Tielens, 1994). In contrast, eggs emerging from the host face an environment that is rich in oxygen, and the embryo within must depend on its stored material for energy production because external nutrient sources are not available. In these conditions, aerobic metabolism, including lipid oxidation, is both possible and advantageous in terms of energy yield for the embryo. In fact, the storage and use of lipids could have two advantages for the *T. hydatigena* embryo: first, lipids are lighter to store, and second, they produce double the energy yield after complete oxidation as compared with carbohydrates and proteins (Berg et al., 2003).

The characterisation of the neutral lipid fraction of *T. hydatigena* eggs revealed the presence of five subclasses of lipids: cholesterol esters, free cholesterol, triacylglycerols (TAG), diacylglycerols (DAG) and free fatty acids (FFA). These subclasses are concordant with the majority of the reported lipid fractions for the larval stage (Abidi et al., 1989). These lipid fractions could function as structural components (cholesterol), energy and carbon reserves (TAG) and metabolic intermediates (DAG and FFA) (Nelson and Cox, 2000; Yeagle, 2001).

In the present study, we have demonstrated that free-living eggs are metabolically active. The use of TTC as an artificial final electron acceptor in the respiratory chain supports the hypothesis that aerobic metabolism takes place in the eggs under natural conditions. In addition, a drastic decrease in the metabolic activity and viability of the eggs occurred in the presence of acrylic acid, which is an inhibitor of the acyl-CoA synthase and 3-ketoacyl-CoA thiolase enzymes of the β-oxidation pathway (Alvarez et al., 2004). We also demonstrated that acrylic acid inhibited the mobilisation of TAGs for energy production. In addition, HPTLC analyses showed that the TAG fraction decreased after 7 months of incubation of the eggs under diverse r.h. conditions. Those results suggest that TAGs are metabolised by the *T. hydatigena* embryo outside of the host, and this capability is required for the maintenance of viability. From a physiological point of view, the presence of a TAG reserve in cestode embryos has interesting implications. The TAG depots could liberate organisms from dependency on external energy sources, providing an endogenous reserve that could be utilised when external nutrients are unavailable (Coleman and Douglas, 2004). In addition, TAGs have been shown to protect against toxic substances, provide a source of intermediates for the synthesis of diverse lipids and may also function for endogenous storage of metabolic water (Alvarez and Steinbüchel, 2002; Haunerland, 2003; Coleman and Douglas, 2004).

Prior to this study, there was no evidence that the lipid depot in Taeniidae eggs was utilised as energy source and/or was metabolised in any way. Based on transmission electron microscopy studies of the oncosphere stage of *Mosgovoyia ctenoides*, an anoplocephalid cestode, Młocicki (2007) proposed that lipids are important for embryonic development. The authors observed changes in the concentration of lipid droplets during development of the oncosphere, detecting a large increase in the number of lipid droplets over the course of differentiation of the oncospheral envelopes. The results obtained in our study suggest that the metabolic activity and viability of the embryo contained in the egg is supported, at least in part, by utilisation of endogenous TAG reserves.

Species such as *T. hydatigena,* which do not infect humans, can be safely used as models of biochemical studies (Lawson and Gemmel, 1990). Therefore, it could be useful to understand some key aspects of how the viability of the Taeniidae embryo is preserved the environment. In this way, the present work will serve as a starting point for further studies in this field.

5. Conclusion

The occurrence of neutral lipids in *T. hydatigena* eggs, including free cholesterol, esterified cholesterol, triacylglycerols, diacylglycerols and free fatty acids, was demonstrated for the first time. This study provides evidence that the *T. hydatigena* egg has aerobic metabolic activity when it is outside of the host and that the viability of the embryo is supported, at least in part, by endogenous utilisation of stored triacylglycerols. Further studies are necessary to determine the cellular and molecular mechanisms involved in the survival of the egg in natural environments. The understanding of the biological responses of free-living stages in representative *Taenia* organisms is of great importance for predicting parasite activities in the environment and for establishing effective prevention and control measures in the field.

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