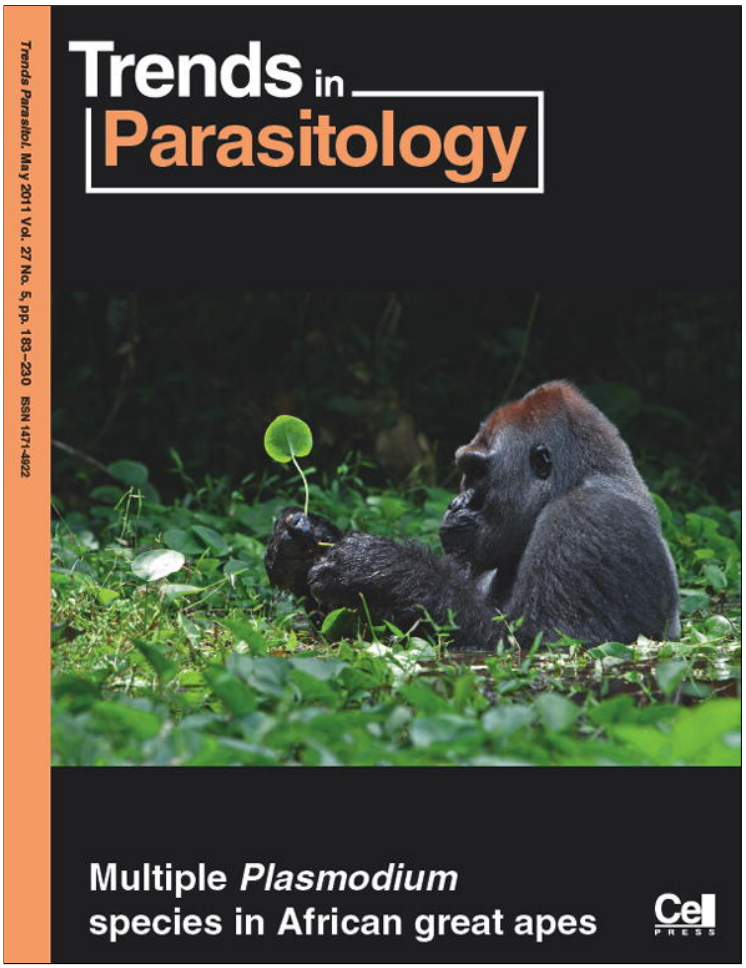

Review

Understanding the laminated layer of larval *Echinococcus* I: structure

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Echinococcus **larvae are protected by a massive carbohydrate-rich acellular structure, called the laminated layer. In spite of being widely considered the crucial element of these host–parasite interfaces, the laminated layer has been historically poorly understood. In fact, it is still often called 'chitinous', 'hyaline' or 'cuticular' layer, or said to be composed of polysaccharides. However, over the past few years the laminated layer was found to be comprised of mucins bearing defined galactose-rich carbohydrates, and accompanied, in the case of *Echinococcus granulosus*, by calcium inositol hexakisphosphate deposits. In this review, the architecture and biosynthesis of this unusual structure is discussed at depth in terms of what is known and what needs to be discovered.**

Echinococcus and hydatid disease

The genus *Echinococcus* of cestode parasites [1] belongs to the family Taeniidae, which includes well-known tapeworms such as *Taenia saginata*. The life cycles of *Echinococcus* species always involve a definitive host harboring the adult worm, and an intermediate host carrying the larva or metacestode. The (carnivore) definitive host becomes infected by ingesting protoscoleces contained within the bladder-like metacestode lodged in intermediate host viscera. Protoscoleces develop into 3-mm-long gut-dwelling adult tapeworms, which produce eggs. The intermediate host acquires infection through the accidental ingestion of eggs passed out with the feces of the definitive host. Infection by larval *Echinococcus* is termed hydatid disease.

Four species are traditionally recognized in the genus, namely *Echinococcus granulosus*, *Echinococcus multilocularis*, *Echinococcus oligarthrus* and *Echinococcus vogeli*. This is being revised, with *E. granulosus* no longer considered a single species [2]. In this article the traditional denominations will be used, with the note of warning that some important observations on *E. granulosus* were actually made on *Echinococcus equinus*, the former 'horse strain' [3–7]. All species in the genus use canids as definitive hosts

Glossary

Brood capsule: a vesicular, fluid-filled structure bounded by a thin layer of cells, originated by inward budding of the germinal layer of *Echinococcus* metacestodes, and which in turn gives rise to protoscoleces in its interior.
Electron-dense, electron-lucent: contrast in transmission electron microscopy is achieved on the basis of the different degrees to which cellular structures scatter electrons, thus restricting their passage through the sample. Structures that scatter electrons strongly or weakly are said to be electron-dense or electron-lucent, respectively. Biological samples are formed mostly from light chemical elements, which scatter electrons poorly. Thus, contrast is enhanced by staining them with heavy metal atoms (lead, uranium). In unstained biological specimens, calcium-containing biomineralized structures appear as *naturally* electron-dense, because calcium is heavier than the major chemical elements that make up cells.
Glycocalyx (plural glycocalyces): the layer of carbohydrate that surrounds animal cells. It is formed by many individual carbohydrate chains (glycans), covalently attached to extracellular-facing plasma membrane proteins/protein domains and lipids.
Glycosylphosphaditylinositol (GPI) anchors [37]: glycolipid moieties that anchor certain proteins to the extracellular-facing leaflet of cell membranes. The C-terminus of the protein is connected via an ethanolamine phosphate bridge to the GPI glycan, and it in turn is connected to a phosphatidylinositol (phospholipid) moiety. The glycan almost always contains three mannose and one glucosamine residue, and can feature a wide diversity of additional carbohydrate decorations. Proteins destined for GPI incorporation bear a characteristic C-terminal GPI signal peptide, which is eliminated in the endoplasmic reticulum concomitantly with the addition of the GPI anchor. GPI-anchored proteins can be released from their membrane-bound form by phosphatidylinositol-specific phospholipases; the sites of cleavage are such that the GPI glycan/inositol moieties remain associated with the proteins.
Hydatid cyst: the tissue-dwelling larva of taenid parasites from the genus *Echinococcus*. More strictly, the name applies to the larvae of *Echinococcus granulosus*, *Echinococcus vogeli* and *Echinococcus oligarthrus*, which develop as bladder-like, roughly spherical structures that grow by concentric enlargement. For larval *Echinococcus multilocularis*, which develops as multiple irregular chambers intermixed with the host fibrous reaction and liver parenchyma, the term 'larval mass' is normally used instead; also, the term 'vesicle' is used for discrete, approximately spherical units as can be obtained *in vitro*.
Inositol hexakisphosphate (InsP6) [44,46]: a member of the soluble inositol phosphate family, which includes the well-known signaling molecule inositol (1,4,5)-trisphosphate (IP3). In InsP_6, all six positions of the *myo*-inositol ring are phosphorylated. InsP_6 is the most

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abundant member of the family, and it is found in concentrations of 10–100 μM in the cytosol and nucleus of all eukaryotic cells studied. However, its functions are still unclear.

Microtriches: specialized microvilli (plasma membrane protrusions) that occur only on the body surface (tegument) of cestodes. Each microtriche normally features an electron-dense spine at its tip. However, the microtriches of the *Echinococcus* germinal layer tegument lack spines, thus being referred to as 'truncated'. Microtriches are thought to increase the surface area of the tegument, through which cestodes absorb their nutrients.

Mucins [28,68,69]: proteins with many sites of glycosylation on Ser/Thr residues, i.e. *O*-glycosylation. By virtue of their extensive glycosylation, the peptide backbones of mucins (termed apomucins) adopt extended, as opposed to globular, conformations. More strictly, this applies to their heavily glycosylated domains, as apomucins can additionally have N- and or C-terminal 'conventional' globular domains. The peptide sequences of glycosylated domains of apomucins can be recognized on the basis of their high content of serine, threonine and proline. Mammalian mucins are either secreted or cell-surface proteins containing transmembrane domains. The protein sequences of the secreted mucins are usually longer, and contain imperfect repeats of a particular subsequence (tandem repeats). Heavily glycosylated mucin-like domains in otherwise 'conventional' proteins also exist.

Mucin-type *O*-glycans [68]: *O*-linked carbohydrates sharing certain specific structural patterns, which set them apart from non-mucin types of *O*-glycosylation. Mucin-type *O*-glycans are built from the monosaccharides *N*-acetylgalactosamine (GalNAc), galactose (Gal), *N*-acetylglucosamine (GlcNAc), fucose, and (in vertebrates) sialic acid. More specifically, animal mucin-type *O*-glycans are based on a limited number of characteristic structures (termed cores) for the two to three monosaccharide residues closest to the peptide backbone. The most common of these are core 1 (Galβ1-3GalNAc1α-Ser/Thr) and its derivative core 2 (Galβ1-3[GlcNAcβ1-6]GalNAc1α-Ser/Thr), but further ones exist. Beyond the cores, much more structural diversity exists. Mucin *O*-glycans are synthesized by stepwise addition of activated monosaccharides to the apomucins in the Golgi apparatus. Because of this strategy of synthesis, they present extensive heterogeneity, with many related structures potentially occupying each glycosylation site in the population of mucin molecules.

Protoscolex (plural protoscoleces): the *Echinococcus* stage infective for the carnivore (essentially canid) definitive host. It measures approximately 0.1 mm, and comprises a scolex featuring hooks and suckers, and a soma that upon establishment in the host gut gives rise to the adult worm proglottids (segments). Within the metacestode, protoscoleces are normally invaginated so that the scolex is hidden within the soma. A single metacestode can produce thousands of protoscoleces.

Secondary infection: the development of *Echinococcus* metacestodes from protoscoleces. When naturally or experimentally released within mammalian tissues or peritoneal cavity, protoscoleces are capable of reverse development into metacestodes indistinguishable from those generated after infection with onchospheres.

Syncytial tegument: a syncytium is a biological structure defined by a single plasma membrane and comprising many nuclei that share a single cytoplasm. The outermost cellular layer of platyhelminths, called tegument, has a syncytial organization. This syncytial tegument comprises what are called distal and proximal cytoplasms. The former is a continuous layer of cytoplasm exposed at the surface of the helminth. The proximal cytoplasm is made up of discrete cell bodies (tegumentary cytons), comprising nuclei and connected to the distal cytoplasm through thin cytoplasmic bridges.

(with the minor exception of the lion strain of *E. granulosus*). A range of mammalian species, mainly domestic ungulates, are intermediate hosts for *E. granulosus*, whereas wild rodents and lagomorphs fulfill this role for the other three species.

Humans are accidental intermediate hosts for the genus. Human hydatid disease is the major concern associated with *Echinococcus*. It comprises mainly cystic hydatid disease, caused by *E. granulosus*, and alveolar hydatid disease, caused by *E. multilocularis* [8]. Cystic hydatid disease can go unnoticed for years, as the slow-growing hydatid cyst usually causes pathology only through compression of the host organ, which is most commonly liver or lung, but can be any organ. Thus symptoms are rather non-specific and depend on the precise location. Complications independent of organ compression are also important; these include bacterial superinfection and traumatic cyst rupture, which carries the risks of anaphylactic shock and of initiation of secondary infection. The disease has essentially a worldwide distribution. Its prevalence is high wherever pastoralism is an important activity and political/institutional conditions have not allowed a regular, state-enforced dosage of domestic dogs with the drug praziquantel. Thus, cystic hydatid disease is important in areas of Central Asia, China, South America, and Africa [2]. Alveolar hydatid disease has the liver as the only primary site. After an asymptomatic period lasting several years, the infection manifests itself initially with symptoms such as jaundice and abdominal pain. As the metacestode aggressively invades the host liver parenchyma, and (in cases) further organs, the disease is severe, even fatal unless appropriately treated. This infection is restricted to cold and temperate climates in the Northern Hemisphere, being most significant in the Tibetan plateau and some other remote areas of China, Hokkaido in Japan, and parts of Central Europe [2].

Echinococcus metacestodes

After being ingested by the intermediate host, eggs release embryos (oncospheres) that penetrate the gut wall, travel via blood or lymph, and are trapped in internal organs, where they develop into metacestodes. These are bounded by a thin cellular layer (germinal layer, GL), and filled by liquid (hydatid cyst fluid, or vesicle fluid). This general structure can be thought to allow a permanently low ratio between total parasite cellular volume and host-exposed area, through linear growth that can exceed three orders of magnitude. The GL buds towards the inside giving rise to brood capsules, which in turn generate protoscoleces. This basic structure has variations among species (Figure 1). *E. granulosus* typically develops as a large unilocular, turgid cyst, which grows through an increase in diameter. *E. vogeli* and *E. oligarthrus* develop similarly, but tend to form multichambered cysts. By contrast, *E. multilocularis* grows by outward budding, giving rise to a labyrinth of chambers and tubules.

The GL [9] exposes towards the outside the apical plasma membrane of its syncytial tegument, which carries truncated microtriches. The GL has additional, non-syncytial cell types, including muscle, glycogen-storage and undifferentiated cells. Towards the cyst cavity, there is neither a syncytial organization nor junctional complexes between cells, so that the intercellular fluid of the GL is apparently continuous with the cyst/vesicle fluid [10].

Separating the GL from host cells and/or host extracellular matrix is the laminated layer (LL), an acellular, carbohydrate-rich sheath secreted by the GL. The LL attains approximately 10 to 12 μm in thickness in *E. multilocularis*, up to 400 μm in *E. vogeli*, and up to

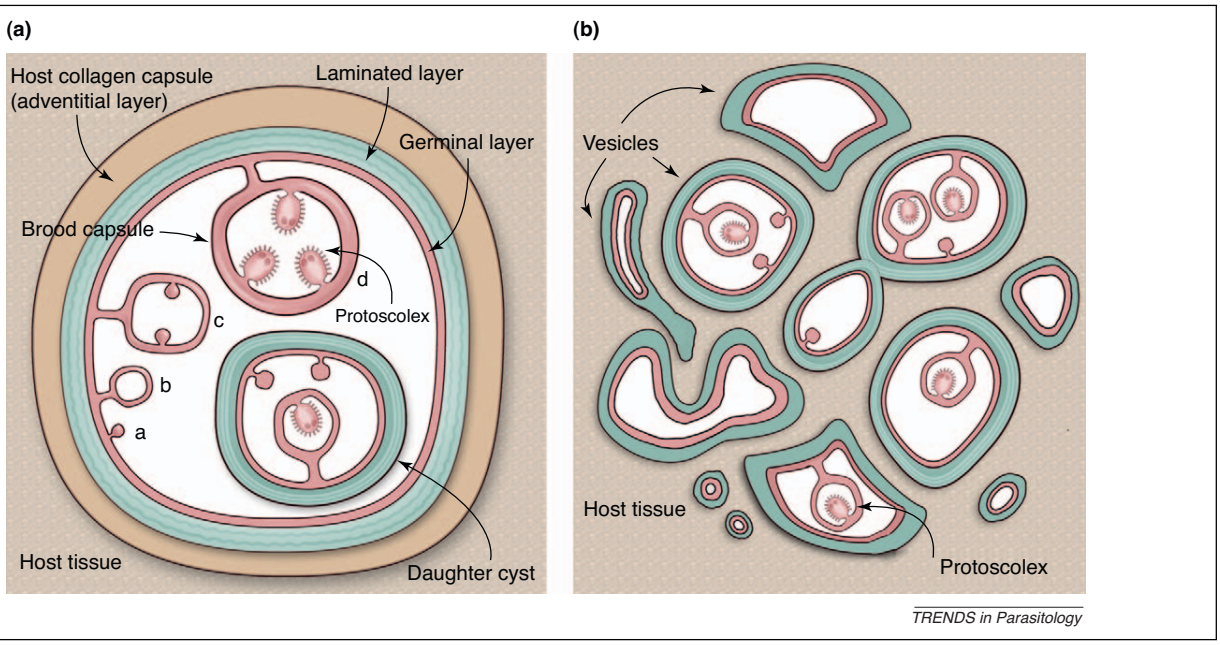


Figure 1. Diagrammatic representation of *Echinococcus* metacestodes. The *E. granulosus* larva **(a)** is a bladder-like structure known as hydatid cyst that grows by concentric enlargement within a host organ parenchyma and can reach tens of cm in diameter. It is bounded by the hydatid cyst wall, which comprises an inner cellular layer (germinal layer) and an outer protective acellular layer (laminated layer). It is usually surrounded by a host-derived collagen capsule (adventitial layer), but can also be encircled by host inflammatory cells. The cyst cavity is filled with hydatid cyst fluid. The germinal layer gives rise towards the cyst cavity to cellular buds that upon vesiculation become brood capsules, and in turn bud towards their inside to generate protoscoleces (phases a through d in the figure). Daughter cysts, with their own complete cyst wall, occasionally form within larger cysts. The *E. multilocularis* metacestode **(b)** has an overall similar organization but instead of forming a well-defined cavity develops as a labyrinth of small chambers intermixed with the host fibrous reaction and liver parenchyma; the whole of the host liver can eventually be affected. Although not depicted in the Figure, the laminated layer is much thicker in *E. granulosus* than in *E. multilocularis*. The metacestodes of *E. vogeli* and *E. oligarthrus* (not shown) are similar to that of *E. granulosus* but form multichambered cysts. Adapted, with permission, from [1].

3 mm in *E. granulosus* [11–13]. The LL is related to cellular glycocalyces. However, only the innermost strata of this massive structure are likely to be covalently anchored to the GL tegumental membrane. Thus, the LL is appropriately described as a specialized extracellular matrix, found only in the genus *Echinococcus*, evolutionarily designed for maintaining the physical integrity of metacestodes and for protecting GL cells from host immunity. It is probably the first of these demands that determines the impressive thickness of the *E. granulosus* LL: live hydatid cysts are turgid, and also pressure from the outside is a significant threat, as illustrated by the occurrence of traumatic cyst rupture [8].

Ultrastructure of the laminated layer

The LL is a macroscopically coherent and elastic structure, based on a microscopic three-dimensional meshwork of hydrophilic, highly hydrated fibrils. Under the transmission electron microscope, these fibrils are irregularly arranged and approximately 10 nm in diameter (Figure 2(a)) [5,9,14]. Although this is not observable in most published micrographs, it is possible that the fibrils adjacent to the GL could be physically connected to the tips of the tegumental microtriches, which project up to 1 μm into the LL [5].

The *E. granulosus* LL is composed, in addition to the fibrillar meshwork, of naturally electron-dense granules, occurring individually or in clusters [9] (Figure 2a). These granules were determined to have a 41 nm size, and to be composed from 8 nm electron-lucent spheres fused together [5]. The granules, conspicuous in *E. granulosus*, have never been reported in *E. multilocularis* or *E. vogeli* [14–16]. As discussed below, for *E. multilocularis* there is biochemical evidence that the granules are genuinely absent.

The LL owes its name to the concentric laminations it shows under the light microscope. Under the scanning electron microscope, these laminations give the sectioned *E. vogeli* LL the appearance of an open book with very thin pages [15], whereas the *E. granulosus* LL appears as more compact [17] (Figure 2(b)). The origin of the laminations is unknown, but by transmission electron microscopy these appear to result from different degrees of compaction adopted by a single type of ultrastructure [9].

Molecular structure of the laminated layer: mucins

The abundant carbohydrates that characterize the LL are found in the fibrillar meshwork [3]. Pioneering works determined that the carbohydrate component could not be separated away from protein(s) [18–20]. In 1962, the carbohydrate-protein complex was defined as a mucopolysaccharide [18]. This was a correct description at the time, although the term mucopolysaccarides today refers to proteoglycans, which the LL appears not to contain. Rather, the LL meshwork is formed by the other major type of highly glycosylated glycoproteins, the mucins. The monosaccharide composition of the LL, only galactose (Gal), *N*-acetylgalactosamine (GalNAc) and *N*-acetylglucosamine (GlcNAc) [18–21], is only compatible with mucin-type *O*-glycans among the forms of glycosylation known in animals. In agreement with a mucin-based composition, threonine is the dominant amino acid of LL preparations

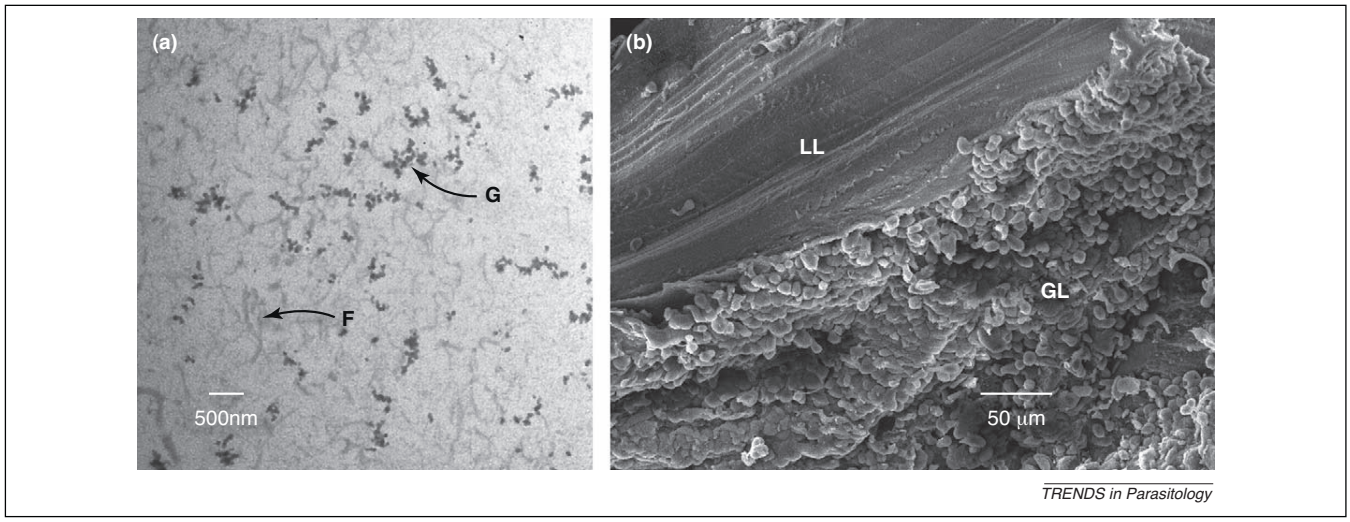


Figure 2. The *E. granulosus* LL under the transmission **(a)** and scanning **(b)** electron microscopes. In **(a)**, the fibrillar meshwork (F) and calcium inositol hexakisphosphate granules (G) are indicated. In **(b)**, the laminated (LL) and germinal (GL) layers are indicated. The micrograph in **(b)** was kindly provided by María Celina Elissondo (CONICET-Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata, Argentina).

[19,20]. As detailed in the following section, in 2002, a mucin from the *E. multilocularis* LL was characterized [22]. Solubilization of the *E. granulosus* LL is achieved by β-elimination [18,20,23,24], a reaction typically undergone by *O*-glycans [25]. The identification of high molecular mass, heterogeneous carbohydrate-containing molecules as major components of the *E. multilocularis* LL [26] is also broadly consistent with a mucin composition. Finally, the solubilization, and visualization by agarose gel electrophoresis and lectin-blotting was recently reported of molecules with the features expected of the *E. granulosus* LL mucins [27].

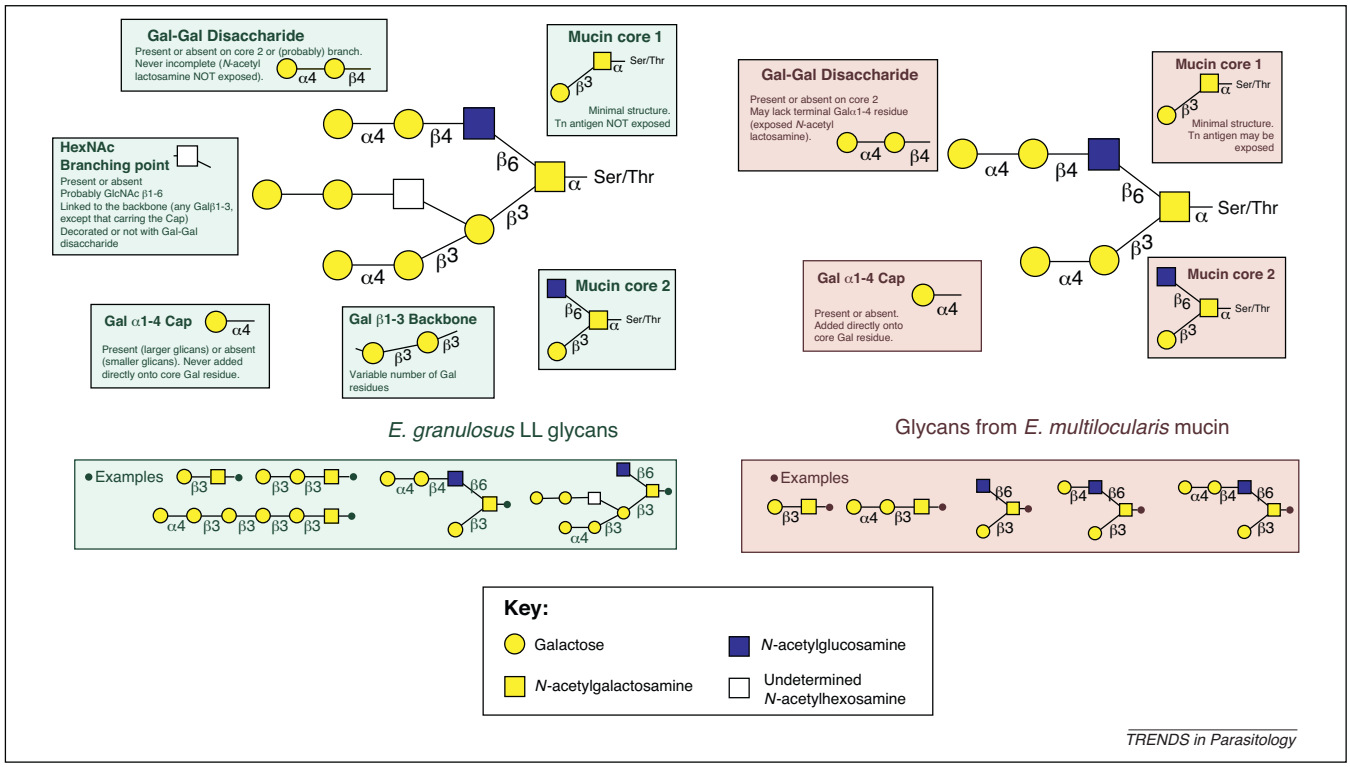


Figure 3. Structure of the glycans present in the *E. granulosus* LL and comparison with known *E. multilocularis* LL glycans. For each species, a 'general structure' is shown on top that encompasses all the structural possibilities/motifs detected, and examples of specific known structures are given below. Note that each specific glycan results from the usage of a different discrete subset of elongation/decoration steps out of the total set of possible monosaccharide additions. However, some biosynthetic intermediates (carrying terminal *N*-acetyl lactosamine, Galβ1-4GlcNAc, or the T_n-antigen, non-decorated GalNAcα-Ser/Thr) seem to be excluded from the final *E. granulosus* mucins. *E. granulosus* structures are from. [24] and (A. Díaz *et al.*, unpublished data). The Gal-Gal-HexNAc branches are currently being studied, and it is hypothesized that they could correspond to P_1-antigen-related motifs (Galα1-4Galβ1-4GlcNAcβ1-6) similar to those found in cases associated with core 2. The *E. granulosus* LL contains glycans up to 18 residues in size, all of which probably fit the 'general structure' shown. *E. multilocularis* structures [22] are those present in the mucin purified by the Em2(G11) antibody, and are therefore not necessarily representative of the *E. multilocularis* LL as a whole. Anomeric configurations in *E. multilocularis* are inferred from comparison with *E. granulosus*.

Box 1. Old questions answered

In 1962, the mucopolysaccharide from the *E. granulosus* LL, solubilized by partial β-elimination in alkaline medium, and the insoluble remainder of the crude LL extracted with hydrochloric acid were observed to have very similar IR spectra [18]. Both of these materials lacked a common set of IR bands, present in the crude LL. In turn, these same missing IR bands (8.9, 10.1 and 11.9 μm, i.e. 1120, 990 and 840 cm^{-1}) were displayed by the (apparently inorganic) residue left behind after digesting the LL in concentrated potassium hydroxide. These IR bands are now known to correspond precisely to those of the calcium salt of InsP_6 [62]. Calcium InsP_6 remains insoluble after alkaline solubilization of the mucins, and conversely is soluble in acidic media [23,63]. InsP_6 solubilization also explains the marked increase in transparency of the LL after acid treatment [18]. Solubilization of InsP_6 further explains the disappearance of the electron-dense granules upon acid treatment, which was observed in 1984 [3]. Calcium InsP_6 also explains the finding made in 1971 that calcium and phosphate are 20% and 47% of the ash of the LL, respectively [59].

In 1974, in studying the reactivity with the LL of infection-elicited antibodies, the LL was observed to be recognized by antibodies against the P_1 blood antigen (found in some human erythrocytes [20]: Galα1-4Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-ceramide [64]. The structures of the LL *O*-glycans now available [22,24] provide the probable structural basis of this reactivity in the Galα1-4Galβ1-4GlcNAc motif (associated with core 2 and possibly other branches; Figure 3), which is identical to the terminal trisaccharide of the P_1 blood antigen. Of note, the P_1 determinant is also present in *E. granulosus* tissues other than the LL, and is carried on glycosphingolipids and *N*-glycans as well as on *O*-glycans [20,29,65–67].

In the studies mentioned above, the following observations were made: (i) antibodies in human hydatid infection sera precipitated from the LL mucopolysaccharide molecules had P_1 reactivity; (ii) these antibodies could be inhibited by α-galactose, i.e. the same residue present at the terminus of the P_1 motif; and (iii) unexpectedly, the same sera did not react with P_1^+ red blood cells [20]. This is most probably explained by human infection sera recognizing terminal Galα1-4Galβ1-3Gal structures (Figure 3) [24], that is, terminal α-Gal containing epitopes different from the P_1-blood antigen-related motif but present in the same mucins or mucin aggregates.

Animal mucins can bear limited numbers of *N*-glycans in addition to the *O*-glycans [28]. However, this does not seem to be the case in the LL constituents, as mannose (invariably present in *N*-glycans) is not detectable in the crude hydatid cyst wall, i.e. the GL plus the LL [24]. Enzymatic release does allow retrieval of *N*-glycans from *E. granulosus* cyst wall preparations [29]. These include parasite-derived (non-sialylated) and host-derived (sialylated) structures, and must be derived from the GL and from nonstructural proteins associated with the LL.

Molecular structure: the mucin glycans

In 2002, a molecule purified from the *E. multilocularis* metacestode using an anti-carbohydrate monoclonal antibody selectively reactive with the LL (Em2(G11) [30,31]) was defined as a mucin, on the basis of its high molecular weight, high threonine content, and decoration with mucin-type *O*-glycans [22]. Recently, the glycome of the *E. granulosus* LL was tackled, revealing only mucin-type *O*-glycans, related to those described in the *E. multilocularis* mucin [22], but reaching larger sizes [24]. The structures of the LL glycans are summarized in Figure 3. The major features are: (i) the construction from cores 1 and 2 (see 'mucin-type *O*-glycans' in the Glossary); (ii) the quantitative dominance of the non-decorated cores with respect to more elaborate glycans also present; (iii) the lack of sialylation, expected in invertebrates; (iv) the lack of fucosylation, which contrasts sharply with the glycobiology of schistosomes [32]; (v) the elongation by $(Galβ1\text{-}3)_n$, previously unknown; and (vi) the capping of glycans by Galα1-4 (probably also present in protoscolex glycoconjugates [33]).

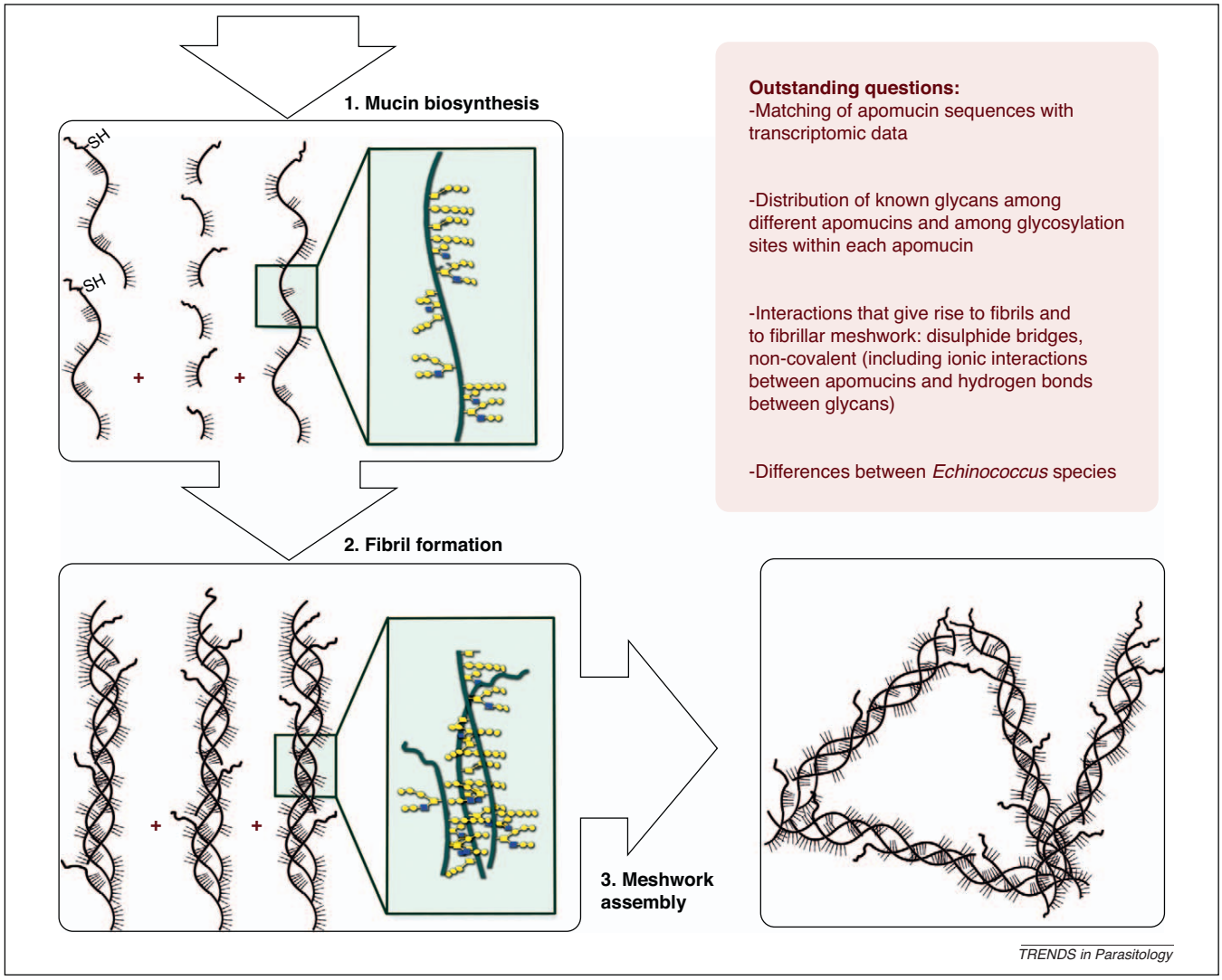
The non-decorated core 1 is probably a major LL glycan across the genus, as suggested by the structural data [22,24] and by the binding of lectins specific for it (PNA, jacalin) to the LL of *E. multilocularis*, *E. vogeli*, and *E. granulosus* [15,26,27]. The virtual absence of *N*-glycans in the *E. granulosus* LL is also probably shared with the other species, as concanavalin A, which binds *N*-glycans, labels the GL but not the LL of *E. multilocularis* [26] (and a similar although less clear-cut result is obtained for *E. vogeli* [15]). By contrast, some carbohydrate motifs present in the LL can be species-specific. For example, neither the antibody Em2(G11) mentioned above nor a polyclonal antiserum against the *E. multilocularis* LL react with the *E. vogeli* LL [15,30]. Also, non-decorated *O*-linked GalNAc is present in the *E. multilocularis* but not in the *E. granulosus* LL [22,24].

In summary, the glycomic data confirm that the LL meshwork is made up from mucins carrying essentially only *O*-glycans, and suggests that there could be LL-specific carbohydrate motifs as well as motifs shared with other stages/tissues (Box 1). Also, both shared *Echinococcus* and species-specific LL glycans probably exist.

Molecular structure: mucin backbones and mucin assembly

The transcriptome of the *E. granulosus* GL (http://www.compsysbio.org/partigene/) contains several apomucin sequences. Some of these are shared between GL and protoscoleces, and are therefore not seen as coding for candidate LL components. The LL is a unique structure among *Echinococcus* tissues, and indeed the mucopolysaccharide fractions from protoscoleces and LL are different, at least in monosaccharide composition [18,20]. Other apomucin sequences (clusters EGC0000317, EGC0004254, EGC0002904, EGC0005092) are instead absent from protoscoleces and additionally constitute the most highly expressed transcripts in the GL altogether. Although these probably code for LL components, no direct evidence supports this speculation so far. These sequences feature no tandem repeats, contain a very high proportion of putative *O*-glycosylation sites, with interspersed charged residues, (in cases) carry unpaired cysteines, and bear signals for addition of glycosylphosphatidylinositol (GPI) anchors. Clear orthologs of EGC0000317 and EGC0004254 are found in the *E. multilocularis* transcriptome (http://www.compsysbio.org/partigene/; http://fullmal.hgc.jp/em/) and genome (www.sanger.ac.uk/cgi-bin/blast/submitblast/Echinococcus; [34]).

As mentioned, the LL fibrils might be associated with the GL tegument when first externalized [5]. If this was the case, the LL apomucins (whether corresponding or not to sequences discussed above) would possess either transmembrane domains or GPI anchors, and would be released from the membrane by phospholipases or proteases. In this hypothesis, a 35 kDa so-called polysaccharide with a typically LL-like lectin-binding profile purified from detergent



Outstanding questions:
-Matching of apomucin sequences with transcriptomic data

-Distribution of known glycans among different apomucins and among glycosylation sites within each apomucin

-Interactions that give rise to fibrils and to fibrillar meshwork: disulphide bridges, non-covalent (including ionic interactions between apomucins and hydrogen bonds between glycans)

-Differences between *Echinococcus* species

Figure 4. Possible assembly of mucins into the fibrillar meshwork of the LL. The clustering of glycosylation sites, the presence of unpaired cysteines, and the possibility of lateral assembly of apomucin molecules of different lengths and charges, are based on the sequences of the putative apomucins found in the GL transcriptome (http://www.compsysbio.org/partigene/). These apomucins are only inferred to be LL constituents, and therefore the structural possibilities mentioned are still hypothetical.

extracts of *E. multilocularis* metacestodes could be such a membrane-bound precursor [35]. Also speculatively, membrane vesicles and whorls sometimes seen to be shed from the tips of the GL microtriches [10,15,36] might represent excess membrane containing the anchors or 'stubs' of released mucins. If the GPI-anchored mucin possibility turns out to be true, an explanation will have to be found for the lack of detectable mannose (a component of all known GPI anchors [37]) in the *E. granulosus* LL [24].

It seems probable that several mucin molecules, especially if some of these are short as suggested by the transcriptomic data, interact to give rise to the fibrils observable by electron microscopy. Then fibrils would interact among them to give rise to the three-dimensional network. Hydrogen bonds between sugars must contribute to these higher structural levels of the LL: 6 M urea disrupts the LL meshwork [26] and solubilizes some mucin components [38]. Interactions between the apomucins must also contribute, including disulphide bonds [3,27], and conceivably, ionic interactions. Overall, the form of assembly must differ radically, quantitatively and/or qualitatively, from that of the mammalian secretory mucins, which also associate through hydrogen bonding and disulfides [39], to allow the formation of a physically coherent structure. The precise assembly might differ among *Echinococcus* species, as suggested by the difficulty, in the case of *E. vogeli* but not in that of *E. multilocularis*, both to observe the fibrils under the electron microscope and to detect the mucins in SDS–PAGE [15]. The knowledge, hypotheses and standing issues relating to LL meshwork assembly are summarized in Figure 4.

The proteome of the LL is a challenge, mainly because of the extraordinary extent of post-translational modification that can be expected for its mucins. Non-mucin proteins could also be structural components of the LL. Discrete non-mucin, non-host proteins have been detected in the LL [40–42].

Molecular structure: inositol hexakisphosphate deposits

By serendipity, the *E. granulosus* cyst wall was found to contain large amounts of *myo*-inositol hexakisphosphate

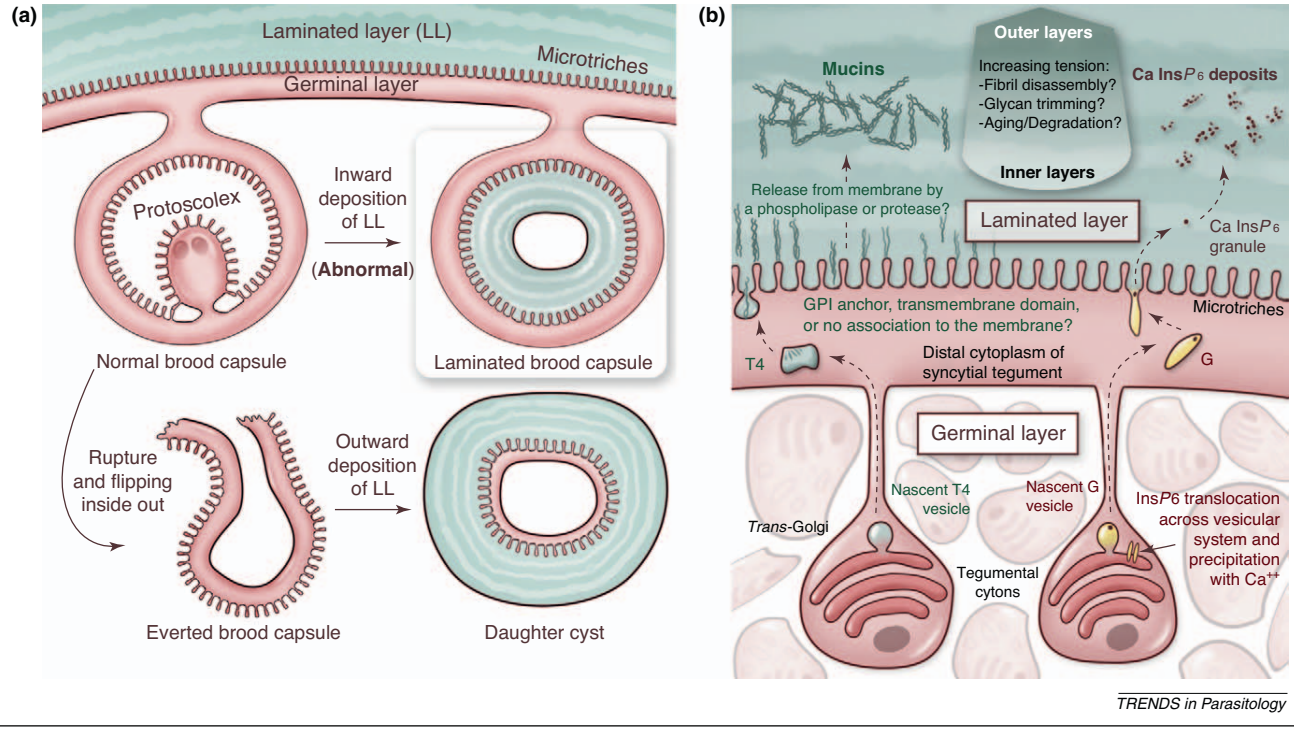


Figure 5. Summary of data and outstanding issues on the biosynthesis and turnover of the *E. granulosus* LL. Issues related to the polarity of the GL and brood capsule tegument are depicted in **(a)**. Note that the microtriche-bearing (and syncitial) surfaces of the GL, brood capsules and protoscoleces are analogous ('tegumental pole'); the opposite, microtriche-free surfaces are continuous between GL and brood capsule (non-tegumental pole) [50]. Cell biological aspects of LL biosynthesis are summarized in **(b)**. For simplicity, the biosynthesis of mucins is presented on the left-hand side of the panel, and that of the calcium InsP_6 deposits on the right-hand side; however, the same GL tegumentary cyton can synthesize both components, which are also intimately laid together in the LL.

(InsP_6, Glossary) [43], a normally cytosolic and nuclear compound [44–46]. InsP_6 also forms, in certain plant tissues, deposits with mineral cations in vesicular cellular compartments [47]. In the *E. granulosus* cyst wall, it was present in the GL, but mostly in the LL, thus constituting the first case of extracellular InsP_6 [43]. The molecule could be solubilized from the LL, together with calcium counterions, by chelating agents. This connected with the previous observation, by X-ray microanalysis, of a significant calcium peak in the electron-dense granules of the *E. granulosus* LL [7]. The granules were then proven to be deposits of the calcium salt of InsP_6, $Ca_5H_2L\bullet16H_2O$ (L representing fully deprotonated InsP_6) [23,48]. The appearance of the granules under the transmission electron microscope [5,23] suggests that they are microcrystalline solids, i.e. solids formed from many individual crystals fused together. Thus the electron-lucent spheres previously described [5] would be crystals and the electron-dense areas in between the so-called grain boundaries. Each crystal was estimated to comprise some 2×10^2 InsP_6 molecules [23]. The deposits are a finely divided solid phase; their solubilization turns the LL from opaque to translucent, implying that the remainder consists of a single physical phase, i.e. an aqueous gel formed by the mucins [48].

Extracellular InsP_6 is not present in the *E. multilocularis* metacestode [43], in coincidence with the lack of microscopical detection of the granules, which as mentioned are also not observed in *E. vogeli*. This lack of conservation across the genus makes the function of InsP_6 accumulation in *E. granulosus* all the more mysterious.

Biosynthesis and turnover of the laminated layer

The deployment of the massive LL, particularly in *E. granulosus*, can be considered a biosynthetic feat by the much thinner GL. It is clear from the available microscopical evidence [7] and from biological commonsense that the GL carries out a polarized exocytic activity that results in LL build-up. More precisely, this is the responsibility of the GL syncytial tegument. This tegument is analogous to that present in the internal surface of the brood capsules and external surface of the protoscoleces, also syncytial and microtriche-bearing (Figure 5(a)). However, the GL tegument is specialized in comparison with that of the brood capsules, being thicker and presenting numerous vesicles and abundant, large mitochondria [49], presumably reflecting necessities associated with LL biosynthesis. The function of the brood capsules is to generate protoscoleces, through inward budding. However, everted brood capsules can synthesize an external LL, and probably give rise to daughter cysts [6]. Even intact brood capsules can synthesize an inward-facing LL, in what is apparently an abnormal developmental pathway [50]. Therefore the normally inward-facing tegumental pole of the brood capsule, analogous to the outward-facing tegumental pole of the GL, has a latent but normally suppressed capacity to secrete LL components. It should be noted that the protoscolex tegument can differentiate into GL tegument and then synthesize LL during reverse development towards metacestode, as it happens in secondary infections.

Synthesis of the LL starts soon after the beginning of cystic development. *E. granulosus* oncospheres developing

in vitro secrete fibrillate material as early as three days after activation, although the first material resembling the mature LL meshwork appears by day six [51]. By day eight, a second wave of fibrillar material has been secreted, which is delineated outwardly and inwardly by particles, probably the InsP_6 deposits. *In vivo*, the LL has been observed to appear 14 or 20 days after infection by *E. multilocularis* oncospheres [12,49,52]. It is probable that when metacestodes develop from protoscoleces, which need to become reprogrammed for reverse development, LL formation is delayed, appearing after approximately 28 days *in vitro* [53], or after 20–40 days *in vivo* [7,54].

In all likelihood, the LL *O*-glycans are synthesized in the Golgi apparatus of the GL tegumentary cytons. The *E. granulosus* GL transcriptome includes sequences that code for two enzymes with the potential to catalyze the first step in this synthesis, that is UDP-GalNAc:polypeptide GalNAc transferases, one of which has been characterized [55]. This transcriptome also contains sequences for galactosyltransferases and for a Golgi membrane transporter for UDP-Gal. In spite of these clues, the biosynthetic machinery of the LL glycans is essentially entirely unknown. It can be anticipated that this machinery will be highly analogous among different *Echinococcus* species; for example, the two sets of glycans shown in Figure 3 could result from the actions of the same set of glycosyltransferases as long as the relative activities of individual enzymes differed.

The exocytic vesicles in appearance responsible for the discharge of the LL mucin component were termed T4 vesicles [7]. They originate in the Golgi apparatus and contain material very similar to that of the LL meshwork, which cannot be easily discerned as membrane-bound or otherwise. The first 3 μm of LL adjacent to the *E. granulosus* GL feature fibrils that instead of forming a meshwork are arranged loosely and approximately perpendicular to the tegument surface [5]. This suggests that incorporation into the meshwork takes place some time after externalization, perhaps after the mucins are released from a hypothetical anchor in the tegumental membrane.

The InsP_6 deposits of the *E. granulosus* LL can be easily matched to their intracellular precursors present within GL tegumentary cytons [5,48]. The intracellular deposits are contained within so-called G vesicles, which are apparently Golgi-originated, carry a single granule each, and can be occasionally seen participating in exocytosis at the tegumental surface. Therefore, precipitation of the insoluble calcium salt of InsP_6 takes place in an intracellular vesicular compartment and the resulting particles are then exocytosed. Calculations based on equilibrium constants predict that InsP_6 will precipitate with available Ca^{2+} under a range of possible conditions expected in exocytic pathway compartments [23]. Although InsP_6 synthesis, which is cytosolic and nuclear, is thought to be conserved throughout animals [56], it is translocation to a vesicular compartment that is of particular interest in relation to *E. granulosus*. InsP_6 translocation is common in plants, and an ABC-family transporter responsible for it has been recently identified [57]. The existence of an InsP_6 transporter in the endoplasmic reticulum of animal cells has been suggested on the basis that the only enzyme known to dephosphorylate InsP_6 (multiple inositol phosphate phosphatase, MIPP) is found exclusively in the lumen of this compartment, yet influences cytosolic levels of this compound [58]. It can therefore be speculated that the biosynthesis of the InsP_6 deposits in the *E. granulosus* GL tegument is carried out by a pathway that is common to animals, but which in this structure features a particularly high activity of the InsP_6 transporter and/or low MIPP activity.

The LL must be somehow remodeled and/or turned over. Successive laminations are formed from the inside of pre-existing ones, pushing these towards the outside. As the parasite can grow from approximately 30 μm in diameter at the time of commencement of LL synthesis [51] to tens of cm (for *E. granulosus*), the external strata of the LL must always be under tension. This is reflected in the turgidity of normal *E. granulosus* cysts, and in isolated LL pieces curling up with the opposite concavity to that found in the intact cyst [4]. Although the LL is elastic, elasticity alone cannot explain growth by up to six orders of magnitude in linear dimension, and therefore mechanical and/or chemical loosening of the structure must take place. Figure 5b summarizes what is known on LL structure, synthesis, turnover, as well as unresolved issues.

Concluding remarks

Because the LL is difficult to work with, and occurs only in a genus of neglected disease-causing organisms, large time gaps have separated successive steps in its elucidation: in the late 1990 s and 2000 s, Gottstein, Hemphill, Kohler, and their collaborators (in *E. multilocularis* and *E. vogeli*; [15,22,26,40]), and ourselves (in *E. granulosus;* [23,24,27,43,48]) have given continuation to pioneering work carried out in the 1960 s to 1980 s [5,7,18–21,59]. It has been fascinating to arrive at explanations for observations that lay forgotten in papers published decades back (Box 1). These advances notwithstanding, transcriptomic and genomic data still needs to be matched with future proteomic information on the apomucins to complete a basic understanding of the LL.

As will be discussed in a forthcoming Review [60], the LL appears to be evolutionarily shaped for eliciting non-inflammatory responses from the host immune system. This makes it a promising study subject in the area of helminth-derived immunosuppressive molecules [61]. Such immunological studies must be grounded in a molecular-level understanding of this peculiar structure, in part already available and in part to be obtained in the coming years.

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