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C0066 Biology and Systematics of *Echinococcus*

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Contents

Abstract

The biology of *Echinococcus*, the causative agent of echinococcosis (hydatid disease) is reviewed with emphasis on the developmental biology of the adult and metacestode stages of the parasite. Major advances include determining the origin, structure and functional activities of the laminated layer and its relationship with the germinal layer;

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and the isolation, in vitro establishment and characterization of the multipotential germinal cells. Future challenges are to identify the mechanisms that provide *Echinococcus* with its unique developmental plasticity and the nature of activities at the parasite–host interface, particularly in the definitive host. The revised taxonomy of *Echinococcus* is presented and the solid nomenclature it provides will be essential in understanding the epidemiology of echinococcosis.

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1. INTRODUCTION

p0010

[Q3]

Echinococcus Rudolphi, 1801 (see Chapter 1), is a small endoparasitic flatworm belonging to the Class Cestoda (Table 1). It is a 'true tapeworm' (Subclass Eucestoda) and as such exhibits the features that characterize this group (Table 1; Fig. 1). It has no gut and all metabolic interchange takes

t0010 **Table 1** Classification of *Echinococcus*

Phylum Platyhelminthes

Soft-bodied, triploblastic and acoelomate; dorsoventrally flattened with cellular outer body covering; excretory system protonephridial

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Class Cestoda

Endoparasites; gut absent; outer body covering a living syncytial tegument with microtriches

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Subclass Eucestoda

True tapeworms; adults characteristically with elongated body (strobila) consisting of linear sets of reproductive organs (proglottids); specialized anterior attachment organ, a scolex; hermaphrodite with indirect life cycles

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Order Cyclophyllidea

Scolex with four muscular suckers and a rostellum usually armed with hooks strobila consisting of proglottids in various stages of development and each proglottid clearly demarcated by external segmentation; eggs round, not operculate, containing nonciliated six-hooked oncosphere

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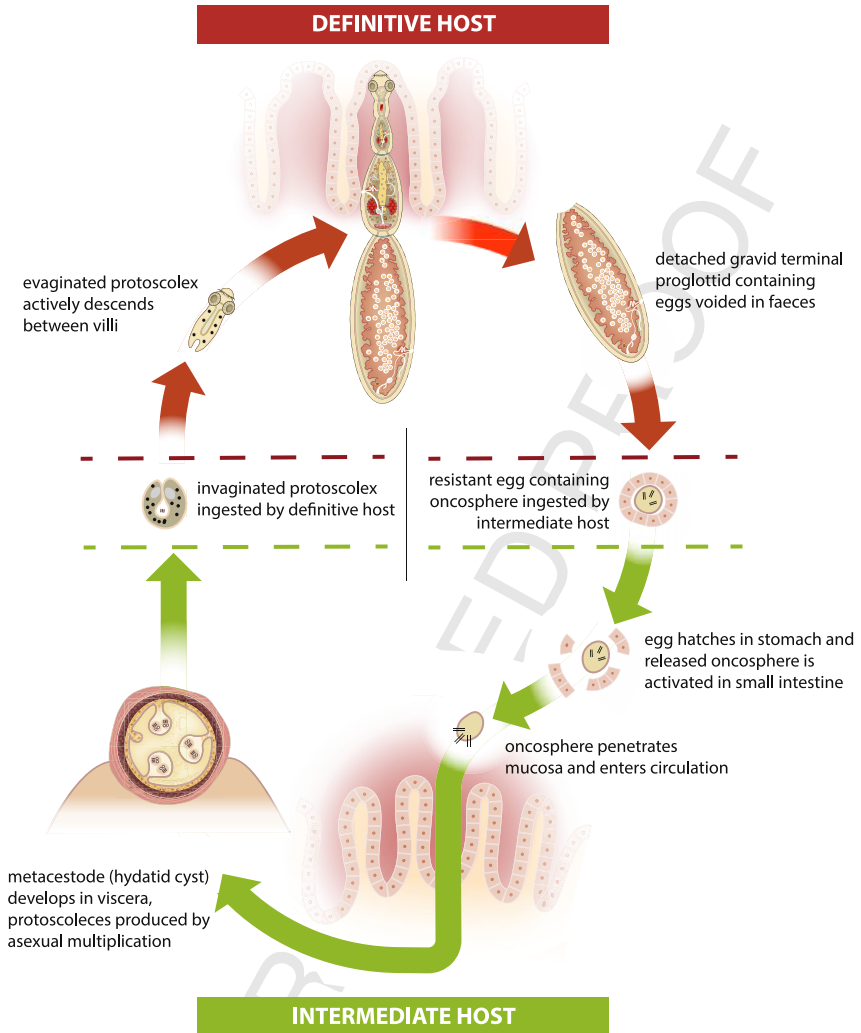*Family Taeniidae*

[Q17] Adults in small intestine of carnivores and man; intermediate hosts all mammalian; scolex with rostellum usually armed with double row of hooks; genitalia unpaired in each proglottid with marginal genital pore irregularly alternating; eggs with radially striated hardened 'shell' (embryophore) metacestode a cysticercus, coenurus, **hydatid** or strobilocercus
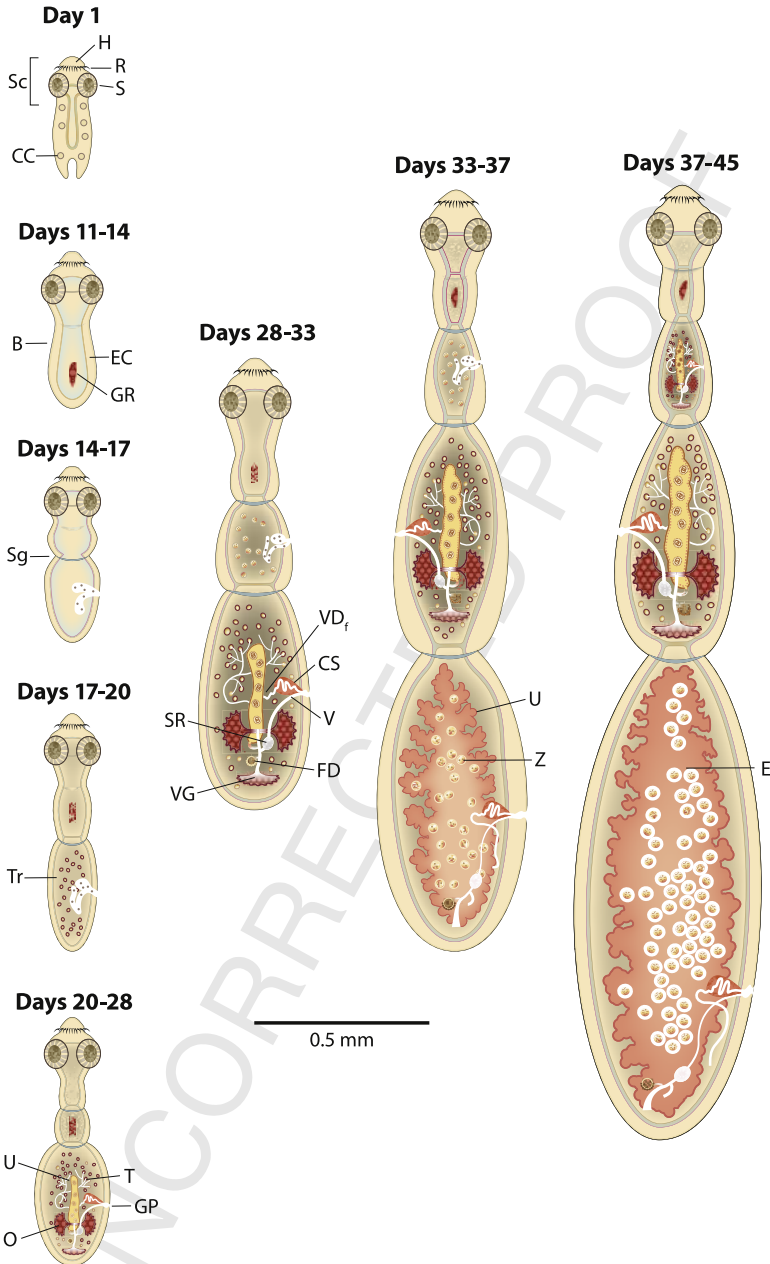

DEFINITIVE HOST

evaginated protoscolex
actively descends
between villi

detached gravid terminal
proglottid containing
eggs voided in faeces

invaginated protoscolex
ingested by definitive host

resistant egg containing
oncosphere ingested by
intermediate host

egg hatches in stomach and
released oncosphere is
activated in small intestine

oncosphere penetrates
mucosa and enters circulation

metacestode (hydatid cyst)
develops in viscera,
protoscoleces produced by
asexual multiplication

INTERMEDIATE HOST

Figure 1 Basic life cycle of *Echinococcus*.

place across the syncytial outer covering, the tegument. Anteriorly, the adult possesses a specialized attachment organ, the scolex, which has two rows of hooks on the rostellum, and four muscular suckers (Fig. 2). A narrow 'neck' region separates the scolex from the rest of the body, or strobila, which is 'segmented' and consists of a number of reproductive units (proglottids) (Fig. 2). *Echinococcus* has an indirect, two host life cycle in which the sexually reproducing adult is hermaphrodite and the larval metacestode stage, the hydatid cyst, proliferates asexually (Fig. 1).

Figure 2 Stages of development of adult *Echinococcus granulosus* in the definitive host. (The periods at which various stages appear may vary and are dependent on 'strain'/ isolate of parasite and various host factors). Day 1: Protoscolex has evaginated and elongated; contains numerous calcareous corpuscles. Days 11—14: calcareous corpuscles

p0015   Apart from its size, only a few millimetres long, and the possession of rarely more than five proglottids, *Echinococcus* is a typical taeniid cestode requiring two mammalian hosts to complete its life cycle (Fig. 1); a carnivorous definitive host in which the adult cestode develops in the small intestine, and a herbivorous or omnivorous intermediate host in which the metacestode develops, usually in the viscera. Unlike *Taenia*, the metacestode exhibits a low degree of host specificity and has a much greater reproductive potential. The definitive host is always a carnivore. The metacestode is a fluid-filled cystic structure that undergoes asexual multiplication to produce large numbers of scolices, termed protoscoleces. There may be several thousand protoscoleces within a single cyst, and each one is capable of developing into a sexually mature adult worm. Following sexual reproduction adult worms produce fertilized eggs, each containing a single embryo (oncosphere). Proglottids containing fully developed eggs are voided with the faeces of the definitive host. They may attach to the perianal region of the definitive host or contaminate the environment. The eggs that are released from proglottids are surrounded by a thick, resistant outer covering and are capable of surviving in the environment for extended periods.

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have disappeared; lateral excretory canals are conspicuous; genital rudiment present denoting formation of first proglottid; constriction and clear area below the neck ('Banding') marks the site of the first segment. Days 14–17: genital rudiment has divided into two and extends unilaterally; first segment fully formed. Days 17–20: rudimentary testes appear in first proglottid; initial stages in formation of second proglottid. Days 20–28: two-segmented worm; male genitalia – testes, cirrus and vas deferens – have developed; female genitalia – ovary, Mehlis' gland and vitelline gland – still developing; uterus appears as a streak; both cirrus and vagina open to exterior via lateral genital pore. Days 28–33: male and female genitalia in terminal proglottid fully mature; uterus still dilating; penultimate proglottid has developing genitalia; either a band or third segment appears. Days 33–37: ovulation and fertilization in terminal proglottid; fully dilated uterus contains dividing zygotes; male and female genitalia degenerating in terminal proglottid; mature genitalia in penultimate proglottid and developing genitalia in ante-penultimate proglottid; strobila divided by three or four segments. Days 37–45: gravid with embryonated eggs in uterus of terminal proglottid – eggs have fully formed embryophore ('thick-shelled') and contain embryo (oncosphere); zygotes in uterus of penultimate proglottid and maturing genitalia in ante-penultimate proglottid; strobila divided by three, four or five segments. *B*, band; *CC*, calcareous corpuscles; *CS*, cirrus sac; *E*, embryonated eggs; *EC*, excretory canal; *FD*, female reproductive ducts; *GP*, genital pore; *GR*, genital rudiment; *H*, Hooks, *O*, ovary; *R*, rostellum; *S*, sucker; *Sc*, scolex; *Sg*, segment; *SR*, seminal receptacle; *Tr*, rudimentary testes; *T*, testes; *U*, uterus; *V*, vagina; *VD$_f$*, vas deferens; *VG*, vitelline gland; *Z*, zygotes. Scale bar, 0.5 mm. *Based on an original drawing by L.M. Kumaratilake.*

Numerous species of herbivorous or omnivorous intermediate hosts are susceptible to infection with the metacestode following accidental ingestion of the eggs.

s0015 2. TERMINOLOGY

p0020   Infection with *Echinococcus* may be naturally transmitted between man and other animals. It thus claims membership of the most significant group of communicable diseases, the zoonoses. The clinical and economic significance of the parasite are almost completely confined to infection with the metacestode. Hydatid disease, hydatidosis and echinococcosis are all terms used to refer to infection with the metacestode. Strictly speaking, the terms hydatid disease and hydatidosis should be restricted to infection with the metacestode, and echinococcosis to infection with the adult stage. This is the convention with *Taenia* infections in which the terms cysticercosis and taeniasis apply to infection with the metacestode (cysticercus) and adult, respectively. However, more recently a consensus has been reached to use the term echinococcosis for infections with the metacestode of *Echinococcus* to clarify the distinctness between the diseases in humans caused by *Echinococcus granulosus* and *Echinococcus multilocularis*, cystic echinococcosis (CE) and alveolar echinococcosis (AE), respectively (Table 2). *Echinococcus oligarthra* and *Echinococcus vogeli* both cause polycystic echinococcosis (PE) in humans.

p0025   The term strain was introduced during the period of taxonomic uncertainty to refer to intraspecific variants of *Echinococcus* with defined phenotypic and subsequently genotypic characteristics (Lymbery and Thompson, 2012; Thompson and Lymbery, 1990, 1996). The term strain has largely been replaced by genotype as this enabled a numerical system to be developed to refer to the different strains (Table 2). However, the majority of strains/genotypes are now recognized as distinct species and the revised classification is shown in Table 2.

s0020 3. TAXONOMY

s0025 3.1 Species, strains and species

p0030   There has been a long history of taxonomic confusion at the species level in the genus *Echinococcus*. This has been reviewed extensively over the years and it is not intended to reiterate the history here. In summary,

t0015 **Table 2** Current taxonomy of *Echinococcus*

Species	Strain/genotype	Known intermediate hosts	Known definitive hosts	Infectivity to humans	Disease
Echinococcus granulosus	Sheep/G1	Sheep (cattle, pigs, camels, goats, macropods)	Dog, fox, dingo, jackal and hyena	Yes	CE
	Tasmanian sheep/G2	Sheep (cattle?)	Dog, fox	Yes	CE
	Buffalo/G3	Buffalo (cattle?)	Dog, fox?	Yes	CE
Echinococcus equinus	Horse/G4	Horses and other equines	Dog	Probably not	CE?
Echinococcus ortleppi	Cattle/G5	Cattle	Dog	Yes	CE
Echinococcus canadensis	Cervids/G8,G10	Cervids	Wolves, dog	Yes	CE
Echinococcus intermedius	Camel/Pig/G6/G7	Camels, pigs, sheep	Dog	Yes	CE
Echinococcus felidis	Lion/?	Warthog, (zebra, wildebeest, bushpig, buffalo, various antelope, giraffe Hippopotamus?)	Lion	?	–
Echinococcus multilocularis	Some isolate variation	Rodents, domestic and wild pig, dog, monkey, (horse?)	Fox, dog, cat, wolf, racoon–dog, coyote	Yes	AE
Echinococcus shiquicus	?	Pika and ?	Tibetan fox and?	?	AE? [Q18]
Echinococcus vogeli	None reported	Rodents	Bush dog	Yes	PE
Echinococcus oligarthra	None reported	Rodents	Wild felids	Yes	PE

AE, alveolar echinococcosis; *CE*, cystic echinococcosis; *PE*, polycystic echinococcosis.
Data from Thompson, R.C.A., Lymbery, A.J., Constantine, C.C., 1995. Variation in *Echinococcus*: towards a taxonomic revision of the genus. Adv. Parasitol. 35, 145−176; Thompson, R.C.A., McManus, D.P., 2002. Towards a taxonomic revision of the genus *Echinococcus*. Trends Parasitol. 18, 452−457; Jenkins, D.J., Romig, T., Thompson, R.C.A., 2005. Emergence/re-emergence of *Echinococcus* spp.—a global update. Int. J. Parasitol. 35, 1205−1219; Thompson, R.C.A., 2008. The taxonomy, phylogeny and transmission of *Echinococcus*. Exp. Parasitol. 119, 439−446; Thompson, R.C.A., Jenkins, D.J., 2014. *Echinococcus* as a model system. Int. J. Parasitol. 44, 865−877.

many species have been described and just as many invalidated on sound taxonomic grounds. What has been clear, however, from the earliest descriptions of the parasite is that the genus exhibits considerable variability at the species level in terms of host specificity, morphology, antigenicity, development rate, and cycles of transmission (Lymbery and Thompson, 2012; Thompson and Lymbery, 1988; Thompson and McManus, 2001, 2002; Thompson, 2008). However, concerns about the genetic basis of phenotypic differences, particularly with respect to morphology, intermediate host specificity and evidence of reproductive isolation, have been the principle reasons for questioning the taxonomic status of some species [Q4] (also see Chapter 3).

p0035 One of the most important observations in the recent history of *Echinococcus* taxonomy was made as a result of studies on the in vitro cultivation of the parasite, in which protoscoleces collected from hydatid cysts in horses failed to develop in the same way as those of sheep origin. Protoscoleces from horses evaginated and increased in length but failed to undergo proglottisation or segmentation, even though they were grown in exactly the same diphasic medium (Smyth and Davies, 1974a). This fairly simple observation resulted in radical shifts in our understanding of the epidemiology of echinococcosis and transmission of the aetiological agents as well as their taxonomy and phylogenetic relationships (Howell and Smyth, 1995; Thompson and Lymbery, 2013; Thompson and Jenkins, 2014). The results demonstrated that there were fundamental physiological differences between *E. granulosus* of sheep and horse origin and the coining of the term 'physiological strain differences' (Smyth and Davies, 1974b; Smyth, 1982). This had a broad influence beyond *Echinococcus*, and in particular the importance of combining phenotypic and genetic differences in the characterization and description of parasites at the intraspecific level (Lymbery and Thompson, 2012; Thompson and Lymbery, 1990, 1996; Thompson and Jenkins, 2014).

p0040 The observation of physiological differences between the two parasites of sheep and horse origin complemented earlier epidemiological and taxonomic studies on *Echinococcus* of horse origin (Williams and Sweatman, 1963). These demonstrated morphological differences between the two forms that were considered to be taxonomically significant, and to reflect differences in host specificity and their life cycles. The sympatric occurrence of distinct sheep and horse dog cycles in several European countries (Gonzalez et al., 2002; Thompson and Smyth, 1975; Thompson, 2001) supported the existence of two separate host–adapted species. In addition,

epidemiological evidence not only demonstrated distinct differences in intermediate host specificity but also that, unlike the sheep strain (= *E. granulosus*), the horse strain (= *Echinococcus equinus*; Table 2) does not appear to be infective to humans (Thompson and Lymbery, 1988; Thompson, 1995, 2008).

p0045   The outcomes of this research caused an attitudinal shift in studies on *Echinococcus* that were not constrained by taxonomic issues with the growing realization that a 'strain' was an acceptable term when describing variability at the phenotypic, and subsequently genotypic levels within species of parasites (Lymbery and Thompson, 2012; Thompson and Lymbery, 1990, 1996). As such, research on the horse and sheep strains of *E. granulosus* led to similar studies on *Echinococcus* of cattle, pig, camel and cervid origin with the description and characterization of several new strains/genotypes (Thompson and McManus, 2002; Thompson, 2008, Table 2). These studies not only confirmed the existence of a number of host–adapted life cycles in different parts of the world but also provided additional data on developmental differences between strains which may impact on control (Lymbery and Thompson, 2012; Thompson, 2001, 2008; Thompson and Lymbery, 1988; Thompson and McManus, 2002). These informal groupings were retained for many years but with the advent of molecular characterization they were shown to be genetically distinct (Thompson and McManus, 2001). PCR–based techniques using a variety of genetic loci, and sequencing of nuclear and mitochondrial DNA, coupled with molecular epidemiological studies in endemic areas, confirmed the genetic and morphological distinctness of the host–adapted strains and revealed phylogenetic relationships which support a robust, meaningful taxonomy based on a previously documented nomenclature (Table 2; Bowles et al., 1994; Cruz-Reyes et al., 2007; Harandi et al., 2002; Huttner et al., 2009; Jenkins et al., 2005 Lavikainen et al., 2003, 2006; Moks et al., 2008; Nakao et al., 2013; Pednekar et al., 2009; Romig et al., 2006, 2015; Saarma et al., 2009; Thompson et al., 1995, 2006, 2014; Thompson, 2001, 2008; Thompson and McManus, 2002; Thompson and Jenkins, 2014; Tigre et al., 2016). Interestingly, the nomenclature used for these 'species' conforms to that proposed by observational parasitologists in the 1920s–60s, before molecular tools were available to confirm and support their morphological descriptions and epidemiological observations (Thompson et al., 1995, 2014; Thompson and McManus, 2002; Thompson, 2008). Importantly, these molecular epidemiological studies have given confidence to the morphological characters used for species discrimination, which now offer a simple, cost-effective

means of parasite identification in endemic foci where the application of molecular tools may not be practical or cost effective (Harandi et al., 2002, 2012; Lymbery and Thompson, 2012; Sharbatkhori et al., 2011).

p0050   Interestingly, in terms of considering the life cycles of these host-adapted species, Rausch (1997) considered that a uniform, typical larval structure, with long survival without degenerative change and high protoscolex production, are characteristic of metacestodes of recognized species in their natural intermediate hosts, and this is the case with *E. granulosus, E. multilocularis, Echinococcus equinus, Echinococcus ortleppi, Echinococcus canadensis, Echinococcus intermedius* and *Echinococcus felidis* (Table 2). In the future, it is possible that new species of *Echinococcus* will be discovered, particularly as more studies investigate the parasites of wildlife in areas such as Africa and China. Molecular tools will undoubtedly play a role in these studies, as for example the finding a novel genotype of *E. granulosus* in a human patient in Ethiopia (Wassermann et al., 2016). It will be important to ensure that descriptions of new strains or species do not rely solely on molecular data and that as much biological information is obtained to support the epidemiological significance of such discoveries.

4. EPIDEMIOLOGICAL SIGNIFICANCE OF INTRA- AND INTERSPECIFIC VARIATION

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p0055   Differences in host specificity between strains and species of *Echinococcus* is clearly important in areas where there are different host assemblages and cycles of transmission (Lymbery and Thompson, 2012; see

[Q5]   Chapter 5). Developmental differences may also impact on the control of *Echinococcus* in different regions, particularly if the regular drug treatment of dogs is used to prevent transmission with the frequency of treatment less than the time required for worms to reach patency. Research has demonstrated that a standard treatment frequency may not 'break the cycle of transmission' since the maturation rate and onset of egg production varies between strains and species of *Echinococcus* (Lymbery and Thompson, 2012; Thompson, 2001; Thompson and McManus, 2001, 2002). Similarly, differences in development and maturation between species of definitive hosts must be taken into account in areas where more than one species is involved in transmission, e.g., *E. multilocularis* in foxes, dogs and cats (Kapel et al.,

[Q6]   2006; Thompson et al., 2006). Evidence of differences in infectivity for humans, clinical manifestations and pathogenicity are also clearly important (Lymbery and Thompson, 2012; Thompson, 2001). As described earlier, all

available epidemiological evidence supports the conclusion that humans are not susceptible to infection with *Echinococcus equinus*. With *E. canadensis*, it has long been thought that the clinical consequences of infection in humans are negligible compared to infection with *E. granulosus* (Thompson, 2015). In part, this may be due to the long progression of the disease in humans, often without symptoms, and the nonspecificity of symptoms when they do occur. However, the limitations of serological tests used to diagnose cystic infections caused by *E. canadensis* have contributed to human cases being underdiagnosed (Jenkins et al., 2013; Thompson et al., 2014; Thomposn, 2015). There has been a reliance on tests developed for *E. granulosus* and there are known to be antigenic differences between *E. canadensis* and *E. granulosus* (Jenkins et al., 2011, 2013; Schurer et al., 2013, 2014). The two genotypes of *E. canadensis* also appear to vary in virulence in humans with G8 more pathogenic than previously considered, with two severe cases recently reported (Jenkins et al., 2011; Thompson, 2015). Some species of *Echinococcus* develop very differently in different species of intermediate host. *Echinococcus granulosus* produces viable, fertile, cysts in sheep whereas in cattle and pigs, cysts are usually sterile and these hosts play little role in transmission of *E. granulosus*. *Echinococcus granulosus* preferentially affects the lungs of wild macropod marsupials and establishes clinically significant infections in contrast to the seemingly benign infections that develop in the liver and lungs of sheep infected with this species (Barnes et al., 2011; Thompson, 2013).

p0060   As mentioned earlier, differences in the antigenic characteristics between species of *Echinococcus* will have a bearing on the development of immunodiagnostic procedures in different countries (Cameron, 1960; Gottstein et al., 1983; Huldt et al., 1973; Jenkins et al., 2013; Lightowlers et al., [Q7] 1984; Thompson, 2015; see also Chapter 9). It was proposed that an obvious corollary to this must be the assumption that a vaccine developed against one particular species or strain of *Echinococcus* may not protect against infection with another strain (Thompson, 1995, 2001). This has now been confirmed with recent investigations showing that the EG95 vaccine antigen developed to protect against infection with *E. granulosus* is immunologically different in *Echinococcus intermedius* (Alvarez Rojas et al., 2014). It may therefore be necessary to develop 'genotype-specific' vaccines in the future (Alvarez Rojas et al., 2014). It will also be interesting to see whether species and strains of *Echinococcus* vary in their response to particular chemotherapeutic regimes. Albendazole is the most widely used drug to treat cystic and AE, and as with other helminths β-tubulin is believed to be the target. Recent

research has demonstrated some variation in the β-tubulin gene of *E. granulosus* (Pan et al., 2011). Further, Brehm and Koziol (2014) have shown that in *E. multilocularis*, germinal cells express a β-tubulin isoform with limited affinity to benzimidazoles (See Chapter 4).

5. DEVELOPMENT OF *ECHINOCOCCUS*

Most of what we know about the developmental biology of *Echinococcus* and the host—parasite relationship has been obtained from studies on the metacestode. This is because it is relatively straightforward to maintain larval stages in axenic culture, and the availability of rodent models for maintaining cystic infections following either primary or secondary infection (Howell and Smyth, 1995). The practical and ethical difficulties, as well as safety concerns, of undertaking in vivo studies in the definitive hosts of *Echinococcus* have been a major limiting factor in studying the development of the adult parasite. If it was not for the pioneering studies of Smyth (Howell and Smyth, 1995; Smyth et al., 1966; Smyth and Davies, 1974a; Thompson and Lymbery, 2013) in developing in vitro systems for studying the developmental biology of both larval and adult stages, the underlying principles of differentiation, host—parasite relationships and evolutionary biology (Smyth, 1969; Smyth et al., 1966) are unlikely to have been discovered at a formative era in parasitology in which hypotheses were developed that could influence future, emerging research in the genomics area (Thompson and Lymbery, 2013). As such, Smyth (1969) saw the potential of exploiting *Echinococcus* as a novel model system for studying parasitism as distinct from a model for studies on evaluating anthelmintics or other antiparasitics. He thus expanded the definition of a 'model' to embrace studies on all the biological activities that supported the parasite life style of *Echinococcus*, as well as the potential of this model system for broader studies of a more fundamental nature in biology, for example, developmental plasticity and stem cells (heterogeneous morphogenesis), gene regulation and evolutionary developmental biology (Cucher et al., 2011; Smyth, 1969, 1987; Thompson and Lymbery, 2013; Thompson and Jenkins, 2014; see later and Chapter 4).

5.1 Adult

5.1.1 Establishment in the definitive host

The definitive host acquires infection by ingesting viable protoscoleces. These may be ingested still within the hydatid cyst and the masticatory

actions of the host will assist in tearing open the cyst and freeing the brood capsules. The process of excystment — removal of brood capsule and other cystic tissue — is further assisted by the action of pepsin in the stomach. Prior to ingestion, the apical region of the protoscolex (suckers, rostellum and hooks) is invaginated within the mucopolysaccharide-coated basal region of the protoscolex tegument (Fig. 1). This protects the scolex until it is stimulated to evaginate. In dogs experimentally infected with evaginated protoscoleces of *E. granulosus*, far fewer worms establish than in dogs infected with invaginated protoscoleces (Thompson, 1995).

p0075   The precise nature of the stimulus for evagination is not known. Protoscoleces are sensitive to environmental changes and evaginate in response to variations in temperature and osmotic pressure, and to agitation (Thompson, 1995). However, in an intact hydatid cyst, protoscoleces will remain viable, with the majority invaginated for several days depending on the temperature (Thompson, 1995), thus enhancing transmission in sylvatic cycles reliant on predation or scavenging by definitive hosts. Aerobic conditions appear to be essential for evagination but specific enzyme or bile is not essential although the rate of evagination is increased in the presence of bile (Smyth, 1967, 1969).

p0080   In the definitive host, the time required for evagination is variable, with the majority of protoscoleces evaginated after 6 h but complete evagination takes up to 3 days (Thompson, 1977). Following evagination, protoscoleces are initially very active as they have to rapidly locate and attach to the mucosal surface in the crypts of Lieberkuhn to avoid being swept out of the small intestine, with some actually within the crypts by 6 h after infection (Thompson, 1977). As such, motility is enhanced by a well-developed nervous system and glycogen energy reserves (Brownlee et al., 1994; Camicia et al., 2013; Hemer et al., 2014; Smyth, 1967). The evaginated protoscoleces are rich in glycogen which acts as an energy reserve although this is rapidly used up, usually within 3 h (Smyth, 1967). Activity then declines as energy reserves are replenished, accounting for a lag phase in growth during [Q9] the first 3 days of infection in dogs (Thompson, 1975). Developing worms attach mainly by grasping substantial plugs of tissue with their suckers (Smyth et al., 1969; Thompson et al., 1979; Thompson and Eckert, 1983, Fig. 3). The hooks only superficially penetrate the mucosal epithelium but their shape ensures that they act as anchors to assist in preventing the worm being dislodged.

p0085   The intestinal distribution of the adult worm appears to be similar for *E. granulosus* and *E. multilocularis* with worms dispersed unevenly along
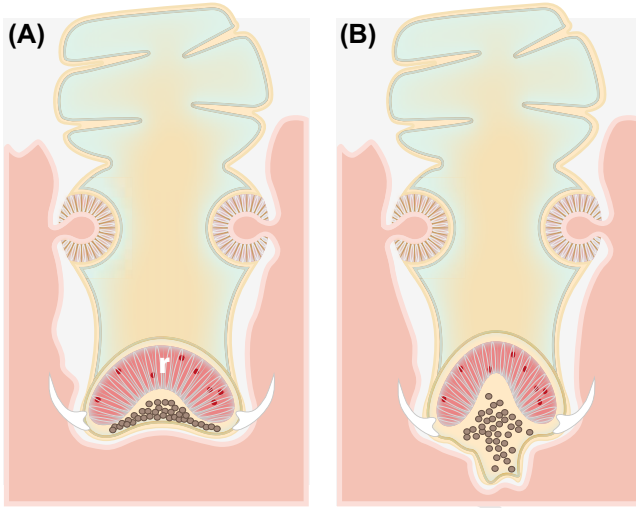
f0020 **Figure 3** Diagram illustrating adult *Echinococcus* in situ in the intestine, with suckers on the scolex grasping the epithelium at the base of the villi. Rostellum is deeply inserted into a crypt of Lieberkühn (A and B) and extensibility of the apical rostellar region is shown in (B). Rostellar gland with secretory material is anterior to the rostellar pad (r). *From Thompson, R.C.A., Jenkins, D.J., 2014.* Echinococcus *as a model system. Int. J. Parasitol. 44, 865—877.*

the intestine with most worms located in the proximal regions (Constantine et al., 1998; Thompson et al., 2006). It is not known to what extent the developing adult *Echinococcus* migrates within the small intestine, although it does move between adjacent villi. Constantine et al. (1998) found that adult *E. granulosus* in dogs did change location in the gut to form aggregates which led to differences in worm density along the intestine. Whether this is a consequence of attraction between worms and/or to particular microenvironmental sites is unclear. These local high densities of worms may promote rapid development but as the worms grow, competitive interactions or local immune responses by the host inhibit development with a change from cytosolic to more energetically efficient mitochondrial metabolism (Constantine et al., 1998).

s0050 ### 5.1.2 Activities at the interface
p0090 Observations that contact the scolex of *E. granulosus* with a proteinaceous–base-stimulated strobilate development led to studies on the nature of the interface both in vitro and in vivo and the discovery of the rostellar gland which comprises a modified group of tegumental cells situated in the apical
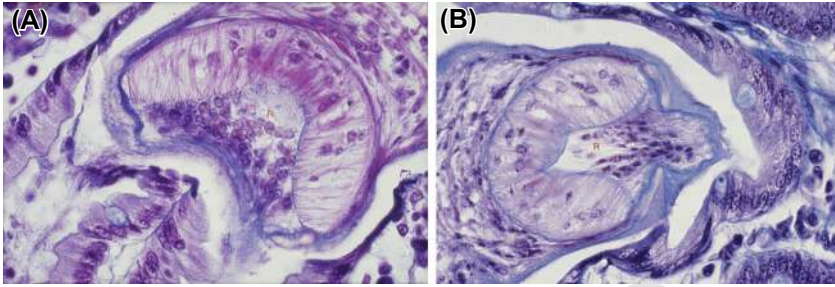
Figure 4 Section of mucosal wall of a dog's small intestine showing rostellum of *Echinococcus granulosus*, deeply inserted in a crypt of Lieberkuhn showing apical rostellar gland (R) retracted (A) and extended (B). Section stained with Martius Scarlet Blue ×60.

rostellum (Smyth, 1964a; Smyth et al., 1969; Thompson et al., 1979; Thompson and Eckert, 1983, Figs 3 and 4). However, subsequent studies demonstrated that *E. multilocularis* can differentiate sexually in monophasic media (without a solid serum base) (Howell and Smyth, 1995; Smyth and Davies, 1975; Smyth, 1979) suggesting that the stimulus for strobilate development must be more complex than previously thought (Constantine et al., 1998; Thompson et al., 1990, 2006).

p0095   Although an adult *Echinococcus* may alter its position and move up and down and between adjacent villi during development, this may not occur once the worm reaches maturity (Constantine et al., 1998). Between 20 and 35 days after infection, respectively, *E. multilocularis* and *E. granulosus* are found in a position characteristic of the mature worm. The rostellum is deeply inserted into a crypt of Lieberkuhn with the mobile apical rostellar region usually fully extended, the hooks superficially penetrating the mucosal epithelium and the suckers grasping the epithelium at the base of the villi (Smyth et al., 1969; Thompson et al., 1979; Thompson and Eckert, 1983, Figs 3 and 4). Invasion of the crypts of Lieberkuhn by the mature worm is of particular physiological significance to *Echinococcus*. It is a characteristic not shared by other adult taeniids, which achieve only a relatively superficial attachment to the mucosa of the definitive host (Beveridge and Rickard, 1975; Featherstone, 1971), presumably because of their greater size. *Echinococcus* has a very mobile and extensible apical rostellar region. Extension of this region into the crypts coincides with the commencement of secretory activity of the rostellar gland (Figs 3 and 4) and the release of secretory material by a holocrine process into the interface between parasite and host (Smyth, 1964a,b; Smyth et al., 1969; Thompson et al., 1979; Thompson and Eckert, 1983; Thompson and Jenkins, 2014). The secretion

is proteinaceous containing cystine and lipid. It is not known if there are one or more proteins secreted but Siles-Lucas et al. (2000) demonstrated that the secretion contains a regulatory protein (14–3-3) that is released into the host–parasite interface. The origin and site of synthesis of the secretion has not been determined, although large amounts occur in both the perinuclear and distal cytoplasm of the tegument as well as in the tegumental nuclei (Thompson et al., 1979; Herbaut et al., 1988). Recently, Kouguchi et al. (2013) used a surface glycoprotein from *E. multilocularis* as a vaccine in dogs which induced significant protection when administered via a mucosal route and demonstrated antibodies raised by their vaccine on the surface of the suckers, rostella and hooks. More research is required to determine whether this glycoprotein is related to the secretions from the rostellar gland.

p0100   The crypts of Lieberkuhn may represent a site of particular nutritional significance for mature *Echinococcus*. Nutrients could be derived from the lysis of host cells but there is no evidence that the rostellar gland secretion is histolytic or has any enzymatic activity. An important factor to be considered is the timing of secretory activity, which coincides with a levelling off in growth of the worm at around 30 days after infection and the commencement of egg production (Thompson et al., 1979, 2006). The secretion may therefore be associated with the maturation of ova and/or subsequent release of the gravid proglottid (apolysis). Gland activity could be recurrent with a cycle of activity associated with the maturation and release of each proglottid. The rostellar gland of *Echinococcus* is seemingly unique to *Echinococcus* (Thompson and Jenkins, 2014). Although rostellar secretions have been described in larval *Taenia crassiceps* (Krasnoshchekov and Pluzhnnikov, 1981), no gland has been described. Rostellar glands have been described in other adult cestodes, particularly proteocephalids but their function is also unclear and structurally they are different to the rostellar gland in *Echinococcus* (McCullough and Fairweather, 1989; Zd'arska and Nebesarova, 2003). Modified glandular parts of the scolex tegument have been described in some other cestodes and the most-favoured role for their secretions is one of attachment (Hayunga, 1979; Ohman-James, 1973; Richards and Arme, 1981; Sawada, 1973; Specian and Lumsden, 1981). In *Echinococcus* perhaps firm attachment is a prerequisite for apolysis, since unattached gravid worms in vitro do not shed their terminal proglottids (Thompson and Eckert, 1982). An adhesive function to assist in retention of the worms most adequately accounts for the special location of the rostellar secretory cells, site of release, and timing if apolysis necessitates particularly firm positioning.

p0105   Mature *E. granulosus* possesses two morphologically distinct types of microthrix (Thompson et al., 1982; Irshadullah et al., 1990). On the strobila, they are bladelike and rigid for most of their length and probably serve to keep the absorptive surface of the parasite and host apart, thus maintaining a free flow of nutrients at the interface between the two absorptive surfaces. On the apical rostellum and scolex the microtriches are long, slender filamentous types apparently flexible for most of their length, thus allowing the scolex and rostellum to achieve close contact with the host, perhaps to enhance adhesion (Mettrick and Podesta, 1974; Thompson et al., 1979).

p0110   One other possible function for the rostellar gland secretion is that of protection. It is feasible that the secretion may protect the worm either by inhibiting or inactivating host digestive enzymes or by interfering with [Q10] the host's immune effector mechanisms (see also Chapter 7). However, it would be reasonable to expect such a protective mechanism to operate throughout the life of the adult worm, unless, as seems to be the case, it is only after maturity that a permanent and very intimate association is achieved between parasite and host. Recent studies have demonstrated a Kunitz-type protease inhibitor (EgKI-2) in the *E. granulosus* genome, which is highly expressed in the adult worm and may play a protective role in preventing proteolytic enzyme attack thereby ensuring survival in the definitive host (Ranasinghe et al., 2015). Further research is required to determine whether EgKI-2 is a component of the rostellar gland secretions.

p0115   *Echinococcus* seldom engenders a morphologically apparent host response, although occasionally in heavy infections, there may be an excessive production of mucus (Thompson, 1995). As emphasized by Heath (1995), the scolex is in intimate contact with the systemic circulation even in the Payer's patches and would appear to maintain its privileged integrity by suppression of cytotoxic and effector cell activity in the region of the scolex. The host tissue that is grasped by the suckers is usually necrotic, but the hooks cause little damage (Thompson et al., 1979). Observations at the ultrastructural level have shown that hook damage is restricted to columnar cells with an associated loss of some host microvilli (Thompson et al., 1979). The epithelium of parasitised crypts is commonly flattened and there may be occasional rupture of a crypt wall with release of host cells into the crypt (Smyth et al., 1969). Adult worms have been observed to invade the lamina propria, but this appears to be a rare event. No substantial pathology or evidence of a host cellular reaction has been observed in infections with adult *E. granulosus* or *E. multilocularis* (Thompson et al., 1979; Thompson and Eckert, 1983).

However, the presence of the adult worm does not go unnoticed by the host and a specific humoral response with the production of circulating IgG antibodies does occur (Jenkins and Rickard, 1986). Deplazes et al. (1993) also demonstrated local humoral, IgG and IgA, and cellular reactions in the intestine of dogs experimentally infected with *E. granulosus*, emphasizing the importance of Peyer's patches in localized, specific immune responses.

p0120   The intimate association of the rostellar gland and its secretions suggests a role(s) that enhances the host—parasite relationship in favour of the parasite, which may be regulatory, nutritional and/or protective. The relationship between rostellar gland activity and localized humoral and cellular reactions (Deplazes et al., 1993) is not known but such localized reactions demonstrate stimulation of host immune effector mechanisms. The rostellar gland secretory molecules would seem to be obvious candidates for exploitation in vaccine studies since a focus on prophylaxis of the definitive host may be more attractive than the intermediate host, particularly for the control of *E. multilocularis* (Thompson, 1995; Thompson and Jenkins, 2014).

p0125   There is clearly a need for more studies on the interface and attachment of adult *Echinococcus*, as well as other cyclophyllidean cestodes (Pospekhova and Bondarenko, 2014; Thompson and Jenkins, 2014).

s0055 ### 5.1.3 Differentiation

p0130 The development of the adult parasite involves germinal and somatic differentiation and can be divided into the following processes: proglottisation; maturation; growth; segmentation (Thompson, 1995; Thompson et al., 2006). Germinal differentiation comprises proglottisation, which refers to the sequential formation of new reproductive units (proglottids), and the maturation of the proglottids. Somatic differentiation consists of growth, i.e., increase in size, and the somatic delineation of each proglottid by segmentation (strobilisation). Segmentation in cestodes is not to be confused with true mesodermal segmentation (metamerism) which occurs by distal growth not proximally as in cestodes (Freeman, 1973). In some cestodes, including *Echinococcus* (see later), proglottisation may occur without segmentation. Thus both terms are necessary for a full comprehension of the process of development (Freeman, 1973), but should not be referred to interchangeably. Segmentation in cestodes, including *Echinococcus*, does not involve the formation of any separatory structure or 'interproglottid' membrane between adjoining proglottids (Mehlhorn et al., 1981). The demarcation of each proglottid is purely an external phenomenon caused by an infolding of the tegument which gives rise to the characteristic constricted appearance.

It also appears that the microtriches in the infolded regions of the tegument may be linked together thus stabilizing the infoldings (Mehlhorn et al., 1981).

p0135   The four developmental processes described earlier take place independently. This has been demonstrated by studies on *E. granulosus* and *E. multilocularis* in vitro (Smyth, 1971; Smyth and Davies, 1975; Smyth and Barrett, 1979), and *E. granulosus* in vivo (Thompson, 1977; Constantine et al., 1998; Thompson et al., 2006). Further, in the adult parasite, somatic and germinal differentiation are independently associated with a transition from cytosolic to mitochondrial energy metabolism (Constantine et al., 1998). This very complicated process of cytodifferentiation was considered to indicate the possible existence of several primitive cell lines as in *Hymenolepis diminuta* (Sulgostowska, 1972, 1974). However, preliminary studies on cytodifferentiation in adult *E granulosus* suggested that only one primitive cell type exists located in the neck region of the adult worm (Gustafsson, 1976) (see later and Chapter 4). In vitro studies on *Echinococcus* demonstrated that this so-called 'germinative' (germinal) cell was also extremely sensitive to environmental and/or nutritive conditions (Smyth and Barrett, 1979; Howell and Smyth, 1995). In some cultures mainly germinal cells were produced, leading to proglottisation and maturation but no segmentation, whereas in other cultures more somatic cells were produced leading to growth without sexual maturation. These observations from in vitro studies have been complemented by studies in vivo, comparing the development of adult *E. multilocularis* in foxes, raccoon dogs, cats and dogs. Thompson et al. (2006) compared developmental processes in the different definitive hosts, and by examining germinal and somatic differentiation, confirmed that these processes can be influenced by their environment; in this case the small intestine of different carnivore host species. In cats, the investment by worms in the somatic processes of growth and segmentation was not complemented in terms of maturation, in contrast to foxes, dogs and raccoon dogs, demonstrating the fine balance that exists which can easily be upset if environmental factors are not correct (Thompson et al., 2006).

s0060 5.1.4 Sequential development

p0140 The newly evaginated protoscolex contains an abundance of calcareous corpuscles (Fig. 2), which consist of an organic base and inorganic material (Smyth, 1969). They are of cellular origin with the characteristic concentric layers of mineral deposition increasing with age (Ohnishi and Kutsumi, 1991; Pawlowski et al., 1988). They develop from living cells and two

different mechanisms of formation coexist with corpuscles originating from the nucleus or cytoplasm. Their function may be that of a buffering system or a source of inorganic ions, CO_2 and phosphates (Smyth, 1969), but their transitory nature suggests an association with cell death (Thompson, 1995) and recently they have been shown to be associated with autophagy and catabolic processes (Loos et al., 2014). Within 3—4 days after infection, the lateral excretory canals of the young worm are clearly evident and by the end of the first week a posterior excretory bladder is seen (Smyth and Davies, 1974a). The excretory system of *Echinococcus*, like all other cestodes, is based on the platyhelminth protonephridial system with the lateral excretory canals (Fig. 2) acting as collecting ducts for numerous flame cells distributed throughout the parenchyma. However, the physiology of excretion has not been investigated. Evidence that the excretory ducts of some pseudophyllidean cestodes are capable of absorption (Lindroos and Gardberg, 1982) raises the possibility that the excretory system of *Echinococcus* could also function as a distributive system.

p0145   The sequence of development described later and illustrated in Fig. 2 refers to *E. granulosus*. Although it is essentially the same in other species, the rate of development varies, particularly in relation to growth, onset of egg production and number of proglottids produced (Kapel et al., 2006; Thompson et al., 2006). The first sign of proglottisation is the appearance of a genital rudiment or anlagen which may appear as early as 11 days after infection, separated from the scolex by a clear band. By 14 days the first proglottid is clearly evident as a darkly staining body demarcated from the scolex by the transverse infolding of the tegument which delineates the first segment. Within 1—2 days a lateral branch forms from the genital rudiment which will eventually open to the exterior via the genital pore. Subsequent stages of maturation follow the general cestode pattern and are summarized in Fig. 2. Growth, as determined by total worm length, exhibits a steady log linear increase throughout the first 35 days of infection apart from a lag period during the first 3 days (Thompson, 1995). Growth also levels off prior to egg production (Kapel et al., 2006; Thompson, 1995; Thompson et al., 2006).

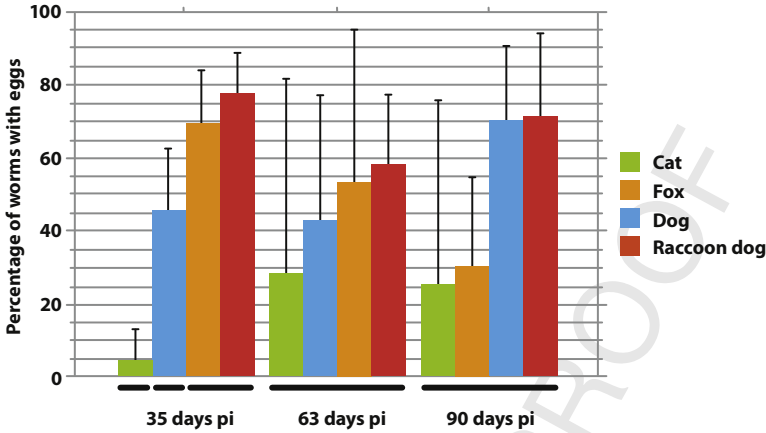s0065   ### 5.1.5 Sexual reproduction

p0150   Mature *Echinococcus* is hermaphrodite (Fig. 2) and capable of both self- and cross-insemination (Smyth and Smyth, 1969) although it is predominantly self-fertilizing (Lymbery et al., 1997; Lymbery and Thompson, 2012; see Chapter 3). It is not known whether cross-insemination between two

individuals takes place. Hermaphroditism combined with self-insemination is obviously an advantage to a small worm such as *Echinococcus*, which might find it difficult to find another worm, particularly in light infections. Furthermore, such a reproductive mechanism has a significant evolutionary potential (see Chapter 3). The requirements for self-insemination in *Echinococcus* appear to be extremely complex as suggested by the repeated failure to achieve fertilization in vitro (see Howell and Smyth, 1995; Smyth and Davies, 1974a; Smyth, 1979; Thompson and Jenkins, 2014). It has been suggested that there may be specific or nonspecific factors in the intestinal secretions of the definitive host which activate the cirrus to commence its copulatory movements and that without such stimulation self–insemination may not occur (Smyth, 1982).

s0070 5.1.6 Egg production and subsequent development

p0155 The initial onset of egg production varies between species and even between strains. In *E. granulosus* it ranges from 34 to 58 days, whereas *E. multilocularis* has a far more rapid rate of maturation with egg production commencing between 28 and 35 days after infection (Kapel et al., 2006; Thompson and Eckert, 1982; Thompson et al., 1984; Thompson et al., 2006).

p0160   Although development up to the initial onset of egg production has been extensively studied, there have been few studies of subsequent development. Thompson et al. (2006) studied the maturation of adult *E. multilocualris* in experimentally infected foxes, dogs, raccoon dogs and cats, at 35, 63 and 90 days postinfection. They found that egg production was a continuous process throughout the 90 day period (Fig. 5). The number of eggs produced is uncertain, with reports varying between 100 and 1500 per proglottid (Heath and Lawrence, 1991; Rausch, 1975; Thompson and Eckert, 1982), with *E. multilocularis* producing fewer eggs per proglottid than *E. granulosus*. A study by Kapel et al. (2006) found that approximately 114, 42 and 27 eggs per worm were excreted in the faeces of dogs, raccoon dogs and foxes, respectively, experimentally infected with *E. multilocularis*, over a 90-day period. However, it is not known how often species of *Echinococcus* produce gravid proglottids. Based on the rate of development during the first 40 days of infection, it has been estimated that gravid proglottids of *E. granulosus* are produced and detached every 7—14 days (Schantz, 1982; Smyth, 1964a). However, without further investigation it is impossible to conclude whether the rate of proglottisation after apolysis in *Echinococcus* is constant or declines. It is also not known how long the adult parasite may survive in the definitive host. It has been reported that adult worms become

senescent after 6–20 months, although worms may live for 2 years or longer (Schantz, 1982). In the absence of accurate information on the rate of production and release of gravid proglottids and the life span of the adult parasite, it is impossible to accurately determine the reproductive potential of *Echinococcus* in the definitive host.

s0075 ## 5.2 Egg

p0165 Taeniid eggs are spherical to ellipsoid in shape and usually range in size from 30 to 50 μm and from 22 to 44 μm in their two diameters. They are morphologically indistinguishable at the light microscope level and ultra-structural studies of the eggs of *E. granulosus, E. multilocularis* and various *Taenia* species have shown that they possess similar structures consisting of several layers and membranes (Fig. 6) (Morseth, 1965; Sakamoto, 1981; Swiderski, 1982). The embryophore is the principal layer affording physical protection to the embryo, or oncosphere, since the vitelline layer ('egg shell' or outer envelope) is passively removed from the egg before it is liberated. The embryophore is relatively thick and impermeable, consisting of polygonal blocks composed of an inert keratin-like protein, which are held together by a cementing substance (Morseth, 1966; Nieland, 1968; Sakamoto, 1981).
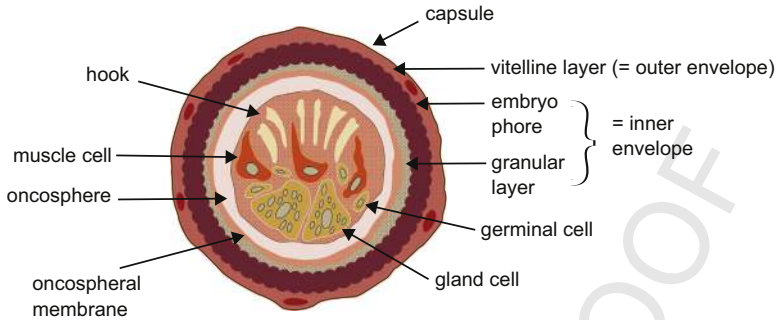
p0170   When released from the definitive host, the egg of *Echinococcus* is presumed to be fully embryonated and infective to a suitable intermediate host. However, taeniid eggs at the time of expulsion are probably at different stages of maturation and immature eggs may mature in the environment under appropriate conditions (Gemmell and Roberts, 1995).

p0175   *Echinococcus* eggs are extremely resistant enabling them to withstand a wide range of environmental temperatures for many months (Gemmell et al., 1986; Schantz et al., 1995; Thevenet et al., 2005; Veit et al., 1995). Dessication is lethal and the end points for temperature are approximately +40°C to −70°C (Gemmell and Roberts, 1995). However, the availability of moisture is a limiting factor in survival and recent research has shown the eggs of *E. multiloculalris* suspended in water could survive for 2 h after exposure to a temperature of +65°C (Federer et al., 2015).

[Q11]

[Q12]

s0080 5.2.1 Hatching and activation

p0180 When ingested by a suitable intermediate host, viable eggs of *Echinococcus* hatch in the stomach and small intestine. Hatching is a two-stage process involving (1) the passive disaggregation of the embryophoric blocks in the stomach and intestine and (2) the activation of the oncosphere and its liberation from the oncospheral membrane (reviewed by Holcman and Heath, 1997; Jabbar et al., 2010; Lethbridge, 1980). Disaggregation of the embryophoric blocks appears to require the action of proteolytic enzymes, including pepsin and pancreatin, in the stomach and/or intestine but does not depend on any one specific enzyme. The oncosphere plays no part in disaggregation of the embryophore and remains essentially dormant until activated. Evidence suggests the oncosphere may be stimulated to free itself from the

oncospheral membrane following changes in membrane permeability brought about by the surface active properties of bile salts. This led to the proposal that bile may play a part in determining intermediate host specificity, since its composition varies between different species of vertebrate (Smyth, 1969). However, the situation is certainly not as straightforward since eggs of *E. granulosus* were shown to hatch in extraintestinal sites including the lung, liver and peritoneal cavity of sheep and rodents inoculated experimentally by tracheostomy or intraperitoneal injection (Blood and Lelijveld, 1969; Borrie et al., 1965; Colli and Williams, 1972; Kumaratilake and Thompson, 1981; Williams and Colli, 1970). Eggs inoculated into the peritoneal cavity were rapidly surrounded by adhering neutrophils and macrophages which probably released hydrolytic enzymes causing dissolution of the embryophore. Eggs of *Echinococcus* can also be hatched and activated in vitro using chemicals and enzymes not derived from a particular species of intermediate host. Thus hatching requirements do not depend on the physiological characteristics of the definitive host gut and are not specific. Consequently, factors which regulate whether eggs of a particular taeniid species will or will not develop in a particular intermediate host must operate on the oncosphere either during the invasive or establishment phases (Thompson, 1995).

s0085 5.2.2 Penetration and tissue localization

p0185 The liberated, activated oncosphere exhibits intricate rhythmic movements involving the body and hooks, the coordinated movement of the latter effected by a complex muscular system (Swiderski, 1983). The so-called 'penetration glands' are also prominent at this stage. Studies in sheep and rabbits have shown that the oncospheres of *E. granulosus* penetrate the tips of the villi in the jejunal and upper ileal region of the small intestine (Heath, 1971). The oncospheres initially attach to the microvillous border of the villi, presumably using their hooks as anchors. Studies on several taeniid species, including *E. granulosus*, have shown that oncospheres rapidly migrate through the epithelial border of the villi, reaching the lamina propria within 3–120 min after hatching (reviewed by Lethbridge, 1980; Jabbar et al., 2010). Penetration appears to involve hook and body movements presumably assisted by the penetration gland secretions. Stainable material in the penetration glands is totally extruded from between the hooks at the time, and in the place where the oncosphere is actively engaged in penetration (reviewed by Fairweather and Threadgold, 1981; Jabbar et al., 2010). Degeneration of host tissue also occurs in the vicinity of the invading

oncosphere (Heath, 1971). It is therefore assumed that penetration gland secretions must aid the penetration process by causing lysis of host tissue. However, the putative enzymatic nature of the secretion has yet to be established, although oncospheral penetration glands are the source of the EG95 antigen used to vaccinate against CE (Jabbar et al., 2011). Alternatively, penetration may be purely mechanical, involving hook and body movements. The secretion may have other functions such as to assist adhesion, act as a lubricant or afford protection against host digestive enzymes or immunological factors (Fairweather and Threadgold, 1981; Lethbridge, 1980). Ultrastructural studies (Swiderski, 1983) demonstrated that the oncosphere of *E. granulosus* has three types of gland cells (Fig. 6), thus several secretions with different functions may be produced during penetration. Harris et al. (1989) found that not all penetration gland secretions are necessarily shed during penetration and much secretory material is retained in the oncospheral epithelium where it appears to be involved in the formation of transitory microvilli which disappear within 6 days and may have a digestive function. As in the adult worm, the oncosphere has also been shown to release a Kunitz–type protease inhibitor, EgKI-1, which is highly expressed in the oncosphere and is a potent chymotrypsin and neutrophil elastase inhibitor that binds calcium and reduces neutrophil infiltration (Ranasinghe et al., 2015). EgKI-1 may be involved in host immune evasion by inhibiting neutrophil elastase and cathepsin G once this stage is exposed to the mammalian blood system (Ranasinghe et al., 2015).

p0190   The factors that determine the final localization of the metacestode of *Echinococcus* in a given host are not clear but probably include anatomical and physiological characteristics of the host as well as the species and strain of parasite. Heath (1971) provided strong circumstantial evidence that oncospheres of *E. granulosus* are capable of completing a lymphatic or venous migration. He further postulated that since the lymphatic lacteals of the villus differed in size between different hosts, the size of the oncosphere in relation to the venules and lacteals in various animals may determine the distribution of cysts between the liver and lungs. It has also been suggested that the microvilli on the surface of the developing metacestode may assist in initial retention in the liver and lungs (Harris et al., 1989).

s0090 ### 5.2.3 *Postoncospheral development*

p0195 Once the oncosphere attains a site of predilection, postoncospheral development proceeds leading to the formation of the metacestode. The oncosphere

of *Echinococcus* very rapidly undergoes a series of reorganizational events during the first 14 days, involving cellular proliferation, degeneration of oncospheral hooks, muscular atrophy, vesicularization and central cavity formation, and development of both germinal and laminated layers (Heath and Lawrence, 1976; Rausch, 1954; Sakamoto and Sugimura, 1970). Slais (1973) demonstrated that postoncospheral development was initiated by the growth and division of primary germinal cells. Slais (1973) and Swiderski (1983) described five pairs of these cells in the posterior pole of the oncosphere. Although the complexity and plasticity of developmental processes in the adult and metacestode stages initially led to the belief that several primitive cell lines must exist (see earlier), all available evidence supports the existence of only one primitive morphological cell type, as a pool of uncommitted, undifferentiated multipotent germinal, or stem, cells in both the adult and metacestode (Gustafsson, 1976; Koziol et al., 2014; Smyth, 1969; Thompson et al., 1990; Thompson, 1995; Thompson and Lymbery, 2013; Thompson and Jenkins, 2014), although there are subpopulations with different gene expression patterns (Koziol et al., 2014; and see Chapter 4). The germinal cells are a component of the syncytial germinal layer of the metacestode and neck region of the adult worm. Ultrastructural studies reveal unremarkable rounded cells of variable size of around 4 μm (Albani et al., 2010; Gustafsson, 1976; Mehlhorn et al., 1983). Cell proliferation derives from the continuous replicative activity of these dividing stem cells located in the germinal layer of the metacestode or neck region of the adult worm (Galindo et al., 2003; Gustafsson, 1976). They have considerable proliferative potential (Eckert et al., 1983; Galindo et al., 2003; Martínez et al., 2005; Mehlhorn et al., 1983) and are the only proliferating cells in *Echinococcus* (Koziol et al., 2014). This is particularly well illustrated by the capacity of the parasite for indefinite perpetuation in the larval stage by the passage of protoscoleces or germinal layer material in rodents (secondary hydatidosis; Howell and Smyth, 1995). In AE caused by the metacestode of *E. multilocularis*, the proliferating larval parasite has an infiltrative capacity to establish distant foci of infection due to the distribution via blood or lymph of detached germinal cells (Ali-Khan et al., 1983; Ammann and Eckert, 1996; Eckert et al., 1983; Mehlhorn et al., 1983, Fig. 7; and see later).

p0200   Problems with host cell contamination dogged early attempts to establish germinal cell lines of *E. granulosus* and *E. multilocularis* (reviewed in Howell and Smyth, 1995). In addition, their isolation from the germinal layer, and their in vitro propagation, could have been hampered by the fact that the germinal layer is a syncytium. However, the establishment and
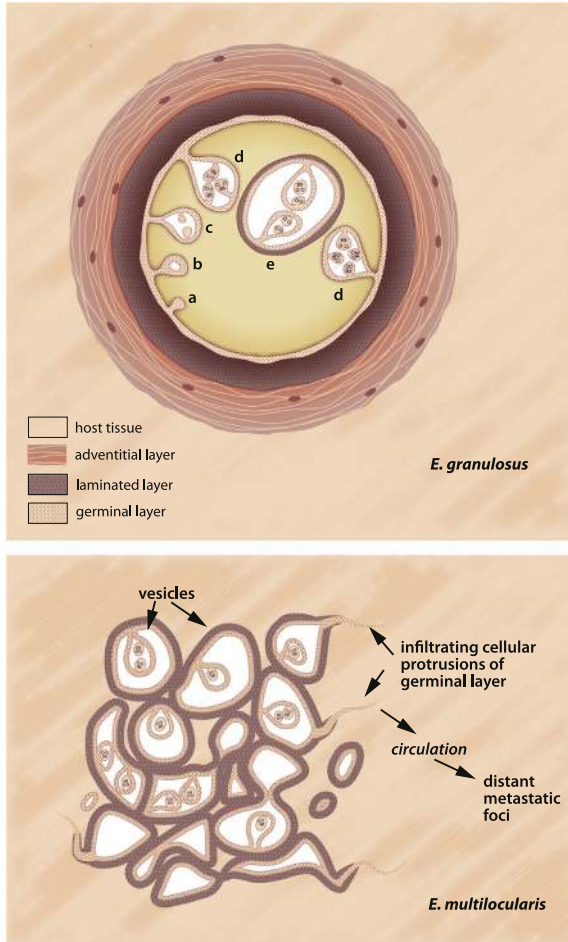
Figure 7 Diagram illustrating the structural differences between the metacestodes of *Echinococcus granulosus* (a–d stages in development of protoscoleces and brood capsule, and e daughter cyst) and *Echinococcus multilocularis*. *From Thompson, R.C.A., Jenkins, D.J., 2014.* Echinococcus *as a model system. Int. J. Parasitol. 44, 865–877.*

long-term perpetuation of *Echinococcus* germinal cells has now been achieved for both species (Albani et al., 2010; Spiliotis and Brehm, 2009; Spiliotis et al., 2008; Yamashita et al., 1997). The germinal cells behave very much like classical stem cells with the formation of cell aggregates and clusters with cavity formation, and there is cytological evidence of transformation (Albani et al., 2013; Spiliotis et al., 2008; see Chapter 4).

s0095 ## 5.3 Metacestode

p0205 Metacestodes of the four species of *Echinococcus* have certain basic features in common which can be illustrated by a detailed examination of *E. granulosus*. Differences exhibited by the other three species will then be discussed.

s0100 ### 5.3.1 Structure
s0105 #### 5.3.1.1 Echinococcus granulosus
p0210 The fully developed metacestode of *E. granulosus* is typically unilocular, subspherical in shape, fluid–filled and exhibits the least complex structure of the four species (Cameron and Webster, 1969; Moro and Schantz, 2009; Rausch et al., 1981; Schantz, 1982; Thompson, 2001). The cyst consists of an inner germinal or nucleated layer supported externally by a tough, elastic, acellular laminated layer of variable thickness, surrounded by a host–produced fibrous adventitial layer (Fig. 7). Typically *E. granulosus* produces a single-chambered unilocular cyst in which growth is expansive by concentric enlargement. Asexual proliferation of the germinal layer and brood capsule formation takes place entirely endogenously. Pouching of the cyst walls may occur giving rise to secondary chambers communicating with the central cavity (Vanek, 1980). Sometimes the central cavity may be partly separated from the secondary chambers by incomplete septa. Occasionally cysts may abut and coalesce, forming groups or clusters of small cysts of different size. In some hosts, particularly man, where unusually large cysts may develop, daughter cysts may form within the primary cyst (Moro and Schantz, 2009; Thompson, 2001, Fig. 7).

p0215 The germinal layer is similar in structure to the tegument of the adult worm, consisting of a distal cytoplasmic syncytium from which microtriches project into the overlaying laminated layer (Bortoletti and Ferretti, 1973, 1978; Lascano et al., 1975; Morseth, 1967). The cell bodies and nuclei comprise the perinuclear, or proliferative cell layer, which contains several cell types including tegumental, muscle, glycogen-storing and undifferentiated cells. The tegumental cells are multinucleated, which may be indicative of their rapid growth (Rodriguez-Caabeiro and Casado, 1988). Cytoplasmic connections between the two layers maintain continuity. The undifferentiated cells of the perinuclear layer are proliferative and are responsible for the formation of brood capsules which originate endogenously as small nuclear masses, or buds, which proliferate towards the cystic cavity (Fig. 7; Slais, 1973; Thompson, 1976), whereas in *E. multilocularis* recent evidence suggests they arise from an invagination of the germinal layer (Koziol et al.,

2016). Brood capsules enlarge, vacuolate and become stalked. Within their lumen, a repetition of the asexual budding process takes place, leading to the production of numerous protoscoleces. The formation of protoscoleces is asynchronous with a number of different developmental stages being present in a brood capsule at the same time (see Thompson, 1995). Fully developed protoscoleces are characterized by the possession of hooks on the invaginated rostellum. The spines of microtriches are the precursors of the hook blades which become enveloped by the rostellar tegument with subsequent formation of the guard and handle (Rogan and Richards, 1987) with subsequent addition of hook material in the adult worm (Hobbs et al., 1990). Hook formation must be subject to environmental factors of host origin given the variability in hook number and size in the same species of *Echinococcus* from different hosts.

p0220   In addition to its proliferative activity, the germinal layer is involved in secretory activity and in this respect, Monteiro et al. (2010) identified several molecules in hydatid cyst fluid that could play a role in host evasion that presumably were secreted by the germinal layer. Irigoin et al. (2001) showed that myo–Inositol hexakisphosphate (IP(6)) which is present in the germinal and laminated layers of *E. granulosus* inhibits complement activation and Breijo et al. (2008) indicated that the establishment and survival of the hydatid cyst is associated with the control of complement and, consequently, of local inflammation.

p0225   The delicate germinal layer is supported externally by the acellular and elastic laminated layer. All species of *Echinococcus* are characterized by the possession of a laminated layer which, because it is periodic acid–Schiff positive (Kilejian et al., 1961), provides a useful diagnostic marker. The laminated layer also undoubtedly assists in supporting the cyst and allows an often considerable intracystic tension to develop (Cameron and Webster, 1969; Slais, 1973). It is a remarkable and specialized interface in the intermediate host providing a physiochemical barrier with apparent multi–functionality and a structure whose biosynthesis has become a model system for carbohydrate chemistry (Diaz et al., 2011a,b; Parkinson et al., 2012; Thompson and Jenkins, 2014). The laminated layer comprises a specialised extracellular matrix unique to *Echinococcus* (Fig. 7), whose synthesis is a major metabolic activity of the much thinner germinal layer (Diaz et al., 2011a; Lin et al., 2012; Parkinson et al., 2012). It is a carbohydrate—protein complex of highly glycosylated mucin glycoproteins (Kilejian and Schwabe, 1971). Ultrastructurally it can be seen to be a microfibrillate, three-dimensional meshwork matrix in which aggregates of electron-dense

material occur (Richards et al., 1983). The origin of the laminated layer was controversial for some time, but electron microscopy and in vitro studies have unequivocally demonstrated that it is entirely of parasite (germinal layer) origin (see Thompson, 1995).

p0230   Considerable metabolic activity in the germinal layer is required to synthesize and maintain the interfacial barrier of the laminated layer (Parkinson et al., 2012). The role of the laminated layer would appear to be one of protection by modulating the host—parasite interface, since cyst survival is dependent upon its integrity (Gottstein et al., 2002; Stadelmann et al., [Q13] 2010). Whether this is purely physical or if there is selective permeability is not known. It may protect the cyst from immunological attack by offering an immunogenically inert barrier which can deny access to host defence cells (Coltorti and Varela-Diaz, 1974). Immunoglobulin, however, can pass through the laminated layer but the capacity to regulate penetration of macromolecules into the cyst appears to be a function of the germinal rather than the laminated layer (Coltorti and Varela-Diaz, 1974).

p0235   Smyth (1969) commented on the significance of the presence of a human blood group P-like substance in the laminated layer of *Echinococcus* and its significance as a model system in better understanding the immunological basis of the host—parasite relationship. This P1 blood-antigen motif has since attracted much attention and has been further characterized as a protein—carbohydrate, trisaccharide/mucin complex containing galactosamine, yet no biological function has been described to date (Lin et al., 2012). Recently, however, Nicolao et al. (2014) described the expression of an ATP-dependent transporter, P-glycoprotein in *E. granulosus* (Eg-Pgp). How this may relate to the laminated layer is not clear but given its clinical significance in humans as a transporter involved in the efflux of a wide variety of lipophilic substrates, such as toxins and xenobiotics, and its role in the ineffective therapeutic treatment of cancer cells and microbial pathogens (Nicolao et al., 2014), it seems likely to have a role in enhancing the survival of the *Echinococcus* metacestode. Recent research has shown that the laminated layer protects *E. granulosus* against the nitric oxide protective response of the host by increasing arginase activity in macrophages, which counteracts the nitric oxide production (Amri and Touil-Boukoffa, 2015). Stadelmann et al. (2010) investigated the molecular and functional characterization of *E. multilocularis* phosphoglucose isomerase (EmPGI), which is a component of the laminated layer, and proposed that besides its role in glycolysis, EmPGI could also act as a factor that stimulates parasite growth and potentially induces the formation of novel blood vessels around the

[Q14] developing metacestode in vivo. Noya et al. (2014) also demonstrated that mucinlike peptides from *E. granulosus* induce antitumour activity. In studies on the identification and characterization of Emp53, the homologue of human tumour suppressor p53, from *E. multilocularis*, Cheng et al. (2015) concluded that since the parasite develops in host organs, it must have evolved a stress defence system, which could involve Emp53, to cope with various genotoxic and cellular stresses that may cause DNA damage and genomic instability.

p0240   The host fibrous capsule (adventitial layer) which typically surrounds fully developed, viable cysts of *E. granulosus*, is the product of a three-layered host cellular inflammatory reaction initiated in the early stages of postoncospheral development (Cameron and Webster, 1969; Slais and Vanek, 1980; Smyth and Heath, 1970). The initial intensity of this reaction varies between hosts and governs the fate of the developing metacestode. If too intense it will cause the degeneration and eventual death of the parasite, whereas in suitable intermediate hosts the initial reaction resolves, leaving a fibrous capsule. The latter situation is common where a stable host—parasite relationship has evolved (Rausch, 1997), as appears to be the case, for example, between *Echinococcus equinus* and the horse (Roneus et al., 1982; Thompson, 1977) and *E. granulosus* in sheep. In contrast, *E. granulosus* rarely produces protoscoleces in cattle and the inflammatory response does not resolve causing the destruction of the developing cyst (Thompson, 2008).

s0110 ### 5.3.1.2 Echinococcus multilocularis

p0245 The metacestode of *E. multilocularis* is the most complex and develops quite differently to that of *E. granulosus* (Braithwaite et al., 1985; D'Alessandro et al., 1979; FAO, 1982; Ohbayashi et al., 1971; Wilson and Rausch, 1980). It is a multivesicular, infiltrating structure with no limiting host—tissue barrier (adventitial layer), consisting of numerous small vesicles embedded in a dense stroma of connective tissue (Thompson, 1995, Fig. 7). The larval mass usually contains a semisolid matrix rather than fluid. Proliferation occurs both endogenously and exogenously and is attributable to the undifferentiated cells of the germinal layer (Mehlhorn et al., 1983; Moro and Schantz, 2009; Sakamoto and Sugimura, 1970). The metacestode consists of a network of filamentous solid cellular protrusions of the germinal layer which are responsible for infiltrating growth (Fig. 7) transforming into tubelike and cystic structures (Eckert et al., 1983; Mehlhorn et al., 1983; Vogel, 1978). Furthermore, the detachment of germinal cells from infiltrating cellular protrusions and their subsequent distribution via the lymph

or blood can give rise to the distant metastatic foci characteristic of *E. multilocularis* (Ali-Khan et al., 1983; Eckert et al., 1983; Mehlhorn et al., 1983; Thompson, 2001).

p0250   Since the first report of the metacestode of *E. multilocularis* in extraintestinal sites in dogs and cats in 1990 from Germany, there have been a growing number of cases reported from Europe and Canada (Corsini et al., 2015; Deplazes et al., 1997; Geisel et al., 1990; Losson and Coignoul, 1997; Pergrine et al., 2012; and reviewed in Weiss et al., 2010). It is not known whether such infections resulted directly from the ingestion of eggs or indirectly by autoinfection as a result of a previously acquired worm burden, but they illustrate the unusual developmental potential of *E. multilocularis* (Thompson, 2001).

s0115   5.3.1.3 *Echinococcus vogeli* and *Echinococcus oligarthra*

p0255   The metacestodes of *E. vogeli* and *E. oligarthra* (= *E. oligarthrus*) have been less studied but exhibit developmental and structural characteristics considered intermediate to those of *E. granulosus* and *E. multilocularis* (Moro and Schantz, 2009; Rausch et al., 1981). The metacestodes of both species are termed polycystic since they are characterized by the internal division of fluid-filled cysts to form multichambered cyst masses (D'Alessandro et al., 1979; Morales et al., 1979; Gottstein and Hemphill, 1997; Moro and Schantz, 2009; Rausch et al., 1981). *Echinococcus vogeli* produces cysts varying greatly in size from 2 to 80 mm, which may occur singly, in small groups, or occasionally in dense aggregations in which each cyst is enclosed by its separate adventitia. In *E. vogeli*, endogenous proliferation and convolution of both germinal and laminated layers leads to the formation of secondary subdivisions of the primary vesicle with production of brood capsules and protoscoleces in the resultant chambers, which are often interconnected. In *E. oligarthra*, there is less subdivision into secondary chambers and the laminated layer is much thinner than that of *E. vogeli* (Sousa and Thatcher, 1969; Rausch et al., 1981). Exogenous proliferation has been reported in both species but, at least in *E. vogeli*, it appears to be abnormal and restricted compared to *E. multilocularis*, and does not occur in the natural intermediate host (Gottstein and Hemphill, 1997).

s0120   **5.3.2 Asexual reproduction and differentiation**

p0260   The asexual reproduction exhibited by species of *Echinococcus* has a potential unsurpassed by other tapeworms and is of particular evolutionary significance (see Chapter 3). The metacestode has a potentially unlimited

sequential generative capacity (reviewed by Whitfield and Evans, 1983). Although some germinal cells initiate the production of new brood capsules and protoscoleces, a pool of uncommitted, undifferentiated, germinal cells remain. This fact makes possible the indefinite perpetuation of larval *Echinococcus* in rodents by repeated intraperitoneal passage of protoscoleces or germinal layer material (secondary hydatidosis), and the development in man and other animals of secondary cysts following the rupture of a primary cyst (see Chapter 10). Thus undifferentiated cells retained in the germinal layer and protoscolex are capable of initiating new cycles of asexual multiplication. Apart from being able to initiate the production of new protoscoleces, a protoscolex has a dual capability (heterogeneous morphogenesis) since if ingested by a suitable definitive host it will develop into an adult worm (Cucher et al., 2011; Thompson, 1995, 2001). However, even the adult worm must retain some undifferentiated multipotential germinal cells, since adult worms can de-differentiate in a cystic direction under unfavourable conditions (Smyth, 1969).

[Q15]

s0125   5.3.3   *Rate of development*

p0265   Although in both *E. granulosus* and *E. multilocularis*, initial reorganization of the oncosphere and formation of the germinal and laminated layers occurs rapidly, usually within the first 14 days (see earlier), the rate of subsequent development differs markedly. In *E. granulosus*, it is slow and variable and dependent on a number of factors including the strain of parasite, the species and strain of host and the degree of infection. Heath (1973) concluded that *E. granulosus* cysts increase in diameter by between 1 and 5 cm per year depending on factors yet unresolved. The time taken for brood capsule formation is also extremely variable. The earliest recorded is 195 days in mice following oral infection with eggs (Colli and Schantz, 1974). In pigs, 10–12 months has been reported (Slais, 1980), whereas in sheep reports range from 10 months to 4 years (Heath, 1973; Gemmell et al., 1986). The production of brood capsules and protoscoleces does not seem to depend on cyst size and in mice it has been found that it is not always the largest cysts which develop protoscoleces (Colli and Schantz, 1974). In horses, fertile cysts as small as 2 mm in diameter have been reported (Edwards, 1981). The life span of hydatid cysts of *E. granulosus* can be as long as 16 years in horses (Roneus et al., 1982) and 53 years in man (Spruance, 1974).

p0270   In contrast to *E. granulosus*, *E. multilocularis* develops rapidly in its natural intermediate host, producing protoscoleces in only 2–4 months, an adaptation to the short-lived arvicoline rodents it utilizes (Rausch, 1975;

Woolsey et al., 2015). Thereafter, proliferation of vesicles is curtailed and there is little if any further increase in size (Rausch and Wilson, 1973). In man, growth is very different. Proliferation continues indefinitely although there are few if any protoscoleces produced (Moro and Schantz, 2009; Rausch and Wilson, 1973). The larval mass proliferates peripherally and at the same time regressive changes occur centrally (Ammann and Eckert, 1996; Moro and Schantz, 2009). Thus a progressively enlarging mass of necrotic tissue with a relatively thin zone of viable proliferating parasite is produced. The term 'alveolar hydatid' was initially used to describe this form of growth, which is not a feature of the development in natural intermediate host species.

s0130

p0275

6. PERSPECTIVES FOR THE FUTURE

In terms of the biology of *Echinococcus*, there have been major advances made since I last expressed some hopes and predictions (Thompson, 1995). Most notable have been (1) determining the origin, structure and functional activities of the laminated layer and its relationship with the germinal layer; and (2) the isolation, in vitro establishment and characterization of the multi-potential germinal cells (see Chapter 4). The latter is a particularly remarkable achievement and along with the sequence data that is now available, we are well placed to build on Desmond Smyth's legacy (Thompson and Lymbery, 2013) and identify the mechanisms that provide *Echinococcus* with its unique developmental plasticity, as well as providing answers to questions such as what governs host specificity and the nature of activities at the parasite—host interface, particularly in the definitive host. For example, discovering the nature and function(s) of rostellar gland secretions in the adult worm would be a major breakthrough in understanding the host—parasite relationship. However, this presents a challenge given the lack of an ethically acceptable model for maintaining the adult parasite in vivo, given the difficulties in undertaking experimental infections in the definitive hosts. However, in vitro techniques are established for growing the adult parasite from protoscolex to maturity and with the advanced imaging and analytical tools now available, they should be exploited to investigate the functional aspects of the rostellar gland. 'New tools' per se, will be valuable in discovering novel aspects of the biology of *Echinococcus* but unless we 'rediscover' the fundamental issues that were laid down by earlier workers we may miss clues to guide data mining.

p0280   The last two decades have proved particularly productive in terms of elucidating the phylogenetic relationships of species and intraspecific variants within the genus *Echinococcus* (see Chapter 3). The development and application of appropriate molecular tools have been instrumental in this regard and have underscored a taxonomic revision of the genus. We now have a sound nomenclature, which is essential in understanding the epidemiology of echinococcosis. The revised taxonomy has been built on the basis of phenotypic (morphology, host specificity) and genotypic data. It is important that we maintain such a holistic approach in characterising species and strains in the future and do not rely solely on molecular data alone.

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