1 The Discovery of *Fasciola hepatica* and its Life Cycle

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1.1 Introduction

Fasciolosis is a parasitic disease of humans and their livestock caused by digenean trematodes of the genus *Fasciola*, referred to as liver flukes. The two species most commonly implicated as the aetiological agents of fasciolosis are *F. hepatica* and *F. gigantica* (subfamily Fasciolinae, family Fasciolidae). *Fasciola* species are located within the Digenea subclass of the class Trematoda, phylum Platyhelminthes.

The Digenea are characterized by a complex life cycle involving one or more intermediate hosts. Many variations on the life cycle exist, but each typically includes a molluscan primary or intermediate host in which larval asexual multiplication occurs, and a vertebrate final or definitive host in which sexual reproduction occurs. Members of the family Fasciolidae are hermaphroditic and self-fertilization can occur, although preferential cross-fertilization is usual.

Fasciolosis has the widest longitudinal, latitudinal and altitudinal distribution of any helminth disease; it is present on every continent excluding Antarctica (Mas-Coma *et al.*, 2019). *F. hepatica* has a worldwide distribution but predominates in temperate zones, while *F. gigantica* is found on most continents but primarily in tropical regions, in particular Asia and Africa. However, where climatic conditions favour both parasites, they can coexist and hybridize. Recent animal trade has also influenced their distribution, especially in Asia and Africa (Mas-Coma *et al.*, 2005, 2019). The two species are believed to have diverged 5 million years ago close to the Miocene–Pliocene boundary (Choi *et al.*, 2020).

Fasciolosis is an economically important disease of domestic livestock, particularly cattle, sheep and water buffalo, and the cost of the disease to the global farming community is conservatively estimated at more than US$3 billion (Spithill *et al.*, 1999; Beesley *et al.*, 2018). The parasite also infects many wild animals, such as rabbits, hares, deer, coypu, rats, horses and camels, some of which can be regarded as important reservoir hosts. The potent immunomodulatory effects of *Fasciola* spp. on the host immune system may compromise the host's ability to resist other microbial infections, such as tuberculosis, salmonella and clostridiosis (Claridge *et al.*, 2012; Cwiklinski *et al.*, 2016), adding further impairment to animal health, welfare and productivity.

Since the 1990s, fasciolosis has emerged as an important food-borne disease of humans and a major public health issue in a growing number of countries. Estimates of human infections are still very approximate and range from 2.4 to 17 million people (Mas-Coma *et al.*, 2019), with 91–180 million people at risk of infection (Mas-Coma, 2005; Keiser and Utzinger, 2009).

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Countries where human infections are highest are Bolivia, Peru, Ecuador, Egypt, Iran, China, Vietnam and, less so, Portugal and Spain. Recently, the World Health Organization has classified fasciolosis as a 'Neglected Tropical Disease' (WHO, 2020).

The disease caused by *F. hepatica* and *F. gigantica* is known within the literature under various synonymous names, namely fasciolosis and fascioliasis. In keeping with the World Association for the Advancement of Veterinary Parasitology standardized nomenclature of parasitic diseases, the term fasciolosis will be used within this text (Kassai, 2006).

1.2 The Discovery of the Life Cycle

An overview of the historical discovery of the life cycle of *F. hepatica* is given below, together with the main biological factors relevant to the successful completion of the cycle. Further information and perspectives can be found in past literature, including Taylor (1937, 1964), Reinhard (1957), Jefferies and Dawes (1960), Pantelouris (1965), Dawes (1968), Smyth and Halton (1983), Andrews (1999) and Wilson (2020).

A list of the principal discoveries in the life cycle of *Fasciola* is shown in Table 1.1. The earliest description of fasciolosis in the literature is contained in a book entitled *Black Book of Chirk*, published circa 1200, in which reference is made to liver fluke in sheep (Froyd, 1969). Within this book, a reference is drawn from an earlier text that may describe fasciolosis in the Gwentian Code of Wales, describing the laws enacted by Howel the Good in the 10th century (Froyd, 1969).

The first observation of liver fluke was made by Jean de Brie in 1379. While he was preparing a treatise on wool production and sheep management for Charles V of France, de Brie made mention of the disease 'liver rot' in sheep, though he did not actually describe the appearance of the worm in this treatise (Huber, 1890). Unfortunately, the original account of his work has been lost and his observations are only known from various editions published between 1542 and 1594. Like many other early writers, de Brie did not associate the liver fluke with the disease 'liver rot', but thought that this was a consequence of the liver being affected by toxic substances produced by certain plants eaten by the sheep.

According to Cole (1944) another recognizable description of liver fluke appeared in a book entitled A newe Tracte or Treatyse moost profytable for all Husbandemen which was published in 1523 by Sir Anthony Fitzherbert. Twenty-six years later, in 1549, reference was

Table 1.1. Milestones in the discovery of the life cycle of *Fasciola hepatica*.

Person/year	Milestone
De Brie, 1379	First to observe the liver fluke *F. hepatica*
Redi, 1688	Disproved the theory of spontaneous generation
Swammerdam, 1737	First to see cercariae dissected from a snail (see Swammerdam, 1758)
Müller, 1773	Observed cercariae swimming in water
Zeder, 1803	Described a miracidium hatching from fluke egg
Nitzsch, 1807	Observed cercariae encysting
Bojanus, 1818	Described redia and development of cercariae
Steenstrup, 1842	Published theory of alternation of generations (see Steenstrup, 1845)
La Valette St George, 1855	Observed infection of a snail by a miracidium
Wagener, 1857	Observed penetration of a snail by miracidium and subsequent development of redia
Weinland, 1875	Suggested that the larval stages of the liver fluke occur in *Lymnaea truncatula*
Leuckart, Thomas, 1882	Confirmed *L. truncatula* as intermediate host and worked out life cycle of *F. hepatica*
Lutz, 1892; Lutz, 1893	Confirmed that herbivorous animals acquire adult fluke by ingestion of metacercariae
Sinitsin, 1914	Confirmed route of migration of *F. hepatica* to the liver

made in a book entitled *De lumbricis alvum occupantibus* to an observation made by an Italian physician by the name of Fanensi Gabucinus who described worms resembling pumpkin seeds in the blood vessels of the liver of sheep and goats. Additional observations on liver fluke disease were recorded during the second half of the 16th century, namely those of Conrad Gesner (1551) and Cornel Gemma (1575). Nevertheless, it was still commonly thought at the time that the feeding of particular plants to sheep was responsible for the disease.

It was not until 1688 that this theory was challenged by Francesco Redi, after whom the redia stage in the digenetic life cycle was named. He was a physician in Italy, who, by showing that parasites lay eggs, destroyed the false doctrine of spontaneous generation, i.e. the hypothetical process by which living organisms arise from inanimate matter. It is uncertain in which parasite this observation was first made, as Redi described a number of different types of worms from many kinds of animals, but he was the first to publish a picture of the liver fluke – a sketch from a specimen removed from the liver of a castrated ram.

The rejection of the theory of spontaneous generation was a significant breakthrough and was to stimulate a new wave of research. Prominent in this new research was Govert Bidloo, a professor of anatomy at The Hague and physician to William III. Bidloo (1698) observed worms in the bile ducts of sheep, stags and calves and recalled having seen similar worms in the livers of humans. This followed the observation by John Faber in 1670, who was the first to state that the liver fluke lives in the bile ducts, not in the blood vessels (Reinhard, 1957). Bidloo also observed eggs inside the living worm and thought that sheep probably became infected by swallowing the worms or their eggs and that the worms got to the liver in the blood rather than by passing via the small intestine. His results were reported in the form of a memoir to Antony van Leeuwenhoek, which stimulated Leeuwenhoek to investigate further. Leeuwenhoek thought that the worms lived in water and that sheep became ill by drinking this water, but he could not explain why he was unable to find such creatures in water samples taken from ditches in fields near the city of Delft. His observations were recorded in letters sent to the Royal Society that were subsequently published in the Philosophical Transactions (Leeuwenhoek, 1700, 1704).

It was not until the end of the 18th century that reference was made in the scientific literature to the intermediate stages of the life cycle. It was a chance observation by a Dutchman named Johann Swammerdam (1758) who, while dissecting a snail (*Paludina vivipara*) in order to examine its internal structure, saw living things that he thought were not of snail origin. Examination of his illustrations of these 'worms' clearly shows them to be the cercariae of some trematode. Later, in 1755, Frank Nicholls, a physician and prominent anatomist in England, presented a report to the Royal Society in which he remarked that the bile ducts in the livers of bullocks infected with 'liver rot' were blocked by 'a wall of stone' around the flukes – this was the first reference to calcification of the bile ducts and thus the earliest insight into the pathology of the disease.

The next contribution towards an understanding of the life cycle was made almost 20 years later by Otto Müller. In 1773, he wrote of finding microscopic tadpole-like creatures swimming in the water of ponds and called them cercariae, a generic name which he gave to all of these creatures characterized by having a tail. He mistakenly thought that these cercariae were Infusoria (a term applied to microscopic organisms, including various Protozoa and Rotifera, found in infusions of organic substances), as did many others who subsequently followed in his footsteps. Several different kinds of cercariae were observed and described, including those depicted by Johann Eichhorn (1781) and by Johann Hermann (1783). However, at this time the life cycle of flukes was still a mystery and no one even suspected that more than one animal host was required for its completion. The first person apparently to have an inkling was Peter Abildgaard of Copenhagen (1790). Ahead of his time, the results of his experiments (which provided evidence that the development of the tapeworm *Diphyllobothrium* sp., from larval to adult stage, required the larval stage to pass from a fish host to a bird) were soon forgotten and the idea that an immature form of a parasitic worm can require a different host from that of the adult had to wait another 60 years (Küchenmeister, 1852) before being accepted.

In 1803, Johann Zeder reported observing the hatching of eggs from a number of different species of trematode, although not of *F. hepatica*, and the subsequent escape of a ciliated embryo (miracidium) into the water. After Zeder's observation, Christian Nitzsch (1807) followed with the first account of cercariae encysting. He had been watching some cercariae swimming in water and had noticed that after a while they attached themselves to a substrate, then lost their tails prior to becoming covered by a gelatinous substance. Having lost all means of movement he assumed that he had witnessed the cercariae dying. Nitzsch continued working with cercariae and nine years later he had described several new cercariae (in Dawes, 1968). In doing so, he noticed the similarity between the anterior end of a cercaria and a distome fluke (Nitzsch, 1817), although he still thought that cercariae were different from flukes and from all other known forms of pond life.

During this period, despite such advances, the only thing that was definitely known about the life history of the liver fluke, or any other fluke, was that a ciliated larval form emerged from the eggs. Although many observations were recorded on other stages of the cycle, such as cercariae and rediae, a connection between them remained elusive. The next observation of relevance was made by Ludwig Bojanus who, in 1818, unaware of Swammerdam's work published in 1737 (see Swammerdam, 1758), rediscovered the redial stage of a trematode. Unlike Swammerdam, who failed to grasp the significance of his finding, Bojanus noted the resemblance between rediae, cercariae and adult flukes. By observing the birth of cercariae from the rediae, Bojanus put forward the idea that there may well be a connection between cercariae and flukes. However, like Swammerdam, Bojanus did not work on the snail *Lymnaea truncatula* and therefore did not observe the larval stages of *F. hepatica*. Despite these publications, the consensus at this time remained that cercariae were independent forms of life.

E. Mehlis, a German medical practitioner, made the next significant contribution in 1831 by describing the hatching of 'ciliated embryos' from the eggs of trematodes, although it was Friedrich Creplin (1837), six years later, who observed such forms (miracidia) hatching from the eggs of *F. hepatica*. Not only did Mehlis observe the hatching of miracidia but he also noted their energetic swimming in water and suggested that this behaviour might be associated with the need to find something that would enable them to develop to a stage that could eventually infect the final host. Such a theory went against the more popularly accepted theory that the final host became infected by the ingestion of the egg stage. However, in 1852 the latter theory was finally disproved by Professor James Simonds of the Royal Veterinary College, London. Simonds (1880) reported that he failed to find a single fluke or any signs of liver disease in an experimental sheep infected six months earlier with thousands of fluke eggs.

By the middle of the 19th century most of the individual parts of the life history of many species of trematode had been noted. Johannes Steenstrup was the first person to start to bring together the various pieces of the story. In 1842, he published his work *On the Alternation of Generations* in Danish; the same year saw the publication of the German edition, which was translated into English (Steenstrup, 1845). He fitted the theory to various forms of life, including trematodes; most of his work on trematodes described echinostome and stylet cercariae with only a brief mention of *F. hepatica*. However, the term 'alternation of generations' was not novel, having been previously used to describe the life cycle of tunicates (benthic invertebrates) in 1819 by the Franco-German poet and naturalist, Louis Charles Adelaide de Chamisso (in Jefferies and Dawes, 1960).

Carl von Siebold (1854) provided more evidence to support the theory. He found rudimentary sex organs in some encysted cercariae and suggested that it was likely that this stage, which occurred in invertebrates, was the infective stage for vertebrates, in which the sexually mature fluke occurred. The first suggestion of a definite connection between a specific cercaria and an adult fluke was probably made by Rudolph Leuckart in 1852 (in Taylor, 1937). He observed the similarity between a certain fluke found in the intestine of a predacious fish and of encapsulated cercariae found in the gills of its prey. Adolphus von La Valette St George (1855) demonstrated, by feeding experiments, that certain encysted cercariae from water snails developed into sexually mature flukes in birds, and that cercariae which had not encysted were not infectious.

Further pieces of the jigsaw were put in place by Guido Wagener (1857) who observed the penetration of miracidia into snails and the subsequent development of rediae. German helminthologist David Weinland (1875), according to Reinhard (1957), was the first person to suspect that larval stages of liver fluke occurred in *Lymnaea truncatula*. In 1875, Weinland found 'cercaria sacs' in the digestive gland of *L. truncatula* snails and also noted that cercariae showed a strong inclination to leave water and climb on to foreign objects. He conjectured that cercariae encysted on grass in order to be eaten by sheep and that these cercariae were in fact young liver flukes. Twenty years later *L. truncatula* was confirmed as the common intermediate host of *F. hepatica* (other species of snail may also be infected, see for example Boray, 1969). The discovery was made independently by Algeron Thomas (1881, 1882a,b, 1883a,b) in the UK and by Leuckart (1881, 1882) in Germany. However, it was Thomas who established the right to be acknowledged as the first person to make the discovery, details of which were first published in Nature (Thomas, 1882b). The work of Thomas and Leuckart was summarized by Reinhard (1957).

Despite the work of Thomas and Leuckart, certain parts of the life history were still uncertain and required experimental evidence. For example, proof was still required that herbivores acquired the parasite by swallowing metacercariae. The discovery of the exact migration route by which young flukes reached the liver of the final host was also still to be elucidated. Experimental data confirming the first issue were generated by Adolpho Lutz (1892, 1893) who successfully infected guinea pigs, a rabbit, a goat and a brown rat by adding metacercariae to their food. However, according to Joseph Alicata (1938), the species of liver fluke with which Lutz was working was *F. gigantica* rather than *F. hepatica*. The final piece of the jigsaw was added by the Russian helminthologist Dimitry Sinitsin in 1914. Sinitsin proved that young flukes in the rabbit, after liberation from their cysts in the small intestine, penetrated the wall of the gut and migrated to the liver via the peritoneal cavity. This observation was supported by subsequent investigations by Shirai (1927), Susuki (1931), Shaw (1932), Schumacher (1939) and Krull and Jackson (1943).

1.3 The Life Cycle

The life cycle of *F. hepatica* consists of five phases as shown in Fig. 1.1. They are: (i) passage of eggs from the host to the outside environment and their subsequent development; (ii) hatching of miracidia, their search for and penetration of the intermediate snail host, usually *Galba (Lymnaea) truncatula*; (iii) development and multiplication of the parasites inside the snail; (iv) emergence of the cercariae from the snails and their encystment; and (v) ingestion of infective metacercariae by the final hosts and development to adult worms.

1.3.1 Development and survival of the fluke egg

Liver fluke eggs are passed from the common bile duct into the duodenum and subsequently are passed with the faeces. The eggs consist of a fertilized ovum surrounded by a large number of yolk granules. They are yellowish brown in colour, oval in shape, 130–145 μm long by 70–90 μm wide and have an indistinct operculum (Fig. 1.2A–C). The eggs that are passed out in the faeces on to pasture are undeveloped and, therefore, undergo embryonation outside the host.

Although partial development of the egg can occur while still inside moist or wet faeces, complete development and hatching will only occur after the egg has been liberated from the faeces, a requirement that is normally facilitated by factors such as the action of heavy rain, the deposition of faeces in water and the trampling action of animals. The inhibitory effect of faeces is likely to be caused by a number of factors, for example competition for oxygen by micro-organisms or the presence of toxic substances. Eggs, however, can remain viable in faeces from 3 weeks to several months, according to various conditions and the time of the year. Egg viability persists for a longer period during the winter than in the summer months and tends to decrease with increasing dryness (Rowcliffe and Ollerenshaw, 1960). Several physico-chemical factors, especially temperature, humidity and oxygen tension, are known to influence embryonation.

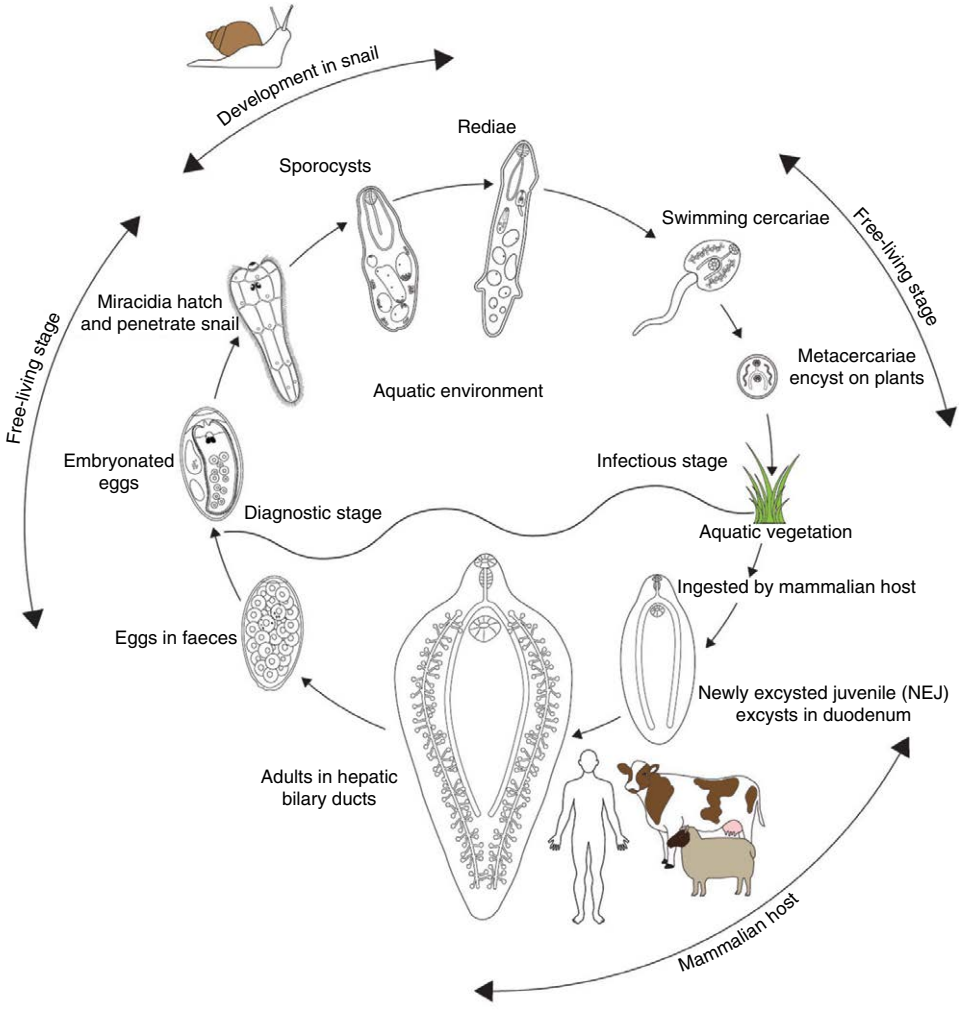


Fig. 1.1. The life cycle of *Fasciola hepatica*. (Drawn by Fiveprime Design.)

1.3.1.1 Temperature

A temperature of at least 10°C is necessary for embryonation (Ross and McKay, 1929). Under laboratory conditions, the rate of development of the egg increases with temperature within the range 10–30°C. Thus, at 10°C development of the egg takes about 6 months but at 30°C it is completed in 8 days. Above 30°C, development is increasingly inhibited and at 37°C it does not occur at all. Mortality increases the longer the eggs remain at 37°C, with 100% mortality being reached after about 24 days (Rowcliffe and Ollerenshaw, 1960).

At temperatures below 5°C, development of the egg stops but can be resumed if the temperature is increased to 13°C. Eggs refrigerated (2–10°C) for 2.5 years remain viable, although undeveloped, and, after being kept at room temperature for 18 days following refrigeration, they will hatch over a period of 14 days (Krull, 1934). Similar findings were recorded by Boray (1969) who kept eggs of *F. hepatica* in the refrigerator at 4°C for at least 2 years that, after

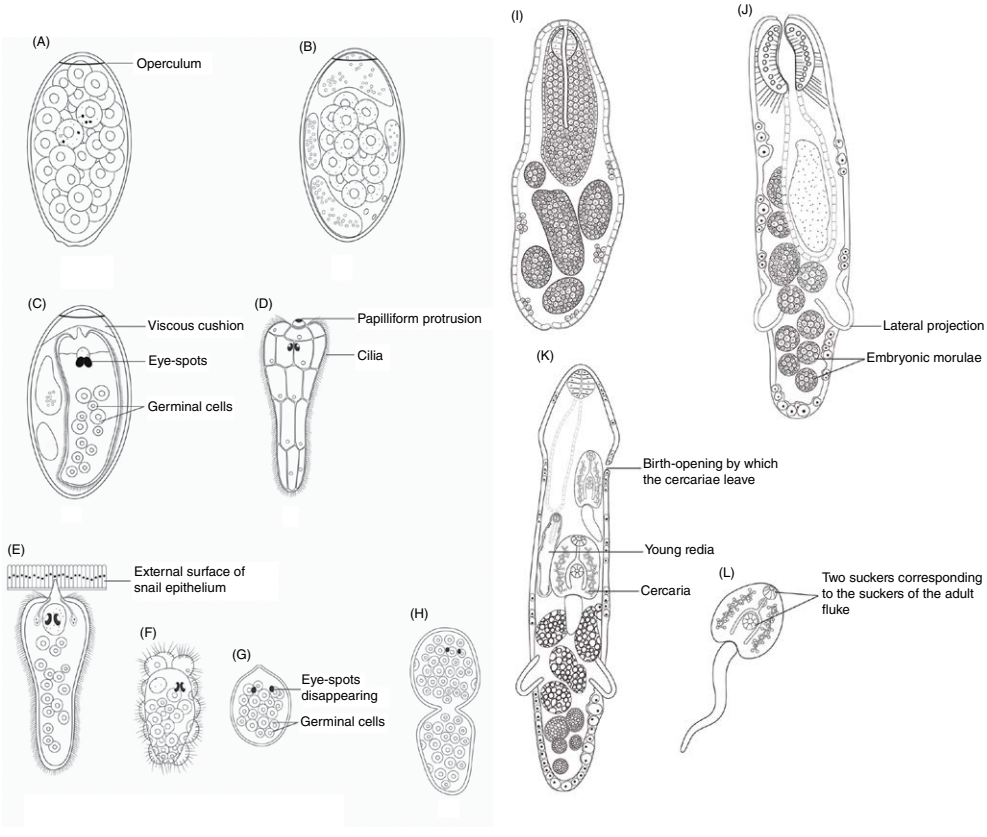


Fig. 1.2. Stages in the life cycle of *Fasciola hepatica*. (A) Undeveloped egg – note operculum (cap) and difference between embryonic cells (small mass in upper central part of egg) and yolk-bearing cells. (B) Morula – embryonic cells have grown at the expense of the yolk cells. (C) Fully developed egg ready to hatch – miracidium – note cilia and eye-spots. (D) A free-swimming miracidium, external view. (E) A miracidium penetrating a snail, internal view. (F) After penetrating the snail, the miracidium loses its cilia and becomes a sporocyst. (G) Sporocyst. (H) The sporocyst dividing. (I) The sporocyst forming redia (form with sucker and primitive gut). (J) A more mature redia – note other embryonic morulae in the body cavity. The two lateral projections are characteristic of this stage. (K) A fully mature redia showing developing redia and cercariae (the forms with the tail). (L) The cercaria; the free-swimming form which encysts on vegetation. (Drawn by Fiveprime Design.)

incubation, hatched miracidia that were infective to snails (*Galba tomentosa*).

1.3.1.2 Moisture

Water is required for embryonation. Maintenance of a surface film of moisture around the egg for at least 3 weeks is essential. Eggs on soil will develop without the presence of free surface water, provided that the soil is saturated (Ollerenshaw, 1959). Eggs in moist faeces can survive for at least 10 weeks in the summer and 6 months in the winter in the UK. However, if the faecal mass dries out, there is rapid mortality of the eggs (Ollerenshaw, 1971).

1.3.1.3 Oxygen tension and pH

Eggs do not develop in a concentrated faecal suspension, although eggs will survive twice as long in aerobic conditions compared with anaerobic conditions. Eggs kept in cultures without faeces show little variation in mortality, but those in aerobic conditions hatch in one-fifth of the time taken for those at a lower oxygen tension (Rowcliffe and Ollerenshaw, 1960).

Eggs incubated at 27°C will develop and hatch within a pH range of 4.2–9.0, but development is prolonged above pH 8.0 (Rowcliffe and Ollerenshaw, 1960). The optimum pH for embryonation appears to be 7.0 (Al-Habbib, 1974).

1.3.2 Hatching of the miracidium from the egg and penetration of the intermediate snail host

1.3.2.1 Hatching of the egg

As originally noted by Thomas (1883a,b), fully embryonated eggs exposed to the same conditions do not always hatch together, with hatching occurring on successive days for some weeks, even months. Such a strategy is obviously of practical importance to the parasite but renders a suitable habitat dangerous for grazing animals for a long period of time. It is generally accepted that light and temperature affect the hatching of eggs of *F. hepatica*. Experiments carried out by Roberts (1950) showed that eggs of *F. hepatica* incubated for 14 days in the dark hatched only on exposure to light. It is common laboratory practice to induce mass hatching of miracidia by placing incubated eggs in strong light. A method for hatching eggs for teaching and research purposes is shown in Fig. 1.3.

1. Add eggs to a 12-well plate to give approx. 50 eggs/well then add sterile water to a total volume of 1 ml (all wells in duplicate).
2. Incubate eggs for 14 days at 26°C, wrapped in foil.
3. For EMBRYONATION ASSESSMENT:
    a. Expose plate to bright light for 30 min at room temperature.
    b. Using an inverted microscope assess egg embryonation.
    c. To assess egg hatch follow hatch protocol from (4b) onwards.
4. For EGG HATCH ASSESSMENT:
    a. On day 14 put plate at 4°C overnight.
    b. Day 15 remove plate from fridge and incubate at 26°C for 30 min, followed by 1 h incubation at room temperature under a bright light.
    c. Using an inverted microscope assess egg embryonation.

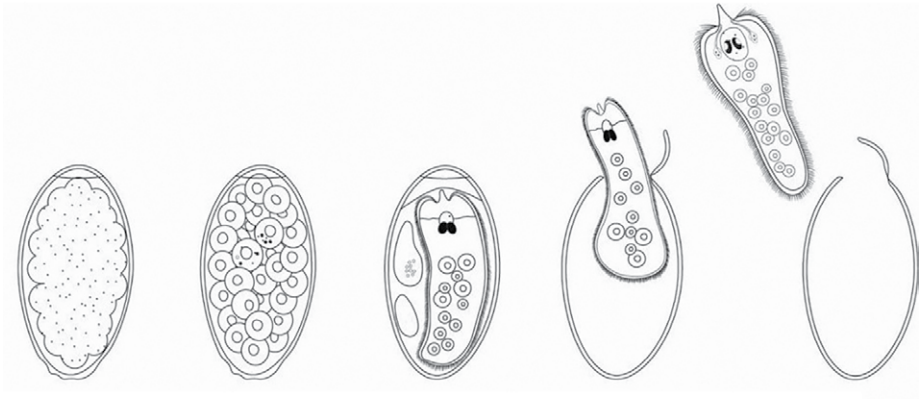


Fig. 1.3. *Fasciola hepatica* egg hatch assay and schematic detailing the stages of egg development. (Schematic drawn by Fiveprime Design.)

1.3.2.2 Survival of miracidia

The miracidium is about 130 μm in length, broad anteriorly and tapering posteriorly to a blunt end. The cuticle is ciliated, and there is an anterior papilliform protrusion and a pair of darkly staining eye-spots visible near the anterior end of the body (Fig. 1.2D,E). Once hatched from the egg the miracidium becomes active, immediately starting to swim at great speed (on average, 1 mm/s; Wilson and Denison, 1970). Characteristic swimming movements have been used as a means of determining the infectivity of miracidia. Those swimming in circles are not usually infective. Boray (1969) suggested that speed of movement of the miracidia after hatching has also been found to be a good indicator of their infectivity; those taking about 30 s to move 1 cm do not infect snails, while those requiring 4–12 s to travel 1 cm are generally infective.

The need to find a suitable host to penetrate is an urgent one, as miracidia failing to do so generally die within 24 h (Thomas, 1883a,b; Hope Cawdery *et al.*, 1978), at a rate which is age dependent (Smith and Grenfell, 1984). Such age-dependent mortality is a common feature of short-lived, non-feeding larval stages in the Digenea subclass and is associated with the depletion of finite energy reserves (Anderson *et al.*, 1982). It has been shown, using histochemical techniques, that glycogen levels in the miracidia of *F. hepatica* decline with age (Wagner, 1965). Although miracidia are capable of covering long distances during their short lifetime (over 50 m at temperatures of 10–15°C), in practice they are often confined to small bodies of water just a few centimetres across, most of which do not contain snails (Smith, 1978).

There appears to be no evidence that miracidial mortality varies with pH of the medium, at least in the range 6–8 (Smith and Grenfell, 1984). On the other hand, these authors showed that mortality does vary both with the temperature of the medium and with the age of the miracidia. The mean expected lifespan of miracidia decreases from about 36 h at 6°C to about 6 h at 25°C. At 10°C the mean lifespan is about 1 day (Al-Habbib, 1974). However, in his early reports Thomas (1883b) kept miracidia alive for 3 days in a slightly alkaline solution of peptone.

1.3.2.3 Location and penetration of the snail host

The behaviour patterns of miracidia have been extensively studied, especially with regard to their phototactic, thermotactic, geotactic and chemotactic responses. Three main areas have received special attention: (i) the extent to which a miracidium is 'attracted' towards a snail; (ii) whether such responses (if they occur) are specific for a particular species of snail; and (iii) whether the general responses of a miracidium are merely responsible for bringing a miracidium into an ecological niche similar to that of the snail so that contact is readily established (Smyth and Halton, 1983; Haas and Haberl, 1997).

The sequence by which a miracidium finds its snail host can be divided into three main steps (Wright, 1959; Ulmer, 1971; Saladin, 1979; Christensen, 1980): (i) host habitat selection – the newly emerged miracidium makes its way to the environment of its specific snail host; (ii) a period of random movement in the host habitat; and (iii) host finding – the miracidium orientates towards the host using tactic or kinetic mechanisms. The miracidium of *F. hepatica* is strongly phototropic, which is generally believed to be an adaptation for locating the snail host.

The intermediate snail hosts are amphibious. In Europe and some parts of Asia, the intermediate snail host is usually *Galba truncatula*, though elsewhere several other species are implicated (see Chapter 2). Their habitat is typically along the edge of small ponds, ditches and marshy land in areas that are subject to alternate flooding and desiccation. The ability of the miracidium to move towards the light ensures that it will not waste vital energy reserves by exploring the bottom of ponds where the snails are not to be found. An interesting comparison can be made with the miracidium of *F. gigantica*, which infects different species of snail. Studies on the infection of snails show this species of *Fasciola* to be less host-specific than *F. hepatica* (Kendall, 1954; Boray, 1966). In Africa, where *F. gigantica* primarily infects *Lymnaea natalensis* (a species that lives in deeper water), the miracidium is actively repelled by light and therefore dives further down into the water, where the snail is more likely to be found (Taylor, 1964).

The presence of a wide range of potential stimulant molecules in the mucus of snails is

well documented (see for example, Wilson, 1968b; Wilson and Denison, 1970; Wilson *et al.*, 1971). Concentrations of glucose, 16 amino acids and various lipids have been identified from the mucus of *G. truncatula* (Wilson, 1968). Short-chain fatty acids C7–C9 (> 0.1 mM) will stimulate miracidia of *F. hepatica* to attach to *G. truncatula* but such treatment appears to have a detrimental effect on the miracidia after about 10 min; chain lengths of between C6 and C9 (> 0.01 mM) stimulate a turning response by the miracidium (Wilson *et al.*, 1971). Experiments by Nansen *et al.* (1976) and Christensen *et al.* (1976b) confirmed the existence of a special chemical attraction of miracidia of *F. hepatica* not only towards *G. truncatula* but also towards other snail species, which is not affected by an existing infection. These chemoattractant molecules allow the *Fasciola* spp. to distinguish between different snail species, enhancing host-finding of susceptible snails (Bargues *et al.*, 1997; Kalbe *et al.*, 1997). Recent studies have shown that glycoproteins/glycoconjugates within the snail mucus play a role in the attraction and attachment of *Fasciola* miracidia with their snail host (Kalbe *et al.*, 2000; Haas, 2003; Georgieva *et al.*, 2019). These glycoproteins also play a role for the stages within the snail, as they are used in a host-mimicry fashion to protect the parasite stages from immune recognition (Georgieva *et al.*, 2012, 2014, 2016).

Although light is known to stimulate the hatching process, and the miracidium is positively phototropic, light does not appear to be a stimulus for the infectivity of the miracidium (Christensen, 1975). Infectivity of the miracidium is independent of pH (in the range 5.4–8.4), but dependent on water temperature. At or below 5°C, the miracidium is not infective, regardless of the exposure time. Optimum temperature is in the range of 15–26°C and a clear inverse relationship has been demonstrated between the environmental temperature and the duration of the host-finding capacity of the miracidia. At 8°C, 16°C and 24°C the host-finding capacity ceased after 24–30, 20–24 and 13–20 h, respectively (Christensen *et al.*, 1976a).

Penetration of the miracidium of *F. hepatica* into *G. truncatula*, and its transformation into a sporocyst, has been described by a number of works, including, at the light microscope level, by Thomas (1883a,b), Faust (1920), Eales (1930), Mattes (1949), Roberts (1950), Dawes (1959), Jefferies and Dawes (1960), and Southgate (1970); and at the transmission and scanning electron microscope level, by Wilson *et al.* (1971), Blankespoor and van der Schalie (1976), Køie *et al.* (1976) and Coil (1977). The penetration process involves a mechanical boring action by the miracidial anterior papilla and is also likely to be facilitated by the secretion of proteolytic enzymes (Smyth and Halton, 1983). Tissue at the point of penetration, generally near the branchial aperture, is observed to be degraded (Wilson *et al.*, 1971).

1.3.3 Development and multiplication inside the snail

One of the most favourable factors contributing to the successful completion of the life cycle, and thus the propagation of the liver fluke, is parthenogenetic multiplication within the snail. It was first demonstrated by Krull (1941) that a snail infected with a single miracidium can produce about 4000 metacercariae. Similar experiments performed by Hodgkinson *et al.* (2018) revealed a range of about 500–3200 metacercariae recovered following single snail/miracidia infections for six different *F. hepatica* isolates. Molecular analysis of the resulting metacercariae of the six isolates using microsatellite markers confirmed that genetic clonal expansion occurs within the snail host (Hodgkinson *et al.*, 2018).

Although a number of snail species can act as intermediate host for *Fasciola* spp. (Váquez *et al.*, 2018) (see also Chapter 2), the snail species can impact the dynamics of *Fasciola*–snail infection, with varying numbers of rediae and metacercariae being produced. For example, *Pseudosuccinea columella*, an important vector for fasciolosis in South America, has been shown to generate larger numbers of *F. hepatica* metacercariae when compared with *G. truncatula* (Dar *et al.*, 2014; Vignoles *et al.*, 2015). For details of some of the factors affecting the development of the fluke within the snail, see for example Faust (1920), Ross (1930), Rees (1931), Schumacher (1939), Kendall (1949, 1953), Kendall and McCullough (1951), Kendall and Parfitt (1959), Boray (1963, 1966, 1967a,b), Kendall and

Ollerenshaw (1963), Hodasi (1972), Rondelaud and Barthe (1987), and Graczyk and Fried (1999).

Once inside the snail, the young sporocyst (Fig. 1.2F) migrates via the blood vessels or lymph channels primarily to the digestive gland (often referred to as the liver), which is situated in the upper spirals of the shell. Here the sporocyst (Fig. 1.2G,H) begins to grow. Consisting initially of a minute ball of tightly packed germinal cells in which remnants of the eyespots can be seen, each germinal cell gives rise to a ball of new germinal cells from which the next larval stages, the rediae, develop. Ultimately, the sporocyst, distended by rediae (Fig. 1.2I), ruptures, liberating the rediae into the digestive gland. The rediae move about more actively than the parent sporocysts and cause considerable damage to this gland.

The redia is roughly cylindrical in shape and possesses a pair of marginal lappets (bulging projections) at the posterior end and a raised collar-like structure just behind the anterior end of the body (Fig. 1.2J,K). There is a mouth which leads into a muscular pharynx and posteriorly to a simple unbranched intestine. The body of the redia contains numerous germinal cells which, like those in the sporocyst, multiply to form germinal balls from which the final larval stage, the cercaria, is produced. Between 16 and 20 of these germinal balls are produced within each redia. Under adverse conditions, such as high or low temperatures or drought, two redial generations may occur from which the cercariae arise but the reasons for this to occur are not clearly understood. This unusual further multiplication phase was first observed by Thomas (1883a,b) (see also Fig. 1.2K). However, comparable to *F. gigantica*-infected snails where five redial generations have been observed (Dinnik and Dinnik, 1956, 1964), up to four generations of *F. hepatica* may arise during snail infections (Rondelaud *et al.*, 2009). The differences between *F. gigantica* and *F. hepatica* result in a larger number of rediae being generated for *F. gigantica* in comparison with *F. hepatica* (Dreyfuss and Rondelaud, 1995) (see Chapter 2).

The mature rediae measure 1–3 mm in length and are capable of considerable movement. Their migrations can cause serious damage and, in heavy infections, death of the snail. Normally, however, the snail shows a remarkable power of regeneration. When the cercariae are fully developed, they escape from the redia by way of the birth pore, which is situated laterally behind the anterior collar. Snails larger than 5 mm are more likely to shed cercariae than smaller snails (Olsen, 1944) (see also Chapter 2). Consistent with other parts of the *Fasciola* spp. life cycle, temperature plays a role in the maturation and development of the stages within the snail. Experiments by Dinnik and Dinnik (1964) revealed that *F. gigantica* redial development to cercariae did not occur at temperatures of less than 16°C but could be reverted when the temperatures rose to 20°C.

The cercariae are tadpole-like with a discoidal body and a long tail. The body measures 250–350 μm and the tail is twice as long. An oral sucker and a ventral sucker are in the centre of the body (as in the adult fluke). Leading from the oral sucker there is a pharynx, on either side of which are very conspicuous cystogenous glands, an oesophagus and a forked intestine (Fig. 1.2L). The mobile cercariae generally leave the snail 4–7 weeks after infection by migrating through the tissues. Different larval stages may coexist in a single snail (Agersborg, 1924). It follows, therefore, that the cercariae do not mature at the same time and, as demonstrated by Faust and Hoffman (1934), leave the snail over a period of time.

1.3.4 Emergence of cercariae from snails and their encystment

For a short time after they have emerged from the snail the cercariae swim freely in the water. They are very active and frequently change direction, both horizontally and vertically, although tending to keep near to the surface rather than going down into deeper water. The process of encystment and the structure of the cyst wall are complex and have been described by Wright (1927), Stirewalt (1963), Dixon and Mercer (1964), Dixon (1965), Køie *et al.* (1977) and Smyth and Halton (1983). Alicata (1938) described the structure of the cyst wall of *F. gigantica*.

1.3.4.1 Encystment

During a few minutes to 2 h after emergence, the cercaria settles on various objects, including blades of grass, and attaches by means of the

ventral sucker. Encystment may also take place upon the surface of the water (Wright, 1927) (see Chapter 2). Once attached, the body contracts inwards, releasing the outer layer of the cyst. Simultaneously, as the embryonic 'epithelium' is shed and the outer layer is laid down, the tail separates from the body. The tail is sometimes shaken off before the encystation begins but, as a rule, the tail remains in connection with the body during the process (Thomas, 1883a,b). The cyst is white when laid and is almost immediately infective to the definitive host. After a day or two the cyst gradually becomes yellow in colour, due to the presence of quinine, and darkens as it hardens.

1.3.4.2 Structure of the metacercarial cyst

The structure of the cyst wall consists of an outer cyst and an inner cyst. The outer cyst is composed of an external layer of tanned protein and an underlying fibrous layer of mucoprotein. The inner cyst has a complex mucopolysaccharide layer subdivided into three, and an additional layer (layer IV) of laminated or keratinized protein. A region of layer IV is specialized to form the ventral plug. The outer cyst wall probably acts as a barrier against bacterial and fungal infections and is also important for attachment to the substrate, normally grass (Dixon, 1965). Strong adhesion to grass for long periods is important for the survival of metacercariae and the infection of the final host. As the cysts may survive for long periods and remain infective if the outer wall is removed, the inner cyst walls must play a more important part in the survival of the metacercariae (Boray, 1963).

1.3.4.3 Longevity of metacercariae

Metacercariae may survive for more than 1 year on pasture (Soulsby, 1965). Survival is mainly dependent on sufficient moisture and moderate temperatures. Metacercariae are resistant to freezing between −2°C and −10°C but lose their infectivity at −20°C (Boray and Enigk, 1964). They can retain their infectivity after being maintained at −2°C for 8 weeks (Taylor, 1949) and may survive for as long as 11 months at temperatures varying from −3°C to 2°C if the average temperature remains above freezing (Shaw, 1932). Approximately 50% of metacercariae encysting on pasture in September can survive winter conditions in the UK to infect animals in the spring (Ollerenshaw, 1967). Survival rates and subsequent viability for parasites in silage and hay are less well known, given the varying practices of ensiling and storage as recently reviewed by John *et al.* (2019). Temperature of stored silage, bacterial proliferation during the fermentation processes, presence of oxygen and moisture content of stored hay all play a role in the viability of metacercariae present (John *et al.*, 2019).

Metacercariae produced by *L. tomentosa* can survive for 6 months at temperatures of 12–14°C, with 25% surviving for 8 months. When stored at between 2°C and 5°C, only 10% will survive for 1 year; others will survive for 8 weeks at 20°C, but none will survive for more than 6 weeks at 25°C (Boray, 1963). Under natural conditions in the USA, it has been shown that metacercariae are destroyed by heat and drought during the four summer months (Olsen, 1947). Similarly, metacercariae will not survive a typical Australian summer (Boray and Enigk, 1964). The infectivity of metacercariae depends not only on various climatic conditions but also on the temperature during their development through the larval stages in the snail (Davtyan, 1956; Boray, 1963).

1.3.5 Ingestion of infective metacercariae

Within an hour of ingestion by the definitive host, metacercariae begin to excyst in the small intestine. Within 2 h following infection these have bored through the wall of the intestine and can be found in the abdominal cavity en route to the liver.

1.3.5.1 Excystation

The process of excystation is likely to involve extrinsic factors (such as elevated temperatures, reducing conditions, pH, pCO_2 and the presence of bile salts) and intrinsic factors such as secretions by the fluke. There are basically two phases of excystation: a passive activation

phase followed by an active emergence phase. These have been described by Dixon (1966) and Smith and Clegg (1981) and reviewed by Sukhdeo and Mettrick (1987). Activation is believed to occur in the stomach or rumen and is a prerequisite to emergence. Conditions that stimulate activation *in vitro* are high pCO_2, temperature about 39°C and reducing conditions. During activation, the metacercariae rotate vigorously for a while before the quiescent phase (Dixon, 1966), during which time the predominant activity appears to be the emptying of their caecal contents (Sukhdeo and Mettrick, 1986). During excystment the parasites secrete cathepsin L and B-like cysteine proteinases which likely assist their emergence, as inhibition of these enzymes prevents this event (Robinson *et al.*, 2009; Cwiklinski *et al.*, 2018).

Excystation occurs in the small intestine of the host below the opening of the *ductus choledochus* or common bile duct. However, Hughes (1959) and Dawes (1961) showed that young flukes can emerge from cysts injected into the peritoneal cavity of hosts, suggesting that, apart from a temperature of about 39°C and possibly the presence of a low concentration of carbon dioxide, excystation may require very little additional stimulus. However, the emergence phase is probably triggered by bile and its presence may activate an enzyme secreted by the parasite, inducing muscular movements of the young fluke (Dixon, 1966). A method for the *in vitro* excystment of metacercariae for teaching and research purposes is shown in Fig. 1.4.

1.3.5.2 Migration to the liver

Details of the process of penetration of the intestinal wall, movement through the abdominal cavity and penetration of the liver have been described in great detail by Schumacher (1956), Dawes (1961, 1962, 1963), Dawes and Hughes (1964), Boray (1969) and Fairweather *et al.* (1999) and will be covered in more depth in Chapter 3.

After the metacercariae have excysted in the small intestine, the newly excysted juvenile flukes rapidly penetrate the intestinal mucosa, in doing so breaking down epithelial cells, connective tissue and unstriped muscle fibres, and move into the peritoneal cavity. Once in the peritoneal cavity, the flukes apparently browse on whatever tissue is available, occasionally penetrating organs, including the local lymph nodes. The liver appears to be reached by random wanderings, although evidence suggests that newly excysted juveniles may migrate towards the liver in response to some stimulus, an orthokinesis (Sukhdeo and Mettrick, 1987). Once through the liver Glisson's capsule (a process that typically takes place between 4 and 6 days after infection), the young flukes burrow through the liver for between 5 and 6 weeks, causing extensive haemorrhage and fibrosis. Significant growth of the fluke occurs during this period (see Chapter 3).

There is evidence that young flukes prefer to feed on hepatic cells rather than blood, although some blood is inevitably ingested. The flukes eventually reach the bile ducts, beginning about 7 weeks after infection, in which they grow to adults and become permanently established. Occasionally, immature flukes may be found in unusual sites, e.g. lungs, pancreas, lymph nodes and thymus, and some may infect the fetus in pregnant animals. From 8 weeks after infection, eggs are found in the bile, and later in the faeces, thus completing the life cycle.

Some adult flukes may live for a considerable time in the liver (Fig. 1.5 illustrates the adult fluke). For instance, flukes 11 years of age have been recorded in sheep by Durbin (1952), each producing up to 20,000 eggs per day. It is noteworthy that fertile eggs can be obtained from an animal infected with a solitary fluke (Hughes, 1959). Recent genomics, transcriptomics and proteomic studies have shed light on the molecular changes that take place when the parasites migrate through the host tissues and have identified the major molecules (proteinases, proteinase-inhibitors and antioxidants) that they secrete to affect this migration (Robinson *et al.*, 2009; Cwiklinski *et al.*, 2015, 2018; Zhang *et al.*, 2019).

Finally, many methods and procedures for the study of the biology and molecular biology of various stages of *F. hepatica* can be found in the book *Fasciola hepatica*: *Methods and Protocols*, edited by Cancela and Maggioli (2020).

Removing the outer cyst wall

1. Fill the lid of a Petri dish with 2% sodium hypochlorite solution. Scrape the encysted metacercariae from the visking tubing into the sodium hypochlorite solution.
2. Remove the outer cyst wall with agitation by pipetting the metacercariae up and down in the glass watch glass using a 1 ml pipette for no more than 10 min.
3. Once the outer walls have been removed, transfer the cleaned metacercariae to a watch glass with distilled water. Wash the metacercariae in distilled water by sedimentation several times to remove all traces of sodium hypochlorite.
4. Do not vortex or centrifuge the metacercariae as the parasites will burst out of the cysts.
5. The metacercariae can be stored at 4°C in water until required.

Excystment protocol

1. Prepare the excystment media solutions in separate tubes.
   a. 5 ml of a pre-made stock of 0.9% NaCl and 1.2% $NaHCO_3$ (for 100 ml: 0.9 g NaCl + 1.2 g $NaHCO_3$ in 100 ml dH_2O/RO) + 40 mg sodium tauroglycocholate
   b. 5 ml of N/20 HCl (4.75 ml of dH_2O/RO + 0.25 ml 1N HCl of pre-made stock; 1N solution – 50 ml: 4.9 ml 37% HCl + 45.1 ml H_2O) + 40 mg L-cysteine
2. Warm the excystment solutions at 37°C.
3. Transfer the metacercariae to a glass watch. Remove as much of the water as possible.
4. Mix the excystment media solutions together by inversion. The solution should effervesce when combined, but remain a translucent yellow (with bubbles on top). If the media does not effervesce or turns cloudy the metacercariae will not excyst (likely that too much HCl has been added).
5. Add the excystment media immediately to the watch glass, to completely cover the cysts. Place another watch glass on top and place the watch glasses into a humidity box, to prevent the excystment solution evaporating.
6. Incubate at 37°C for no more than 3 h. At approximately 1 h 15 min check for movement of NEJ inside the cysts and any parasites that have emerged. Check at 10/15 min intervals for excystment.
7. Once the NEJ emerge, collect using a 10 µl pipette and place in another watch glass with pre-warmed RPMI media.
8. Wash the recovered NEJ with RPMI media to remove traces of excystment media.

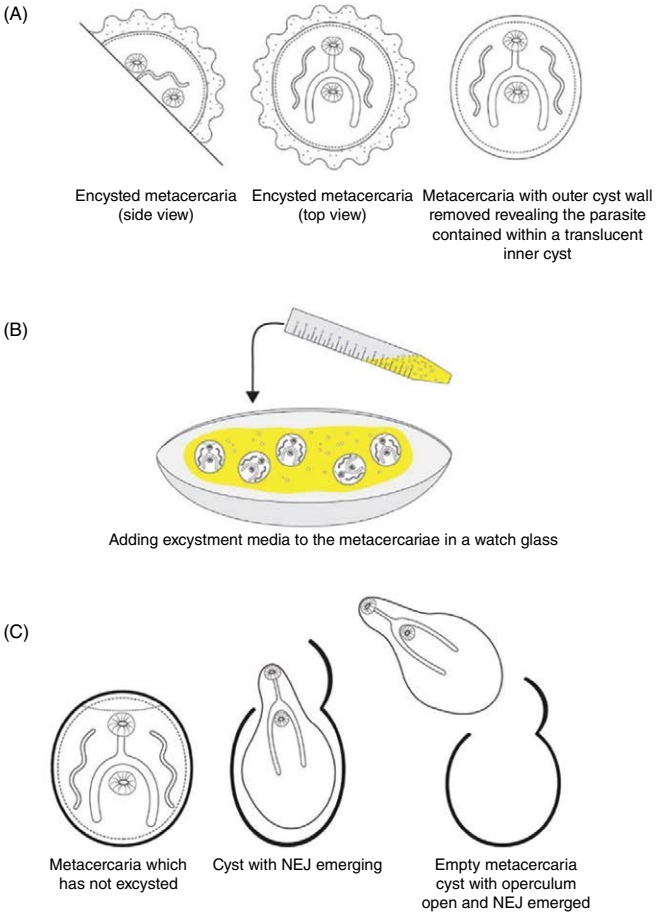
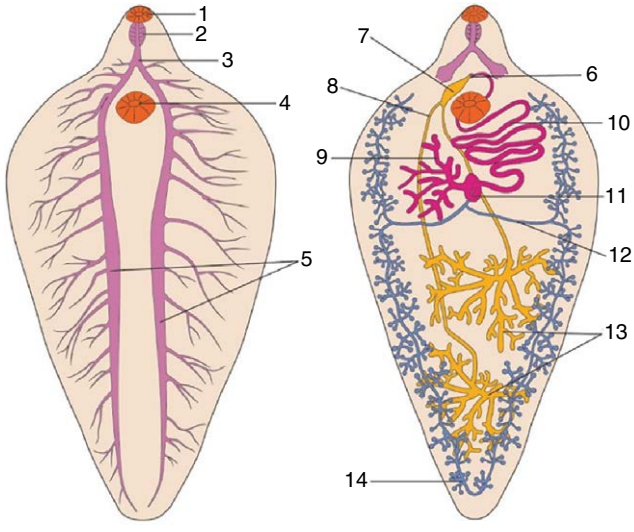


Fig. 1.4. *In vitro* excystment of *Fasciola hepatica* metacercariae and schematic detailing the stages of the *in vitro* excystment protocol. (A) Metacercaria with and without the outer cyst wall. (B) The addition of the effervescent excystment media to the metacercariae within a watch glass. (C) The excystment process with the NEJ emerging from the metacercaria cyst. (Schematic drawn by Fiveprime Design.)



Internal structures of *Fasciola hepatica*

1. Oral sucker
2. Pharynx
3. Oesophagus
4. Ventral sucker
5. Caeca
6. Genital pore
7. Cirrus sac
8. Vas deferens
9. Ovary
10. Uterus
11. Ootype
12. Vitelline duct
13. Testes
14. Vitelline gland

Fig. 1.5. Diagram of an adult *Fasciola hepatica* showing major structures. (Drawn by Fiveprime Design.)

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