Chapter 2
Laboratory Rearing of *Biomphalaria glabrata* Snails and Maintenance of Larval Schistosomes In Vivo and In Vitro

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Abstract This chapter describes the methods used by the current authors and numerous other investigators to optimize conditions for successfully growing and maintaining both uninfected and infected *Biomphalaria glabrata* in the laboratory, with particular emphasis on the optimal physical, chemical, and biological factors that affect successful snail rearing and/or breeding, and the ways that they can be measured. Consideration is also given to incidental organisms that can alter aquarium ecology and affect both the snails and their intended trematode parasites. Various methods of infecting snails and maintaining snail tissues and cells in vitro are also described. The goal of this chapter is to aid researchers who have the need or desire to maintain the life cycle of this pulmonate and its trematode parasites in the laboratory.

2.1 Introduction

Schistosomiasis and other snail-transmitted trematode infections continue to be parasitic scourges of humanity; difficulties studying them in the laboratory include the proper rearing and maintenance of their snail intermediate hosts. Snails thrive in nature under diverse and changing environmental conditions, but the exact physicochemical factors for their survival are difficult to duplicate in the laboratory, where diurnal and seasonal fluctuations in light, temperature, salinity, and other unknown ecological conditions may not be fully understood or cannot be replicated (Berrie 1970; Webbe and James 1971).

Because the maintenance of the schistosome life cycle can be quite involved in terms of manpower, space, and other resources, the National Institutes of Health – National Institute of Allergy and Infectious Diseases (NIH-NIAID) provides snails

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and developmental stages of schistosomes. Depending upon the laboratory's research focus and requirements for snail-derived schistosome stages (i.e., sporocysts, cercariae), it may sometimes be preferable to maintain the life cycle in the laboratory, which requires sustaining both the intermediate and an appropriate definitive host. When resources cannot be committed, and experiments can be timed and planned in advance, both snails and definitive hosts with prepatent infections can be procured free of charge. Both schistosome and snail cDNA libraries and a *Biomphalaria glabrata* (M-line) embryonic (Bge) cell line are also available (Lewis et al. 2008).

This chapter is intended for researchers who have the need and resources to maintain the entire life cycle of schistosomes or other trematodes in their laboratory. It attempts to describe the optimal parameters and methodologies for rearing and maintaining the snail intermediate hosts, and both classical and surgical techniques for infecting the snails with schistosomes. It describes the methods for maintaining snail tissues and cells to support in vitro cultivation and the growth of intramolluscan developmental forms of schistosomes and other trematodes. It also attempts to highlight some of the experimental studies on rearing and maintaining *Biomphalaria* snails that have dealt with the challenges of maintaining and studying trematodes in the laboratory.

2.2 Snail Rearing and Maintenance

B. glabrata snails are bred and maintained in the laboratory for several purposes. They are most often used to support the life cycles of schistosomes and other trematodes, and to generate a supply of schistosome sporocysts or cercariae (Lee and Lewert 1956; Lewis et al. 1986). They are also used in the laboratory as first and second intermediate hosts, particularly for echinostomatid trematodes whose cercariae encyst in snails (Huffman and Fried 1990; Fried and Huffman 1996), and as a source of organs and cells for in vitro culture (see Sect. 2.4). In addition, *Biomphalaria* snails are used in undergraduate research and laboratory teaching exercises.

2.2.1 Measures of Successful Snail Rearing

Successful snail rearing is the first requirement for maintaining the schistosome life cycle. Growth rate, fecundity, shell size, survival, and death rates are important indicators of the physiological state of the snail host. Large snails produce greater numbers of cercariae and lay more and large egg masses (or clutches), even though snail size does not appear to influence cercarial infectivity (Eveland and Ritchie 1972).

2.2.1.1 Survival and Longevity

In nature *B. glabrata* can live for 12–18 months, but their survival in the laboratory under closely monitored conditions may or may not exceed 12 months. Since the generation time is approximately 5 weeks, this snail can undergo several generations over a 12-month period (Ritchie et al. 1963; Loker 2006).

2.2.1.2 Growth and Maturation

Although it is impractical to determine the exact growth and maturation potentials of *Biomphalaria* snails in nature, theoretical growth curves have been developed for *B. glabrata* and several other species of snails in their natural habitats (Leveque and Pointier 1976). An approximation of those potentials has also been reported under laboratory conditions (Ritchie et al. 1963, 1966). When three different strains of *Biomphalaria* snails were cultivated individually in 200 ml of water, they grew more slowly and began egg-laying later than when cultured in a circulating water system. The best mean growth in the stagnant system was 19.7 mm in 180 days, and in the circulating system snails reached 27 mm in 150 days. Also, in the circulating system egg-laying began sooner than in the stagnant system (Ritchie et al. 1963). Another study reported better growth and fecundity of *Biomphalaria pfeifferi* in nonaerated water that was renewed weekly, and better snail growth but less fecundity in nonaerated water that was changed monthly (Frank 1963). *B. glabrata* growth parallels that of another planorbid snail (*Helisoma trivolvis*) maintained under identical laboratory conditions (Schneck and Fried 2005).

2.2.1.3 Fecundity

B. glabrata is hermaphroditic and is capable of both self- and cross-fertilization, although the latter is the preferred mechanism. Under ideal conditions, the egg to egg cycle may be as short as 1 month (Ritchie et al. 1963). A mature snail may lay 10,000 or more eggs/year. The eggs are laid in gelatinous masses of several eggs each that are found on the wall of the laboratory aquaria or other, e.g., filtration devices or the glass-covered heaters. We have used small 2–3×4–5″ cellophane sheets (rafts) that float in aquaria and snails deposit egg masses on their under surface; the sheets are easily transferred to other aquaria. We have observed that snails laid more eggs when maintained in plastic trays with shallow water.

2.2.2 Physical and Biological Factors that Affect Rearing

Although snails are prolific in nature, a number of important physical and biological factors must be taken into consideration for their successful cultivation in the laboratory, including but not limited to temperature, diet, crowding, and parasitism.

2.2.2.1 Temperature

Numerous studies have been conducted to determine optimal temperatures for *Biomphalaria* growth and development (Webbe and James 1971). Although minor differences in both growth and fecundity have been reported in studies on different *Biomphalaria* spp., uninfected snails grow best over a temperature range of 24–28°C, with 25°C being optimal for *B. glabrata* (Chernin and Schork 1959). *B. glabrata* grows more slowly in the laboratory at 20°C (Sturrock and Sturrock 1972). However, infected snails can be kept at a somewhat higher temperature (26–28°C) to allow for the rapid development of the trematode stages. Noninfected snails can be maintained in rooms with a relatively constant temperature, and submerged aquarium heaters can be used to achieve higher temperatures in aquaria containing infected snails. In rooms where constant temperature cannot be easily maintained, thermostatically controlled heaters should be used in aquaria, and the room temperature should be kept slightly lower than that of the aquaria.

2.2.2.2 Diet

As stated above, shell size is an important determinant of snail fecundity and cercarial production, although it does not influence cercarial infectivity in mice. The snails can live solely on a lettuce diet, but they grow much larger and lay more egg masses when their diet is supplemented. Romaine lettuce is preferable to iceberg, and maximum growth was obtained when lettuce was supplemented with a formula consisting of young grass shoots from wheat, barley, and rye crops, mixed together with an extract of thyroid and endocrine glands, wheat germ and powdered milk in a ratio of 4:2:2:1. This formula results in snails nearly 3 times larger than the lettuce-fed snails, and the larger snails lay 7 times more egg masses. Snails fed on lettuce supplemented with Gaines Meal dog food or commercial chicken food grow nearly twice as large as those fed on lettuce alone, and lay 6 and 4 times as many egg masses, respectively. The egg masses from snails on the supplemented diets are also larger and contain more embryos (Eveland and Ritchie 1972). Infected *B. glabrata* grows twice as large when fed on Purina Rat Chow for 7 weeks than when fed on boiled lettuce (Mecham and Holliman 1972). Cumulative cercarial outputs have also been shown to be higher from snails fed Romaine lettuce than from those fed iceberg lettuce, but cercarial output was not affected by adding the formula mentioned above to the diet (Cohen 1984). We and others (Boston et al. 1994) have also supplemented lettuce diet with Tetramin fish food.

It has been documented that some snail fauna are scarce in calcium-poor British freshwaters (Boycott 1936). *B. glabrata* growth rates, relative shell weights, fecundity correlate with increasing calcium concentrations (Thomas et al. 1974), and snails maintained in water with <2 mg/L Ca^{++} have smaller and extremely fragile shells (Mishkin and Jokinen 1986). Snail mortality is significantly greater when calcium levels are very low (1.5 mg/L) or very high (75 mg/L), with 30 mg/L being the optimal calcium concentration for the greatest snail fecundity (Mishkin and Jokinen 1986). Deleterious effects of high aquarium calcium concentrations on *B. glabrata* fecundity,

growth, and survival have been reported (Frank 1963). Small pieces of chalk (calcium carbonate, $CaCO_3$) have been added to aquaria as a calcium source (MacInnis 1970; Ulmer 1970) with the aim of promoting snail growth and shell hardening. In fact, calcium reserves in *B. glabrata* increase when snails are infected with *Schistosoma mansoni* (Shaw and Erasmus 1987; Ong et al. 2004). Hemolymph calcium levels of snails kept in aquaria with chalk sticks for 3 days were higher than those of snails maintained without chalk (Chernin 1963). Another study that compared calcium content of *B. glabrata* maintained with and without chalk documented higher calcium levels in both soft tissues and shells of snails raised without chalk (Boston et al. 1994). Also, *B. alexandrina* and *Bulinus truncatus* with patent *S. mansoni* and *Schistosoma haematobium* infections were found to have relatively lower calcium content in shells and higher calcium content in soft tissues than the uninfected snails (Mostafa 2007). It seems that the substantial amount of calcium in both artificial spring water (Ulmer 1970) and lettuce (Boston et al. 1994) is apparently enough to meet the calcium requirement of most snails.

2.2.2.3 Crowding

Extreme crowding may influence both snail growth and fecundity. However, uninfected *Biomphalaria* grow and reproduce well over a wide population density range from 2 to 50 snails per unit volume of water when adequate nutrition is provided and the water is properly conditioned, either by the use of an activated charcoal and/or a water circulation system such as that mentioned in Sect. 3.2.1.2 on *Growth and Maturation* (Wright 1960; Ritchie et al. 1963). Due to their more fragile condition, infected snails should be kept at between 15 and 20 snails per liter. When 20 uninfected *B. glabrata* were maintained for as few as 4 days in one liter of stagnant water, the average O_2 uptake was considerably lower than for uncrowded snails (Coelho et al. 1977).

2.2.2.4 Parasitism

Many bacteria (Michelson 1961; Richards 1978; Ducklow et al. 1979, 1981; Bean-Knudsen et al. 1988), ichthyosporea (Hertel et al. 2002, 2004), microsporidia (Bayne et al. 1975; Lai and Canning 1980), fungi (Bagy et al. 1992), protozoa (Richards 1968), and numerous metazoa have been described living in or on *B. glabrata* collected from the field; also, many larval trematodes use *B. glabrata* as their first intermediate host. Occurrence of many diverse organisms in snails is not surprising since their natural habitats are shared by a variety of flora and fauna. Their relationship with *B. glabrata* remains imprecisely defined; most are probably symbionts and commensals although some may be pathogenic. Some may even exclusively parasitize trematode parasites of snails without causing apparent injury to the latter and may qualify as hyperparasites. Properly maintained snail populations seldom show signs of distress (declining snail number or reduced breeding potential) even when multiple species are present, but poorly maintained aquaria may permit unchecked growth of microorganisms that harm young and adult snails. Because

B. glabrata are maintained in the laboratory to support life cycles of schistosomes and other trematodes, the effect of unintended trematode infections on the growth, survival, and fecundity of snails is of wide interest.

Trematodes (Schistosomes and Other Trematodes)

Growth: Influence of infection on snail growth is obviously complex and varies with host and parasite species and strains, nutrition, and a number of other unknown factors (Thompson 1983). For example, one study using *S. mansoni*-infected *B. pfeifferi* provided evidence of "parasitic gigantism," or accelerated shell growth during the prepatent period of infection (Sturrock 1966), while another reported that *S. mansoni* infection of *B. glabrata* retards growth (Meier and Meier-Brook 1981).

Survival: Schistosome infections adversely affect snail survival, and the average life span of *B. glabrata* infected with *S. mansoni* is 39 days (Barbosa 1962). In one study, 77% of *S. mansoni*-infected *B. glabrata* died within 18 weeks postinfection, compared with only 11% mortality in noninfected controls (Pan 1965). Another study with these species demonstrated that infected snail deaths rose at a steady rate following infection until it reached 30% per week by 12 weeks following infection (Schwanbek et al. 1986). While infections cause profound mortality in *Biomphalaria* spp. regardless of their age, some studies report more deleterious effects in young snails, while others indicate that *S. mansoni* causes more damage to older snails (Sturrock 1966; Meier and Meier-Brook 1981). Other studies suggest that schistosome-infected snails live as long or longer than noninfected snails (Short 1952; McClelland and Bourns 1969; Fryer 1986). The number of miracidia used for infection can also profoundly affect snail survival. In a study with *S. haematobium* in *Bulinus truncatus,* 53% of uninfected snails survived to week 22, two miracidia per snail resulted in 10% survival and none of the snails exposed to 5 or 20 miracidia survived (Chu et al. 1966).

Fecundity: Schistosomes and other trematodes adversely affect the reproductive system of their snail hosts. *S. mansoni*-infected *B. glabrata* lays fewer eggs and their viability is greatly reduced, although complete cessation of egg-laying does not occur (Barbosa 1962). Reduction in or cessation of egg-laying has been observed in *B. glabrata* and *B. pfeifferi* infected with *S. mansoni* around the time infection becomes patent, and its extent may depend upon factors, such as the age of the snails at the time of infection or strains of parasites and snails (Pan 1965; Etges and Gresso 1965; Sturrock 1966). This effect has often been referred to as *parasitic castration*, even though it may or may not imply gonadal invasion and physical damage. Also, despite experiencing a dramatic decline in egg production, *S. mansoni*-infected *B. glabrata* continues to produce and transfer sperm to uninfected snails (Cooper et al. 1996). Other studies have shown increased egg-laying in *B. glabrata* infected with *S. mansoni* during the first 5 weeks of infection (Rodgers et al. 2005).

Other possibilities for the reduction in host fecundity include interference with host development or sexual maturation by nutrient depletion, or deleterious effects on

the host accessory glands or endocrine function (Hurd 1990). The larval schistosomes may compete for scarce resources such as glucose required by the albumin gland of the snail host (von Brand 1979). They may also cause physiological stress, preventing the host from feeding and/or utilizing its nutritional intake (Becker 1980; Thompson 1983). Schistosomes produce substances that cause endocrine dysfunction and thus interfere with the host gonad development or oogenesis (Meulman 1972; Meier and Meier-Brook 1981). For example, substances present in hemolymph as well as excretory–secretory products from secondary sporocysts of *S. mansoni* suppress both polysaccharide and galactogen synthesis in *B. glabrata* (Crews and Yoshino 1990). The schistosome-induced host-derived factor, schistosomin, interferes with glucose and galactogen incorporation by the snail albumin gland (Dictus et al. 1987). This heat stable, pronase-labile factor also suppresses both male and female reproduction in *Lymnaea stagnalis* infected with the avian schistosome *Trichobilharzia ocellata* (de Jong-Brink et al. 1988, 1992). In this system, schistosomin appears and fecundity decreases at the same time that cercariae begin to differentiate in daughter sporocysts (Shallig et al. 1991). Schistosomin is believed to be a gonadotropin-antagonist that interferes with the neuroendocrine mechanisms in the host (Hurd 1990; de Jong-Brink 1995). However, in *B. glabrata* snails infected with *S. mansoni* or *Echinostoma paraensei,* a schistosomin-like substance was detectable during early snail development but did not become elevated when mature snails were infected, suggesting that schistosomin may not be responsible for castration in *B. glabrata* (Zhang et al. 2009) and that some other factor(s) may be involved. Effects of larval schistosomes on the snail are more completely covered in Chap. 5.

Organisms Other than Trematodes

Ichthyosporea and Microsporidia: One of the first microsporidia found in *B. glabrata* epithelial cells, *Coccospora brachynema* (Richards and Sheffield 1970) was renamed *Steinhausia brachynema* (Sprague et al. 1972). Transovarial transmission of a related species *Steinhausia mytilovum* in bivalves without apparent pathogenesis has been described (Sagrista et al. 1998; Matos et al. 2005). Microsporidia use both horizontal and vertical transmission (Dunn et al. 2001), making it more likely for these organisms to have survived in laboratory-reared *B. glabrata* that were isolated from the field many years ago. *Pleistophora* sp. found in cultured cells from the heart and gonads of juvenile *B. glabrata* do not impair host reproduction (Bayne et al. 1975; McClymont et al. 2005). *Nosema algerae*, a parasite of mosquitoes, was found to experimentally infect *S. mansoni* sporocysts in *B. glabrata* but not snail cells (Lai and Canning 1980).

Originally described as an ameba, *Nuclearia* sp. isolated from *B. glabrata* hearts attaches to and kills both *S. mansoni* sporocysts and *B. glabrata* embryonic cells (Stibbs et al. 1979; Owczarzak et al. 1980). This organism has been redescribed as a unicellular eukaryotic symbiont *Capsaspora owczarzaki* (Hertel et al. 2002). It is more prevalent in laboratory-maintained snails that are resistant to *S. mansoni*, but may not be the sole reason for the resistance (Hertel et al. 2004). Another

Table 2.1 Directly observed metazoa found associated with *Biomphalaria glabrata* or in aquaria with snails[a]

Taxonomic group	Organism	Comment
Ostracoda	*Cypridopsis vidua*	Detritus feeder; robs snail nourishment
Turbellaria	*Macrostomum* sp.	Consumes young snails
Rotifera	*Rotaria rotatoria* *Philodina acuticornis*	May not affect snails directly but indicate poor aquarium conditions; reduce cercarial output, motility and infectivity
Copepoda	*Cyclops* sp.	May not affect snails directly but indicate poor aquarium conditions
Oribatida (water mite)	*Hydrozetes lemnae*	
Bryozoa	*Plumatella* sp.	
Oligochaeta	*Chaetogaster l. limnaei*	Ingests miracidia and cercariae
	Tubifex sp.	Consumes dead snails; keeps aquaria clean
Hirudinida	*Helobdella* sp.	Feed on snails; reduces snail fecundity

[a] Adapted from Webbe and James (1971) and Lewis et al. (1986)

unicellular eukaryotic symbiont (*Anurofeca* sp.) was originally described as an alga that inhibits anural tadpole growth (Richards 1949; Hertel et al. 2004). Based on DNA sequence homology, two ciliates (*Paruroleptus* sp. and *Trichodina* sp.) were also identified in field-collected *B. glabrata* in Brazil (Hertel et al. 2004).

Eukaryotic Metazoa: Of the many metazoa associated with *B. glabrata*, the few properly identified and documented are listed in Table 2.1. Perhaps the most closely examined is *Chaetogaster limnaei limnaei*. It has been found in association with *B. glabrata* and many other snail species, and has been regarded as a commensal or a symbiont. A large number of studies have demonstrated that *C. l. limnaei* protects *B. glabrata* against infection with *S. mansoni* either by ingesting miracidia or preventing their penetration into the snail (Khalil 1961; Michelson 1964; Wajdi 1964), but does not interfere with *B. glabrata* growth or fecundity (Rodgers et al. 2005). It also ingests cercariae of *Fasciola hepatica* when living in *Lymnaea tomentosa* (Rajasekariah 1978) and those of *Echinostoma trivolvis* and *Zygocotyle lunata* when living in *Helisoma trivolvis* (Fried et al. 2008). Immersion of infested snails in 1% urethane for 10–20 min has been reported helpful in partial elimination of *C. l. limnaei* (Michelson 1964), however, complete elimination of this and other infestations can only be accomplished by thorough cleaning of aquaria, including an ethanol rinse, and using new water, gravel, and known clean snails (see Sect. 2.2.3).

2.2.3 Methods of Snail Rearing

Snails reared for experimental purposes are typically maintained in monocultures (i.e., one snail species in one container) to prevent the introduction of competitor snails, commensals and pathogens, including trematode cercariae that encyst in these molluscs. They are most often kept in a room where temperature, humidity, and light can be regulated. Temperature and humidity are usually kept constant and the room with uninfected breeding snails is illuminated 12–14 h daily with fluorescent light

(Lee and Lewert 1956). Snails require 8 h of light for breeding (Webbe and James 1971). Schistosome-infected snails are maintained either in a separate room which is kept dark or in tanks that have been covered with dark or black paper or other material that prevents light from reaching snails and thus cercarial shedding.

Containers: The choice of containers depends on the number of snails to be maintained and the laboratory space available for this purpose. In developing countries, where ambient summer temperatures are high and the cost of temperature regulation is prohibitive, both field isolates and experimental snails are maintained in large earthen pots that may hold 2–10 Ga of water. Earthen pots are porous and have a large surface area that allows much greater evaporation, thus lowering the water temperature. These vessels require frequent replenishment of water.

In laboratories where temperature regulation is feasible, snails are maintained in glass or plexiglass tanks of various capacities depending on the shelving or storage arrangement. 10-Ga tanks are used more often because they are easy to clean and allow access to snails or debris at the tank bottom. The 10-Ga aquaria are also more suitable because of the ease of availability of various devices such as aerators, filters, and immersed heaters that fit and operate with these tanks.

Water: Snails do not usually survive for more than a day or two in freshly collected chlorinated water from municipal supplies. Tap water can be made usable for snail rearing by storage in aquaria with large surface area or repeatedly filtered through activated charcoal, which eliminates harmful substances such as chloramines. Charcoal-filtered water from aquaria with healthy snails (snail-conditioned water) can also be added. Where available, investigators have reliably used well water. For maintaining small number of snails or for experimental studies, deionized or distilled water can be reconstituted with different combinations of salts to prepare artificial spring water or "snail water" (Hopf and Muller 1962; Ulmer 1970; Cohen et al. 1980; Thomas 1986). Poorly conditioned new aquaria adversely affect snail survival, growth, and reproduction, but these effects may not be noticed over several days. Introduction of guppies (*Lebistes reticulatus*) to new aquaria conditions water and their survival indicates that snails would thrive unaffected in these aquaria. The fish also eat *Chaetogaster* sp. that may contaminate the tanks. Guppies should be removed once the snails have been established because they will also eat snail egg masses. Growth of snails is also believed to be enhanced because snails will feed on the organic waste of the fish (Ulmer 1970).

pH: Studies have shown that pH in the range of 4.9–8.9 does not adversely affect snail rearing and breeding (Chernin and Schork 1959; Webbe and James 1971). Optimal pH has been reported to be 7.0 ± 0.2 (Etges and Ritchie 1966), and we maintain aquaria over a narrow pH range of 6.9–7.1.

Aeration and Filtration: Aquaria with standing water and those with water circulating through a series of aquaria are in use in different laboratories. In either case, the water is filtered through filter wool and activated charcoal or beds of sand which also aerates water; direct aeration of aquaria is often unnecessary. A variety of aeration and filtration devices that run on electricity or pressurized air are available

from biological supply houses. When pressurized air is used, it is prudent to use an in-line air filter to trap oil and debris. Besides aeration, properly operational filters remove much of the snail feces, thus facilitating the cleaning of the aquaria; the settled snail feces and decaying lettuce remains still require removal by nets or siphoning off using a suction device. Care must be exercised not to remove the juvenile snails. Aquatic plants may also be added to aquaria; some provide favorite sites for snails to deposit their egg masses (Standen 1951).

2.2.3.1 Axenic Snail Rearing

Axenically grown snails may be used in studies of their nutritional requirements and for the screening of microbial agents which may prove useful in snail control. *B. glabrata* have been successfully grown axenically from individual eggs separated from egg masses (Chernin 1957). The snails were maintained in either aquarium water or a "handling solution", and fed autoclaved brewer's yeast and formalin-killed *Escherichia coli* (Chernin 1957; Chernin and Schork 1959). Some of the snails maintained in aquarium water reached adult size, their growth was suboptimal, and they never laid eggs. This retarded growth and development was thought to be due to the limited food supply (Chernin and Schork 1959). Also, the snails did not grow when either brewer's yeast or *E. coli* was missing, and the growth was dependent upon the concentration of *E. coli*. Snails did not grow when *E. coli* were heat-killed, suggesting the presence of a heat-labile essential growth factor(s) in bacteria. Although penicillin had no adverse effect on the snails, streptomycin severely inhibited their growth (Chernin and Schork 1959).

A subsequent study using a medium containing both a solid and a liquid portion demonstrated optimal *B. glabrata* growth in the absence of egg-laying, but when vitamin E (α-Tocopherol) was added to the medium all of the seven noncontaminated snails laid eggs (Vieira 1967). Another study using an even more complex medium with both solid and liquid portions containing multiple vitamins, including vitamin E, also demonstrated optimal snail growth and egg-laying. This study used two slightly different solid media: medium one was used for growing snails for up to 8 weeks, and medium two was used thereafter. The solid media were sterilized either by autoclaving or by irradiation, and the liquid medium was autoclaved. The snails grew larger and laid many more eggs when the solid portion of the medium was sterilized by irradiation (de Souza et al. 1977).

2.3 Methods of Infecting Snails

2.3.1 Miracidial Infection

Ideally, one should infect snails using as few miracidia as necessary because polymiracidial infections may result in shorter snail longevity (see Sect. 2.2.1.1).

However, high infection rates are often difficult to obtain with monomiracidial infections. Using increasing numbers of miracidia generally results in increased parasite load within the surviving snails, although this increase is not necessarily linear. One study reported a mean of 34 secondary sporocysts resulting in *B. glabrata* monomiracidially infected with *S. mansoni*, but when the miracidia number was increased to two and four per snail the numbers of secondary sporocysts were 54 and 52, respectively (Christie and Prentice 1978). Snail mortality was not reported in this study.

Preparation of Miracidia: The organs with the largest number of schistosome eggs (e.g., liver) are surgically recovered (or harvested) from animals 7–10 weeks after exposure to 100–300 cercariae. The animal tissue is homogenized in a glass homogenizer or in a blender for 20–30 s in 0.85% NaCl (salt prevents hatching of miracidia). The homogenate is poured into a flask and allowed to settle for 20–30 min by which time eggs sink to the bottom of the flask. The supernatant is poured off. This washing step is repeated for two or more times as required by adding 0.85% NaCl and pouring off the supernatant until it becomes clear. In the final step, spring or freshwater is added instead of saline, and the entire flask contents are transferred into a side-arm flask which is carefully filled to the rim with water. The flask is placed in bright light for 5 min to stimulate miracidial hatching and then covered with foil or other suitable material, leaving the side-arm uncovered. A bright light is directed at the side-arm to attract miracidia which can then be transferred using a Pasteur pipet (MacInnis 1970).

Infection: For monomiracidial infections, individual snails should be incubated in small clean glass containers with a single miracidium in just enough water to cover the snail for at least 1 h. For polymiracidial infections, 20–25 snails can be put together for 1 h in a large Petri dish containing 100–200 miracidia and enough water to cover the snails. To achieve accurate counting, heavy suspensions of miracidia may first need to be serially diluted before precise numbers can be retrieved using a pipet. From 50 to 100 infected snails should then be maintained in well-aerated 10-Ga aquaria covered with brown paper or contact paper throughout the prepatent period.

2.3.2 Transplantation of Parthenitae

Trematode parthenitae recovered from infected snails and transplanted into non-infected snails thrive and undergo usual/normal/natural asexual multiplication. The first successful transplantations included those of *S. mansoni* sporocysts in *B. glabrata* (Chernin 1966) and *Echinoparyphium dunni* rediae in *Lymnaea rubiginosa* (Heyneman 1966). It should be noted that echinostome rediae produced patent infections following their introduction into body cavities of recipient snails, whereas schistosome sporocysts required implantation into tissues of recipient snails via the cephalopedal sinus. Other studies on transplantation of echinostome rediae have also been reported (Dönges 1963, 1968, 1971; Dönges and Götzelmann 1988).

The original transplantation technique (Chernin 1966) has undergone minor modifications and has helped elucidate the aspects of intramolluscan development of schistosomes. For example, daughter sporocysts of *S. mansoni* when transplanted into noninfected snails produce an additional generation of sporocysts (DiConza and Hansen 1972), while normal intramolluscan development of *S. mansoni* involves continuous multiplication of sporocysts (Jourdane et al. 1980).

Perhaps the technique's greatest benefit has been the perpetual maintenance of schistosome clones (Jourdane and Théron 1980; Nojima et al. 1980; Cohen and Eveland 1984; Jourdane 1984, 1990). In addition to circumventing the requirement for a vertebrate-host (Jourdane 1990), schistosome clones obtained by this technique can be maintained and biologically characterized (Cohen and Eveland 1988). Schistosome clones are biologically different from one another and from respective developmental stages resulting from polymiracidial infections (Rowntree and James 1977; Smith and Clegg 1979; Cohen 1984; Cohen and Eveland 1988; Al-Adhami et al. 2001). One study has revealed genetic heterogeneity among individuals within clones (Bayne and Grevelding 2003).

Sporocysts of other schistosome species have also been transplanted into their respective snail hosts: *S. haematobium* in *Bulinus truncatus* (Jourdane et al. 1981); *S. bovis* in *Bulinus truncatus* (Jourdane et al. 1984); and *S. japonicum* in *Oncomelania hupensis* (Jourdane et al. 1985; Jourdane and Xia 1986; Xia et al. 1998).

Although these techniques offer obvious advantages for the maintenance of clones of various schistosome species and strains, they also present some challenges. For example, they involve excision of schistosome sporocysts which normally are entangled in snail tissues, preparation of sporocysts for implantation, anesthetization of recipient snails to prevent them from retracting into their shells, surgical implantation of sporocysts, and postsurgical care of recipient snails. Some of these procedures used in earlier studies are summarized in Table 2.2.

The donor monomiracidially-infected snails are swabbed with 70% ethanol prior to the removal of soft tissues into a balanced salt solution that approximates the snail hemolymph composition (Chernin 1963; Cohen 1984; Cohen and Eveland 1984; Jourdane et al. 1985). This balanced salt solution has been used with various combinations of antibiotics (Chernin 1963; Cohen and Eveland 1984; Jourdane et al. 1985). Diluted Schneider's Drosophila medium (110 mOsm/Kg H_2O) was used in one study (Nojima et al. 1980). Parasitized tissues (hepatopancreas and ovotestis) are excised and cut into 1–2 mm^3 fragments using iris scissors (Chernin 1966; Cohen 1984).

Early trials of anesthetizing molluscs with chloral hydrate, cocaine, carbon dioxide, chlorotone, nembutal, and aqueous and alcoholic solutions of menthol were unsatisfactory. However, urethane (ethyl carbamate) was reported to be a successful snail relaxant (see Michelson 1958). Aqueous solutions of urethane (0.75–1.5%) administered for 2–5 h have been used to anesthetize 10–20 mm snails (Chernin 1966; Cohen and Eveland 1984). A 5-h treatment in urethane was required for adequate anesthetization and treatments longer than 8 h resulted in snail mortality (Cohen and Eveland 1984). Other studies have used 0.8% nembutal

Table 2.2 Summary of procedures used in the transplantation of larval schistosomes in snails

Schistosome species	*S. haematobium*	*S. japonicum*	*S. mansoni*
Miracidial infection of donor snail	Polymiracidial	Polymiracidial	Polymiracidial
Recipient snail species	*Bulinus truncatus truncatus*	*Oncomelania hupensis hupensis*	*Australorbis glabratus*[a]
Anesthetic	Nembutal[b] (0.15%)	Nembutal[b] (0.08%) for 7 h	Urethane (1.25–1.50%) for 2–5 h
Medium in which sporocysts excised	CBSS	CBSS	CBSS
Route/site of implantation	Cephalopedal sinus	Cephalopedal sinus	Cephalopedal sinus
Postoperative snail care	Aerated water containing nitrofurantoin for 48 h; then in aerated water at 28°C	Tap water containing nitrofurantoin for 24 h; then in water at 26°C with 12 h light and dark cycles	Aerated water in beakers and petri dishes
Outcome measures	84.6 and 33.3% infected on 34th day	80% survival at 48 h; 75% infected	77% infected
Comments	Higher infection rate for the sympatric combination and lower for the allopatric		Other strains and species of snails transplanted
Reference	Jourdane et al. 1981	Jourdane et al. 1985	Chernin 1966

CBSS Chernin's balanced salt solution
[a]*Australorbis glabratus* (=*Biomphalaria glabrata*)
[b]Nembutal = sodium pentobarbital

(sodium pentobarbital) for 5–8 h (Jourdane and Théron 1980) or 0.08% nembutal for 7 h (Jourdane et al. 1985). Exposure to urethane for 2 h affects acid phosphatase in hemocytes and numbers of circulating hemocytes but has little or no effect on phagocytosis of mouse erythrocytes or snail susceptibility to *S. mansoni* infection (Granath and Yoshino 1985).

While most investigators have implanted donor snail fragments into the exposed cephalopedal sinus of anesthetized recipient snails, sporocysts have also been implanted into the cephalopedal sinuses of nonanesthetized snails via a 1.5-mm hole in the shell (Nojima et al. 1980). A later study found success of transplantation (determined by resultant patent infections) to be greater in anesthetized snails with exposed cephalopedal sinuses than in nonanesthetized snails implanted through a hole in the shell (Cohen and Eveland 1984). Holes in snail shells were sealed with "Dental Cyanon" (α-cyanoacrylate) (Nojima et al. 1980) or with plasticene (Cohen and Eveland 1984). In addition, implantation of sporocysts developed in vitro in the

presence of *B. glabrata* embryonic (Bge) cells or in Bge cell-conditioned medium resulted in patent infections (Ivanchenko et al. 1999; Kapp et al. 2003).

2.4 In Vitro Culture of Snail Tissues and Cells

The intramolluscan development of trematodes with multiple larval generations is far too complex to permit the analysis of the host-parasite interaction in vivo. In vitro cultivation is essential for understanding nutritional requirements and metabolic pathways of both the snails and trematodes and the factors that affect parasite development and snail susceptibility. Development of schistosomes in *B. glabrata* has been studied most intensively and our present knowledge is the result of incremental gains from studies of each of the successive larval generations from miracidium to cercaria (Basch and DiConza 1973; Ivanchenko et al. 1999; Coustau and Yoshino 2000).

Prior to 1949, maintenance of molluscan explants (organ culture) succeeded in maintaining healthy explants for only a few days (Bayne 1976). Early attempts to culture *B. glabrata* tissues used tentacles (Benex 1961, 1967), the usual sites of miracidial penetration, and hearts (Chernin 1963). Tentacle ciliary and muscular activity continued for 3 weeks of culture in a complex medium (Benex 1961). *S. mansoni* miracidia penetrated the cultured tentacles and transformed into mother sporocysts that differentiated into daughter sporocysts (Benex 1967). The snail hearts were cut into fragments, trypsinized, and cultured in a balanced salt solution (Chernin's balanced salt solution based on hemolymph analysis) containing bovine amniotic fluid, lactalbumin hydrolysate, yeast extract, beef embryo extract, horse serum, penicillin, and streptomycin. The hearts continued to beat up to 47 days in this solution and released amebocytes and "epithelial" cells, which did not divide (Chernin 1963). Also, digestive gland and ovotestis explants derived from *B. glabrata* infected with *S. mansoni* 40 days previously yielded infective cercariae for 1–2 weeks. When snail tissues were explanted 19 days after infection, cercariae emerged after a lag of 8–13 days demonstrating cercarial development in vitro (Chernin 1964). Thus, snail organ culture has permitted development in vitro of vboth early (Benex 1967) and late (Chernin 1964) schistosome larval stages (Bayne 1976).

The complex Medium 199 supplemented with salts, *Helix pomatia* extract (prepared by homogenizing the snail tissues in distilled water), fetal calf serum, streptomycin, and other antibiotics sustained explanted *B. glabrata* gonads at pH 7.0 and muscle tissue at pH 8.5 for 60 days and the cells that dissociated from the sheared tissue were subcultured (Burch and Cuadros 1965). However, another laboratory could not replicate these results using the same medium (Basch and DiConza 1973).

A study to establish primary cultures of *B. glabrata* cells used snail embryos at the trochophore stage with rudimentary digestive tracts and circulatory systems whose hearts had just begun to beat. Embryos of an albino strain of *B. glabrata* (Newton 1955) were freed from their capsules into a saline solution of six inorganic salts, and glucose, and then teased into fragments. The salt solution was replaced

with one of 120 culture media tested, four of which were found to be superior. One of these media was based on an analysis of adult *B. glabrata* hemolymph. It consisted of salts, amino acids, coenzymes, vitamins, nucleic acid precursors, glucose, and antibiotics, including streptomycin, inactivated fetal calf serum, and whole egg ultrafiltrate. The medium had an osmolality of 155 mOsm/Kg H_2O which was higher than that of the *B. glabrata* hemolymph (112 [range: 108–115] mOsm/Kg H_2O]. The cultures were healthy during 6 weeks of observation, had numerous mitotic figures, and cells differentiated into five cell types: fibroblast-like; epithelial-like; contractile muscle cells; macrophage-like; and hemocytes (Basch and DiConza 1973). Another study reported values of 102–119 mOsm/Kg H_2O for the hemolymph of normal adult *B. glabrata* (Lee and Cheng 1972).

Primary cultures of *B. glabrata* (albino strain) trochophore embryos were more extensively examined in another study in which individual eggs were removed from masses, and incubated at 23°C in autoclaved aquarium water containing antibiotics, including streptomycin, until embryos reached the trochophore stage. They were then teased out of capsules into buffered saline with antibiotics (no streptomycin), trypsinized and transferred to a medium based on Schneider's *Drosophila* medium supplemented with fetal calf serum and several antibiotics, including streptomycin. The cultures were incubated at 25°C, 27°C, or 30°C. The cultures grew dense fibroblast-like cells, small irregular cells, and epithelioid patches. Several subcultures were made following trypsinization in two slightly different media consisting of buffered salts, amino acids, sugars, vitamins, nucleic acid precursors and fetal calf serum, peptone, and lactalbumin hydrolysate. At or about 3 weeks small colonies of cells were observed and subcultured. These cells grew well and were designated Bge (*B. glabrata* embryo) cells. By the seventh passage, Bge cells were cultured in another medium which lacked vitamins, nucleic acid precursors, and peptone but was supplemented with yeast hydrolysate. This medium was also based on diluted Schneider's medium, and was used for all subsequent cultures (Hansen 1976).

Bge cells are clear and approximately 20 µm in diameter when rounded, and have relatively large nuclei measuring about 9 µm. When attached, they are diploid (2n = 36), fibroblast-like or irregular in shape, with two to five protoplasmic extensions; the population doubles in 18–20 h at 27°C. Other subcultures with slightly different characteristics were also described (Hansen 1976).

The previously mentioned penetration of *S. mansoni* miracidia, their transformation into mother sporocysts, and differentiation of daughter sporocysts in *B. glabrata* tentacles maintained in vitro (Benex 1967) has been replicated for *S. japonicum* in cocultures of Bge cells (Coustau et al. 1997). *S. mansoni* daughter sporocysts have also been developed from mother sporocysts in Bge cocultures (Yoshino and Laursen 1995).

These and many other attempts employing modifications in procedures, medium composition, and the gaseous atmosphere have resulted in complete development and continuous propagation of snail-associated developmental stages of *S. mansoni*, from miracidium to cercariae, in Bge cell cocultures and in Bge cell – conditioned medium (Ivanchenko et al. 1999). Specifically, mercaptoethanol was used which

Table 2.3 Survival and development of various trematodes in vitro with and without *Biomphalaria glabrata* embryonic (Bge) cells[a]

Trematode	Natural snail host	Maximum survival (days) in medium only	Maximum survival (days) in medium + Bge cells	Development of daughter sporocysts/ rediae	References
Schistosoma mansoni	*Biomphalaria*	18	Continuous	+	Yoshino and Laursen 1995; Ivanchenko et al. 1999
S. japonicum	*Oncomelania*	28	210	+	Coustau et al. 1997
S. mattheei	*Bulinus*	7	25	–	Coustau and Yoshino 2000
S. intercalatum	*Bulinus*	21	63	–	Coustau and Yoshino 2000
Echinostoma caproni	*Biomphalaria*	14	119	–	Ataev et al. 1998
Fascioloides magna	*Lymnaea*	12	>90	+	Laursen and Yoshino 1999

[a] Adapted from Coustau and Yoshino 2000

possibly reduced oxidative stress or chelated metals ions, and nitrogen was used to modify the O_2 and CO_2 concentrations. The in vitro-derived cercariae were infective to hamsters and the sporocysts were infective to *B. glabrata* when implanted through a hole in the shell (Ivanchenko et al. 1999). Larval trematodes that develop in vitro in the presence of Bge cells are listed in Table 2.3.

It is noteworthy that Bge cells also support the in vitro development of snail-associated stages of trematodes that normally use a snail other than *B. glabrata* (Coustau et al. 1997; Laursen and Yoshino 1999).

In addition, primary cultures of *B. glabrata* ovotestis (Iwanaga 2002; Barbosa et al. 2006) and amebocyte-producing organ (APO) (Barbosa et al. 2006) have been carried out. The ovotestis cultures grew two cell types (Iwanaga 2002) and those of the APO grew three cell types (Barbosa et al. 2006).

2.5 Concluding Remarks

Because of its essential role as the intermediate host of *S. mansoni*, *B. glabrata* has been maintained and studied for almost a century in numerous laboratories throughout the world. Even though a great deal has been learned about its biology from both field and laboratory studies, laboratory rearing and maintenance of this species remain quite challenging and extremely labor intensive. However, numerous studies done in our laboratory and others have succeeded in defining the best possible conditions for breeding and maintaining the snails

under artificial conditions. Of the many factors that determine success, diet, temperature, proper aeration, and water quality factors such as proper pH and salt content appear to be the most important. Also important in maintaining healthy snails is the early recognition and elimination of unintended organisms from the aquaria.

The more recent success in schistosome cloning and their maintenance by microsurgical transplantation of sporocysts has been highlighted in this chapter. Development of successive snail-associated schistosome stages, from miracidial transformation into sporocysts to the generation of infective cercariae in Bge cell culture is discussed as a major achievement. Schistosome clones maintained in snails by microsurgical transplantation of sporocysts should also make a major contribution to our efforts at dissecting and understanding the human immune response in schistosomiasis, which may ultimately help in designing a vaccine that will reduce the human misery caused by schistosomiasis in endemic areas of the globe.

Efforts at deciphering and elucidating the *B. glabrata* genome are progressing rapidly (see Chap. 9). This knowledge will further our understanding of the biology of the snail and its relationship with its trematode parasites. Another potential benefit of this understanding could be to control the natural populations of *B. glabrata*, thus reducing or eliminating transmission of schistosomiasis.

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