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In vitro culture of marine trematodes from their snail first intermediate host

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ABSTRACT

The ability to culture parasites outside their host (i.e. in vitro) is essential for several aspects of parasitological research. Here, a culture medium for marine trematode parthenitae was optimized using *Philophthalmus* sp. rediae from the intermediate snail host, *Zeacumantus subcarinatus*. The medium was optimized by sequentially testing the suitability of different levels of osmolality, different commercially available media, and different concentrations of supplemented chicken serum, while controlling for genetic variation among cultures. *Philophthalmus* sp. rediae survived up to 56 days in cultures of the best tested medium, remaining active and continuously shedding cercariae. The broader suitability of the culture medium was tested using five other trematode species from different families (using either the same or other marine snails as first intermediate hosts): *Galactosomum* sp., *Acanthoparyphium* sp., *Maritrema novaezealandensis*, *Curtuteria australis*, and an undescribed species of the family Opecoelidae. Survivorship of rediae and sporocysts from these species ranged from eight days to 42 days. The culture procedures developed here can therefore be used in the future as a system under which to culture marine trematode parthenitae for experimental studies.

1. Introduction

In ecological parasitology research, the ability to culture trematode parthenitae (sporocysts or rediae, depending on the species) in vitro is essential to answer questions about the complex life cycle of trematodes, the life-history strategies of the parthenitae in the intermediate host, and possible interspecific and intraspecific interactions in cases of multiple infections. The great advantage of in vitro methods is that, at least in principle, trematode colonies in culture can be exposed to a range of altered experimental conditions that cannot be manipulated for colonies within snails.

Previous attempts at in vitro culturing of trematode larvae from their first intermediate host have met with varying levels of success and focused mainly on parasite parthenitae infecting freshwater gastropods: miracidia, sporocysts, and cercariae of *Schistosoma mansoni* and *Schistosoma japonicum* from *Biomphalaria glabrata* (Coustau et al., 1997; Ivanchenko et al., 1999); rediae and cercariae of *Fasciola hepatica* from *Lymnaea truncatula* (Augot et al., 1997; Pullin, 1973); rediae and cercariae of *Echinostoma caproni* from *B. glabrata* (Loker et al., 1999); miracidia and sporocysts of *Trichobilharzia ocellata* from *Lymnaea stagnalis* (Mellink and van den Bovenkamp, 1985), among others. Only one study focused on trematode parthenitae using a marine snail as first intermediate host: rediae of *Himasthla elongata* from *Littorina littorea* (Gorbushin and Shaposhnikova, 2002). Two other studies developed culture protocols for larval stages in second intermediate marine hosts (metacercariae through to egg production): *Maritrema novazealandensis* (Fredensborg and Poulin, 2005) and *Microphallus turgidus* (Pung et al., 2009). There is therefore comparatively little information available on marine trematodes; because of fundamental physiological differences, culture methods developed for freshwater species cannot simply be used for marine cultures.

Each of these previous studies focused on only one trematode species. While the culture method and media are roughly similar across studies, a general and standardized system for culturing trematode parthenitae is lacking. The goal of the present study was to optimize a culture method using rediae of the marine trematode *Philophthalmus* sp. from its intermediate snail host, the mud snail *Zeacumantus subcarinatus* (Batillariidae). The broader viability of the method was then tested by using it to culture three other trematode species from different families which all use the same intermediate host (rediae of *Galactosomum* sp., rediae of *Acanthoparyphium* sp., and sporocysts of *M. novaezealandensis*) as well as two other trematode species from two very different gastropod hosts: rediae of *Curtuteria australis* from the whelk *Cominella glandiformis* (Buccinidae) and rediae of an undescribed trematode species in the family Opecoelidae from the topshell *Diloma subrostrata* (Trochidae). In total, six trematode species from five families were tested with the same culture protocol, providing a robust test of a general system under which marine trematode parthenitae can be cultured in the future.

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2. Materials and methods

2.1. Snail collection, screening, and care

Z. subcarinatus mud snails, *D. subrostrata* topshells, and *C. glandiformis* whelks were collected from Lower Portobello Bay, Otago Harbor, South Island, New Zealand (45°52′ S, 170°42′ E). Mud snails were collected in June–July 2010 (snails used for a particular experiment were taken from the same collection date), whelks were collected in February 2011, and topshells were collected in April 2011. *Z. subcarinatus* snails were screened for infection by *Philophthalmus* sp. (Philophthalmidae), *Galactosomum* sp. (Heterophyidae), *Acanthoparyphium* sp. (Echinostomatiadae), or *M. novaezealandensis* (Microphallidae) by incubating individuals overnight at 26 °C in wells of a 12-well culture plate filled with natural sea water (~950 mOsm). Individuals that shed cercariae were kept in plastic containers (170 cm × 170 cm) filled with natural, aerated sea water, 2 mm sand, and sea lettuce, *Ulva lactuca*. Containers were cleaned, and water changed once weekly. *C. glandiformis* whelks and *D. subrostrata* topshells were kept in larger containers (350 cm × 170 cm) under the same conditions. Screening the whelks and topshells for infection by *C. australis* (Echinostomatidae) and an undescribed trematode in the family Opecoelidae, respectively, by incubation as described above proved unreliable. Therefore, infected whelks and topshells could only be identified by dissecting rediae out of the snail tissue.

2.2. Optimizing media

2.2.1. Philophthalmus sp. rediae isolation, media preparation, and culture procedure

In order to avoid contamination, infected mud snails were wiped with 95% ethanol and soaked in artificially prepared sea water, ASW (1.56 g Instant Ocean/50 ml autoclaved water) + 100 μg/ml gentamicin (Sigma G1914) before dissection. Snail tissue was removed from the shell, placed in a sterile Petri dish, and soaked in ASW + 100 μg/ml gentamicin. Uninfected snail tissue was discarded from the dish as this tissue contains bacteria. Rediae were removed from the snail tissue with forceps and isolated by gently swirling the water. They were transferred through three subsequent washes of ASW + 100 μg/ml gentamicin by pipetting.

Generally, the media described below were prepared by adding commercially available media powder to a mix of ASW and autoclaved water in 50 ml conical tubes, supplemented with 2% penicillin–streptomycin-neomycin antibiotic solution (Sigma P4083), and kept at 4 °C. At the time of culturing, the medium was supplemented with chicken serum (Invitrogen) and an additional 2% penicillin–streptomycin–neomycin solution (4% total concentration in culture). It was then filter sterilized through a 32 mm 0.2 μm syringe filter. In a laminar flow hood, ~10 rediae/well were cultured in 12-well culture plates containing 1 ml medium. Medium was changed every three to four days also in the laminar flow hood. Culture plates were kept in the dark at room temperature.

2.2.2. Parameters tested

Success of redial cultures of the same species dissected out of different snails is highly variable (Gorbushin and Shaposhnikova, 2002). For this reason, rediae were dissected out of an infected snail and allocated equally across treatments of each experiment, i.e. each treatment within a given experiment included two culture wells of rediae from each snail used. Three or four snails were used per experiment. Due to limitations in number of rediae in each infected snail, parameters had to be optimized separately. This basic set of procedures applied to all tests described below.

Preliminary culture experiments started with the recipe reported from the only previous study aimed at culturing marine rediae: 800 mg Leibovitz-15 (L-15) powder, 20 ml distilled water, 80 ml sterile sea water, 1% penicillin–streptomycin–neomycin solution; ~780 mOsm; pH 7.8 (Gorbushin and Shaposhnikova, 2002). Previous studies in which trematode parthenitae were cultured all used variations of this general medium with respect to osmolality, type and concentration of commercially available media, supplementation of BME vitamins and amino acids, and supplementation of a food source. In the present study, the following parameters were optimized individually and sequentially: 1. osmolality (four levels), 2. concentration of chicken serum as a food source (three levels), 3. three commercially available media at half strength, 4. the same three commercially available media at full strength, and 5. full and half strength L-15 media supplemented with BME vitamins and amino acids (see Table 1). Also tested separately was the commercially unavailable Medium F, which was chosen because of its success in allowing cultures of *S. mansoni* sporocysts to proliferate indefinitely (Ivanchenko et al., 1999; Stibbs et al., 1979; Voge and Seidel, 1972). The recipe for Medium F is given in Supplementary material.

Osmolalities used in previously described media ranged from 135 mOsm for freshwater trematodes (Mellink and van den Bovenkamp, 1985) to 780 mOsm for marine trematodes (Gorbushin and Shaposhnikova, 2002). In the present study, four osmolalities were tested, measurements taken with an osmometer (VAPRO volume pressure osmometer model 5520). The osmolality of medium prepared with 400 mg L-15 powder in 50 ml autoclaved water was 180 mOsm and was the lowest osmolality tested. This osmolality is similar to that used in cultures of freshwater trematodes and served to test if trematodes of marine hosts require a culture medium which is different from freshwater trematodes. Higher osmolalities were tested by altering the ratio of ASW to autoclaved water: 774.5 mOsm (3:2 ASW: autoclaved water), 975.5 mOsm (4:1 ASW: autoclaved water), and 1175.5 mOsm (all ASW). Osmolalities higher than the previously reported media were used because water from the Otago Harbor is highly saline (personal observation). It should be noted that osmolality was the first parameter to be optimized and the final concentration of penicillin–streptomycin–neomycin solution was only 2%. Cultures became quickly contaminated, therefore in subsequent experiments the final concentration was increased to 4%.

Previous studies have supplemented cultures with a variety of trematode food sources including human, horse, rabbit, duck, and

Table 1
Summary of the design and number of infected snails used in each experiment.

Parameter tested	Treatments	No. snails	Parameters held constant
Osmolality (mOsm)	180,[a] 774.5, 975.5, 1175.5	4	L-15, 20% serum
Serum Concentration	0%v/v, 20%v/v, 50%v/v	3	L-15, ~962 mOsm
Media half strength	L-15, NCTC-135, medium 199	3	~930 mOsm, 20% serum
Media full strength	L-15,[a] NCTC-135,[a] medium 199[a]	3	~900 mOsm, 20% serum
L-15 ½ and full strength	½ L-15, full L-15, ½ L-15 + BME vitamins and amino acids, full L-15 + BME vitamins and amino acids.	3	~900 mOsm, 20% serum

[a] Indicates treatments where cultures were not successful enough to produce cysts and were thus excluded from the statistical analysis.

fetal calf serum (Mellink and van den Bovenkamp, 1985); other species of trematode parthenitae (Basch and DiConza, 1975); or snail hemolympth and tissues (Mellink and van den Bovenkamp, 1985). The basic recipe on which we based our preliminary experiments did not include a food supplement; surviving rediae simply fed on dead neighboring rediae (Gorbushin and Shaposhnikova, 2002). In a preliminary experiment, we tested cultures either without food supplements, with 20%v/v chicken serum, or with snail tissue. Only the rediae cultured with chicken serum survived beyond 7 days. In subsequent experiments, different concentrations of chicken serum were compared (0%v/v, 20%v/v, and 50%v/v).

Various commercially available media bases have been used in previous studies, most commonly Leibovitz-15 (powder with L-glutamine, Sigma L4386), medium 199 (powder with L-glutamine, Sigma M5017), and NCTC-135 (powder with L-glutamine, Sigma N3262) at either half or full strength (Fredensborg and Poulin, 2005; Park et al., 2006; Pullin, 1973). In the present study, three tests of media bases were conducted to allow several comparisons: 1. commercially available L-15, medium 199, and NCTC-135 at half strength (it should be noted that L-15 was used at slightly above half strength in accordance with a previous study (Gorbushin and Shaposhnikova, 2002) while the other media were used at half the manufacturer's recommended strength), 2. these same three media at full strength, 3. full strength L-15, half strength L-15, full strength L-15 supplemented with BME vitamins (Sigma B6891) and BME amino acids (Sigma B6766), and half strength L-15 supplemented with BME vitamins and amino acids.

Medium F was prepared according to the recipe available in Supplementary material (obtained from C. Bayne, Oregon State University), supplemented with 2% penicillin–streptomycin–neomycin, and kept at 4°. It was used in culture in a 1:1 ratio with half strength L-15 at 954 mOsm and supplemented with an additional 2% penicillin–streptomycin–neomycin solution (4% total concentration in culture) and 20% chicken serum.

2.3. Culturing other species

Sporocysts and rediae of other trematode species infecting *Z. subcarinatus* (*M. novaezealandensis, Galactosomum* sp., and *Acanthroparyphium* sp.) were dissected, washed, and prepared for culture in the same way as *Philophthalmus* sp. rediae. Identification of infection in the whelk by *C. australis* and the topshell by an undescribed opecoelid species was achieved during dissection. All whelks and topshells were wiped with 95% ethanol and soaked in ASW + 100 μg/ml gentamicin. Once rediae were isolated, they were washed and prepared for culture as above. Rediae or sporocysts were cultured in what proved to be the best medium for *Philophthalmus* sp., i.e. L-15 media (~900 mOsm) supplemented with 20%v/v chicken serum and 4% penicillin–streptomycin-neomycin antibiotic solution (final concentration).

2.4. Data analysis

For all experiments and species, survivorship of rediae was scored based on whether or not a redia moved within 30 s of observation. In the case of *M. novaezealandensis*, sporocyst survival was confirmed when live cercariae continued to be released. In the case of *Philophthalmus* sp. rediae, survivorship was generally equal across all treatments, i.e. all rediae in the cultures survived until the cultures were terminated due to contamination. Therefore, the numbers of cysts per well were counted as a measure of culture success since *Philophthalmus* sp. cercariae, after leaving a redia, encyst on the well bottoms as metacercariae that accumulate over time. Each day, the cumulative number of cysts was counted and standardized by dividing it by the number of rediae in the culture on day zero. For each experiment involving *Philophthalmus* sp., significant differences between treatments were tested using a repeated measures ANOVA in the statistics program SPSS, where time (day) was the within-subjects variable and snail identity and treatment were between-subjects variables. Sphericity was tested using Mauchly's test. When sphericity could not be assumed, the Greenhouse–Geisser correction was used. These analyses included only data from the first several days of each experiment, since after that the cysts which appeared in the first days started to deteriorate.

3. Results

Overall, *Philophthalmus* sp. rediae survived in culture under almost all conditions (except for one or two rediae per culture in some cases) until the culture became contaminated with bacteria or fungus. Rediae shed cercariae which in turn encysted as metacercariae. Growth and development of rediae was observed: immature or underdeveloped rediae (marked by not having any visible cercariae formed within the body at time of dissection from the snail) developed cercariae in culture which were in turn shed from the redia. Occasionally daughter rediae were released, however, not frequently. Cultures reliably survived 30–40 days and the longest surviving cultures lasted 56 days.

3.1. Osmolality

The test aimed at optimizing the level of osmolality was the first experiment and only used 2% penicillin–streptomycin–neomycin solution. Cultures became contaminated after only nine days, and therefore the final concentration of penicillin–streptomycin–neomycin solution was increased to 4% (2% when mixing culture medium and an additional 2% when preparing the culture) in subsequent experiments.

Rediae in cultures with an osmolality similar to a freshwater system (180.0 mOsm) died within a few hours of culturing, indicating marine trematodes require a different culture medium than freshwater trematodes. Furthermore, cysts were not counted throughout the experiment. Survivorship of rediae in cultures in the range 774.5–1175.5 mOsm was equal over the nine days during which the cultures remained free of contamination. Numbers of cysts/redia were not significantly different across treatments (Fig. 1). However, numbers of cysts/redia were significantly different between cultures of rediae dissected from different snails (Table 2), as was expected.

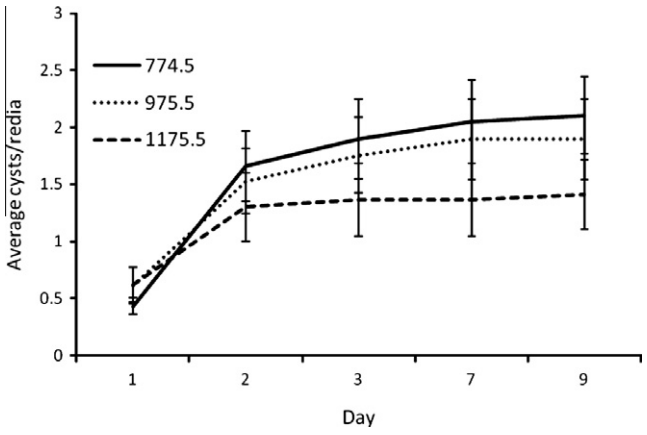

Fig. 1. Mean (±SE) cumulative number metacercarial cysts per redia of *Philophthalmus* sp. over time in cultures held at different osmolalities (774.5, 975.5, or 1175.5 mOsm).

Table 2
Effect of osmolality (774.5, 975.5, or 1175.5 mOsm) and snail identity on metacercarial cyst production of *Philophthalmus* sp. rediae in culture. Results of the repeated measures ANOVA (with multivariate and within subjects results).

Factor	df	MS	*F*	*P*
Between subjects				
Snail	3	11.930	7.030	.006
Treatment	2	1.906	1.123	.357
Snail * treatment	6	1.519	0.895	.528
Error	12	1.697		
Within subjects				
Time	1.194	21.743	78.260	<0.0001
Time * snail	3.581	1.484	5.342	0.009
Time * treatment	2.387	0.913	3.286	0.060
Error	14.325	0.278		

Table 3
Effect of varying chicken serum concentrations (0%, 20%, or 50%) and snail identity on metacercarial cyst production of *Philophthalmus* sp. rediae in culture. Results of the repeated measures ANOVA (with multivariate and within subjects results).

Factor	df	MS	*F*	*P*
Between subjects				
Snail	2	2.369	21.826	<0.0001
Treatment	2	8.907	82.066	<0.0001
Snail * treatment	4	2.651	24.426	<0.0001
Error	9	0.109		
Within subjects				
Time	1.598	0.408	16.546	<0.0001
Time * snail	3.197	0.108	4.385	0.020
Time * treatment	3.197	0.408	16.546	<0.0001
Error	14.386	0.025		

3.2. Chicken serum

Rediae in cultures supplemented with 0% chicken serum did not produce any cysts and slowly died throughout the experiment. Rediae in cultures supplemented with 50% chicken serum survived throughout the experiment but only one cyst was produced across the six cultures (~10 rediae per culture). Rediae in cultures with 20% chicken serum produced a significantly higher number of cysts per redia than rediae in the other two treatments (Fig. 2). Differences in numbers of cysts/redia between cultures from different snails were also significant, as was the interaction between treatment and snail (Table 3). The interaction effect was seen because only one cyst from one culture was observed throughout the duration of the experiment.

3.3. Media base

When culture success was compared between three commercially available media at half strength, rediae in L-15 remained healthy and highly mobile throughout the experiment (27 days). Those in NCTC-135 and medium 199 remained alive but were less mobile and deteriorated slightly. However, survivorship of rediae was roughly equal across all treatments. When comparing average cysts/redia among media, cultures in L-15 medium produced the most cysts (Fig. 3). Numbers of cysts/redia were significantly different between cultures of rediae dissected from different snails (Table 4).

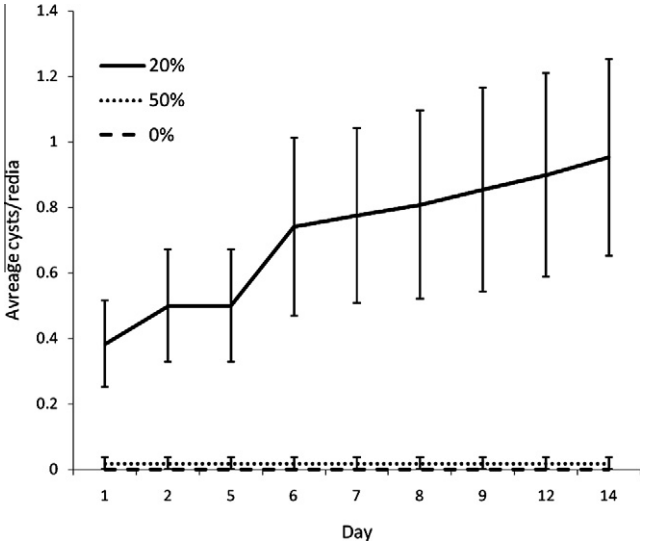

Fig. 2. Mean (±SE) cumulative number metacercarial cysts per redia of *Philophthalmus* sp. over time in cultures supplemented with different levels of chicken serum (0, 20 or 50%).

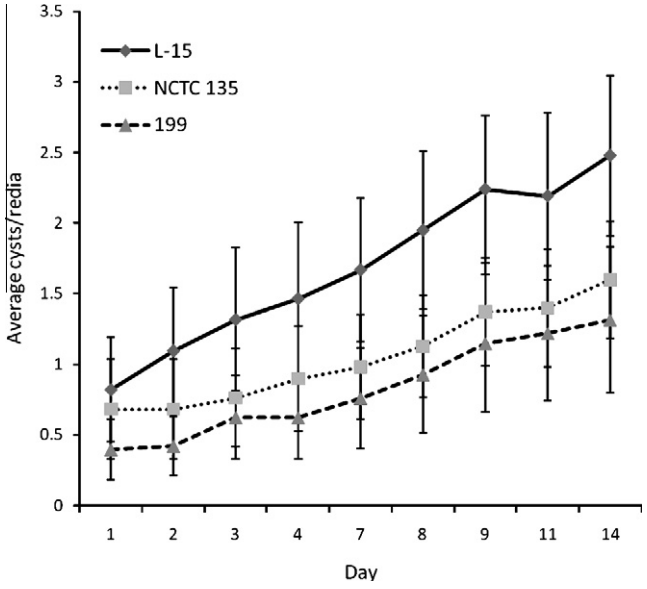

Fig. 3. Mean (±SE) cumulative number metacercarial cysts per redia of *Philophthalmus* sp. over time in cultures held in different commercial media (L-15, NCTC-135, medium 199).

Table 4
Effect of media (L-15, NCTC-135, medium 199) and snail identity on metacercarial cyst production of *Philophthalmus* sp. rediae in culture. Results of the repeated measures ANOVA (with multivariate and within subjects results).

Factor	df	MS	*F*	*P*
Between subjects				
Snail	2	63.468	114.510	<0.0001
Treatment	2	11.029	19.898	<0.0001
Snail * treatment	4	1.750	3.157	0.070
Error	9	0.554		
Within subjects				
Time	2.516	9.919	100.987	<0.0001
Time * snail	5.033	0.798	8.128	<0.0001
Time * treatment	5.033	0.390	3.976	0.010
Error	22.647	0.098		

3.4. Concentration of media

Rediae in preliminary tests of full strength media (L-15, NCTC-135, and medium 199) survived well only in L-15 full strength medium. Cultures in both NCTC-135 and medium 199 became very easily contaminated and died, thus cysts were not counted for statistical analysis. In the final test, comparing full and half strength L-15 with or without BME amino acids and vitamins, rediae survived

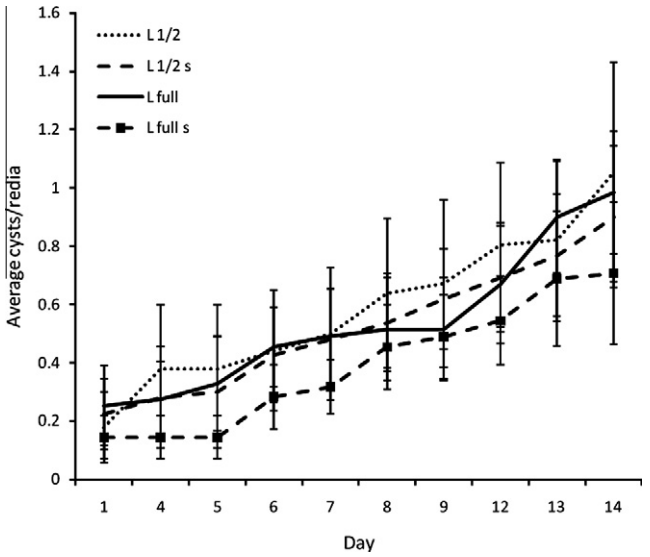

Fig. 4. Mean (±SE) cumulative number metacercarial cysts per redia of *Philophthalmus* sp. over time in cultures held in different media: L ½ indicates L-15 at half strength; L ½ s indicates L-15 at half strength supplemented with 1X BME vitamins and 1X BME amino acids; L full indicates L-15 at full strength; L full s indicates L-15 at full strength supplemented with BME vitamins and amino acids.

equally well and numbers of cysts/redia were not significantly different across all treatments (Fig. 4). Again, numbers of cysts/redia were significantly different between cultures of rediae originating from different snails (Table 5).

Also tested was the commercially unavailable Medium F because of its success in allowing cultures of *S. mansoni* sporocysts to proliferate indefinitely (Voge and Seidel, 1972). Rediae in Medium F cultures shed cercariae which encysted as metacercariae, however, daughter rediae were not reliably produced. Nevertheless, rediae in these cultures had the longest survivorship of all cultures throughout the various experiments (up to 56 days).

3.5. Within subjects effects

Time and the interaction between time and snail identity were significant in all experiments (Tables 2–5). The interaction between time and treatment is significant in all experiments except the tests of osmolality and concentration of media (Tables 2 and 5). The significant effect of time is the mere product of the accumulation of cysts in each well over the number of days the culture remained healthy; numbers could only increase over time. Furthermore, the significance of the interactions between time and both snail identity and treatment was expected because in the first few days of the experiment, all wells uniformly had only a few cysts and differences between wells in terms of numbers of cysts only arose as the experiment unfolded. These interactions were weaker in the experiment comparing concentrations of media because the differences between treatments were so slight.

Table 5
Effect of the concentration and supplementation of media (L-15 at half strength, L-15 at half strength supplemented with 1X BME vitamins and 1X BME amino acids, L-15 at full strength, and L-15 at full strength supplemented with 1X BME vitamins and 1X BME amino acids) and snail identity on metacercarial cyst production of *Philophthalmus* sp. rediae in culture. Results of the repeated measures ANOVA (with multivariate and within subjects results).

Factor	df	MS	*F*	*P*
Between subjects				
Snail	2	9.271	11.364	0.002
Treatment	3	0.418	0.512	0.682
Snail * treatment	6	1.208	1.480	0.265
Error	12	0.816		
Within subjects				
Time	1.764	6.724	44.000	<0.0001
Time * snail	3.528	1.166	7.630	0.001
Time * treatment	5.293	0.072	0.472	0.802
Error	21.1712	0.153		

3.6. Culturing other species

Sporocysts of *M. novaezealandensis* and rediae of *Acanthoparyphium* survived for 42 and 29 days, respectively, in L-15 medium (~900 mOsm) supplemented with 20% serum and 4% penicillin–streptomycin-neomycin antibiotic solution (final concentration). Rediae of *Galactosomum* sp., *C. australis*, and the undescribed species of Opecoelidae survived for 8, 14, and 16 days, respectively, in the same culture medium. In an unsuccessful attempt to keep the rediae alive longer, the medium was supplemented with 50%v/v chicken serum and BME vitamins and amino acids in separate trials, with no detectable impact on survival.

4. Discussion

In the present study, a culture medium for the in vitro study of marine trematode parthenitae was developed by sequentially testing the suitability of different components of the medium. It was shown suitable for six trematode species belonging to five different families, and originating from snails representing three different families, indicating the potential use of the method for other marine trematode species.

Under sterile conditions, it is possible to easily and reliably culture *Philophthalmus* sp., *Acanthoparyphium* sp., and *M. novaezealandensis* parthenitae for up to 30 days using the following basic steps. First, 400 mg L-15 powder and 1.56 g instant ocean must be combined in 50 ml autoclaved water to achieve an osmolality of ~900 mOsm. Second, the medium must be supplemented with 2% penicillin–streptomycin–neomycin, to limit the risk of fungal and bacterial infection. Third, the medium must be kept at 4 °C until used in culture. Fourth, when culturing, fresh 2% pen-strep-neo (4% total concentration in culture wells) and 20% chicken serum must be added anew. Fifth, the medium must be filter sterilized through a 32 mm 0.2 μm syringe filter. Finally, in a laminar flow hood, the medium in wells must be replaced with freshly prepared and filtered medium every three to four days.

Rediae of *Philophthalmus* sp. remained healthy and mobile and shed cercariae which encysted as metacercariae for weeks in culture. In addition, immature or underdeveloped rediae developed to also shed cercariae. However, the number of rediae in an individual culture would only occasionally increase by one or two, but not consistently.

The longest surviving culture of *Philophthalmus* sp. rediae was 56 days in medium F/L-15, 954 mOsm, supplemented with 20% chicken serum and a final concentration of 4% pen-strep-neo, where all rediae in the culture survived. This longevity compares favorably to that achieved by other trematode species in other studies that used a different medium (Augot et al., 1997; Ivanchenko et al., 1999; Loker et al., 1999). The procedures outlined here should be used for any proposed experiment that requires a very long culture period. However, preparing medium F is complicated and it is possible to use a simpler medium for shorter experiments.

As avoiding contamination is the greatest challenge in long term culturing experiments, careful sterile techniques will increase the longevity of cultures. When dissecting rediae from snails, it is essential to clean the snails with 95% ethanol and soak them in ASW + 100 μg/ml gentamicin. After cracking open the shells, uninfected snail tissue should be discarded immediately. Preliminary

tests indicated that it is crucial to wash rediae three times in ASW + 100 μg/ml gentamicin by transferring individual redia with ~7 μl water to a sterile Petri dish. Rediae should then be transferred to culture dishes containing 1 ml culture media in a laminar flow hood, with the media changed every three to four days also under the laminar flow hood. All the above must be done using only sterilized equipment.

Of the other species tested, *M. novaezealandensis* and *Acanthoparyphium* sp. survived longest in culture (42 and 29 days, respectively). Rediae of *Galactosomum* sp. and *C. australis*, and the undescribed Opecoelidae survived for only 8, 14, and 16 days, respectively. It was predicted that trematode species infecting *Z. subcarinatus* would survive longer in the culture medium optimized for *Philophthalmus* sp. than those infecting other snail hosts, since it had been developed to suit a species sharing the same within-host living conditions, their microhabitat being the snail gonad. However, that was not the case. Perhaps the species with lower survivorship require something not provided in the medium for long term survival, growth, and development. Nevertheless, all species tested survived in culture much longer than they would otherwise being kept in only ASW, which would be the saline concentration encountered within the snail as marine and littoral gastropods are isosmotic (Avens and Sleigh, 1965).

Of these species, *M. novaezealandensis* and the Opecoelidae shed normal looking cercariae. *Galactosomum* sp., *C. australis*, and *Acanthoparyphium* sp. shed underdeveloped cercariae (bodies and tails). Unlike *Philophthalmus* sp., the species subsequently tested all require a second intermediate host for cercariae to encyst. Therefore, numbers of cysts could not be counted in the cultures as a measure of realized fitness. Overall, though, because of its suitability for a range of phylogenetically unrelated trematode species, using a range of unrelated snail first intermediate hosts, the culture medium developed and tested in this study will provide a useful tool for experimental studies of trematode biology requiring the long-term in vitro investigation of live parthenitae.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.exppara.2011.07.009.

References

Augot, D., Rondelaud, D., Dreyfuss, G., Cabaret, J., 1997. *Fasciola hepatica*: in vitro production of daughter rediae and cercariae from first- and second-generation rediae. Parasitol. Res. 83, 383–385.

Avens, A.C., Sleigh, M.A., 1965. Osmotic balance in gastropod molluscs–I some marine and littoral gastropods. Comp. Biochem. Physiol. 16, 121–141.

Basch, P.F., DiConza, J.J., 1975. Predation by Echinostome rediae upon Schistosome sporocysts in vitro. J. Parasitol. 61, 1044–1047.

Coustau, C., Ataev, G., Jourdane, J., Yoshino, T.P., 1997. *Schistosoma japonicum*: in vitro cultivation of miracidium to daughter sporocyst using a *Biomphalaria glabrata* embryonic cell line. Exp. Parasitol. 87, 77–87.

Fredensborg, B.L., Poulin, R., 2005. In vitro cultivation of *Maritrema novaezealandensis* (Microphallidae): the effect of culture medium on excystation, survival and egg production. Parasitol. Res. 95, 310–313.

Gorbushin, A., Shaposhnikova, T., 2002. In vitro culture of the avian echinostome *Himasthla elongata*: from redia to marita. Exp. Parasitol. 101, 234–239.

Ivanchenko, M.G., Lerner, J.P., McCormick, R.S., Toumadje, A., Allen, B., Fischer, K., Hedstrom, O., Helmrich, A., Barnes, D.W., Bayne, C.J., 1999. Continuous in vitro propagation and differentiation of cultures of the intramolluscan stages of the human parasite *Schistosoma mansoni*. PNAS 96, 4965–4970.

Loker, E.S., Coustau, C., Ataev, G.L., Jourdane, J., 1999. In vitro culture of rediae of *Echinostoma caproni*. Parasite 6, 169–174.

Mellink, J.J., van den Bovenkamp, W., 1985. In vitro culture of intramolluscan stages of the avian schistosome *Trichobilharzia ocellata*. Z. Parasitenkd. 71, 337–351.

Park, Y.K., Hwang, M.K., Chung, P.R., 2006. Encystment and metacercariae development of *Echinostoma cinetorchis* cercariae in an in vitro culture system. J. Parasitol. 92, 1010–1013.

Pullin, R.S.V., 1973. Preliminary experiments with a defined culture medium for larval *Fasciola hepatica*. J. Helminthol. 47, 181–189.

Pung, O.J., Burger, A.R., Walker, M.F., Barfield, W.L., Lancaster, M.H., Jarrous, C.E., 2009. In vitro cultivation of *Microphallus turgidus* (trematoda: microphallidae) from metacercaria to ovigerous adult with continuation of the life cycle in the laboratory. J. Parasitol. 95, 913–919.

Stibbs, H.H., Owczarzak, A., Bayne, C.J., DeWan, P., 1979. Schistosome sporocyst-killing amoebae isolated from *Biomphalaria glabrata*. J. Invertebr. Pathol. 33, 159–170.

Voge, M., Seidel, J.S., 1972. Transformation in vitro of miracidia of *Schistosoma mansoni* and *S. Japonicum* into young sporocysts. J. Parasitol. 58, 699–704.