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*In Vitro Cultivation* of the Microphallid Trematode  
*Gynaecotyla Adunca*

Jenna West

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IN VITRO CULTIVATION OF THE MICROPHALLID TREMATODE

*GYNAECOTYLA ADUNCA*

by

JENNA WEST

(Under the Direction of Oscar Pung)

ABSTRACT

It is important to study the cultivation of parasites *in vitro* for many reasons, such as to aid in developing antihelminthic drugs and vaccines, to eliminate the need for vertebrate hosts in parasite culture, and to more easily study the genetics and the biology of parasites. Trematodes have complex life cycles with multiple hosts which makes them difficult to grow *in vitro*. However, microphallid trematodes are excellent candidates for parasite *in vitro* cultivation because they are short lived and progenetic. The goal of my study was to optimize *in vitro* culture conditions for the microphallid trematode, *Gynaecotyla adunca*. I determined the optimal concentration of trypsin to be 0.5% in order to excyst the most metacercariae of *G. adunca* that were obtained from the green glands of the second intermediate host, the fiddler crab *Uca pugnax*. Hunter (1952) reported that *G. adunca* only self-fertilize, however, offered no evidence to support this claim. I decided to test *G. adunca* adult worms to either confirm or refute whether this is true or not. I also tested different culture conditions on adult *G. adunca* worms. I chose 3 parameters to evaluate *in vitro* cultivation experiments. To determine these parameters which included worm longevity, number of worms that produced eggs *in utero*, and the number of eggs deposited, the media DMEM and RPMI-1640 were compared to Hank's Balanced Salt Solution. Different sera were also tested including horse, new-born calf,

and chicken, the best of which was tested at different concentrations. To test the viability of the eggs deposited by worms in culture, they were fed to the marsh snail *Ilyanassa obsoleta*. I also compared HBSS and DMEM plus 5% horse serum as the initial incubation conditions of the freshly excysted worms then observed them 24 hr later before adding them to culture to see if this affected egg production. *G. adunca* worms do self-fertilize. When worms were incubated alone, they showed signs of being fertilized. The percentage of worms with eggs *in utero* was greatest when worms were grown in DMEM. Worms lived longer and deposited more eggs when cultured in DMEM supplemented with 5% horse serum. Snails fed eggs from culture were not successfully infected. There was no significant difference on egg production between initially incubating the worms in HBSS and DMEM plus 5% horse serum within the 24 hr period between excystment and adding them to culture. Future studies will further refine *in vitro* culture conditions for *G. adunca* and investigate the best approach for snail infection.

INDEX WORDS: *In vitro* cultivation, Trematode, *Gynaecotyla adunca*, Parasites

*IN VITRO* CULTIVATION OF THE MICROPHALLID TREMATODE

*GYNAECOTYLA ADUNCA*

by

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# CHAPTER 1

## INTRODUCTION

### ***In vitro* cultivation of trematodes**

*In vitro* cultivation of trematodes, while a challenge, is important for both biological reasons and public health concerns (Colley, et. al, 2001). A good candidate to use for parasite *in vitro* cultivation are the microphallid trematodes because they are progenetic, i.e., they are close to sexual maturity at the metacercarial stage and live for a comparatively short amount of time which allows them to be more easily maintained in the laboratory (Irwin, 1997). Past studies showed that microphallids such as *Microphallus similis* and *Sphaeridiotrema globulus* will grow readily *in vitro* with the only consequence being that the adult parasites develop slower than when grown *in vivo* (Davies and Smyth, 1979; Berntzen and Macy, 1969). However, these studies focused on the growth of the parasite; they did not test infectivity of eggs produced in culture. Most of the time the eggs produced from *in vitro* experiments were abnormal and were most likely not fertilized (Davies and Smyth, 1979). Other *in vitro* studies have focused on parasites of humans, such as *Haplorchis taichui*, a trematode that affects populations in northern Thailand (Chaithong et.al., 2001) as well as the *Schistosoma* species (Ivanchenko, et. al., 1999). Obviously with these species, *in vitro* cultivation would be extremely beneficial especially with the exclusion of the human host. Pung, et. al (2009) cultured the microphallid *Microphallus turgidus* *in vitro* that deposited eggs that were infective to the first intermediate snail host. Very few studies have focused on the infectivity of the eggs produced by parasites grown *in vitro* (Irwin, 1997). It is important

to test the infectivity of eggs from cultured worms to assure that the cultivation technique actually works. In addition, no species of metazoan parasites such as helminths, which includes trematodes, has been successfully grown continuously through *in vitro* techniques (Ivanchenko, et. al., 1999).

Other *in vitro* studies focused on specific parts of the trematode life cycle. Most commonly studies focus on the excystment of the metacercariae. Dunn et. al. (1989) worked with excystment of the trematode *Gynaecotyla adunca*, which is the parasite I chose to work with. Fried and Johnson (2005) focused on the excystment of the metacercarial cysts of the digenean *Ribeiroia ondatrae* which parasitizes the leopard frog, *Rana pipens*. However, to grow and study parasites effectively *in vitro*, cultivation needs to span a substantial part of the life cycle of the organism (Clegg and Smith, 1987). In addition, several different species need to be examined. This is because different species, even ones within the same family, behave differently and require specific culture conditions (Smyth, 1990). For example, Smyth (1990) reported that the trematode *Posthodiplostomum minimum* metacercariae has the highest excystment rate in either a treatment of 1% pepsin solution with a pH of 2.0 (with HCl) or a 5% solution of trypsin at a pH of 8. On the other hand, the metacercariae of the trematode *Parvatrema timondavidi* failed to excyst using pepsin or trypsin. Instead, a simple saline solution was the most effective for excystment (Smyth, 1990). Similarly, when comparing culture conditions, *Microphalloides japonicus* produced the most eggs in NCTC 109 plus calf serum, but worms also thrived in Minimum Essential Medium (MEM) plus calf serum. In contrast, *Amblosoma suwaense* produced the most eggs cultured in NCTC 135 plus 20% egg yolk (Smyth, 1990).

Successful *in vitro* cultures of trematodes also require fertilization of the worms (Davies and Smyth, 1979). Some trematodes have been reported as only engaging in cross-fertilization, such as *M. turgidus*, regardless of their hermaphroditic state (Pung et al., 2011). While others have reported only self-fertilization, such as in *G. adunca*, occurs (Hunter and Vernberg, 1953). It is because of all these differences that many species need to be studied using *in vitro* cultivation.

*In vitro* cultivation of parasites is of value in order to fill in gaps of what is currently not known about parasite life cycles, behavior, and how they affect their host and what role this plays in the environment (Mouritsen and Jensen, 1997). Many different conditions for culture as well as different species of parasite need to be tested for future understanding not only for the benefit of aiding in the cure for human parasitic diseases (Colley, et. al., 2001), but also what role parasites play in the natural environment and what effects they have from an ecological stand point (Lauckner, 1987).

### **Importance of studying trematodes *in vitro***

It is important to gain a full understanding of trematodes because of the diseases they cause in people, domestic livestock, and wild animals (Keiser, 2010). This is especially important in the development of new antihelminthic vaccines and chemotherapy treatments due to the rise in drug resistance (Helmy, et. al., 2008). This might be accomplished more readily if disease-causing trematodes could be cultured *in vitro*. However, over the past few decades *in vitro* cultivation work involving trematodes has declined due to the technical difficulties involved. This is unfortunate considering the benefits of *in vitro* techniques. For example, the ability to grow worms *in vitro* eliminates the need for vertebrate hosts that are expensive to maintain due to strict animal care

guidelines and would also increase the availability of parasites for use in drug and vaccine studies (Irwin, 1997). Thus knowledge of the biology of these trematodes could lead to better control methods. However, their virulent nature presents issues when attempting to grow disease-causing parasites *in vitro* for study. Overcoming these issues that arise are not as much of an issue when cultivating other trematodes (Irwin, 1997). Also better understanding the biology of parasites would be beneficial for studying the role they play in natural environments by affecting their animal hosts (Mouritsen and Jensen, 1997).

It is important to fully understand trematode biology because several are important pathogens in humans and domestic animals (Keiser, 2010). For example, schistosomiasis, a disease caused by trematodes of the genus *Schistosoma*, is second only to malaria in the amount of severe morbidity and mortality the parasite is responsible for in places such as Sub-Saharan Africa. Another example of a disease caused by a trematode is fascioliasis caused by *Fasciola hepatica*. Not only is fascioliasis an important disease in humans, but also results in economic problems by being a serious disease in livestock (Keiser, 2010). These are a couple of examples that reinforce the importance of understanding trematode biology and how they function in the multiple stages of their life cycle. Studies such as mine may hopefully facilitate future research with antihelminthic drugs. Also, researchers will be able to gain knowledge of how these parasites function in nature and help provide education to the general public for prevention of disease. My study has its basis in the study by Pung, et. al (2009) in which the trematode *M. turgidus* was grown *in vitro* through the metacercarial stage to infection of the first intermediate host *Spurwinkia salsa* with eggs produced by the adult worms.

Another reason to study trematodes *in vitro* is to understand how they affect their hosts to better understand the role this plays in the natural environment (Mouritsen and Jensen, 1997). For example, Fried and Johnson (2004) experimented with excysting the metacercariae of *R. ondatrae*, a parasite of the leopard frog, *Rana pipens*. Infected frogs suffer from limb malformation possibly resulting in increased predation of these frogs. In another study of the effect of parasites on their hosts, the male amphipod *Corophium volutator* infected with *G. adunca* parasites increase their reproductive efforts and are more likely to crawl out onto mud flats, increasing their risk of predation (McCurdy et.al., 1999). *In vitro* cultivation of these and other parasites that cause changes in their hosts could help researchers understand the mechanisms responsible by viewing parasite behavior in the laboratory.

### ***Gynaecotyla adunca***

The trematode I chose to study is the microphallid, *G. adunca*. The first intermediate host of this parasite is the Eastern Mud Snail *Ilyanassa obsoleta* (Hunter and Vernberg, 1953). The snail is infected by eating worm eggs from the fecal material of the definitive host, marsh and shorebirds (Hunter, 1952). In the gut of the mud snail, the egg hatches releasing the miracidium which develops into a mother sporocyst that embeds in the gut wall. From the mother sporocyst, the parasite develops through one generation into a daughter sporocyst in the reproductive system of the snail. The daughter sporocyst then produces multiple cercariae. The cercariae leave the snail and crawl to the second intermediate host that includes several different species of fiddler crabs (Hunter and Chait, 1952) in the genus *Uca*, which they enter by burrowing into the efferent branchial sinuses (Hunter and Vernberg, 1953). The cercariae then enter the next stage of the life

cycle, the metacercariae, and encyst in the green glands of the crab. The metacercariae remain encysted until the crab is consumed by the definitive host. They then excyst and mature into adult worms in the small intestine of the bird definitive host (Hunter, 1952).

## **Objectives**

For my study, I tested different conditions to optimize *in vitro* growth and egg production of *G. adunca*. The infectivity of *G. adunca* eggs produced from the optimal conditions was also tested.

The first objective of my study was to find the best way to acquire and excyst *G. adunca* metacercariae to obtain the maximum number of adult worms. To do this, fiddler crabs of the species *Uca pugnax* were collected by hand in the salt marsh along the Skidaway River off the coast of Southeast Georgia. *G. adunca* metacercarial cysts were dissected from the green gland of the crabs. The metacercariae of *G. adunca*, unlike other microphallids such as *M. turgidus* (Pung, et. al, 2009), do not excyst spontaneously but will excyst in the presence of trypsin (Dunn, et. al., 1990), therefore, the optimum concentration needed to be determined.

Successful completion of the life cycle from metacercariae to the reproductive adult stage of trematodes in the laboratory requires that the adult worm be fertilized. My second objective was to compare self- and cross-fertilization in *G. adunca*. Some microphallids, such as *M. turgidus* are reported by Pung, et. al.(2009) as incapable of self-fertilization. However, Hunter (1952) reported that *G. adunca* does self-fertilize, but offered no data to back up their claim. I tested this claim by incubating excysted worms by themselves or in groups.

As described by Smyth (1990), different parasites require different culture conditions. My third objective was to test the main culture conditions, medium and serum supplements, to promote worm growth and egg production. Upon the determination of the optimum medium, serum supplement, and concentration of serum in medium, I tested the infectivity of the eggs produced by the worms in culture by attempted infection of the first intermediate host *I. obsoleta*.

## CHAPTER 2

### MATERIALS AND METHODS

#### Crab collection

Atlantic marsh fiddler crabs, *Uca pugnax*, were collected from a *Spartina* marsh adjacent to the Rodney Hall boat ramp on the Skidaway River on the coast of Southeast Georgia (31° 56' 53.52" N, 81° 04' 13.32" W). Specimens were captured by hand at low tide and stored in 5 gal buckets for transport. Enough crabs were captured for 1 mo worth of testing, i.e. 70-100 individuals. After 1 mo, any remaining crabs were euthanized by freezing and new ones were captured. In the laboratory, the crabs were maintained in 25 gal aquaria (25-30 animals per aquarium) containing aerated artificial brackish water (Instant Ocean® Aquarium Systems, Inc, Mentor, Ohio) prepared with dechlorinated tap water with salinity adjusted to 22 parts per thousand (ppt). One end of each aquarium was elevated approximately 1 cm and sufficient water was added to cover about half of the tank bottom. A wadded up paper towel was added to provide additional dry surface area. The crabs were fed to satiation with tropical fish food flakes (TetraMin®, Tetra Werke, Melle, Germany) and provided fresh water twice a wk.

#### Crab dissection

Prior to dissection, crabs were placed in a small container of cold Instant Ocean, which was placed in a larger, ice-filled container for at least 10 min. Using microdissecting scissors, a shallow, vertical cut was made through the carapace between the eyestalks to sever the brain. To remove the green glands, a shallow, lateral cut was made under the eye stalks and around the carapace. The dorsal carapace was then peeled off and the green glands were removed and placed in a petri dish containing 0.7% NaCl.

Microdissecting needles were used to tease apart the green gland and expel *Gynaecotyla adunca* cysts from the tissue. Cysts were then washed 1 time before trypsin treatment by transfer to a 60 x 15 mm petri dish containing 10 ml Hank's Balanced Salt Solution (HBSS; Hyclone Laboratories, Logan, Utah) with penicillin and streptomycin (50 units/ml and 50 µg/ml, respectively).

### **Routine preparation of parasites for cultivation**

Unless noted otherwise, worms were prepared for cultivation as follows.

Metacercarial cysts were obtained as described above. Washed cysts were then allowed to excyst in 10 ml of 0.5% porcine trypsin (Hyclone Laboratories, Logan, Utah) in HBSS incubated for 30 min at 40 C, 100% humidity. Excysted worms were washed twice by transfer to 60 x 15 mm petri dishes containing 10 ml HBSS. Washed worms were incubated overnight at 40 C in 15 ml conical bottom polypropylene centrifuge tubes (50 worms per tube). Twenty-four hrs later the worms and the HBSS were transferred to a 15 x 100 mm plastic petri dish. Approximately 500 µl of horse serum was added to prevent nonspecific attachment of the worms to the petri dish bottom. A sample of approximately 50 worms was then examined using a compound microscope to check for fertilization criteria. Worms were considered fertilized if sperm were visible in the uterus and/or the uterine seminal receptacle. Also, fertilized worms often contain normal-shaped eggs whereas unfertilized worms often contain abnormally-shaped eggs.

### **Optimization of excystment of metacercariae**

To determine the concentration of trypsin to use in order to excyst *G. adunca* metacercariae, 20 cysts from freshly dissected crabs were placed in 60 x 15 mm petri dishes containing different concentrations of trypsin diluted in HBSS. The plates were

incubated at 40C, 100% humidity and the number of excysted worms was counted every 30 min for 90 min.

### **Testing need for cross fertilization**

To determine if worms can self-fertilize or require cross fertilization, *G. adunca* cysts were placed in 10 ml of 0.5% trypsin in a 60 x 15 mm petri dish. The dish was warmed on the stage of a dissecting microscope illuminated with a 120 V incandescent light bulb. The cysts were monitored and as each worm excysted, it was immediately removed from the dish and washed 2 times by transfer to the well of a 24 well tissue culture plate containing 2 ml of HBSS followed by transfer to a second well. Each well was used only 1 time, i.e., no more than a single worm was put in any well. Worms were then transferred to 15 ml conical bottom, polypropylene centrifuge tubes containing 10 ml of HBSS. For each trial, 51 tubes were prepared; 1 tube containing 50 worms and 50 tubes, each containing a single worm. The worms were checked for evidence of fertilization after incubation at 40 C for 24 hr.

### **Optimization of culture conditions**

To optimize culture conditions, worms were grown in different culture medium supplemented with different animal sera and different concentrations of these sera. These experiments were performed as follows.

Worms were cultured in air at 40 C, 100% humidity in 48-well tissue culture plates (Falcon polystyrene, Becton Dickinson, Franklin Lakes, New Jersey), 5 worms and 1 ml of HBSS or medium (with or without serum) per well. Peripheral wells were filled with sterile, distilled water to minimize evaporation of liquid from wells containing worms. Worms were monitored daily for survival, *in utero* egg production, and egg

deposition. After all of the worms had died (survival studies) or after 10 days of culture, the plate was removed from the incubator and egg counts were performed as described below.

Two media were compared with HBSS: Roswell Park Memorial Institute 1640 medium (RPMI-1640; Hyclone, Logan, Utah) and Dulbecco's Modified Eagle's Medium (DMEM; Hyclone, Logan, Utah). Both contained HEPES buffer, penicillin, and streptomycin. Worms were also cultured in media supplemented with heat inactivated horse, chicken, and new-born calf serum (GIBCO, Invitrogen, Grand Island, New York). Different concentrations of the serum in which the worms produced the most eggs in medium were then compared.

#### ***In vitro* measurements of culture success**

To determine optimal media, serum, and serum concentrations, 3 parameters of worm fitness were measured: production of eggs *in utero*, worm longevity, and number of eggs deposited in culture. An inverted microscope was used to examine worms in culture for *in utero* egg production. The number of worms with eggs in their uteri was recorded daily for as long as the uterus was visible (about 10 days). Worm longevity was also recorded daily until all worms had died. Worms were considered dead if they did not move during an observation period of at least 30 sec.

To determine the number of eggs deposited in culture, the contents of each culture plate well was transferred to a preweighed 15 ml centrifuge tube after which the well was rinsed 2 times with 0.7% saline and the rinsate transferred to the centrifuge tube. The tubes were centrifuged at 50 x g for 5 min to pellet the eggs. Next, most of the

supernatant was removed, the tubes reweighed, the eggs resuspended by vortexing, and the number of eggs in at least 2 samples of 10  $\mu$ l was counted on a hemocytometer.

### **Optimization of initial 24 hr incubation conditions**

For all previously mentioned experiments, after *G. adunca* cysts were excysted, they were incubated for 24 hr in a 15 ml polyethylene centrifuge tube containing 10 ml of HBSS and placed in culture medium. Since HBSS contains no nutrients, this method was compared to incubating the worms in DMEM plus 5% horse serum. The same culture methods were used as those described above, the only difference being the solution the worms were incubated in for 24 hr prior to experimentation.

### **Snail infection**

To determine if eggs deposited by *G. adunca* in culture were infective to the snail, *Ilyanassa obsoleta*, 3 plates of worms (375 individuals) were cultured for 10 days using optimal culture conditions determined as described above. Contents of plate wells were then collected and pooled in 50 ml polyethylene centrifuge tubes. Eggs were allowed to settle for 1 hr and then counted on a hemocytometer. Half of the eggs were then transferred to a glass dish that contained 25 snails and 2 L of brackish water (Instant Ocean, 23 ppt) at 30 C on a 12 hr light/dark cycle. The other half of the eggs was incubated at 30 C in brackish water for 10 days and then fed to a second group of 25 snails. All snails were fed concentrated microalgae (Shellfish Diet 1800™, Instant Algae®, Reed Mariculture Inc, Campbell, California) and given fresh water twice a wk for 6 wk. Snails were then dissected and examined under a dissecting and an inverted light microscope for the presence of *G. adunca* cercariae.

## Statistical analyses

Data were analyzed with JMP® Pro 9.0.0 (Copyright © 2010) software. *G*-tests were used to compare the concentrations of trypsin and the percent changes of worms with eggs *in utero*.  $X^2$ -tests were used to analyze self- versus cross-fertilization data. To compare the effects of different media, sera, and serum concentrations on the number of eggs deposited and on worm longevity, a one-way analysis of variance (ANOVA) was utilized and the means were compared using Tukey-Kramer honest significant difference tests. In some cases data were not parametric and were analyzed following logarithmic transformation. Each experiment was performed at least twice; and yielded comparable results each time. Analyses and figures represent data from the last trial performed.

## CHAPTER 3

### RESULTS

The percentage of *G. adunca* metacercariae excysted was significantly affected by trypsin concentration (*G*-test,  $G=94.8$ ,  $df=4$ ,  $P<0.0001$ , Table I). The concentration of trypsin that yielded the highest excystment percentage over the shortest amount of time of 30 mins was 0.5%. I also found that percent excystment in 0.5% trypsin was higher in freshly collected crabs than in crabs maintained in the laboratory for longer than 1 mo (data not shown).

Self-fertilization and cross-fertilization were compared to determine the ratios of occurrence. One trial showed that cross-fertilized group produced the highest percentage of fertilized worms ( $X^2$ -Test,  $X^2=10.07$ ,  $df=1$ ,  $p=0.0015$ ) and another with no difference between the two groups ( $X^2$ -Test,  $X^2=0.05$ ,  $df=1$ ,  $p=0.8224$ , Table II).

In order to evaluate the most effective medium for the *in vitro* growth of *G. adunca*, 3 parameters were evaluated: worm longevity, *in utero* egg production, and egg deposition. There was a significant difference between all 3 parameters which included worms living longer (ANOVA;  $df=2$ , 338,  $F$  ratio= 18.8;  $P<0.0001$ , Fig 2) in DMEM. More worms produced eggs *in utero* (*G*-Test,  $G=10.5$ ,  $df=2$ ,  $p=0.0053$ , Fig 3) in DMEM and RPMI-1640 than HBSS. Finally, worms also deposited more eggs (ANOVA;  $df=2$ , 21;  $F$  ratio=7.4;  $P=0.0037$ ; Fig 4) in DMEM rather than RPMI-1640 medium and HBSS similar to longevity. All *in utero* tests use the percentages from day 2 for statistical testing because worms showed the highest percent change in egg production *in utero* on this day from day 0 of culture (Fig 1).

To further optimize the culture conditions, worms were grown in DMEM containing horse, new-born calf, or chicken sera. The presence of any type of sera almost doubled worm longevity compared to the absence of serum (ANOVA;  $df=3$ , 236, F value= 733.3,  $P < 0.0001$ , Fig 5). The use of horse and chicken sera significantly increased the number of worms that produced eggs *in utero* (*G*-test,  $G= 20.7$ ,  $df= 3$ ,  $P < 0.0001$ , Fig 6), and egg deposition (ANOVA;  $df=3$ , 44, F value=5.4,  $P=0.0029$ , fig 7) compared to calf serum and DMEM only. Although horse and chicken sera performed similarly, the chicken serum began to form an unknown precipitate after 5 days in medium and increased in severity until approximately day 10. When the various concentrations of serum were tested, the percentage of worms with eggs produced *in utero* (*G*-Test,  $G=22.3$ ,  $df=4$ ,  $p=0.0002$ , Fig 8) increased significantly in 5%, 10% and 20% horse serum. Egg deposition (ANOVA;  $df=4$ , 50, F value=5.5,  $P=0.001$ , Fig 9) increased significantly only in the 5% horse serum and DMEM solution.

Immediate incubation of adult worms after excystment was also compared using DMEM with 5% horse serum and HBSS. Both percentage of worms producing eggs *in utero* (*G*-Test,  $G=1.237$ ,  $df=1$ ,  $p=0.266$ , Fig 10) and egg deposition (ANOVA;  $df=1$ , 30, F value= 0.0015,  $P=0.9698$ , Fig 11) yielded no difference in the numbers between the 2 solutions. When snail infection was tested, none of the 3 groups of snails yielded any positive results to suggest infection was achieved, which would have been the presence of cercariae.

Table I. Effect of different concentrations of trypsin on the excystment of metacercariae of the trematode *Gynaecotyla adunca* (n= 25 parasite/treatment) (G-test, P<0.0001).

Observations made after 30 min of incubation at 40 C.

Trypsin Concentration (%)	Percent of metacercariae excysted
0	0
0.01	60
0.1	88
0.5	100
1	96

Table II. Comparison of freshly excysted *Gynaecotyla adunca* adult worms incubated for 24 hr then observed either individually, i.e., 1 worm per culture tube, or in the presence of conspecifics, i.e., 50 worms per tube, with respect to fertilization.

Trial	Incubation Condition	% Fertilized
1	50 Worms	96 *
	50 Single Worms	73
2	50 Worms	70
	50 Single Worms	67

\* Indicates a significant difference within a trial  $X^2$ -Test, P=0.0015

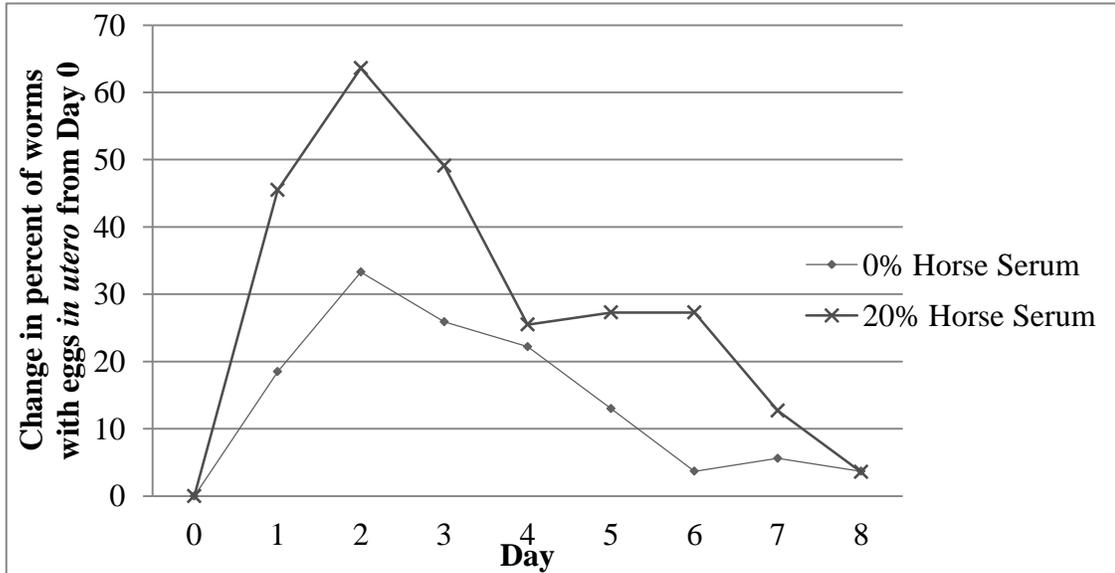


Figure 1. Difference in percentage of worms with eggs *in utero* cultured in DMEM with or without horse serum. The highest percentage of worms that produced eggs *in utero* was consistently observed on Day 2. Consequently, Day 2 results were used in all analyses (n=55 worms/treatment).

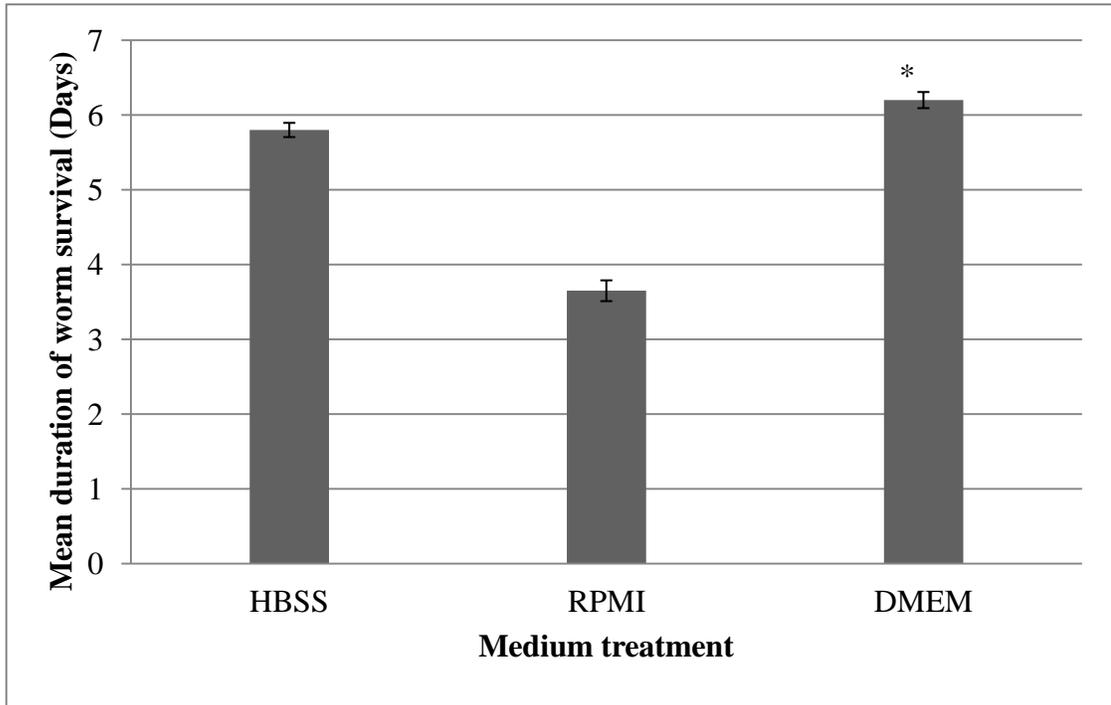


Figure 2. Longevity of *Gynaecotyla adunca* worms cultured in HBSS, RPMI-1640, and DMEM. The worms grown in DMEM on average lived the longest (ANOVA,  $P < 0.0001$ ). Each bar represents the mean survival time  $\pm 1$  SE (n=125 worms/treatment).

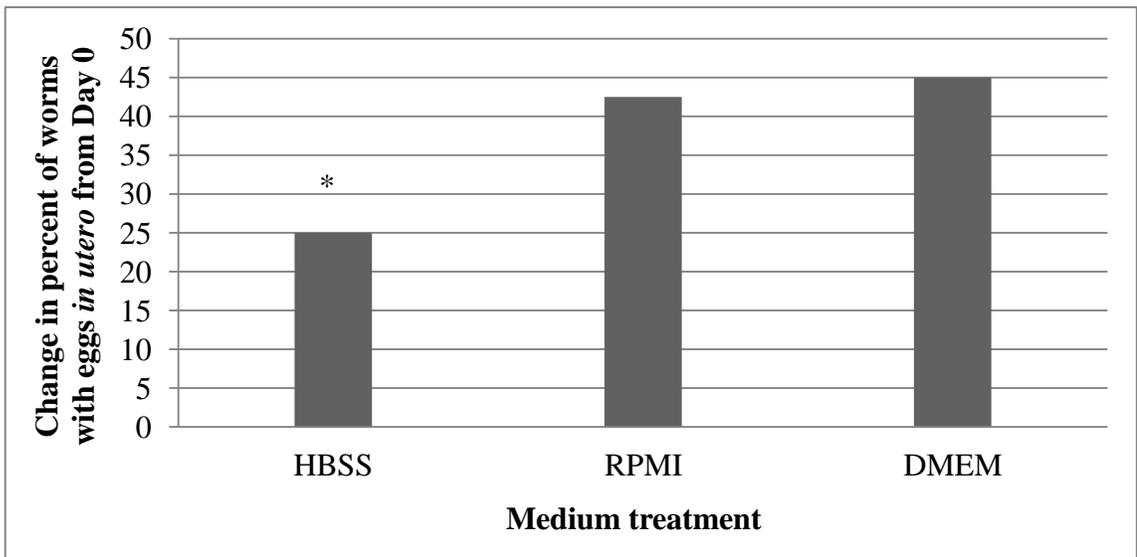


Figure 3. *In utero* production of eggs by the trematode *Gynaecotyla adunca* in HBSS, RPMI, and DMEM. Significantly more worms produced eggs *in utero* when cultured in RPMI-1640 or DMEM than in HBSS ( $G$ -test,  $P=0.0053$ ). Each bar represents the change in the percentage of worms that produced eggs *in utero* from Day 0 on Day 2 of culture ( $n=40$  worms/treatment).

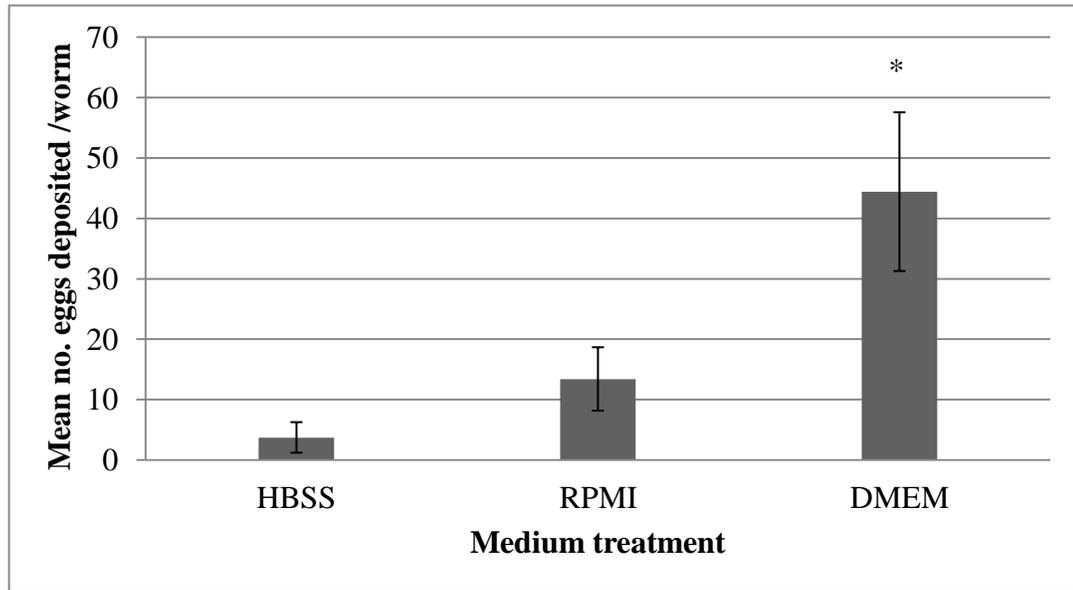


Figure 4. Egg deposition by *Gynaecotyla adunca* in HBSS, RPMI-1640, and DMEM.

The worms deposited the most eggs when cultured in DMEM (ANOVA, P=0.0037).

Each bar represents the mean number of eggs deposited by the worms in each media  $\pm$  1 SE (n $\geq$ 40 worms/treatment).

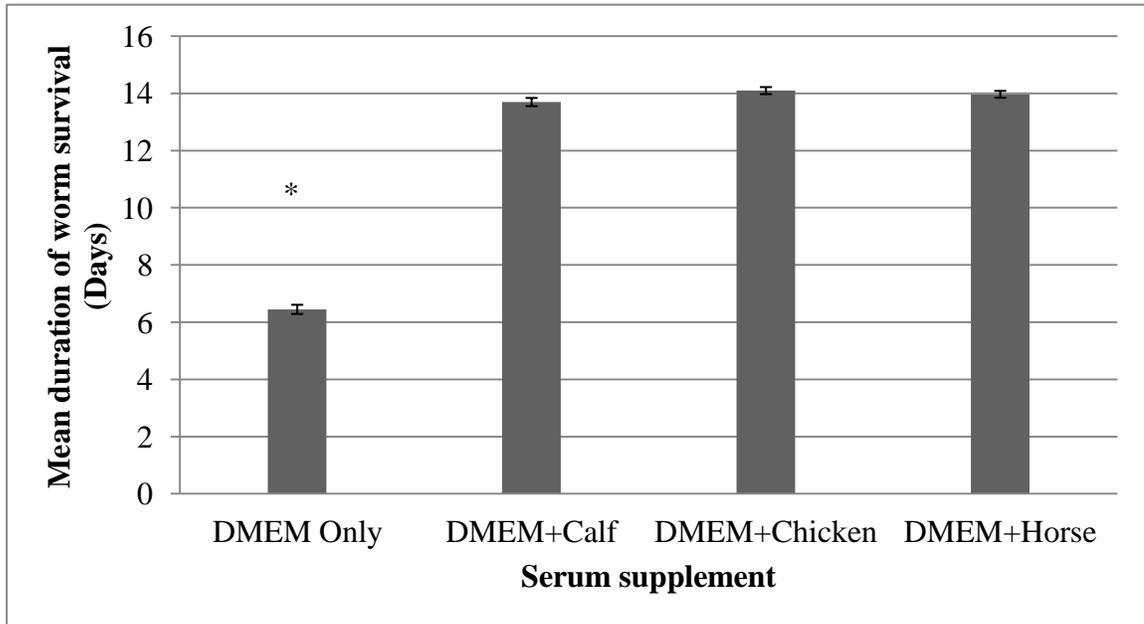


Figure 5. Longevity of *Gynaecotyla adunca* worms cultured in DMEM plus 20% concentration of either horse, new-born calf, or chicken sera or DMEM only. The greatest mean survival occurred in all the treatments containing serum (ANOVA,  $P < 0.0001$ ). Each bar represents the mean survival time  $\pm$  1 SE (n= 240 worms/treatment).

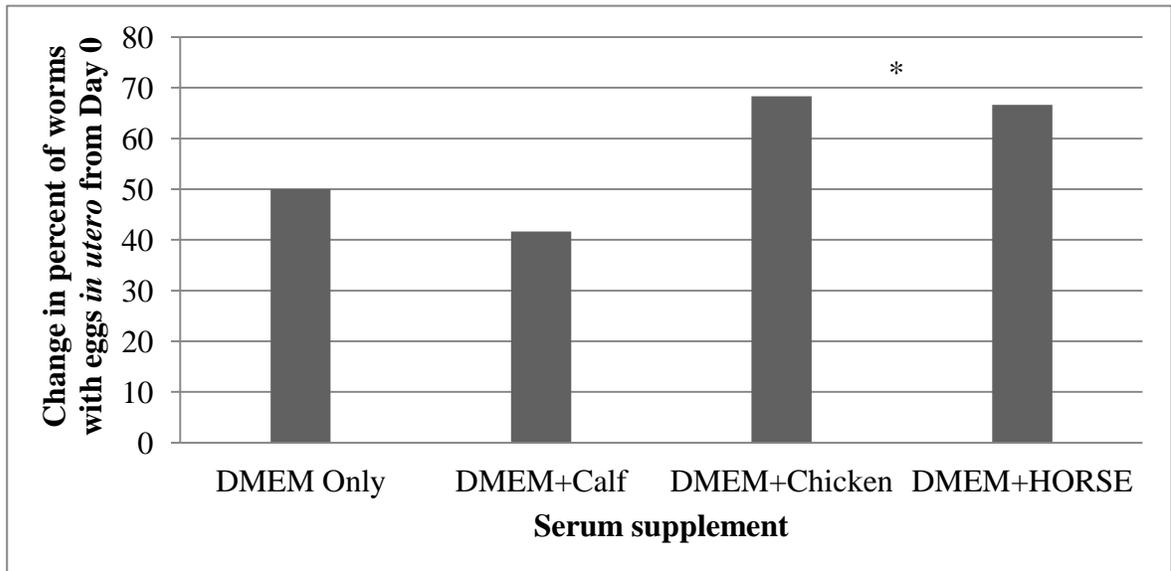


Figure 6. *In utero* production of eggs by the trematode *Gynaecotyla adunca* cultured in DMEM with 20% of either horse, new-born calf, or chicken sera added or DMEM only. There was a significant difference between effects of the 4 treatments on worm *in utero* egg production (*G*-test,  $P < 0.0001$ ). DMEM plus chicken serum had the highest average change, but was not that much different from the solution with horse serum just from comparing the means. Each bar represents the change in percentage of worms that produced eggs *in utero* from Day 0 on Day 2 of culture ( $n = 60$  worms/treatment).

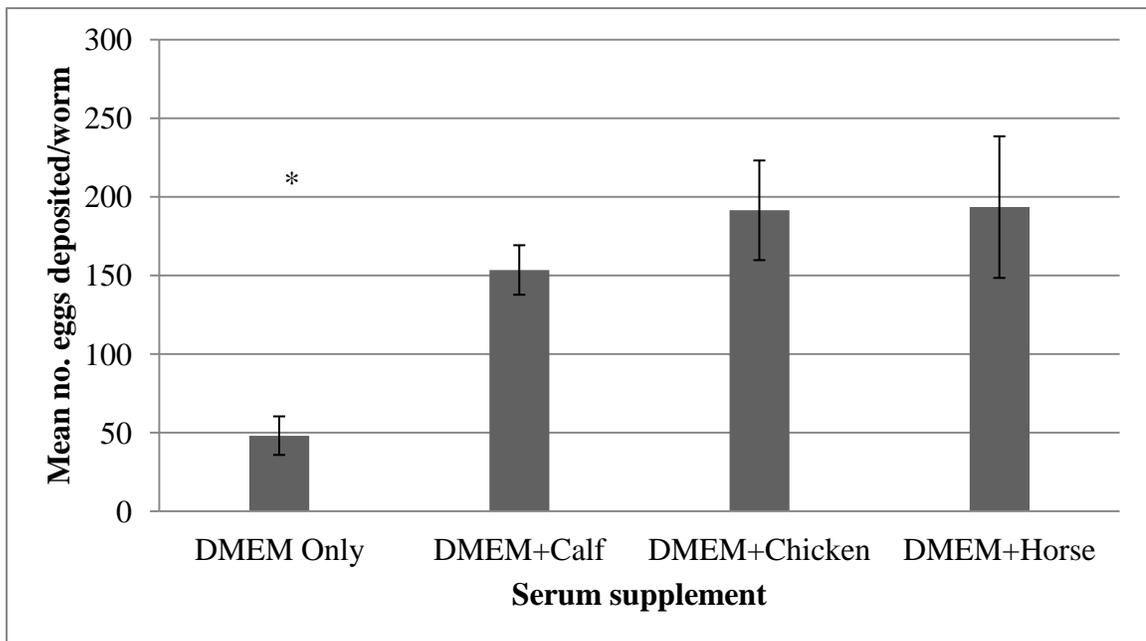


Figure 7. Egg deposition by *Gynaecotyla adunca* in DMEM plus 20% of either horse, new-born calf, chicken sera and DMEM only. The worms deposited the most eggs when cultured in DMEM plus horse and chicken sera (ANOVA,  $P < 0.0029$ ). There was only a slight difference in the means for horse and chicken sera treatments as far as which had the highest mean of egg deposition. Each bar represents the mean number of eggs deposited by the worms in each treatment  $\pm 1$  SE ( $n=60$  worms/treatment).

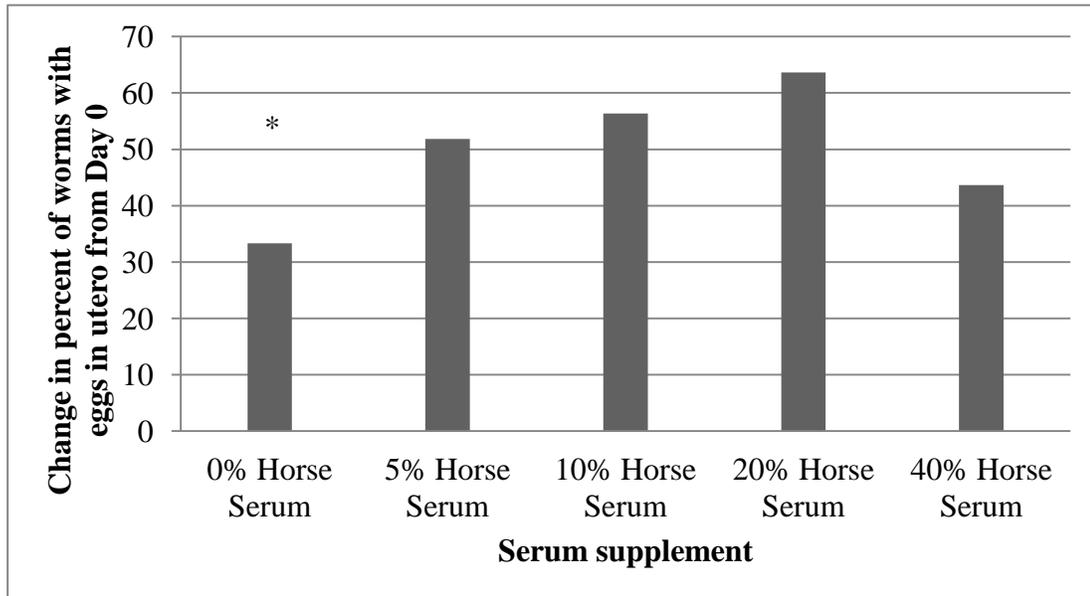


Figure 8. *In utero* production of eggs by the trematode *Gynaecotyla adunca* cultured in different concentrations of horse serum in DMEM. Significantly more worms produced eggs in utero when cultured with a serum supplement (*G*-test,  $P=0.0002$ ). The worms cultured in 20% horse serum showed the greatest change in eggs produced *in utero*. Each bar represents the change in the percentage of worms that produced eggs *in utero* from Day 0 on Day 2 in culture ( $n \geq 54$  worms/treatment).

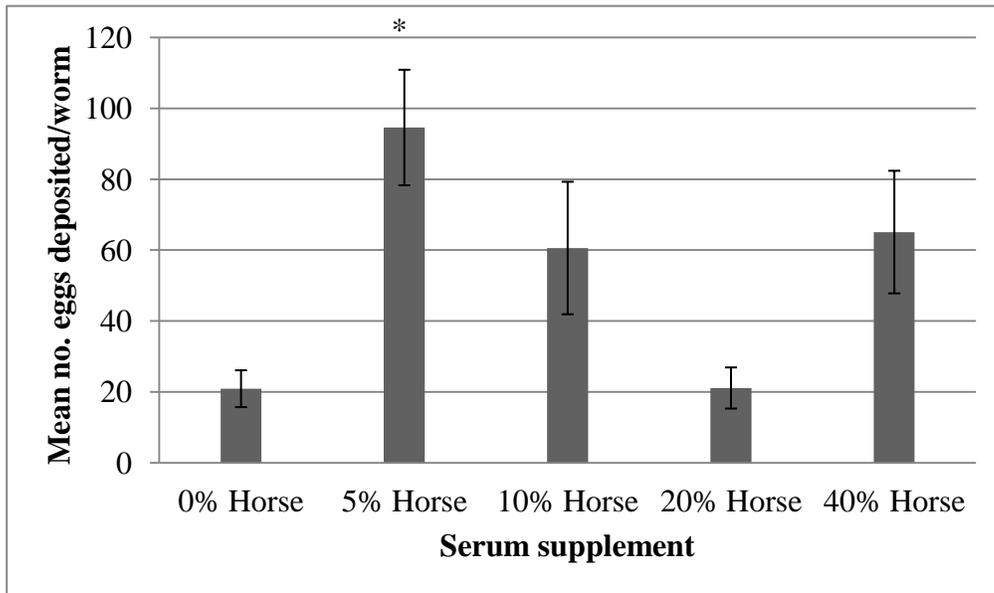


Figure 9. Egg deposition by *Gynaecotyla adunca* in different concentrations of serum in DMEM. The worms deposited the most eggs when cultured in DMEM and 5% horse serum (ANOVA, P=0.001). Each bar represents the mean number of eggs deposited by the worms in each treatment  $\pm$  1 SE ( $n \geq 54$  worms/treatment).

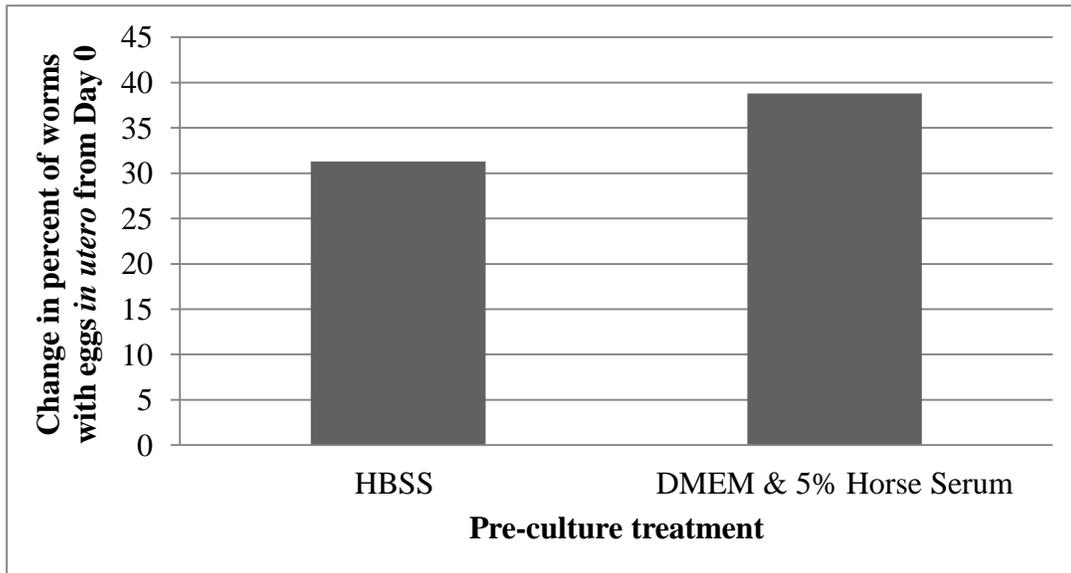


Figure 10. The percent of worms that produced eggs *in utero* after initial incubation for 24 hr in either DMEM plus 5% horse serum or HBSS. There was no significant difference between the 2 treatments. Each bar represents the difference in the percentage of worms that produced eggs *in utero* from Day 0 to Day 2 of culture (n=80 worms/treatment).

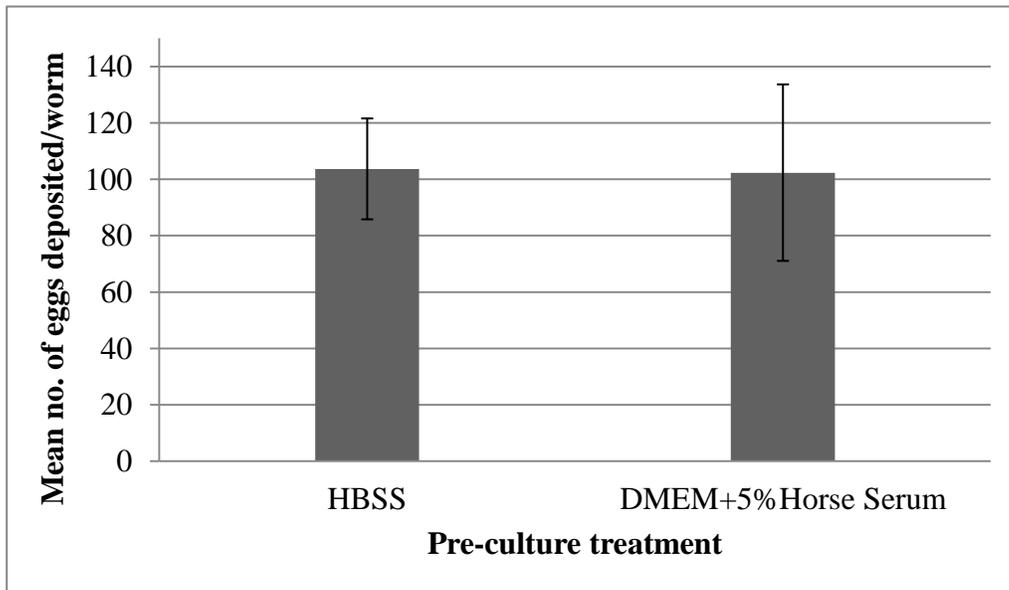


Figure 11. Eggs deposited by *Gynaecotyla adunca* after initial incubation for 24 hr in either DMEM plus 5% horse serum or HBSS. There was no significant difference between the 2 treatments. Each bar represents the mean number of eggs the worms deposited  $\pm$  1 SE ( $n \geq 74$  worms/treatment).

## CHAPTER 4

### DISCUSSION

*In vitro* cultivation of trematodes, while a challenge, is important for both biological reasons and public health concerns (Colley, et. al., 2001). Vaccines and new chemotherapies need to be found for trematode diseases that affect humans and domestic animals, especially since the recent emergence of resistance to the few currently available anti-trematode drugs (Helmy, et. al., 2008). This may be accomplished more readily by studying trematodes *in vitro* (Colley, et. al., 2001). Also, *in vitro* techniques can cut costs and time by eliminating the need for vertebrate hosts (Irwin, 1997). Studying the biology of trematodes may also add to our knowledge as to how they affect their animal hosts and what role this plays in the natural environment (Mouritsen and Jensen, 1997). Since efforts to culture trematodes *in vitro* have diminished in the past few decades (Irwin, 1997), it is more important to begin this research now more than ever.

The aim of my research was to aid in these efforts by studying the techniques needed to culture the microphallid trematode, *G. adunca*. My first objective was to determine the best method of obtaining and excysting *G. adunca* metacercariae. Once the worms were excysted, my next goal was to optimize *in vitro* culture conditions so the worms would produce the maximum number of viable, infective eggs. My final objective was to test the infectivity of the eggs. To accomplish these goals I tested different concentrations of trypsin for excystment. I also tested 3 media (DMEM, RPMI-1640, and HBSS) and 3 different sera (horse, chicken, and new-born calf) on *in vitro* worm longevity and egg production. Finally, I attempted to infect the first intermediate host of

*G. adunca*, the mud snail *I. obsoleta* by feeding them eggs produced by my cultured worms.

### **Excystment of *Gynaecotyla adunca***

Hunter and Chait (1952) published the first studies concerning the excystment of *G. adunca* metacercariae. For their excystment treatment, they used 0.5% pepsin and HCl and incubated the worms for 40-45 min at 40 C. This treatment resulted in 70-90% excystment of the parasite. In another study, Dunn et. al. (1989) reported that *G. adunca* worms excysted in high concentrations of pepsin (10%) died and were partially digested. They also found that more worms excysted in trypsin than in pepsin and remained viable. They used trypsin at concentrations of 0.1% and 5%. Based on the findings of Dunn et. al. (1989) I decided to use trypsin in my excystment experiments rather than pepsin. I found that the concentration of trypsin that induced 100% excystment after 30 min at 40 C was 0.5%. Consequently, I used this concentration in all my other experiments.

During my research on excystment I noted that fiddler crabs can be kept alive in the laboratory for several weeks. This prompted me to ask how long the parasite would remain viable in laboratory maintained crabs. To test this, I compared excystment percentages in parasites from crabs kept in the laboratory less than 1 mo to parasites from crabs kept more than 1 mo. I found that metacercarial cysts in crabs that were kept in the laboratory for longer than 1 mo were less likely to excyst than parasites from crabs maintained less than 1 mo in the laboratory. For all subsequent experiments, I only used cysts from freshly caught crabs to get maximum excystment of the metacercariae.

## Self and cross fertilization

Many trematodes are hermaphroditic, so it makes sense that they might either fertilize themselves when no other worms are available or cross-fertilize in the presence of conspecifics (Trouvé, et. al., 1995). Self fertilization is thought to be favored when there is a low probability of finding a partner, such as cases of low population density in the definitive host. However, it could be assumed that self-fertilization is less preferred than cross-fertilization because it leads to an accumulation of deleterious recessive mutations (Trouvé, et. al., 1995). A number of trematodes, *M. turgidus* for example, are only fertilized in the presence of conspecifics, at least in laboratory conditions (Pung, et. al., 2011).

Hunter (1952) reported that *G. adunca* only self-fertilizes, but offered no evidence to support this claim. I tested this by excysting *G. adunca* and incubating the worms either with conspecifics or individually. The percentage of individual worms that were fertilized was lower than that of worms with conspecifics in one of my trials but not in the second. Regardless, worms incubated individually produced normal eggs and had sperm in the uterus and/or the uterine receptacle indicating that the parasite is capable of self-fertilization. It should be noted that based on my findings alone, we cannot be certain whether the worms incubated with conspecifics were cross- or self-fertilized. The only way to determine this would be through genetic testing. Also, I found it somewhat difficult to assess fertilization in *G. adunca*, and this may have affected my results. The simplest way to determine whether or not a worm is fertilized is to look for the presence of sperm in the seminal receptacle. Unfortunately, *G. adunca* has no seminal receptacle (Hunter, 1952). However, I was able to observe sperm in other parts of the female

reproductive system such as the uterus. Another clue to fertilization is the production of normal-shaped eggs (Davies and Smyth, 1978). I did observe these in the worms incubated individually.

### **Culture experiments**

To create the optimal *in vitro* environment for *G. adunca* to prosper and produce eggs in, I chose to optimize a number of basic culture system components. These included the type of culture medium, the serum supplement, and the concentration of serum. Interestingly, Hunter and Chait (1952) reported that the most effective *in vitro* environment for *G. adunca* culture was 1% seawater at 40 C. However, many other studies have shown the importance of media and sera to the successful *in vitro* growth of trematodes (reviewed by Irwin, 1997).

When determining the success of an *in vitro* experiment, several different parameters can be measured such as worm survival, growth, development, and maturation (Smyth, 1990). With this in mind, I chose 3 parameters to measure the effectiveness of my *in vitro* experiments: worm longevity, the percentage of worms that produced eggs *in utero*, and the number of eggs deposited.

Irwin (1997) discusses how cell culture media such as DMEM and buffered saline solutions such as HBSS are commonly used for trematode *in vitro* studies. Other common synthetic media that can be used include RPMI-1640, NCTC 135, etc. (Smyth, 1990). I chose to compare the buffered saline solution, HBSS, with 2 culture media: DMEM and RPMI-1640. DMEM and RPMI-1640 are both synthetic media originally developed for cell culture. Other investigators have found that DMEM is a good medium for the growth of trematodes, for example, Fujino et. al. (1977) found that *Microphalloides japonicus*

produced twice as many eggs *in utero* when grown in culture medium than they did in HBSS. In addition, worms grown in DMEM produced more eggs than those grown in NCTC 109 medium. Similarly, I found that DMEM was better than HBSS with respect to worm longevity and egg production. Also, *G. adunca* worms lived longer in DMEM than RPMI-1640. Though there was no significant difference between DMEM and RPMI-1640 with respect to egg production, mean values were consistently higher for worms grown in DMEM. This combined with the fact that the worms lived longer in DMEM resulted in my decision to use DMEM rather than RPMI-1640 in subsequent studies.

A food source, normally in the form of an animal serum is usually added to trematode culture media. Chicken serum is frequently used for trematodes whose definitive hosts are birds (Basch, et. al., 1973; Fried and Contos, 1973; Fried, et. al., 1978). For example, in an unsuccessful attempt to culture *Leucochloridomorpha constantiae*, Fried and Contos (1973) used 50% or higher concentrations of chicken serum. Later, Fried et. al. (1978) used NCTC-135 medium supplemented with 50% chicken serum and obtained adult *Cotylurus strigeoides* worms that produced nonviable eggs. Other animal sera used in trematode culture include horse, fetal calf, new-born calf, and lamb sera (Fujno, et. al., 1977; Ivanchenko, et. al., 1999; Park, et. al., 2006; Pung et.al., 2009). Investigators rarely make direct comparisons of the efficacy of sera from different animals. However, Pung et. al. (2009) compared chicken, new-born calf, and horse sera for the culture of *M. turgidus*. They found that this trematode produced more eggs when grown in 20% horse serum. Based on this study, I compared 20% horse, chicken, and new-born calf sera in my preliminary serum studies.

All 3 of these sera improved worm longevity compared to worms grown in DMEM alone. I found that *G. adunca* produced more eggs when cultured in either chicken and horse sera than they did in the new-born calf serum. Worms produced similar numbers of eggs in chicken and horse sera but I decided to use horse serum in my remaining experiments. This was because a precipitate formed in cultures containing chicken serum after 2 days. The precipitate did not seem to affect worm longevity or egg production but the amount of precipitate increased from day to day and made it difficult to observe eggs or worms by Day 9 of culture. The precipitate was tested to determine if it was a microbial contaminant, but no fungal or bacterial growth was detected. I chose horse serum over chicken serum for my remaining experiments because it greatly simplified data collection.

Serum concentration may also affect culture success (Reviewed by Smyth, 1990). Thus, the next *in vitro* culture condition I tested was the concentration of horse serum in DMEM. Pung, et. al. (2009) reported that egg deposition by *M. turgidus* increased until the concentration of horse serum reached 40%. In other studies investigators found that raising serum concentration to as high as 50% had no benefit (Fried et. al., 1978; Fried and Contos, 1973). Based on these studies, I did not test over 40% serum concentration. Interestingly, I found that similar percentages of *G. adunca* worms produced eggs *in utero* when cultured in 5%, 10%, and 20% horse sera whereas the worms deposited more eggs when grown in 5% horse serum than they did in media containing higher concentrations of serum. From these results, I used 5% horse serum for subsequent experiments in DMEM.

The protocol I used for culturing *G. adunca* was based on a protocol originally optimized for *M. turgidus* by Pung et. al. (2009, 2011). In that protocol, worms were excysted, transferred to conical bottom tubes containing HBSS and incubated 24 hr prior to being placed in culture medium supplemented with animal serum. The 24 hr incubation in a conical tube is thought to facilitate copulation and worm fertilization (Pung et. al., 2011). However, because there are no nutrients in HBSS, I decided to test whether or not the use of DMEM plus horse serum during the 24 hr incubation period would enhance egg production by *G. adunca*. To do so, I compared egg production of worms incubated 24 hr in HBSS alone prior to cultivation to worms incubated in DMEM plus 5% horse serum prior to cultivation. There was no difference between either group indicating a measurable benefit to using complete medium during the initial incubation period.

### **Snail infection**

Several researchers have cultured excysted metacercariae in conditions that resulted in growth of the worms into egg-producing adults (reviewed by Smyth, 1990; Irwin, 1997). However, the eggs produced were often malformed or unembryonated (Fried et. al., 1978; Davies and Smyth, 1979; Schnier and Fried, 1980; Fredensborg and Poulin, 2005). This phenomenon may be due to failure of fertilization (Davies and Smyth, 1979). Some investigators have reported *in vitro* production of normal-shaped, miracidium-containing eggs, but did not test the eggs for infectivity to snail hosts (Berntzen and Macy, 1969, Yasuraoka et. al., 1974). To my knowledge, there are only 2 reports of *in vitro* egg production by a trematode combined with the demonstration that the eggs were infective to snails (Basch et. al., 1973; Pung et. al., 2009). In the study by

Basch et. al. (1973), the strigeid *Cotylurus lutzi*, when cultured in medium containing an extract of chicken upper intestinal mucosa, produced infective eggs with miracidia. However, a related strigeid (*Cotylurus strigeoides*) produced abnormal eggs when cultured in the same conditions (Fried et. al., 1978). Pung, et. al. (2009) were the first to culture a microphallid trematode *in vitro* and then infect the first intermediate host, the snail *Spurwinkia salsa* with the eggs produced.

The final goal of my study was to test whether or not eggs produced by *G. adunca* in my optimized culture system were infective to the mud snail *I. obsoleta*.

Unfortunately, none of the snails fed eggs in culture produced *G. adunca* sporocysts or cercariae. One possibility is that the number of eggs fed to the snails, approximately 150 eggs per snail, was too low. Another possibility is that the eggs remained in the embryonic state rather than developing into miracidia. When I examined eggs immediately out of culture and 10 days in further incubation, I observed mostly embryos.

The most striking difference between my research and that of Pung, et. al. (2009, 2011) was that *M. turgidus* produced many more eggs than did *G. adunca*. Pung et. al. (2009) observed that *M. turgidus* produced approximately 400 eggs per worm in RPMI-1640 containing 40% horse serum. On the other hand, *G. adunca* in its optimal conditions of DMEM plus 5% horse serum produced approximately half of this amount. I also noted a good deal of variation from worm to worm with respect to eggs produced *in utero*. Some worms would produce dozens to hundreds of eggs whereas others would produce few to none. Future studies with *G. adunca* should focus on these differences between the 2 parasites. One way to do this could be to focus on other *in vitro* culture conditions not tested in this study. For example, Dunn, et. al. (1989) monitored pH and CO<sub>2</sub> levels

within the treatments of *G. adunca* metacercariae but not the adult worms. These manipulations of pH and the gas phase for culture are quite common for *in vitro* studies (Berntzen and Macy, 1969; Bixler, et. al., 2001). Another factor that can be manipulated is temperature. Fried and Contos (1973) varied their incubation temperatures of *L. constantiae* between 37.5 and 42 C. Ponder and Fried (2004) and Fried, et. al. (2004) examined the effects of different concentrations of glucose on the growth and survival of *Echinostoma caproni* cercariae.

## **Conclusion**

My objectives were to successfully obtain and excyst *G. adunca* metacercariae and to optimize *in vitro* cultivation for worms to produce viable, infective eggs. I captured *U. pugnax* and obtained *G. adunca* metacercarial cysts from their green glands and excysted them effectively using trypsin. I optimized *in vitro* culture conditions by choosing the best medium, serum, and concentration of serum experimentally by analyzing 3 parameters: worm longevity, percentage of worms that produced eggs *in utero*, and the number of eggs that the worms deposited. From this I was able to produce what seemed to be infective eggs, but failed to infect *I. obsoleta*. However, I only attempted snail infection one time due to time restraints and was unable to improve upon my methods.

Future *G. adunca* culture studies could focus on answering this question: Are the eggs produced *in vitro* infectious and if so, how can snail infection be achieved? This could be done by testing other *in vitro* culture conditions such as pH and CO<sub>2</sub> levels (Dunn et. al., 1989). Other media and sera could be tested by examining eggs produced from worms in these culture conditions for presence of miracidia to confirm the

possibility that the eggs are infective. Future experiments could also attempt snail infection multiple times to refine the technique whereas I only attempted snail infection once.

Other *in vitro* studies could focus on multiple stages of the parasitic life cycle (Chaithong, et. al., 2001). This needs to be done with multiple species of parasites such as various trematodes, since individual species require different culture conditions (Smyth, 1990) in order to understand the wide spectra that are trematodes. For example, *M. turgidus* grew best in RPMI-1640 with 20% of either calf or horse sera (Pung, et. al., 2009) whereas I found *G. adunca* to grow best in DMEM plus 5% horse serum. It is because of these differences, as well as examples mentioned previously, that several species of trematode should be grown *in vitro* for further advances in trematode biology.

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