COMMUNICATION

Laboratory Mass Culture of the Freshwater Oligochaete *Dero digitata*

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Abstract

  Proliferative gill disease (PGD) in channel catfish *Ictalurus punctatus* is caused by the myxozoan parasite *Henneguya ictaluri*, which uses the freshwater oligochaete *Dero digitata* as an alternate host. Controlled studies on the transmission of PGD require sustainable *D. digitata* mass cultures. We developed methods to grow and sustain large *D. digitata* populations and compared population growth in aerated versus nonaerated containers. White paper towels (2–3-cm squares) were placed into six 10.7-L dish pans into which 4 L of autoclaved pond water from commercial channel catfish ponds were then added along with 0.1 g of fish food. Each of the six dish pans was initially stocked with 100 *D. digitata* and maintained at 22–25°C; three pans received aeration, and three did not. All worms were counted and returned to their respective pans once each week for 5 weeks. To compensate for evaporative loss, autoclaved pond water was added to the pans weekly, and pond water and paper towels were completely changed after 4 weeks. Over 5 weeks, the mean number of worms per pan increased significantly more in the nonaerated pans than in the aerated pans. We have maintained *D. digitata* mass cultures in our laboratory for over a year, starting with two pans containing 100 worms each. Through routine exchanges of pond water and paper towel squares, these populations have expanded to 16 pans, each supporting 3,000–6,000 worms. We have also removed thousands of worms periodically to give to other researchers or for research in our laboratory. Using methods described here, researchers can maintain *D. digitata* mass cultures and predict population numbers that will be available at given times for studies on myxozoan life cycles and oligochaete control.

  Proliferative gill disease (PGD), commonly referred to as "hamburger gill disease," is one of the most prevalent diseases in farm-raised channel catfish *Ictalurus punctatus*. This disease is caused by the myxozoan parasite *Henneguya ictaluri* (Burtle et al. 1991; Styer et al. 1991; Pote et al. 2000) and is the most prevalent parasitic disease affecting commercial channel catfish production (ADL 2007). In severe outbreaks, mortalities can exceed 50% (Bowser and Conroy 1985). Fish with PGD develop severe branchial inflammation, epithelial hyperplasia, lamellar fusion, and lysis of chondrocytes in gill filaments, resulting in osmoregulatory stress that leads to reduced production and, in severe cases, death (MacMillan et al. 1989).

  The complex myxozoan life cycle requires the oligochaete *Dero digitata* as an alternate host (Styer et al. 1991). However, the collection of large numbers of *D. digitata* from pond sediments is cumbersome, labor intensive, and tedious, and there is no guarantee that the collected population will be parasite free. Therefore, sustainable mass cultures are desirable for use in controlled laboratory studies on the myxozoan life cycle and in studies examining the chemical and biological control of the oligochaete host. Pote et al. (1994) presented a method for isolating and propagating infected *D. digitata*. Although this method was successful in propagating infected worms in petri dishes, the number of worms produced was not reported and the size of the petri dish greatly limits the amount of worm production. Water quality in the small volumes of water (<10 mL) used in petri dishes can deteriorate rapidly, and frequent water changes are required. Additionally, much larger numbers of worms than can be maintained in petri dishes are often needed for research. Therefore, the purpose of this study was to develop a method for the mass culture of *D. digitata* and report the realized population growth.

METHODS

  Initial worm collection.—To obtain initial *D. digitata* for culture, mud samples were collected from channel catfish ponds using an Ekman dredge (Wildlife Supply Company, Saginaw, Michigan). Mud was brought to the laboratory and rinsed through a 2,000-μm copper-mesh screen (number-10 sieve; Fisher Scientific Co., Norcross, Georgia) to remove large debris.

Filtrate that passed through the 2,000-μm screen was caught by a 300-μm copper-mesh screen (number 50) and placed on white plastic cafeteria trays with clear water. Individual worms were collected with a disposable transfer pipette and identified as *D. digitata* (Pennak 1989) under a dissecting microscope.

General culture methods.—Containers used for *D. digitata* culture are 10.7 L (39 × 32 × 14 cm) white dish pans (Rubbermaid, Inc., Huntersville, North Carolina). Three sheets of white paper towels (7 g) are ripped by hand into approximately 2–3-cm squares and placed into the bottom of the dish pans. Nonbleached paper towels have also been used in our laboratory. It appears that there are no differences in worm performance (survival, growth, or reproduction) regardless of the paper towel type used. However, the worms are easier to see and remove from the white paper towels. After the addition of paper towels, 0.1 g of fish food (Finfish Starter, 55% protein, 15% fat, 0.42–0.595-mm meal; Ziegler Brothers, Gardners, Pennsylvania) is added to each pan. Pond water is collected from ponds with moderate to heavy phytoplankton blooms and is autoclaved for 15 min. After cooling, 4 L of the autoclaved pond water is then added to each dish pan. Although fish food is added to the culture pans, most of the sustenance for the worms may come from the killed plankton present in the autoclaved pond water.

Initial cultures are started with 100 parasite-free *D. digitata* individuals identified using previously established protocols (Pote et al. 1994). These cultures are allowed to sit for 4–8 weeks at ambient temperatures (22–25°C) without aeration before all worms are collected and transferred to containers with fresh paper towel media. Additional autoclaved pond water is added as needed to compensate for evaporation and to replenish the food supply (i.e., killed plankton). When transferring worms to containers with new media (every 4–8 weeks), the water and paper towel media are removed and placed on a white cafeteria tray; only aliquots of about 300 mL should be transferred at a time. The worms are easily removed and transferred to the new media with a disposable transfer pipette. When populations in a given container reach about 3,000–5,000 worms, the worms are divided into new containers and numbers are reduced to about 1,000 worms/container. The carrying capacity of the containers is not known, but it appears that with media changes every 4–8 weeks, up to 5,000 worms can be maintained without apparent adverse effects on individual worm health.

Effects of aeration on population growth.—To determine the effects of aeration on population growth, six culture containers were set up as described above using an initial population of 100 *D. digitata* per container and were maintained at room temperature (22–25°C). Large, healthy adult worms from our standing cultures were selected for the aeration study. Additional pond water was added weekly to each container to compensate for evaporative loss and to replenish the killed plankton food supply. Three of the containers received continuous aeration with air stone diffusers, and three of the containers did not receive aeration. Once per week, all *D. digitata* in each container were counted and returned to their respective dish pans, along with the original water and media. After 4 weeks, all worms were transferred to dish pans with new paper towel media and water. Populations increased rapidly, and it became difficult to individually count the worms. Therefore, after 5 weeks, the final population numbers were counted, and the differences in numbers of worms between aerated and nonaerated cultures were compared. The analysis employed a completely randomized design with repeated measures (total numbers of worms) taken on experimental units (culture pans) once per week for 5 weeks. Repeated-measures analysis of variance (mixed models procedure in the Statistical Analysis System version 9.2; SAS Institute, Cary, North Carolina) was used with the first-order autoregressive covariance structure (Littell et al. 1996); mean comparisons were made using a least-significant-difference test with an α of 0.05.

RESULTS AND DISCUSSION

Water quality metrics for aerated and nonaerated pans were generally similar (Table 1). Previous research identified thriving populations of *D. digitata* from commercial channel catfish ponds with a wide range of physicochemical parameters (Bellerud et al. 1995). As such, changes in water chemistry associated with evaporative concentration are not of concern as long as water chemistry remains within normal ranges associated with commercial channel catfish ponds. Additionally, because *D. digitata* inhabit channel catfish ponds with widely varied chemistries, selection of a pond for water collection should focus on the presence or absence of a substantial algal bloom rather than on physicochemical properties.

Dero digitata populations in the nonaerated treatment (mean ± SE = 580 ± 33 worms/pan) were significantly larger than populations in the aerated treatment (223 ± 88 worms/pan; Figure 1). Worm populations continued to increase beyond 5 weeks, but the population study was terminated at 5 weeks because the worms became too numerous to count. There was a significant interaction with treatment (aeration versus no aeration) over time. Populations in the nonaerated pans increased more rapidly over time than did the populations in the aerated pans. It remains unclear whether the agitation of the water caused by aeration is stressful to the worms, thereby reducing reproductive capacity, or whether these benthic oligochaetes prefer a low-oxygen environment similar to what is found at the benthic interface on the bottom of channel catfish ponds. Although dissolved oxygen concentrations were higher in aerated containers, nonaerated pans did not have substantially low dissolved oxygen concentrations (Table 1).

Myxozoan parasites infect oligochaetes from many different aquatic environments. As such, oligochaete cultures for life cycle studies have employed a wide variety of conditions, temperatures, and substrates. For propagation of *Myxobolus parviformis*, tubificid worms were held at 18°C in aerated 4-L tanks with a bottom substrate of coarse-grained sand (Kallert et al. 2005).

TABLE 1. Water quality variables measured weekly in aerated and nonaerated pans used for culture of the oligochaete *Dero digitata*.

		Week					
Variable	Treatment	0	1	2	3	4	Average
Total ammonia nitrogen (mg/L)	Nonaerated	0.6	0.3	0.3	0.3	0.5	0.4
	Aerated	0.5	0.5	0.4	0.5	0.6	0.5
Nitrite (mg/L)	Nonaerated	0.0	0.0	0.0	0.0	0.0	0.0
	Aerated	0.1	0.0	0.0	0.0	0.0	0.0
Chlorides (mg/L)	Nonaerated	6.6	12.3	13.7	15.0	19.0	13.3
	Aerated	8.0	12.3	16.3	16.3	23.0	15.2
pH	Nonaerated	8.9	8.5	8.5	8.6	8.7	8.6
	Aerated	8.9	8.8	8.8	8.8	8.9	8.8
Alkalinity (mg/L, as $CaCO_3$)	Nonaerated	103	154	199	188	233	176
	Aerated	114	160	199	211	228	182
Hardness (mg/L, as $CaCO_3$)	Nonaerated	86	131	160	154	216	150
	Aerated	97	137	165	160	199	152
Dissolved oxygen (mg/L)	Nonaerated	7.6	6.7	6.2	6.4	6.1	6.6
	Aerated	8.0	7.9	8.1	7.9	7.8	7.9
Temperature (°C)	Nonaerated	23.1	22.2	21.7	22.5	22.5	22.4
	Aerated	23.0	22.4	21.8	22.5	22.5	22.4

Limnodrilus hoffmeisteri and *Lumbriculus variegatus* used for the elucidation of the life cycle of the myxozoan *Chloromyxum auratum* were held in containers with 1 L of autoclaved coarse sand and 2 L of constantly aerated water maintained at 14°C (Atkinson et al. 2007). To study the intraoligochaete development of *Myxobolus bramae* and to determine the life cycles of *M. dispar*, *M. hungaricus*, and *M. portucalensis*, the oligochaetes *Tubifex tubifex* and *Limnodrilus hoffmeisteri* were cultured at 18–22°C with sterilized pond mud and regular tap water in either small aquaria (5 L of water) or plastic cups regularly supplied with freshwater to supplement loss from evaporation

FIGURE 1. Increase in mean (±SE) population size of oligochaetes *Dero digitata* reared at 21.7–23.1°C in aerated and nonaerated pans over 5 weeks in the laboratory.

(El-Mansy and Molnár 1997; El-Mansy et al. 1998; Molnár et al. 1999; Eszterbauer et al. 2000). Similar conditions were used to co-culture *Branchiura sowerbyi*, *Tubifex tubifex*, and *Limnodrilus hoffmeisteri* for elucidation of the life cycles of two myxozoans, *Thelohanellus hovorkai* and *Thelohanellus nikolskii* (Székely et al. 1998). The intraoligochaete development of *Myxobolus intimus* was determined by culturing *Tubifex tubifex* and *Limnodrilus hoffmeisteri* in a 500-mL, aerated plastic dish containing a thin layer of sterilized mud and 200 mL of water that was replenished regularly and held at room temperature (Rácz et al. 2004). In the present study, we determined that *D. digitata* prefer nonaerated conditions in the pan size we used, and although their natural habitat is the bottom mud of lakes, ponds, and rivers, use of an artificial substrate (white paper towels) did not prevent proliferation of the population and made subsequent isolation of these oligochaetes easier because they could be easily visualized.

The rapid population growth described in this study suggests that under favorable environmental conditions and in the absence of competing benthic organisms, a cultured population of *D. digitata* can increase very rapidly. The horizontal transmission of myxozoan parasites during the asexual propagation of the oligochaete host (Morris and Adams 2006) suggests that a small population of infected worms can become a large population of infected worms in a relatively short period, which may explain the acute nature of some PGD outbreaks (Wise et al. 2004). Additionally, the suggested rapid propagation of an infected oligochaete population may account for how actinospore concentrations in the closed systems of commercial channel catfish ponds can reach levels significantly higher than those
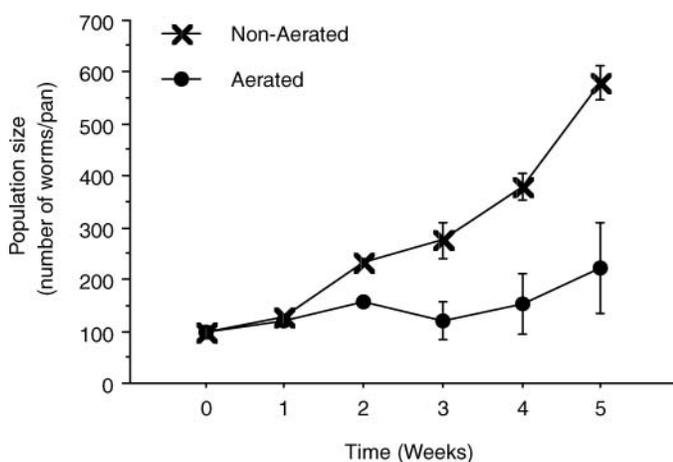
reported for other myxozoans in rivers and streams (Hallett and Bartholomew 2006, 2009; Griffin et al. 2009).

We have expanded our mass cultures of *D. digitata* from 200 individuals to over 70,000 individuals in a little over 1 year. The cultures are easily maintained and thrive under static conditions. Therefore, they are not limited to facilities equipped for aeration and can be housed wherever there is access to pond water and an autoclave and where water temperatures can be maintained at 22–25°C.

The techniques presented here provide researchers with a reliable, low-maintenance method of producing large populations of *D. digitata* in a controlled environment over short periods. Mass cultures produced in the laboratory can provide sufficient numbers of parasite-free individuals for conducting studies of the intraoligochaete development of *H. ictaluri* and studies focusing on the biological and chemical control of the oligochaete host.

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