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Triactinomyxon Production as Related to Rearing Substrate and Diel Light Cycle

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ABSTRACT. The culture of the aquatic worm *Tubifex tubifex*, the alternate host of whirling disease, is necessary to conduct research regarding triactinomyxon (TAM) viability, controlled infection studies, and methods of disease control. Presumed infected worms collected from the field may produce TAMs for several months, but production generally decreases after a few months. To ensure a stable supply of TAMs, we investigated the effects of rearing substrate on TAM production and worm survival. We also analyzed the time of TAM release during 24-h periods divided into 12 h light:12 h dark.

Mixed species of field-collected worms were placed into culture with the following substrates: organic debris, silt, and sand. The controls were reared without a substrate. After nine weeks, worms in the silt substrate produced significantly more TAMs than worms in either the control or sand substrate. The worms in silt also produced significantly more TAMs than the worms in organic debris in four of seven bi-weekly samples where significant treatment differences were found. The estimated average daily TAM production during weeks 9–27 averaged 149 for control worms, 754 for the organic, 231 for the sand, and 2,573 for worms in the silt substrate. By the end of the study (week 27), average total worm weight for the controls was 0.8 g, 3.3 g for the organic, 2.6 g for the sand, and 7.5 g for the silt. The silt treatment had significantly higher worm mass than any of the other treatments, and was the only treatment to increase in mass from the starting weight of 4.5 g.

During two separate periods, worms were also observed for TAM release within 24-h cycles composed of 12 h light:12 h dark cycles. During the first period, there were slightly more TAMs released during the light period (59%) compared with 41% for the dark. For the second period, slightly more TAMs were released during the light period (56%) compared with 44% for the dark. For both tests however, these differences were not significant. These results indicate that, at least in the laboratory, there is no relationship between daylight hours and TAM production.

The culture of the aquatic worm *Tubifex tubifex*, the alternate host of *Myxobolus cerebralis*, has been conducted for several years at the Fisheries Experiment Station (FES, Logan, Utah) to support reseatch on triactinomyxon (TAM) viability, controlled infection, and disease control. Traditionally, presumed infected worms were collected from a field site, brought to the FES laboratory, cleaned of organic debris and sediment, and placed in culture. The worms typically produced TAMs for several months (~20,000 TAMs/culture per d) after which production decreased and the worms were either combined with other cultures or discarded. Using this approach, TAM production could not be predicted, thus compromising research projects. Because of these deficiencies, we undertook several projects to determine if TAM production could be increased, and the survival of worms could be enhanced.

The first study tested the hypothesis that different types of rearing substrates (sand, silt, or organic debris) could enhance worm survival and TAM production. In a stream or river environment, we can generalize that *T. tubifex* is found in higher numbers in shallow regions of low current velocity in substrates rich in fine sediment and organic matter compared with other habitat types (Lazim and Learner 1987). Sauter and Güde (1996) found that in Lake Constance (Germany), sediments composed of 70–100% clay and silt sustained higher densities of *T. tubifex* compared with lower levels of silt and clay. In a more detailed analysis, McMurtry et al. (1983) studied the preferences of *T. tubifex* and *Limnodrilus hoffmeisteri* for

two types of substrate that were similar in physical structure but differed in elemental and organic composition. Mixed species groups and individual *T. tubifex* worms preferred the sediment that was lower in organic matter and elemental content but that had a higher level of bacterial colonization.

For the second part of our research we analyzed the time of TAM release during 24 h periods divided into light and dark cycles. Yokoyama et al. (1993) reported a circadian rhythm in the release of *Echinactinomyxon* spp. spores by the oligochaete *Branchiura sowerbyi*. When kept under a 12 h light and 12 h dark regiment, the highest numbers of spores were released between 2200 and 0200 hours. When the photoperiod was reversed, the release of actinospores was also reversed, indicating that the timing of actinospore release was cued by the daily light:dark cycle.

METHODS

Substrate Test

Worms were collected from a private aquaculture facility that has been infected with *M. cerebralis* since the mid 1990s. The worms were separated from the sediment by rotation on an oscillation table (35 rpm) until a clean collection of worms was obtained. The worms were then divided into 12 separate containers with 4.5 g per container. Worms were not separated by species, but past collections have been composed chiefly of *T. tubifex* and *L. hoffmeisteri*. The containers were plastic 400 mL beakers that contained control and experimental substrate types in triplicate. The substrates included organic debris, silt, and sand. The organic substrate was the debris collected when the worms were separated after field collection. It consisted of pieces of plants, seed husks, decomposed wood, pine needles, and empty clam and snail shells. The average size of the particles was 2.8 × 1.2 mm. The silt was collected from the lower Logan River and was run through a series of sieves so that 92% of particles were less than 125 μm. The sand was purchased from a local landscape company, with the following size composition: 27% less than 250 μm, 32% 250–500 μm, 20% 500 μm-1 mm, 15% 1–2 mm, and 6% greater than 2 mm. The controls had no substrate. All three types of substrate were autoclaved at 100°C for 60 min prior to use, thereby ensuring that bacteria and other living organisms were killed. Each beaker contained 2 cm of substrate and 6 cm of well water.

Beakers were placed into a refrigerator, aerated by an air stone, and maintained at 15°C throughout the study. Lighting was provided by a full spectrum fluorescent bulb on a 12 h light:12 h dark basis. Individual cultures were fed 0.5 g of spirulina pellets on a weekly basis.

For TAM enumeration counts were made three times per week during weeks 1–7, on a weekly basis during weeks 8–12, and on a biweekly basis during weeks 13–27. Regardless of sampling regimen, water exchanges were made on Monday, Wednesday, and Friday of every week. TAM collection and enumeration were accomplished by the following methodology. For TAM collection, water was individually drawn from each replicate container via a siphon tube onto a 20 μm Nitex mesh filter (Aquaculture Research/Environmental Associates, Homestead, Florida). The material retained on the filter was then washed into a 50 mL plastic test tube and the total volume recorded. From each tube, three 50 μL subsamples were withdrawn and placed into individual glass test tubes. This quantity was then stained with crystal violet, prepared as a wet mount, TAM counts made, and total TAM production calculated.

For tracking TAM production over the course of the study, data from weeks 1–12 were pooled into biweekly units and average TAM production was calculated based on the number of sampling events that occurred during that specific two-week period. These discrete biweekly values were then used to test for significance by a one-way analysis of variance (ANOVA), which was also used to test for differences in the final worm weights. For each replicate, all worms were removed from their respective sediments at the conclusion of the test, cleaned of debris, and their gross weight was measured. Thirty worms from each replicate were also preserved in 10% buffered formalin. After seven days they were transferred to 70% alcohol and sent to Aquatic Resources Center (College Grove, Tennessee) for species identification.

Diel Light Cycle Test

For this work, two different sets of experimental cultures were checked for TAM release during two separate time frames. The cultures used during the first period were part of a study on infectivity and TAM recovery on different mesh sizes. For the second period, the cultures from the substrate test were used. For both tests, cultures were maintained at 15°C with aeration provided to the individual cultures. Lighting was provided by a full spectrum

fluorescent bulb on a 12 h light:12 h dark basis. Light intensity was not measured for the first test, but was 73 lux at the air/water interface for the second test. For the first test, water from six cultures was exchanged 12 h before the first TAM harvest beginning at 1930 hours on 13 March 2000. Water was siphoned onto a 100 µm Nitex filter placed over a 20 µm Nitex filter. The 100 µm prefilter was used to remove debris and organisms found in the worm cultures. The retentate was placed into a vial and fixed with 5% formalin, and TAM counts were made the following day according to the methods previously discussed. This procedure was repeated every 12 h at 0730 hours and 1930 hours for each culture through 15 March 2000. The experiment was extended an additional four days, during which time a 10 µm Nitex filter was used for comparison with the 20 µm Nitex mesh.

Concerns about the accuracy of lighting manipulation and worm acclimation time from the first study lead us to repeat the test. For the second test the protocol listed above was repeated during the week of 4 December 2000, using the three silt treatment worm cultures from the substrate study discussed above, as well as one of our main TAM producing cultures. This second test was run for a total of 4 light cycle and 4 dark cycle samples over a period of four days. Samples were examined for TAMs immediately after collection without fixation. For both tests, data from a given 24-h cycle were expressed as a percentage of TAM production for a 12 h light or 12 h dark cycle based on the total production from that 24-h period. Values from a given 24-h cycle were then analyzed for significance by the Wilcoxon Signed Rank Test.

RESULTS AND DISCUSSION

Substrate Test

This test indicated a significant impact of culture substrate type on TAM production and worm survival. Worms in the silt substrate produced significantly more TAMs than either worms in no substrate (controls) or worms in the sand substrate from weeks 9–15, and then again from weeks 19–23 (Figure 1). The worms cultured in the silt substrate also produced significantly more TAMs than the worms in the organic substrate in four of seven bi-weekly samples where differences were

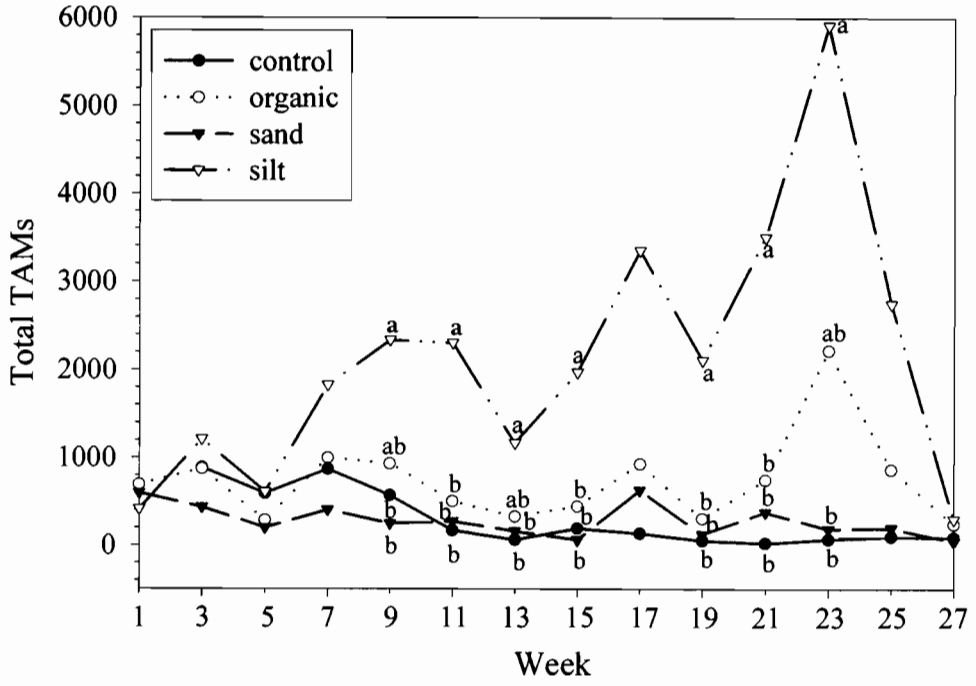

Figure 1. Average (N = 3) biweekly TAM production of tubifex worms reared in organic, sand, silt, or no substrate. A letter next to a treatment symbol indicates a significant difference ($P \leq 0.05$) within a given biweekly period.

found (weeks 11, 15, 19, 21). No significant differences were found in TAM production between treatments during weeks 17, 25, and 27 due to wide variation in TAM production within replicates. The estimated daily TAM ($N = 3$) production during weeks 9–27 averaged 149 TAMs for the controls, 754 for the organic, 231 for the sand, and 2,573 for the silt substrate.

The ability to assess worm survival by gross worm weight was compromised by the apparent reproduction within the silt treatments, although by the end of the study (week 27), total average worm weight for the controls was 0.8 g, 3.3 g for the organic, 2.6 g for the sand, and 7.5 g for the silt. The silt treatment had significantly higher worm mass than any other treatments ($P < 0.001$), and was the only treatment to increase in mass from the starting weight of 4.5 g. There were no significant differences between treatments in the final oligochaete species composition. The largest proportion of worms, 47%, were immature tubificids with hair and pectinate chaetae. Immature tubificids with bifid chaetae comprised 17% of the total. The identifiable adults were broken down by the following percentages: *T. tubifex*, 16%; *Quistadrilus multisetosus*, 9%; *L. hoffmeisteri*, 8%; *L. profundicola*, less than 1%; *Potamothrix bavaricus*, less than 1%.

The ability to explain differences in the treatment responses is compromised by our limited evaluation of the physical composition and size description of the various substrate types. It is clear that worms in the silt substrate produced more TAMs and survived better than the other treatments. Whether this difference can be attributed to the physical makeup of the substrates or to the elemental and bacterial composition is unknown. Lazim and Learner (1987) demonstrated that *T. tubifex* shows a clear preference for silt-clay substrates compared with sand. They also showed that worms demonstrated a preference for substrates that contained leaf litter over substrates that did not. It has been shown by McMurtry et al. (1983) that when tubificids are given a choice of two substrates, one of which contains a richer concentration of elements and organic compounds and the other a higher level of bacteria, the worms preferred the substrate containing the higher concentration of bacteria. In our test all substrates were autoclaved prior to use and all cultures were fed the same amount of spirulina. It is possible that the silt substrate contributed more to microbial colonization and therefore better worm survival.

Diel light cycle

For both tests conducted there was not a strong relationship between the light or dark cycle and TAM production. For the first test there was wide variation in TAM production (Figure 2). Overall, a slightly higher percentage of TAMs was released during the light cycle, 59%, compared with 41% for the dark cycle, although this difference was not significant ($P = 0.219$). Only one of the seven cycles showed a significant difference in TAM production between the two periods. During the fifth cycle, significantly ($P = 0.031$) more TAMs were released during the light cycle (69%), compared with 31% during the dark cycle. The results from the second test were similar to the first (Figure 3). Overall, for the four cycles, an array of 56% of TAMs were released during the light cycles compared with 44% for the dark. For the second 24-h cycle, significantly ($P = 0.022$) more TAMs were released during the light cycle, 72%, compared with 28% for the dark. No other significant relationships were found. Our results indicate that, at least in the laboratory, there does not appear to be a relationship between light or dark hours and TAM production. These results differ from those of Yokoyama et al. (1993) who revealed a circadian rhythm of actinospore release by the oligochaete *B. sowerbyi*. They observed a peak in actinospore release during 2200–2400 hours. It is difficult to determine whether such laboratory results are transferable to the natural environment. In the field, the seasonality of TAM production may be related to a changing diel light cycle, water flows, water temperatures, or timing of worm infection.

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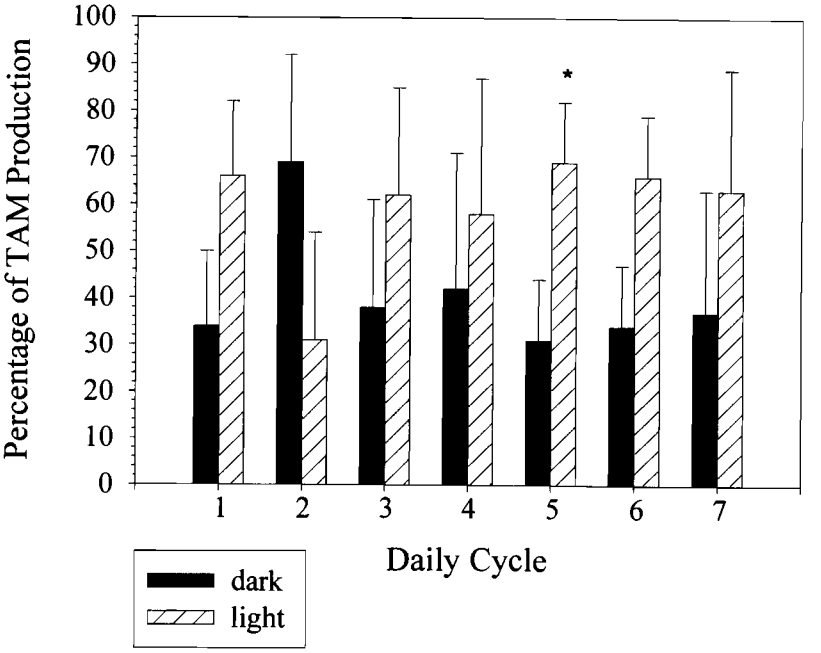

Figure 2. Diel TAM release: Test 1. Average (N = 3, ± SD) percentage of daily TAM release during either the 12 h light or 12 h dark period within a 24-h cycle, sampled over seven cycles. An asterisk above a bar indicates a significant difference ($P \leq 0.05$) within a given 24 h cycle.

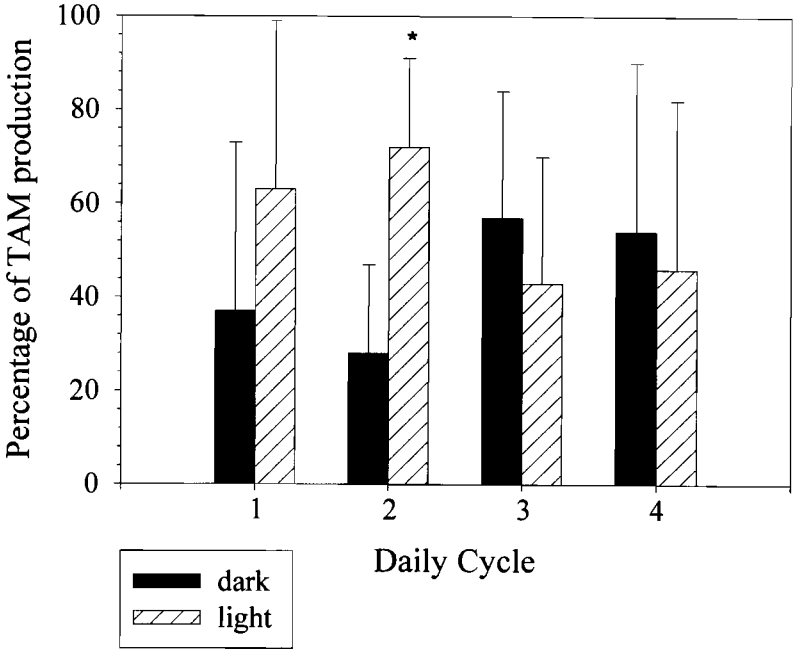

Figure 3. Diel TAM release: Test 2. Average (N = 3, ± SD) percentage of daily TAM release during either the 12 h light or 12 h dark period within a 24 h cycle, sampled over four cycles. An asterisk above a bar indicates a significant difference ($P \leq 0.05$) within a given 24 h cycle.