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Extrusion of Polar Filaments of the *Myxobolus cerebralis* Triactinomyxon by Salts, Electricity, and Other Agents

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ABSTRACT. The ability of several compounds to discharge the polar filaments of polar capsules of the triactinomyxon stage of *Myxobolus cerebralis* was tested. Premature polar filament discharge may provide a means for preventing the infective stage of myxozoan parasites from attaching to fish hosts. The discharge regimes evaluated included high and low pH, chloride and phosphate salts, calcium chelators, direct current, mucus, tricaine methanesulfonate anesthetic, neurochemicals, and chemosensitizing agents that are effective discharge agents for members of the phylum Cnidaria. Polar filament discharge, in response to HCl or NaOH, did not differ from controls until pH levels dropped to 1.1 or increased to 11.7. Among the chloride salts tested (NaCl, KCl, $CaCl_2$, NH_4Cl, $MgCl_2$), discharge increased at concentrations ranging from 3.1 to 100‰. Discharge varied among the salts tested, peaking at 71% for 100‰ KCl; however, the phosphate salts K^+ and Na^+ did not differ in discharge ability. Comparison among KCl, KI, and KPO_4 indicated that Cl^- was significantly more effective at both 6.2‰ (45.6% discharge) and 12.5‰ (57.8%) than the other anions. The calcium chelators sodium citrate and EGTA did not induce any significant increase in discharge, nor did the neurochemicals angiotensin, bradykinin, and acetylcholine chloride. Compounds, such as N-acetyl neuraminic acid, proline, and glutathione, that have been reported as chemosensitizers for cnidae discharge among cnidarians, were ineffective discharge agents for triactinomyxon polar capsules. Mucus from rainbow trout or bovine submaxillary gland failed to significantly increase discharge. Attempts to combine mucus with force (stirring rod) or a 0.45 Gauss magnetic field did not increase discharge rates. However, using an electroporator to administer direct current, the discharge rate increased with pulse length (up to 99 μsec) and the number of pulses (0–25). Maximum discharge (98%) and mortality (100%) was observed after 25 99-μsec pulses of 3 kV. Results with electricity indicate a potential for using direct current as a means of disinfection. The data suggest some similarities and differences with similar research on Cnidaria that is discussed.

Myxozoa is the animal phylum characterized by spores made of several cells forming 1–7 shell valves, 1–2 amoeboid infective sporoplasms, and 2–7 polar capsules (Lom and Dyková 1992). The polar capsules each contain an eversible polar filament that serves to anchor the parasite to the host tissue. The potential for premature polar filament discharge to disarm Myxozoan parasites has been recognized by previous researchers (Hoffman et al. 1965).

The myxozoan parasite *Myxobolus cerebralis* causes whirling disease, an important disease of salmonid fishes. The infective stage of the parasite to fish is known as the triactinomyxon (Markiw 1992a). This stage is also broadly classified as an actinospore, a group of organisms once thought to be separate species but now considered alternate stages of myxozoan parasites (Kent et al. 1994). The triactinomyxon has 3 polar capsules, with eversible polar filaments used to attach to the fish host. Research on the discharge mechanisms of the polar capsules could potentially lead to methods for control of whirling disease and other myxosporean diseases.

Research on polar filament discharge in Myxozoa has primarily been conducted on the myxospore stage of the parasite. A number of agents have been tested, but only a few have been successful. These discharge agents include strong bases, such as KOH (Yasutake and Wood 1957; Uspenskaya 1957; Lewis and Summerfelt 1964), saturated urea (Lom 1964), hydrogen peroxide (Kudo 1918; Lom 1964), and direct pressure on wet mounts (Herrick 1941; Guilford 1963; Iversen 1954).

Discharge of the polar filaments of the actinospore stage of myxozoan parasites has been investigated for a few species. Yokoyama et al. (1995) were able to discharge filaments of raabeia-type

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actinospores of *Myxobolus cultus*, using mucus from a variety of fish species or bovine mucin. Mucus has also been effective for discharge of *Zschokkella nova* actinospores (Uspenskaya 1995) and aurantiactinomyxon, neoactinomyxum, echinactinomyxon, and raabeia forms of actinospore (Xiao and Desser 2000). However, for *M. cerebralis* triactinomyxon actinospore, rainbow trout mucus was ineffective as a discharge agent (El-Matbouli et al. 1999).

The natural discharge mechanisms for both the myxospore and actinospore stages in Myxozoa have yet to be described. Much more research on discharge has been conducted on cnidae, specialized structures with an eversible filament characteristic of the phylum Cnidaria (Mariscal 1974). The development of polar capsules and cnidae are very similar (Lom and de Puytorac 1965), so knowledge gained by researchers studying cnidae may apply to polar capsule discharge. The literature on cnidae discharge is vast (see reviews by Pantin 1942; Picken and Skaer 1966; Mariscal 1974; Tardent 1988; Thorington and Hessinger 1988). Numerous discharge agents have been tested, including various salts, calcium chelating agents, electricity, and strong acids and bases that are the focus of this article (Pantin 1942; Yanagita and Wada 1953; Salleo et al. 1983b). Effects of discharge agents may vary depending upon whether the cnida is still within the cnidocyte (in-situ) or isolated after being artificially expelled from the cnidocyte. For example, in-situ cnidae discharged at pH values below 4 and above 11, whereas isolated cnidae discharged only at pH values of 2 and below (Blanquet 1970). Sodium citrate and EGTA are calcium chelators that have been effective in discharging isolated cnidae (Hidaka and Mariscal 1988). Pantin (1942) found that electrical shocks (240 V from a 2 μF condenser and passed through a 400 Ω potentiometer) stimulated cnidae discharge. Among anions tested by Salleo et al. (1983b), discharge potency of sodium salts varied as follows: $SO_4^{2-} < CH_3COO^- < F^- < Cl^- < Br^- < NO_3^- < I^- < ClO_4^- < SCN^-$. Santoro and Salleo (1991) found that high K^+ concentrations induced massive discharge of cnidae.

Based upon the previous research, discharge of the polar filaments of *Myxobolus cerebralis* triactinomyxons was attempted in a series of tests. The effects of pH, various salts, calcium chelating agents, chemosensitizing agents, neurochemicals, and direct electrical current were evaluated as potential discharge agents. Survival of discharged sporozoites was also of interest. If the sporozoites were still alive, this would be useful for in-vitro tissue culture and research on the sporozoite. If dead, the treatment could potentially be applied to control the parasite.

METHODS

The triactinomyxon stock solutions were made by filtering the supernatant from worm cultures through a 20 μm mesh screen and rinsing the retentate into a vial with hatchery well water. The hatchery well water had a pH of 7.5–8.0, total hardness of 222 mg/L as $CaCO_3$, and total alkalinity of 222 mg/L. For each test, the triactinomyxons were categorized after treatment, as fired, unfired, or empty. Triactinomyxons with at least one of the three polar filaments discharged were categorized as fired. Unfired triactinomyxons were those that retained all their filaments within the polar capsules. The 'empty' category was for triactinomyxons without a spore body. Triactinomyxons recorded as empty were included in the total for percentage calculations. To adequately reflect the effect of pH on discharge, the empty and fired categories were combined for analysis and graphing. Percentage data were arc-sine transformed before analysis, with either *t*-tests or analysis of variance (ANOVA).

Vital staining with propidium iodide (PI) and fluorescein diacetate (FDA) was used to assess viability (Jones and Senft 1985; Markiw 1992b) of triactinomyxons exposed to KCl or direct current. An aqueous stock solution of PI (52 mg/mL) was made and kept frozen (-40°C) in 1-mL aliquots. Vials were then thawed and ready for use on the test day. A concentrated stock solution of FDA (5 mg/mL acetone) was also kept at -40°C. On the day of an experiment, 100 μL of the concentrated FDA solution was added to 8 mL of well water to make a working stock solution of FDA stain.

Effect of pH on Discharge

Acid pH was achieved using HCl and basic pH using KOH. The ranges of pH tested were 3.8–1.1 and 11.7–12.9. Acids and bases were diluted with de-ionized water to achieve the desired pH. These solutions were combined with equal volumes of triactinomyxon stock solution (pH 7.5–8.0) on slides. The resulting pH experienced by triactinomyxons was measured separately in test tubes with a freshly calibrated pH meter (Orion, model SA 720). After mixing triactinomyxon and pH solutions on a slide, the slide was immediately cover slipped and

observed. Time required for reading one slide (i.e., the maximum exposure time) averaged 3.5 min.

Effect of Salts on Discharge

The effects of various chloride salts (NaCl, KCl, $CaCl_2$, NH_4Cl, $MgCl_2$) on polar filament discharge were tested at concentrations ranging from 3.1 to 100‰. In addition, other salts ($NaPO_4$, KPO_4, and KI) were evaluated for a narrower range of concentrations, to determine if certain anions or cations were better discharge agents. Salts were evaluated by combining a salt stock solution of known concentration with equal volumes of triactinomyxon stock solution on glass slides.

For the viability test, 30 µL of triactinomyxon stock solution, 30 µL each of FDA and PI and 90 µL of 20‰ KCl, were added to a microscope slide. This solution was mixed carefully using the edge of a cover slip, producing a 10‰ KCl exposure. The slides had cover slips placed on them and were incubated in an opaque plastic chamber in a refrigerator for 30 min prior to reading.

To determine the effect of calcium ion removal on discharge, two calcium chelators were tested. These were sodium citrate and EGTA (ethelenebis [oxyethylenenitrilo] tetra-acetic acid). A 50-mM solution of sodium citrate was put on three slides with equal volumes of triactinomyxon solution, yielding 25-mM exposure concentrations. Slides were then incubated for 60 min, and discharge was recorded as noted above. In addition to controls (triactinomyxon stock solution only), slides were made in which a NaCl solution of the same molarity was mixed with the triactinomyxon stock solution. This was to compare the discharge effect of sodium between the two solutions. EGTA is only soluble in solutions with relatively high pH, so it was placed in de-ionized water into which NaOH was titrated until all the EGTA dissolved. The resulting pH was 9.4. The EGTA concentration, after dilution with an equal volume of triactinomyxon stock solution, was 12.5 mM. To rule out pH as the factor causing firing, a solution of NaOH and de-ionized water of pH 9.4 was used as a control. Slides were made, incubated, read, and recorded, as in the sodium citrate test.

Electricity and Discharge

Electrical pulses of direct current were also examined as a method for firing triactinomyxon polar filaments using an electroporator (model T 820, BTX, San Diego, California). Electroporation involves administering short, high-voltage pulses of electricity to a cell (Palaniappan et al. 1990). We put 100-µL samples of triactinomyxon stock solution into a 1 mm wide chamber, through which the electricity was pulsed. Voltage (1 or 3 kV), pulse length (0–99 µsec), and the number of pulses (0–25) were manipulated to evaluate effects on triactinomyxon polar filament discharge. Two slides of 30 triactinomyxons each were counted for each treatment combination, but empty triactinomyxons were not included in the total or percentage calculations.

Viability of triactinomyxons was assessed after treating 100 µL of triactinomyxon stock solution to a varying number (1–25) of 3 kV, 99 µsec pulses of direct current. After electrical exposure, the treated solution was put on a slide and stained with 50 µL each of FDA and PI. Two slides of 30 triactinomyxons each were made for each treatment. Cover slips were placed on the slides, which were incubated in a dark chamber in a refrigerator for at least 30 min before reading.

Effects of Mucus and Tricaine Methanesulfonate on Discharge

Three tests were conducted in which mucus from rainbow trout *Oncorhynchus mykiss* was evaluated as a discharge agent. Additional tests were conducted to evaluate the effect of tricaine methanesulfonate (MS-222) or bovine mucin on discharge of triactinomyxon polar filaments. In the first test, mucus was scraped from a 250-mm fish using a microscope slide. A drop of the mucus, applied to a microscope slide, was mixed with 50 µL of triactinomyxon stock solution harvested the same day. The corner of a cover slip was used to gently mix the two solutions on the slide. The slide was cover slipped and viewed at 400×, either immediately or after 30 min. Triactinomyxon discharge was categorized as noted above.

A subsequent test was conducted to determine if mucus in combination with mechanical contact would trigger polar filament extrusion. Mucus, scraped with a glass slide from a rainbow trout, was applied to a glass rod. The rod was stirred for 1 min, within a 1-mL suspension of triactinomyxons in well water. The mucus on the rod was transferred to a microscope slide for examination, and 200 µL of the triactinomyxon solution remaining in the test tube was added to the slide as well. Slides were examined at 400×, and discharge was categorized as noted above.

In the third test, the effect of a refrigerator magnet on discharge was tested with or without mucus. The magnet strength was 0.45 Gauss, as measured by a gaussmeter. Mucus, scraped from the side of a rainbow trout (250 mm) anesthetized with tricaine, was transferred to each of 3 slides. Triactinomyxons, from a stock solution in hatchery well water, were added (100 uL) on top of the mucus. These slides were exposed to the magnetic field for 1 min, by placing the slide directly upon the magnet. Control slides, of triactinomyxon stock only, were not exposed to the magnetic field. Three slides of mucus and triactinomyxons were not exposed to the magnetic field. Three slides were exposed to the magnetic field for 1 min without any mucus present. A total of 50–100 triactinomyxons were viewed on each slide.

Due to contradictory results from the previous tests, the potential for tricaine methanesulfonate to act as a discharge agent was investigated in two series of tests. The first series evaluated discharge at concentrations ranging from 0.091 to 8,182 mg/L (after mixing with triactinomyxon stock). In the second series, tricaine concentrations of 50, 100, 200, and 300 mg/L were evaluated. These solutions were made by diluting appropriate amounts of a 100 mg/mL stock solution with de-ionized water. For each concentration, tricaine and triactinomyxon stock were mixed on each of 3 slides, and 100 triactinomyxons were observed on each slide. Control slides were made by mixing equal amounts of triactinomyxon stock and de-ionized water on each of 3 microscope slides.

Bovine submaxillary mucin (Type 1-S) diluted with de-ionized water was mixed with triactinomyxon stock solution (50 uL of each) to achieve concentrations of 5, 50, 500, and 1000 mg/L. Three slides were observed for each concentration, categorizing 100 triactinomyxons per slide as noted above. Three control slides were made directly from the triactinomyxon stock solution.

Other Discharge Agents

Glutathione was mixed with an equal amount (200 μL) of freshly-harvested triactinomyxons in two microcentrifuge tubes. The glutathione stock solutions were prepared with de-ionized water. After mixing in the tubes, glutathione concentrations were either 0.5 or 0.05 M. After 10–15 min in the tubes, 180 μL of the mix was transferred to a slide. Discharge was categorized as noted above, using 2 slides per concentration. An additional pair of slides was prepared, on which either 1.0 or 0.1 M glutathione was dried on each slide. To each slide, 180 μL of triactinomyxons was added and incubated for 1–3.5 h. Triactinomyxons on the slide were categorized as noted above.

Serial dilutions of a 0.01 M proline solution were prepared using de-ionized water. Equal quantities of proline and triactinomyxon stock solution (50 μL) were mixed on a microscope slide, covered with a cover slip, and immediately examined. The proline concentrations after mixing were 5×10^{-3}, 5×10^{-4}, 5×10^{-5}, and 5×10^{-6} M. Three slides, with a total of 92–100 triactinomyxons per slide, were observed for each concentration.

NANA (N-acetyl neuraminic acid, type VI from *E. coli*) was tested at concentrations (after mixing) of 0.5×10^{-7}, 1.0×10^{-7}, 2.0×10^{-7}, and 4.0×10^{-7} M. On each of 3 slides per concentration, 100 triactinomyxons were observed. For the NANA and proline tests, control slides were made by mixing 50-μL triactinomyxon stock solution with 50-μL de-ionized water. Discharge was categorized as noted above, for replicate slides.

Other agents evaluated included three neurochemicals. A 0.79-mM stock solution of synthetic salmon angiotensin-1 was made with de-ionized water. Serial dilutions with de-ionized water provided test concentrations of 0.395, 0.0395, and 3.95×10^{-3} mM, after mixing equal amounts (50 μL) of angiotensin and freshly harvested triactinomyxon stock on a microscope slide. Two slides, with 32–71 triactinomyxons on each, were observed for each concentration. Acetylcholine chloride was evaluated at concentrations (after mixing with triactinomyxons) of 1.13, 11.3, and 113 mg/L. Bradykinin concentrations (after mixing with triactinomyxons) tested were 5×10^{-4}, 5×10^{-5}, 5×10^{-6}, 5×10^{-7}, and 5×10^{-8} M. For both the bradykinin and acetylcholine chloride tests, 3 slides were observed per concentration, categorizing up to 100 triactinomyxons per slide as noted above. For each of the neurochemical tests, 3 control slides were made in which 50-μL de-ionized water was mixed with triactinomyxon stock.

RESULTS

Effect of pH on Discharge

Discharge of triactinomyxon polar filaments increased toward both extremes of the pH range (Figure 1). However, the percentage discharged

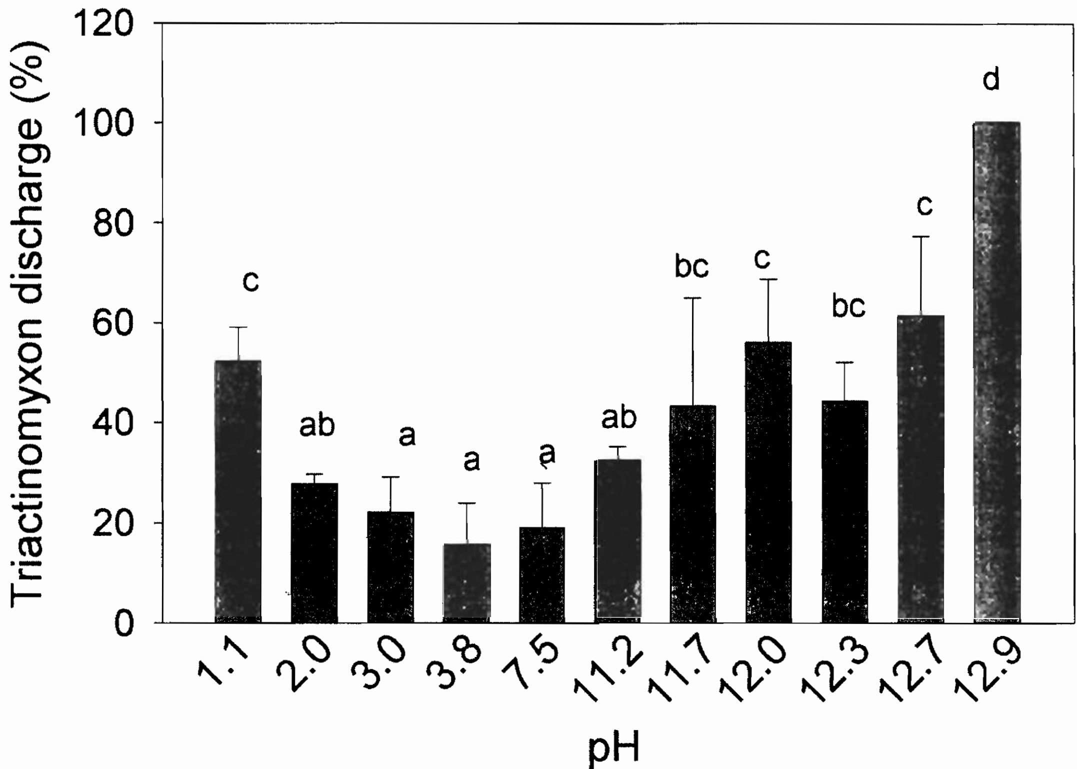

Figure 1. Mean percentage of *Myxobolus cerebralis* triactinomyxons with discharged polar filaments (±SD; N = 3 slides of 30 triactinomyxons each, fired and empty categories combined) after exposure to high and low pH. Significant differences ($P \leq 0.050$) between pH treatments are noted by a different letter.

was greater at the basic end of the pH spectrum. A significant difference ($P \leq 0.050$) from controls was not found until the pH was at or below 1.1 or above 11.7. A pH of 12.9 was needed to discharge 100% of polar filaments.

Effect of Salts on Discharge and Viability

Comparison of discharge among the chloride salts indicated that some cations were significantly more effective than others (Figure 2). K^+ tended to have higher discharge percentages than the other ions, though the difference was significant only at 50‰ (ANOVA, P = 0.039). NH_4^+ had significantly lower discharge percentages at 6.2‰ and 12.5‰ than for K^+, Mg^{2+}, or Ca^{2+}. There was no significant difference in the percent discharged (t-test, P = 0.28) between $NaPO_4$ (21.1 ± 5.1%, SD) and KPO_4 (26.7 ± 5.8%) at 6.2‰. Similarly, at 12.5‰, there were no significant differences in discharge between $NaPO_4$ (30.0 ± 6.7%) and KPO_4 (42.2 ± 8.4%, P = 0.12). Generally, as salt concentration increased, the discharge percentage increased, peaking at 71% for 100‰ KCl. Most of the salts induced discharge, indicating that high osmolarity in general can induce discharge. Survival was compromised by the use of KCl. After exposure to 10‰ KCl for 30 min, only 38% of triactinomyxons remained viable, compared with 74% with controls.

Discharge differences among anions were also significant. Comparison of KCl, KI, and KPO_4 indicated that Cl^- was significantly more effective ($P \leq$ 0.02) at both 6.2 (45.6% discharge) and 12.5‰ (57.8%) than the other anions. Comparison of NaCl and $NaPO_4$, at 12.5‰, also indicated chloride ion was more effective (44.4 versus 30.0% discharged, respectively; t-test, P = 0.02).

Both sodium citrate and NaCl, at tested concentrations, had significantly more fired polar filaments (P = 0.010, 0.015) than did controls (Table 1). However, there was no significant difference between NaCl and sodium citrate treatments (P = 0.336). After 1 h, both the EGTA and pH 9.4 solutions produced significantly more triactinomyxons

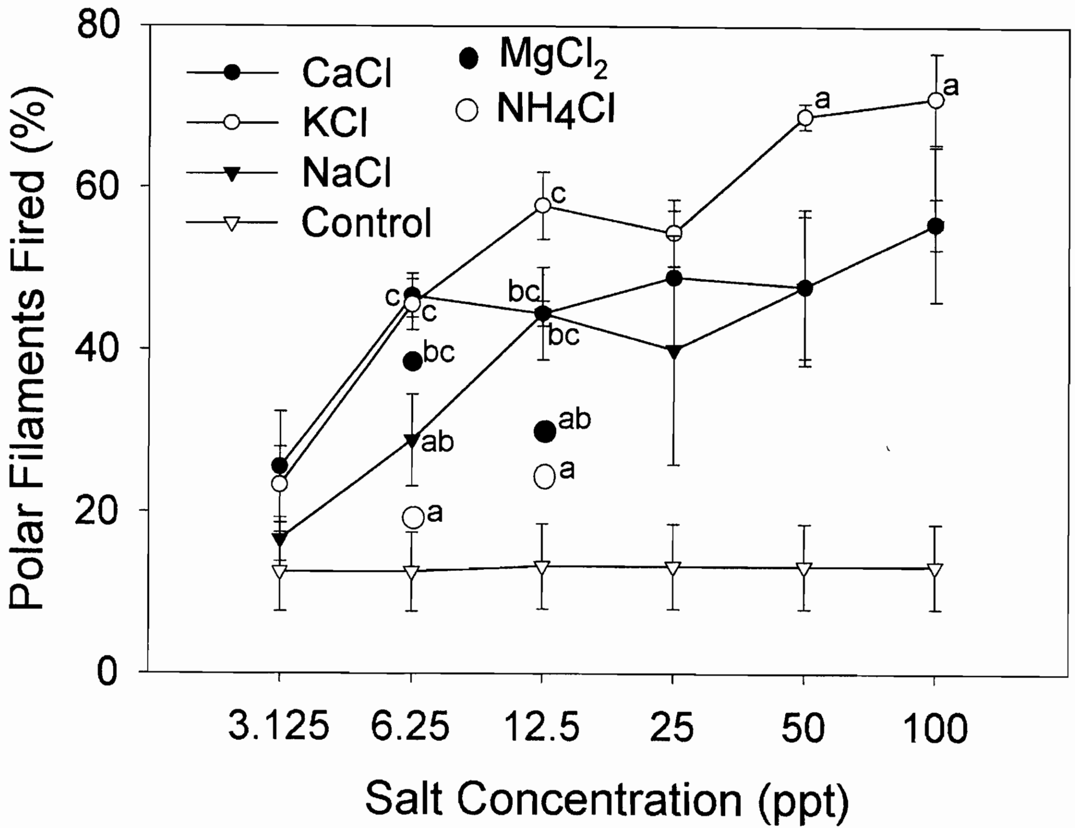

Figure 2. Mean percentage of *M. cerebralis* triactinomyxons with discharged polar filaments (±SD; $N = 3$ slides of 30 triactinomyxons each) after exposure to various concentrations of chloride salts. Within a given concentration, means that are significantly different (ANOVA, $P \leq 0.050$) among the salts are noted by a different letter. Salt concentrations reflect actual concentration after mixing on the slide.

Table 1. The effect of sodium citrate and EGTA on the percentage (Mean ± SD; $N = 3$) of discharged polar filaments of *Myxobolus cerebralis* triactinomyxons after 1 h incubation. Data for controls for the cation and pH effects are shown as well.

Treatment	Polar filament discharge (% ± SD)
25 mM NaCl	32.22 ± 10.99*
25 mM sodium citrate	22.22 ± 6.84*
12.5 mM EGTA (pH 9.4)	35.55 ± 12.27*
NaOH (pH 9.4)	16.66 ± 7.20*
Control	0.33 ± 0.47

*Denotes treatments that are significantly different ($P \geq 0.050$) from control.

with fired polar filaments ($P = 0.015$, 0.033) than controls (Table 1). EGTA treatments extruded more polar filaments (36%) than did the pH 9.4 NaOH solution (17%), but the difference was not significant ($P = 0.134$). It appears that most of the firing observed with EGTA can be explained by the pH of the solution as opposed to the chelating action of EGTA.

Effect of Electricity on Discharge

Polar filament discharge varied with electrical pulse length. At 1 kV, a 1-μsec pulse induced 18% of triactinomyxons to fire polar filaments. Pulse lengths of greater than or equal to 5 μsec significantly increased the percentage of polar filaments fired (Figure 3). When the pulse length was increased to 99 μsec (electroporator maximum), 76.7% of triactinomyxons had discharged filaments after a single pulse. There was no significant difference in the percent discharged between a single 99-μsec pulse of 1 kV (74.0%) and the machine maximum of 3 kV (76.7%; $P = 0.877$). At a constant pulse length of 99 μsec and a voltage of 3 kV, the number of pulses significantly increased the number of triactinomyxons firing polar filaments (Figure 4). There was no significant increase in discharge if the number of pulses increased from 1 to 20 ($P = 0.737$). However, after 25 pulses, 98% of triactinomyxons had fired filaments; this was a significant increase from a single pulse ($P = 0.028$). The correlation between the number of pulses and the percentage fired was significant ($P = 0.002$, $r^2 = 0.50$, ordinary least-squares regression). The correlation between pulse length and polar filament discharge was also significant ($P = 0.015$, $r^2 = 0.24$).

Viability was also affected by electricity. After a single 3-kV, 99-μsec pulse, 83% of triactinomyxons were dead, a significant increase from controls. After treatment with various numbers of pulses of direct current, a significant correlation ($r^2 = 0.914$, $P < 0.001$) was found between the percentage of triactinomyxons with fired polar filaments and the percentage of dead triactinomyxons (Figure 4). As

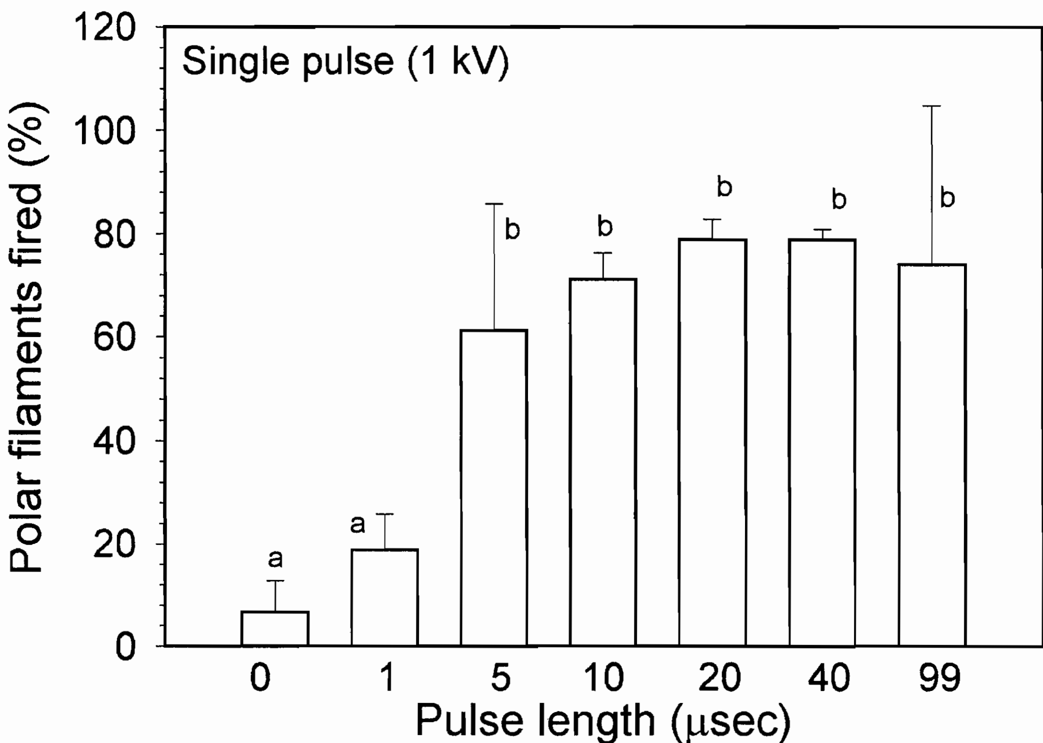

Figure 3. The mean percentage of *M. cerebralis* triactinomyxons with discharged polar filaments (±SD) after exposure to a single 1kV pulse at various pulse lengths. Means for each pulse length ($N = 3$) that are significantly different from each other ($P \leq 0.05$) have different letters.

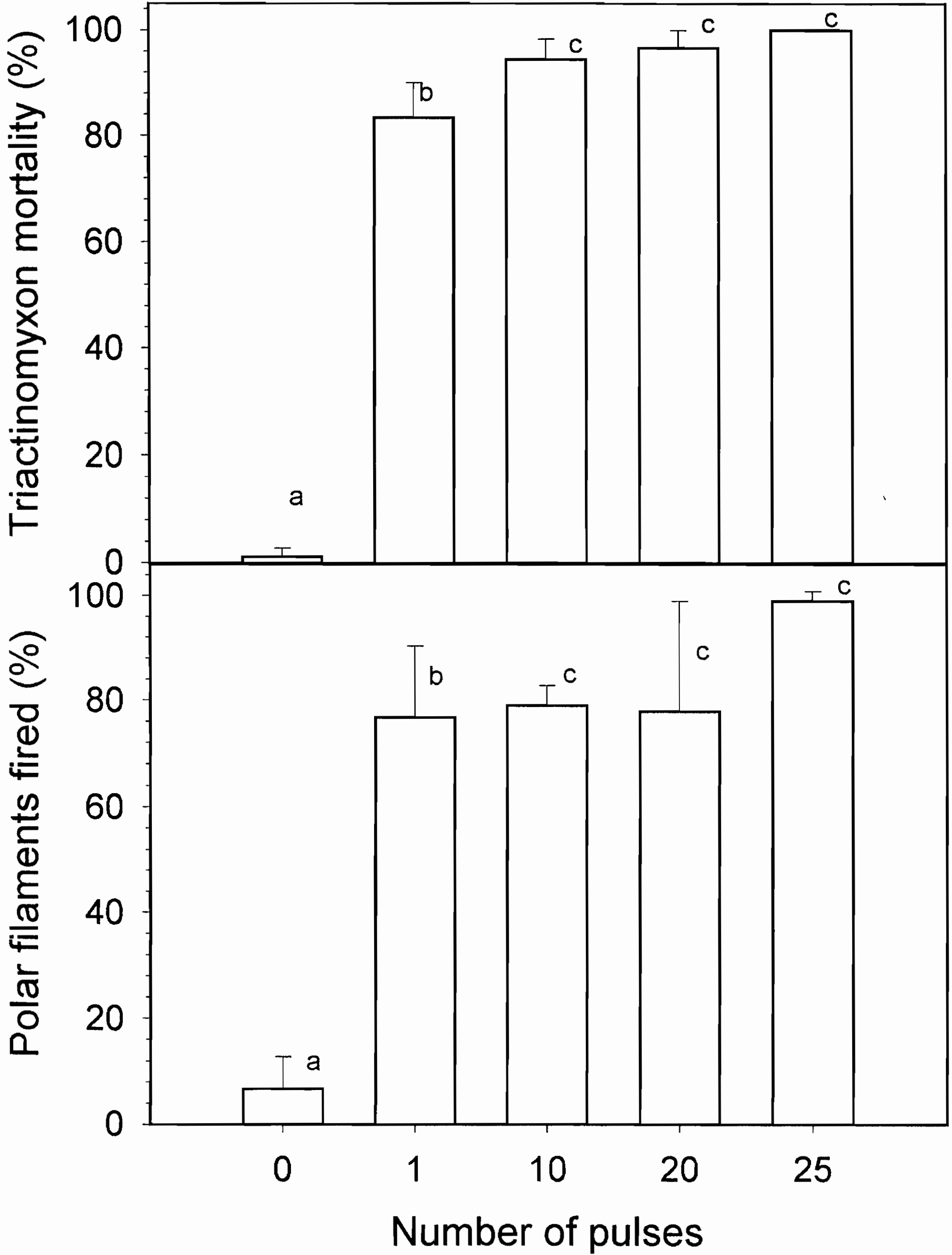

Figure 4. The effect of varying the number of pulses (0–25) on the viability and polar filament discharge of *M. cerebralis* triactinomyxons. The test was conducted at 3 kV and a pulse length of 99 μsec. Results are expressed as mean percentage of dead triactinomyxons ± SD (top) and mean (±SD, $N = 3$) percentage of triactinomyxons with fired polar filaments (bottom). Significant differences ($P \leq 0.050$) between the number of pulses are noted by a different letter.

the number of pulses increased, more triactinomyxons were killed. However, the differences in percent mortality did not differ among triactinomyxons exposed to 10, 20, and 25 pulses. To achieve consistent 100% mortality of triactinomyxons, 25 pulses of 3 kV for 99 μsec were required.

Effect of Mucus and Tricaine Methanesulfonate on Discharge

In the first test, no discharge was observed among triactinomyxons that had been exposed to mucus (N = 29–33 triactinomyxons). If mechanical agitation was used in combination with the mucus, no extruded polar filaments were seen (N = 116 triactinomyxons). Bovine mucin failed to induce higher rates of polar filament extrusion than observed for controls (Table 2). Concentrations of mucin from 5 to 1000 mg/L resulted in only 13.3–22.0% discharge.

The magnetic field test indicated that the percentage of polar filaments discharged in a magnetic field alone (9.0 ± 2.0% discharge) did not differ significantly from controls (4.3 ± 2.3%). However, rainbow trout mucus induced significantly higher (ANOVA, $P < 0.001$) discharge rates, either with (39.3 ± 3.0%) or without the magnetic field present (31.3 ± 16.3%). The effect of mucus in this test was notably different from the previous experience, so use of MS-222 anesthetic in the second trial was suspected.

Further testing indicated that the anesthetic had a significant effect on discharge, although the doses required for discharge were higher than normally used for anesthesia. Tricaine caused significantly higher rates of discharge than observed in controls, but only at concentrations at 909 mg/L and above (Figure 5). Maximum mean discharge was observed at 6,364-mg/L tricaine (29.4 ±

Table 2. Effect of mucin from bovine submaxillary gland on the mean percent discharge (± SD) of polar filaments of the *Myxobolus cerebralis* triactinomyxon.

Concentration (mg/L)				
0.0	5.0	50	500	1,000
13.4 ± 5.6	22.0 ± 6.1	14.0 ± 2.6	13.3 ± 2.9	14.7 ± 8.3

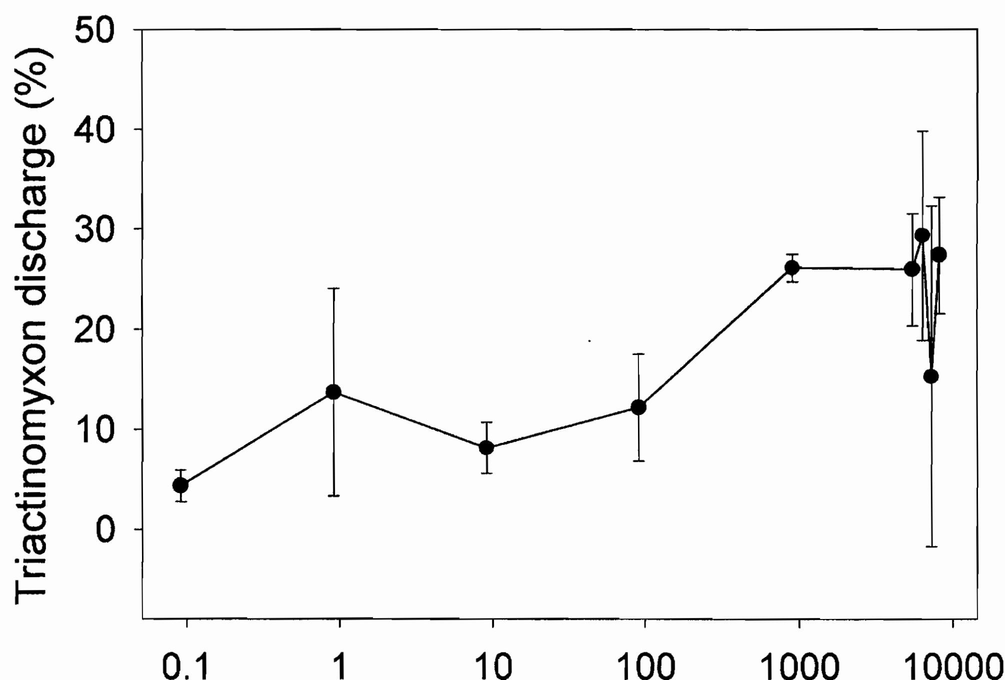

Figure 5. The effect of tricaine methanesulfonate anesthetic (MS-222) on the mean percentage of *M. cerebralis* triactinomyxons with discharged polar filaments (±SD, N = 3 slides of 38–395 triactinomyxons each).

10.5%). At 90.9 mg/L of MS-222, discharge was about 12.2% but did not significantly differ from controls (1.9%). In the second series of anesthetic tests, the percent discharge at MS-222 concentrations, ranging from 50 to 300 mg/L, varied from 16.3 to 32.3%, and did not differ significantly from controls (26.1%; Figure 6).

Other Agents

Glutathione discharge in the 0.05 M treatment averaged 34.2% (N = 24, 37 triactinomyxons) and 26.3% (N = 39, 46 triactinomyxons) in the 0.5-M treatment. Controls averaged 22.5% discharge (N = 20, 25 triactinomyxons). Additional tests with 0.1-M gluthione dried on a slide resulted in an average of 43.1% discharge. Slides coated with1.0 M glutathione were too thick with crystals to adequately observe the triactinomyxons.

Polar filament discharge after exposure to proline concentrations of 5×10^{-6} to 5×10^{-3} M did not significantly differ from controls (Table 3). Similarly, NANA failed to significantly increase the percentage of discharged triactinomyxons (Table 3), varying from only 10.6% to 18.1% discharge among the concentrations.

The discharge percentages among controls varied from 7.7% to 17.7%. The neurochemicals tested did not induce any significantly higher discharge of polar filaments in the ranges of concentration tested. Mean discharge percentages for acetylcholine chloride ranged from only 15.0% to 19.1% (Table 4). For angiotensin and bradykinin, mean discharge percentages were also low, ranging from 5.5% to 15.0% and 8.4% to 10.0%, respectively.

DISCUSSION

pH

In this study, pH effects on triactinomyxon polar filament extrusion were observed only at extreme values. Acid-induced discharge was only signifi-

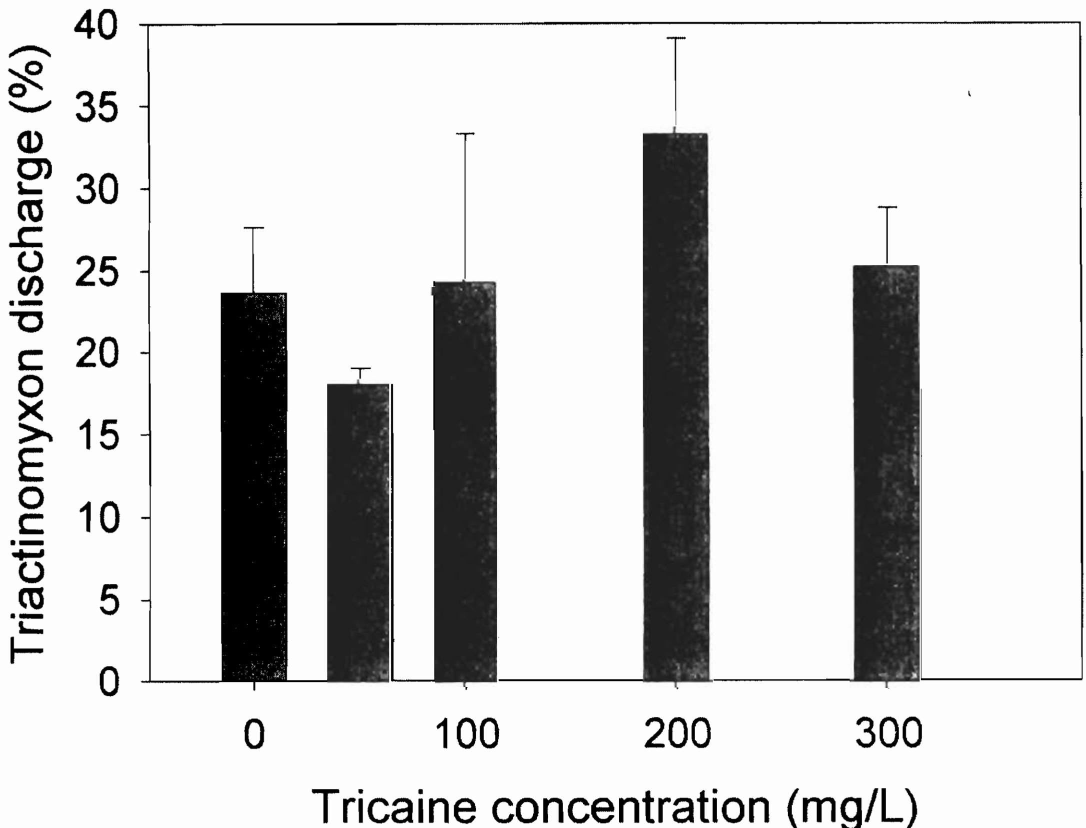

Figure 6. The effect of tricaine methanesulfonate concentrations of 50–300 mg/L on the mean percentage of *M. cerebralis* triactinomyxons with discharged polar filaments (±SD, N = 3 slides of 100 triactinomyxons each).

Table 3. Effect of proline or NANA on the percentage (± SD, $N = 3$) of triactinomyxons (tams) of *Myxobolus cerebralis* that had at least 1 polar filament discharged or were lacking a spore body (empty). The range in the number of triactinomyxons per slide is also presented.

Chemical concentration	Discharged (%)	Empty (%)	Tams/slide (range)
Proline			
0.0 M	5.5 ± 1.5	4.0 ± 1.7	100
5×10^{-6} M	5.4 ± 2.1	6.4 ± 1.1	98–100
5×10^{-5} M	6.2 ± 1.6	5.2 ± 0.8	93–100
5×10^{-4} M	5.1 ± 2.0	2.3 ± 1.1	100
5×10^{-3} M	6.5 ± 2.1	3.8 ± 2.5	92–100
NANA			
0.0 M	7.0 ± 1.0	9.3 ± 2.5	100
1.0×10^{-7} M	17.0 ± 3.5	6.3 ± 2.1	100
2.0×10^{-7} M	13.0 ± 6.6	9.0 ± 2.6	100
4.0×10^{-7} M	14.7 ± 2.9	7.7 ± 1.5	100
8.0×10^{-7} M	9.7 ± 4.0	7.7 ± 4.0	100

Table 4. Effect of various neurochemicals on the percentage (± SD, $N = 3$) of triactinomyxons (tams) of *Myxobolus cerebralis* that had at least 1 polar filament discharged or were lacking a spore body (empty). The range in the number of triactinomyxons per slide is also presented.

Chemical concentration	Discharged (%)	Empty (%)	Tams/slide (range)
Acetylcholine chloride			
0.0 mg/L	12.0 ± 6.0	4.0 ± 1.7	100
1.13 mg/L	18.0 ± 5.0	5.3 ± 2.5	100
11.3 mg/L	15.3 ± 4.0	6.0 ± 3.6	100
113.0 mg/L	14.0 ± 9.5	8.3 ± 4.0	100
Angiotensin			
0.0 mM	14.8 ± 9.3	15.2 ± 7.2	40–72
3.95×10^{-3} mM	5.7 ± 5.4	15.9 ± 6.7	54–63
3.95×10^{-2} mM	5.0 ± 5.3	8.9 ± 0.1	57–78
3.95×10^{-1} mM	13.2 ± 0.6	11.7 ± 1.6	39–66
Bradykinin			
0.0 M	8.7 ± 4.6	6.0 ± 1.0	100
5×10^{-8} M	8.7 ± 4.2	13.0 ± 3.6	10
5×10^{-7} M	7.3 ± 4.0	14.3 ± 4.9	100
5×10^{-6} M	8.3 ± 2.5	11.7 ± 1.5	100
5×10^{-5} M	8.3 ± 4.0	10.0 ± 1.7	100
5×10^{-4} M	8.3 ± 6.1	10.7 ± 2.3	100

cantly higher than controls at pH 1.1 using HCl. Gurley (1894) observed extrusion of *Henneguya creplini* myxospores induced by glacial acetic acid (concentration or pH not given). Gurley (1894) also noted myxospores of a wide variety of myxozoans extruded polar filaments when exposed to sulfuric acid. Sulfuric acid did not give similar results with *Henneguya rupestris*, nor did hydrochloric acid (Herrick 1941). Similar failure of acids to extrude filaments has been noted for *M. cerebralis* (Uspenskaya 1957) and *M. cartilaginis* (Hoffman et al. 1965). However, Herrick (1936) reported that some filaments of *M. kostiri* extruded after using concentrated sulfuric acid. Stomach acids have also generally failed to induce extrusion of myxospore polar filaments in tests with

M. cerebralis (Uspenskaya 1957), *M. muelleri* (Lom 1964), and *M. cartilaginis* (Hoffman et al. 1965). However, Iversen (1954) used the digestive juices of a freshly killed rainbow trout and was successful in extruding polar filaments of *Myxobolus squamalis*.

Bases were more effective discharge agents than acids in this study. This has also been noted for myxospores of other myxozoans (Hoffman et al. 1965). Strong bases such as potassium and sodium hydroxide, at concentrations of greater than 1–2%, have been used to extrude polar filaments in *Myxidium minteri*, *Chloromyxum majori*, *M. cerebralis*, *M. kisutchi*, *M. notemigoni*, and *M. cartilaginis* (Yasutake and Wood 1957; Uspenskaya 1957; Lewis and Summerfelt 1964; Hoffman et al. 1965). The effect of KOH on discharge of the polar filaments was temperature-dependent; using 1.5% KOH, Hoffman et al. (1965) noted that no filaments of *M. cartilaginis* myxospores fired at 6°C, but at 23°C and 40°C, 57% and 96% of polar filaments were extruded. Herrick (1936) used ammonium hydroxide (no concentration or duration noted) but was unsuccessful in extruding polar filaments of *Myxobolus kostiri*. Herrick (1941) was similarly unsuccessful using potassium hydroxide on *Henneguya rupestris*. Durations, pH, temperatures, and concentrations were not reported in most of these studies; therefore, it was difficult to ascertain whether or not there are true genetically-based differences among Myxozoans in susceptibility to acids and bases.

Among Cnidaria, the effects of pH have depended on whether cnidae are isolated from the cnidocyte. In-situ cnidae discharged at pH values below 4 and above 11, whereas isolated cnidae discharged only at pH values of 2 or below (Blanquet 1970). Sulfuric acid solutions in seawater (10%) caused discharge (Parker and Van Alstyne 1932). Exposure of isolated cnidae to sulfuric, citric, hydrochloric, and acetic acids, with mean pH values ranging from 2.9 to 3.3, caused 50% of the cnidae to discharge (Yanagita and Wada 1953). This is higher than the discharge rates observed in this study at similar pH levels (22–28%). Yanagita and Wada (1953) used potassium hydroxide, sodium hydroxide, and ammonium hydroxide, at pH values ranging from 10.8 to 11.0, and caused 50% of the cnidae to discharge. These results are also higher than the rates of discharge observed in this study at a similar pH.

Salts

Anions have varied in their ability to discharge cnidae. Salleo et al. (1983b) noted that the discharge potency of sodium salts isosmotic with seawater followed the lyotropic series: SO_4^{2-} < CH_3COO^- < F^- < Cl^- < Br^- < NO_3^- < I^- < ClO_4^- < SCN^-. In the present study, Cl^- was superior to I^- and PO_4^- for discharge of triactinomyxon polar filaments. Chloride ions, especially with divalent cations ($CaCl_2$ and $MgCl_2$), were effective discharge agents for the isolated cnidae of the anemone *Diadumene*, at concentrations above 6.9‰ (Yanagita 1959). In the present study, Cl- concentrations of 6.2‰ or higher were required for significant increases in discharge.

The overall effect of high salt concentrations on discharge of triactinomyxon polar filaments indicated that the influx of ions can play a role in polar filament discharge. In this study, K^+ was the most effective cation for triactinomyxon discharge. Gurley (1894) also noted the ability of KI to induce discharge for a variety of myxozoan myxospores. The efficacy of K^+ for discharge has also been observed for many cnidarian species (Parker 1905; Yanagita 1960; Santoro and Salleo 1991; Kawaii et al. 1997). This effect was muted in isolated cnidae (Yanagita 1959; Blanquet 1970; McKay and Anderson 1988). The effect of potassium was also dependent on the presence of calcium (McKay and Anderson 1988). Sodium ion depletion has also blocked discharge (Brinkmann et al. 1996).

Among other cations tested, NH_4^+ also stimulated in-situ cnidae discharge (Yanagita 1960). Blanquet (1970) noted that Ca^{2+} and Mg^{2+} did not induce discharge. Similarly, Na^+ and Li^+ as chloride salts were not effective (Yanagita 1960). In this study, NH_4^+ was not as effective for inducing discharge as K^+, Mg^{2+}, or Ca^{2+}, yet still was capable of low levels of polar filament discharge.

The discharge induced by the calcium chelators sodium citrate and EGTA, in this study, did not differ from the salt or pH controls, but discharge increased relative to the triactinomyxon stock controls. This indicated that high osmolality and pH were more conducive to discharge than the loss of Ca^{2+}. Results also indicated that a drop in Ca^{2+} is not needed for polar filament discharge.

In isolated cnidae, contrary to in-situ cnidae, removal of calcium has induced discharge (Lubbock and Amos 1981; Hidaka and Mariscal 1988). Isolated cnidae of *Pelagia noctiluca*, pre-

treated with $CaCl_2$, were inhibited from discharging when exposed to trypsin, an effect not observed in controls or for cnidae treated with $MgCl_2$ (Salleo et al. 1983a).

The effect of calcium ion does not apply to all cnidae. Salleo et al. (1990) noted that very little calcium was discharged from the cnidae of *Calliactis parasitica*. These authors also found that Ca^{2+} and Mg^{2+} free artificial seawater did not induce discharge of isolated cnidae. Mariscal (1984) has similarly observed holotrichous isorhiza cnidae of *Haliplanella luciae* that appear to lack calcium, suggesting that other ions and chemical messengers aid in triggering these.

Mucus and Tricaine Methanesulfonate

Mucus would seem to be a logical candidate for a discharge agent, but results appear to vary among species of Myxozoa. Actinospore forms, such as aurantiactinomyxon, raabeia, neoactinomyxum, and echinactinomyxon, have discharged polar filaments in response to mucus (Yokoyama et al. 1995; Xiao and Desser 2000). The percentage of extruded polar filaments varied with species and actinospore, but no actinospore was species specific (Xiao and Desser 2000). However, Xiao and Desser (2000) noted that some triactinomyxon forms were discharged by mucus from a narrower range of species. For example, for the 'triactinomyxon C' form (Xiao and Desser 1998), discharge was induced in more than 90% of the actinospores, by common shiner *Luxilus cornutus*, fathead minnow *Pimephales promelas*, and golden shiner *Notemigonus crysoleucas* mucus, but discharge was 12% or less for the other species, including the other cyprinid *Semotilus atromaculatus*. Triactinomyxon F had a narrower range, discharging 77% in mucus of common shiner, 12% in golden shiner, and 8% or less in the remaining species. Recent work with mucus has shown that it contains compounds that can alter membrane permeability (Ebran et al. 2000). This may be a part of the signaling mechanism for those Myxozoa that respond to mucus.

Yokoyama et al. (1995) noted that mucin from bovine submaxillary gland (0.1% w/v) was also a discharge agent. In this study, bovine mucin failed to induce extrusion at concentrations from 5 to 1000 mg/L. Similarly, El-Matbouli et al. (1999) failed to extrude polar filaments of *M. cerebralis* triactinomyxons, using rainbow trout mucus. This study also failed to find a link between triactinomyxon discharge and contact with mucus. This was true whether there was a mechanical stimulus or not. It is possible that a larger force may be required than was applied by the stirring rod used in this study. Propulsive forces of 0.0–0.6 N have been recorded for swimming cod *Gadus morhua* (Videler 1993), but none were measured in this study. However, triactinomyxon attachment to the respiratory epithelium and buccal cavity (El-Matbouli et al. 1999), where presumably these forces are small, indicates that other mechanisms may be responsible for discharge.

Tricaine induced significant increases in polar filament discharge, but the concentrations required for discharge were much higher than those used for routine fisheries studies (Eisler and Backiel 1960). At concentrations of 50–300 mg/L no significant increase in discharge was observed. This observation would indicate that the polar filament extrusion for triactinomyxons exposed to mucus were not influenced by anesthetic use for mucus collection. Further study is needed to examine the combined effect of mucus and MS-222.

Electrical potentials are an integral part of living organisms (Crane 1950; McCaig and Robinson 1980). These result from the high electrical resistance of the lipid membrane of cells and charged ions in the fluids on either side of the membrane (Zhelev and Needham 1994). The intercellular electrical currents are generally in the mV range (McCaig and Robinson 1980), so the associated magnetic field produced by a living fish would be much smaller than that provided by the magnet used in this study. The magnetic field had no effect on discharge in this study, either with or without mucus present. The hypothesis that a magnetic field might be required for discharge was therefore rejected.

Electricity

Pulses of direct current in this study were capable of inducing polar filament extrusion. The effect was influenced by pulse length. The longest pulse duration tested (99 μsec) produced the greatest discharge. The number of pulses was also a factor, with maximum mortality and discharge observed after 25 pulses of 3 kV. This is the first reported attempt to use electricity for polar filament discharge in Myxozoa.

Electricity has stimulated cnidae discharge, primarily in-situ. Glaser and Sparrow (1909) found that in-situ cnidae of *Metridium* responded to electricity, but isolated cnidae did not. This effect was later corroborated by Parker and Van Alstyne

(1932), for *Metridium* and *Physalia*, and by Yanagita (1960), for *Diadumene*. The threshold direct current Yanagita (1960) needed to induce extrusion varied from 8 to 20 μA (delivered by a 67.5 V battery through a 100 kΩ potentiometer). Pantin (1942) experimented with *Anemonia sulcata* and found that electrical shocks (240 V from a 2-μF condenser and passed through a 400 Ω potentiometer) stimulated cnidae discharge. Anderson and McKay (1987) found that isolated cnidae of *Cladonema* and *Chrysaora* failed to discharge after electrical stimulation, despite detection of a variety of voltage-dependent ionic currents. In-situ cnidae of *Physalia* similarly failed to discharge (Anderson and McKay 1987). In *Hydra* tentacles, stenotele and desmoneme nematocysts readily discharged, using 24-V DC, but the isorhizas did not (Tardent 1988).

Electrical pulses in this study were deadly. Despite the positive correlation between discharge and triactinomyxon mortality, it is not clear if discharge was the cause of death. Nonetheless, the results do indicate a potential for using electricity as a means of disinfection. The recommended lethal direct current would be 25 99-μsec pulses of 3 kV.

Other Discharge Agents

In sea anemones, two distinct classes of chemoreceptors, located on the supporting cells adjacent to cnidocytes, predispose these to discharge cnidae in the event of prey contact (Thorington and Hessinger 1988; Watson and Hessinger 1987). One class of chemoreceptor binds N-acetylated sugars, and the other binds certain amino compounds, including proline (Watson and Roberts 1994). N-acetylated sugars, which occur as conjugates of the surface mucins or external chitin of prey, sensitize cnidocytes to discharge (Thorington and Hessinger 1988). For example, glycine and N-acetylneuraminic acid (NANA) have chemosensitized cnidae discharge (Thorington and Hessinger 1990; Thorington and Hessinger 1998). Chemoreceptors for N-acetylated sugars may stimulate adenylate cyclase to sensitize and tune mechanoreceptors involved in initiating cnidae discharge (Watson and Hessinger 1992). Other chemosensitizing agents include glutathione (Loomis and Lenhoff 1956), glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate, acetylcholine (1/100,000), and acetylcholine with physostigmine (10^{-5} M, a cholinesterase inhibitor; Lentz 1966).

In this study, mixing proline, NANA, or glutathione with triactinomyxons did not induce polar filament extrusion at concentrations similar to those used for cnidarians. Also, the neurochemicals acetylcholine chloride, angiotensin, and bradykinin were ineffective discharge agents. For hydra, the presence of a primitive nerve network may be the reason that discharge is affected by neurochemicals such as acetylcholine (Lentz 1966). The absence of any nerves in myxozoans may explain the negative results observed in this study. The inability of chemosensitizing agents such as NANA to induce polar filament discharge tends to indicate that the supporting cell complex that is present among cnidarians may not be present in Myxozoa. Further work is needed to elucidate the exact biochemical and physical mechanisms of polar filament discharge.

In summary, there are some similarities and differences between cnidae and polar capsules in their response to discharge agents, including the sensitivity to K^+, electricity, and extreme pH values. However, the discharge rate in response to extreme pH was lower than for cnidarians. Major differences between the two structures include the lack of response by triactinomyxons to neurochemicals and chemosensitizing agents. The loss of Ca^{2+} induced by the chelators tested did not induce discharge of triactinomyxons, whereas this can cause discharge in some cnidarians. Bovine mucin and rainbow trout mucus were not effective discharge agents for triactinomyxons, despite mechanical contact and magnetic fields. Electrical pulses were effective in discharging and inactivating triactinomyxons. Further research is needed to explore the potential for premature discharge of polar capsules as a means of preventing infection of fish by myxozoan parasites.

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