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Author(s): Jessie I. Price, Brian S. Yandell, Warren P. Porter
Source: *The Journal of Wildlife Management*, Vol. 56, No. 2, (Apr., 1992), pp. 274-278
Published by: Allen Press
Stable URL: http://www.jstor.org/stable/3808823
Accessed: 30/07/2008 12:28

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Received 29 April 1991.
Accepted 14 November 1991.
Associate Editor: Nudds.

CHEMICAL IONS AFFECT SURVIVAL OF AVIAN CHOLERA ORGANISMS IN PONDWATER

JESSIE I. PRICE, U.S. Fish and Wildlife Service, National Wildlife Health Research Center, 6006 Schroeder Road, Madison, WI 53711
BRIAN S. YANDELL, Departments of Horticulture and Statistics, University of Wisconsin, Madison, WI 53706
WARREN P. PORTER, Department of Zoology, University of Wisconsin, Madison, WI 53706

Abstract: Avian cholera (*Pasteurella multocida*) is a major disease of wild waterfowl, but its epizootiology remains little understood. Consequently, we examined whether chemical ions affected survival of avian cholera organisms in water collected from the Nebraska Rainwater Basin where avian cholera is enzootic. We tested the response of *P. multocida* to ammonium (NH_4), calcium (Ca), magnesium (Mg), nitrate (NO_3), and ortho-phosphate (PO_4) ions individually and in combination using a fractional factorial design divided into 4 blocks. High concentrations of Ca and Mg, singly or in combination, increased survival of *P. multocida* organisms ($P < 0.001$). We developed a survival index to predict whether or not specific ponds could be "problem" or "nonproblem" avian cholera sites based on concentrations of these ions in the water.

J. WILDL. MANAGE. 56(2):274–278

The epizootiology of avian cholera in wild waterfowl is not fully understood. Zinkl et al. (1977) reported the first occurrence of the disease in Nebraska in 1975 when an avian cholera die-off occurred in the wetlands of the western Rainwater Basin during spring waterfowl migration. Since that time, the disease has recurred annually, often in the same wetlands. Price and Brand (1984) reported recovering virulent avian cholera organisms in water collected from a wetland in the western part of the Rainwater Basin in Nebraska during a large die-off. Windingstad et al. (1985) reported differences in water quality between wetlands located in the eastern and western sections of the Rainwater Basin, finding higher concentrations of Ca and Mg ions in water from the 3 western (Funk, Gleason, and Prairie Dog) wetlands.

We investigated the effects of these and other chemical ions on survival of *P. multocida* organisms at a low temperature in pondwater from the Rainwater Basin. Low and high concentrations of Ca, Mg, NH_4, NO_3, and PO_4 were evaluated in all possible combinations. We conducted the study at 4 C to simulate the ambient winter and early spring temperature in Nebraska during avian cholera die-offs.

We thank R. M. Windingstad and M. K. Moore for field and laboratory assistance. We also thank P. G. Slota for technical services, and K. M. Cunningham, P. M. Crump, and W. R. Hansen for computer operations. W. P. Porter was supported by funding from DE-AC02-76EV02270, and B. S. Yandell was supported by funding from USDA-CSRS 511-100.

METHODS

Pondwater

We collected water samples from the same wetlands described by Windingstad et al. (1985)

in August 1982, and analyzed them for baseline chemical ion concentrations (Table 1). Of the 3 wetlands sampled in the west, Mg and Ca ion variables were lowest in water collected from Prairie Dog; therefore, we selected this wetland for the study to have the greatest difference between the naturally low level of Ca and Mg and the artificially high concentrations added to the water. Two days after the ions were measured on site, 40 L of water were collected from Prairie Dog (20 C, ambient temperature) and refrigerated. We filter-sterilized water to remove extraneous bacteria using cotton, Whatman #2, Whatman #50, and Millipore filters (3.0, 1.2, 0.45, and 0.20 μm) in sequence. We froze the samples in 450-mL aliquots and brought them to the laboratory. Ion concentrations in the Prairie Dog water changed in the 3-week interval between initial testing (Table 1), and post-filtration (Table 2). Freezing and filtering the water altered the levels of the ions. Concentrations of Ca, Mg, and PO_4 decreased; NH_4 increased; and NO_3 remained the same.

We conducted laboratory assessment of *P. multocida* using thawed, post-filtration water from Prairie Dog, with (high) or without (low) adding chemical ions (Table 2). High levels were set substantially higher than those recorded in the western wetlands during spring 1981 and 1982 (Windingstad et al. 1985). We established center point levels by mixing mean concentrations of the 5 ions together. Chemical adjustments were made by addition of stock solutions of each ion to the control water to bring levels of each ion to the concentration needed.

Table 1. Field concentrations (mg/L) of calcium and magnesium hardness, nitrate, ammonia, and ortho-phosphate in the Nebraska Rainwater Basin study ponds, August 1982.

Ion	Study ponds		
	Funk	Prairie Dog	Gleason
Ca	233.0	72.0	146.0
Mg	71.3	28.0	44.0
NO_3	2.9	0.9	0.8
NH_4	2.5	1.9	0.4
PO_4	2.1	3.1	4.1

Bacterial Culture

We used a Type 1 *P. multocida* culture isolated from the carcass of 1 lesser snow goose (*Chen caerulescens caerulescens*) that died during an avian cholera epizootic in the Central Valley of California. Characteristics of the culture were described by Price (1985). Aliquots of the culture for this study were stored in liquid nitrogen.

Due to space limitations, we conducted the experiment in 4 separate blocks each taking about 3 weeks. Five days before each block was run, we removed a vial of culture from the liquid nitrogen. After thawing, a loopful of the material was streaked onto agar and incubated at 37 C for 24 hours. At that time, single colonies were selected and inoculated into Difco Brain Heart Infusion Broth (BHIB) (Difco Lab., Detroit, Mich.) and incubated for 14 hours to allow the culture to develop into a late stationary phase. A 1:1,000 dilution of the culture was prepared at that time by inoculating flasks containing 299.7 mL of the test water for each experimental run with 0.3 mL of the culture growth (approximately 3.0–8.1 $\times$ 10^8 cells). Flasks were subdivided into each of 3 bottles, 100 mL/bottle, which were maintained at 4 C. We determined survival of the *P. multocida* organisms by duplicate spread plate titrations from 2 of the 3 bottles for each sample at 0 (sampled immediately after adding the *P. multocida* organisms and mixing), 8, 24, 48, 120, and every 48 hours thereafter through 648 hours (27 days). Plates then were incubated at 37 C for 24 hours. We counted and determined those dilutions with 300 or less colonies and the number of the colony forming units/mL (CFU). All plates were reincubated at 37 C for another 24 hours and checked again for evidence of delayed growth. One flask containing 99.9 mL BHIB was inoculated with 0.1 mL of the NWHL-B-2 culture, incubated at 4 C and tested along with the pondwater runs by inoculating duplicate spread plates. This flask served as a viability control of the inoculum. We based computation of estimated counts on standard dilution plating assumptions (Finney 1978).

Table 2. Chemical ion concentrations (mg/L) of thawed post-filtration water used for laboratory assessment, 1982.

Chemical ions	Concentrations	
	Low[a]	High[b]
Ca	31.0	600.0
Mg	12.0	180.0
NO_3	0.9	22.0
NH_4	2.3	24.0
PO_4	2.4	44.0

[a] Prairie Dog pond water after filtration and freezing.
[b] After addition of chemical ions.

Chemical Analysis

We analyzed water samples for Ca and total hardness using Hach titration methods and based them on the American Public Health Association Standard Methods procedures (Hach Co. 1982, Am. Public Health Assoc. 1975). Magnesium hardness, as $CaCO_3$, was derived by subtracting Ca hardness from total hardness. We used Hach spectrophotometric procedures (Hach Co. 1982) to analyze the water for NH_4, NO_3, and PO_4 concentrations (Hach Co., Loveland, Colo.). Four days before each block was started, water was removed from the freezer, thawed, and ion concentrations were re-measured.

Statistical Analysis

We used a factorial design to study the effect of the 5 chemical ions and their possible synergism. Main effects and interactions of the ions were estimated and compared using Yate's algorithm. We conducted the experiment in 4 fractions or "blocks" for convenience of working with the organisms in a standardized way, with a 2- to 3-week interval between day 0 of each block. Each block consisted of 11–12 samples containing 8 of the 32 possible ion level combinations, duplicate center points (all ions at a level halfway between high and low), 1 extra control (untreated water), and 1 BHIB, all run in random order. Block 4 had only 11 samples; the control was part of the quarter fraction of the 32 ion combinations.

The 4 fractions of high/low ion level combinations were determined by the generator 13 = ± 245 (PO_4•NH_4 = NO_3•Ca•Mg), leading to a 2_{III}^{5-2} fractional factorial design (Box et al. 1978). Thus, the 2-way interaction of PO_4•NH_4 and the 3-way interaction NO_3•Ca•Mg were both confounded with blocking. However, it was possible to use the center points and extra controls to approximately account for block differences during analysis.

We measured response as raw counts of CFU's, percent viability, and a survival index (SI). Raw counts were analyzed by each recorded time separately and over time assuming an exponential decay due to mortality. Percent viability of *P. multocida* organisms was determined as the ratio of the number of colonies at a specified time to the number at sample time 0. We developed the SI to approximate the time until colony counts were fewer than 10 CFU/mL relative to controls. Specifically for an ion level combination x, $SI(x) = T(x) - [T(M) + T(C)]/2$, where $T(y)$ = days to <10 CFU for level y, M = center point (medium level of all 5 ions) and C = control (untreated water). These 3 responses led to the same conclusions.

RESULTS

Block Differences

Marked differences in the 4 blocks were found by considering only center points, controls, and BHIB samples (Table 3). In the BHIB, 5% viability was reached by 48 hours. The last colonies in BHIB were seen at 120–216 hours with counts of 5–120 CFU/mL. There were no viable cells at the next 2 sampling times in any of the 4 blocks. Survival of *P. multocida* at center points increased in 3 of 4 blocks at the 8-hour sampling time, and then decreased (Table 3). At most, 11% viability was found at 84 hours, with the last colonies visible at 264–648 hours with counts of 3–130 CFU/mL. In controls, 8% viability was found at 84 hours; the last colonies were visible 312–552 hours with counts of 3–8 CFU/mL.

Chemical Ion Main Effects and Interactions

Addition of Ca increased ($P < 0.0008$) the percent viability of *P. multocida* organisms. Magnesium affected percent viability, although results were not consistent ($P = 0.88$ at 24 hr, $P = 0.032$ at 84 hr, and $P = 0.01$ at other times). There was synergism between Ca and Mg, particularly at 0 hours ($P = 0.001$) and again at 24 and ≤120 hours ($P = 0.028$ and 0.023, respectively). Other ion combinations had viability effects (Ca•NH_4•PO_4 at 84 hr, $P = 0.0075$; Ca•NO_3 at 120 hours, $P = 0.0084$) but presented no consistent picture for percent viability. Therefore, we focused on Ca and Mg (Table 4), while remaining cognizant of the possibility of complicated but subtle interactions of several ions on viability of *P. multocida* over time. Adding both Ca and Mg increased viability the most, while the control (pondwater) was least effective. Adding Mg alone decreased viability, whereas adding Ca alone produced a modest increase.

Analysis of the survival index indicated effects only for Ca ($P = 0.0025$), Mg ($P < 0.0001$), and their synergistic interaction ($P = 0.025$). Addition of Ca and Mg together yielded the largest survival index of 15 days (Table 4), Mg alone had a stronger effect (3 days) than did Ca (>1 day). Although Ca elevated the percent viability consistently, it did not alter the survival index;

Table 3. Percent viability of *Pasteurella multocida* cells in Brain Heart Infusion Broth (BHIB), mean concentrations of the 5 ions (center points), and controls (untreated pondwater) for sampling times 0–120 hours for each block.

		% viability (hr)					
	Block no.	0	8	24	48	84	120
BHIB	1	93.5	24.6	3.2	0.5	0.1	0
	2	76.4	28.4	7.1	2.4	0.5	0.1
	3	37.7	44.2	16.2	4.8	0.7	0.1
	4	71.1	42.2	13.8	4.3	0.9	0.1
Center points	1	38.6	32.0	22.2	9.4	6.7	4.0
	2	26.7	49.1	25.2	16.0	7.0	4.6
	3	40.6	61.4	48.0	29.4	10.0	6.8
	4	31.2	50.2	30.4	19.2	11.1	5.7
Controls	1	12.7	21.9	16.4	10.3	7.6	3.8
	2	23.2	28.0	9.8	4.3	2.1	2.0
	3	16.8	56.6	45.7	19.9	7.4	5.3
	4	16.4	27.3	22.3	14.2	5.0	2.9

Mg raised percent viability slightly but increased the survival index. The most potent effect was found when both Ca and Mg were added to pondwater.

DISCUSSION

There is an inverse relationship between temperature and survival of Gram-negative organisms in water (5–15 C) (McFeters and Stuart 1972, Sjogren and Gibson 1981). However, there are conflicting reports in the literature about survival of *P. multocida* organisms in media at lower temperatures. Nobrega and Bueno (1950) reported that *P. multocida* survives longer in nutrient broth at 18 C than at 4 C. Bendheim and Even-Shoshan (1975) reported that *P. multocida* survived for 90 days at 5 C and for 46 days at 18 C. Jensen (1978) indicated that broth cultures of *P. multocida* in the stationary growth phase decreased at variable and unpredictable rates after 48 hours at 4 C. Bredy and Botzler (1989) reported that *P. multocida* organisms survived from >1 year in autoclaved pondwater samples supplemented with 0.5% NaCl and 175 μg/mL protein held at 18 C in the presence of contaminating organisms. They also reported that the concentration of *P. multocida* organisms dropped from 1.83×10^5 CFU/mL to $<10^1$ CFU/mL in the first 24 hours at 2 C. In this study, we observed *P. multocida* survival in BHIB at 4 C from 120–216 hours. Beuchat (1978) hypothesized that bacteria in a complete medium become metabolically injured. This may explain the short-term survival of the *P. multocida* organisms in our BHIB.

The seemingly synergistic effect of Ca and Mg on survival of the *P. multocida* organisms may reflect sustainment of stressed or injured cells by chemical ions as has been previously reported for coliform cells (Postgate and Hunter 1962, Granai and Sjogren 1981). However, until we develop a better procedure for isolating injured cells, we cannot be certain that the time the last colonies are visible is accurate. Consequently, survival times observed in this study may be shorter than they actually are under field conditons. Further studies will have to be conducted to determine whether or not the SI of *P. multocida* predicted herein would be reflected elsewhere.

Table 4. Percent viability and survival index (SI)[a] of *Pasteurella multocida* at low and high levels of Ca and Mg ions over time.

Ions		Time (hr)						SI
Ca	Mg	0	8	24	48	84	120	(days)
High	High	65A[b]	60A	37A	28A	13A	9A	15A
High	Low	18B	49B	45A	21B	10AB	4B	1C
Low	High	20B	37C	23B	11C	5BC	4B	3B
Low	Low	17B	27C	16B	8C	4C	2B	0C

[a] SI = Approximate time colony counts were <10 CFU/mL relative to low levels of Ca and Mg (control, untreated water).
[b] Means sharing same letter in column are not different ($P < 0.05$) using protected LSD.

The minimal survival of *P. multocida* organisms in pondwater in the laboratory suggests that this pathogen may be sustained indefinitely in the environment, especially in areas where Ca, Mg, and their complex exist in high concentrations along with the other unknown variables. Survival of *P. multocida* in control water for 23 days illustrates the hardiness and persistence of this pathogen. The numbers of bacterial cells are constantly changing. If there is a sudden influx of susceptible migratory birds into an area where high numbers of virulent *P. multocida* organisms exist in the water, it seems possible that an avian cholera die-off could be initiated. Cycles of low and high mortality have been described in endemic avian cholera areas (Windingstad et al. 1985).

Research is needed not only to further investigate relationships between chemical ions evaluated in this study and survival of avian cholera organisms, but also to determine whether or not similar results occur when physical variables, such as pH, turbidity, populations of other bacteria, algae, and other temperature regimes are included in the experimental design. We also need to evaluate the virulence of the *P. multocida* organisms after prolonged exposure to these altered physical and chemical variables.

MANAGEMENT IMPLICATIONS

The only methods used to control avian cholera in waterfowl presently are carcass collection to remove foci of infection, and water manipulation. Wetlands are frequently flooded to lure migrating birds away from avian cholera die-off areas, and to provide additional pond acreage to lessen bird density and to reduce transmission of avian cholera organisms. It would be advantageous to be able to determine which ponds are hazardous by measuring water quality (e.g., Ca and Mg) before migration in areas of heavy use by migrating waterfowl. Then birds could be discouraged from using these areas before a die-off starts. If Ca and Mg exist in high concentrations in the endemic areas, it might be possible to use chemical methods to reduce the concentrations of these ions to prevent the development and persistence of virulent *P. multocida* organisms. Water quality control may be one of the strategies that can be developed for managing avian cholera in the endemic areas.

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Received 4 September 1990.
Accepted 8 December 1991.
Associate Editor: Smith.