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Authors: Lehr, Margaret A., Botzler, Richard G., Samuel, Michael D., and Shadduck, Daniel J.

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ASSOCIATIONS BETWEEN WATER QUALITY, *PASTEURELLA MULTOCIDA*, AND AVIAN CHOLERA AT SACRAMENTO NATIONAL WILDLIFE REFUGE

Margaret A. Lehr,[1,4] Richard G. Botzler,[1] Michael D. Samuel,[2,3] and Daniel J. Shadduck[2]

[1] Wildlife Department, Humboldt State University, Arcata, California 95521, USA
[2] U.S. Geological Survey, Biological Resources Division, National Wildlife Health Center, 6006 Schroeder Road, Madison, Wisconsin 53711, USA
[3] Current address: U.S. Geological Survey, Wisconsin Cooperative Wildlife Research Unit, 204 Russell Lab, University of Wisconsin, Madison, Wisconsin 53706, USA
[4] Corresponding author (email: mlehr@stanford.edu)

ABSTRACT: We studied patterns in avian cholera mortality, the presence of *Pasteurella multocida* in the water or sediment, and water chemistry characteristics in 10 wetlands at the Sacramento National Wildlife Refuge Complex (California, USA), an area of recurrent avian cholera epizootics, during the winters of 1997 and 1998. Avian cholera outbreaks (≥50 dead birds) occurred on two wetlands during the winter of 1997, but no *P. multocida* were recovered from 390 water and 390 sediment samples from any of the 10 wetlands. No mortality events were observed on study wetlands during the winter of 1998; however, *P. multocida* was recovered from water and sediment samples in six of the 10 study wetlands. The pH levels were higher for wetlands experiencing outbreaks during the winter of 1997 than for nonoutbreak wetlands, and aluminum concentrations were higher in wetlands from which *P. multocida* were recovered during the winter of 1998. Water chemistry parameters (calcium, magnesium, sodium, and dissolved protein) previously linked with *P. multocida* and avian cholera mortality were not associated with the occurrence of avian cholera outbreaks or the presence of *P. multocida* in our study wetlands. Overall, we found no evidence to support the hypothesis that wetland characteristics facilitate the presence of *P. multocida* and, thereby, allow some wetlands to serve as long-term sources (reservoirs) for *P. multocida.*

Key words: Avian cholera, *Pasteurella multocida,* water characteristics, wetlands.

INTRODUCTION

Avian cholera is an infectious disease caused by *Pasteurella multocida,* a Gram-negative, rod-shaped bacterium, with a bipolar staining characteristic (Rimler and Glisson, 1997). Since the first reported epizootics among North American waterfowl during the winter of 1943–44 in northwestern Texas (USA) (Quortrup et al., 1946) and in the lower delta of the Sacramento and San Joaquin Rivers in California (USA) (Rosen and Bischoff, 1949), mortality from avian cholera has expanded in distribution and magnitude. In North America, epizootics have been reported from the east to west coasts, from Mexico, and from Canada (Friend, 1999). Avian cholera has become one of the most important causes of infectious disease mortality among North American wildfowl (Stout and Cornwall, 1976; Friend, 1999).

Friend (1999) reported four major foci of avian cholera in North American wildfowl: the Central Valley of California, the Tule Lake and Klamath Basins of northern California and southern Oregon (USA), the Texas Panhandle, and the Rainwater Basin of Nebraska (USA). The consistent recurrence of avian cholera in the same wetlands has led to the hypothesis that wetlands may serve as long-term sources (reservoirs) for *P. multocida.* However, others have hypothesized that birds are the primary reservoir for *P. multocida* (Botzler, 1991; Wobeser, 1992).

The viability of *P. multocida* has varied with physical and chemical characteristics of water, both in the laboratory (Bredy and Botzler, 1989; Price et al., 1992) and in wetlands (Titche, 1979). Sodium, protein, calcium, and magnesium have been associated with both the survival of *P. multocida* and with avian cholera mortality. Bredy and Botzler (1989) observed survival of pasteurellae >1 yr in sterilized wetland

water with addition of protein, or sodium, as well as following contamination by other microorganisms. Windingstad et al. (1984, 1988) also found five- to 10-fold higher concentrations of sodium in epizootic wetlands in Nebraska. In addition, increased calcium and magnesium levels have been associated with persistence of *P. multocida* (Price et al., 1992) and avian cholera mortality (Windingstad et al., 1984, 1988). There is also evidence that animal protein enhances survival of *P. multocida* (Rosen and Bischoff, 1949; Titche, 1979; Bredy and Botzler, 1989). In addition, *P. multocida* has been isolated from the water and sediment of wetlands experiencing avian cholera epizootics (Rosen and Bischoff, 1949; Rosen, 1969; Korschgen et al., 1978; Price and Brand, 1984; Backstrand and Botzler, 1986; Samuel et al., 2003), and the bacteria can persist in wetland soil and water (Rosen and Bischoff, 1949; Rosen, 1969; Price and Brand, 1984; Backstrand and Botzler, 1986; Bredy and Botzler, 1989). Thus, regardless of the reservoir for infectious bacteria, wetlands are likely an important source of bacteria during avian cholera epizootics.

We conducted an investigation at the Sacramento National Wildlife Refuge Complex (SNWRC) in northern California, an area of recurrent avian cholera epizootics to evaluate the relationships between avian cholera and wetland characteristics. During the winters of 1997 and 1998, we routinely collected sediment and water samples from 10 wetlands to characterize wetland water chemistries and for isolation of *P. multocida.* To better understand the possible role of wetlands in the epizootiology of avian cholera, we addressed three objectives: 1) to determine whether *P. multocida* was more likely to be isolated from wetlands with observed avian cholera mortality than in wetlands without observed mortality, 2) to determine whether there were differences in water characteristics between wetlands that regularly experienced avian cholera outbreaks of ≥50 birds in the past 6 yr, and those not experiencing regular outbreaks, and 3) to determine whether there were differences in the water characteristics between wetlands from which *P. multocida* were recovered compared with wetlands with no detectable pasteurellae.

METHODS

Study site

The SNWRC consists of six refuges located in the Sacramento Valley of northern California (39°24′N, 122°10′W). The refuge complex includes wetland, grassland, and riparian habitats that are intensively managed to provide wintering habitat for waterfowl. Avian cholera epizootics have consistently recurred on the refuge since 1949 (Mensik, pers. comm.).

During the winters of 1997 (7 January to 20 March) and 1998 (5 January to 2 April), we evaluated 10 wetlands at SNWRC for avian cholera mortality, the presence of *P. multocida* in water and sediment, and physical and chemical water characteristics. Four wetlands at Colusa National Wildlife Refuge (NWR) and six wetland tracts at Delevan NWR were chosen a priori for the study. These wetlands had similar vegetation and water management regimes (e.g., flooding and drawdown schedules), and none experienced direct hunting pressure.

Five of these wetlands (enzootic) were identified as having experienced regular avian cholera mortality during the previous six winters (1991–96), and five wetlands (reference) had little or no history of avian cholera mortality (Table 1). Enzootic wetlands had annual mean mortality ≥63 birds between 1991 and 1996 with high annual variation and annual mortality of ≥50 birds during at least two of the six winters. In contrast, reference wetlands had annual mean mortality ≤37 birds with low annual variation and mortality of ≥50 birds during less than two of the six winters. The magnitude of avian cholera mortality was higher ($\log_{10}$ transformed; $F_{1,8}=11.97$, $P<0.01$) in enzootic wetlands, compared with reference wetlands (nested analysis of variance, ANOVA, with enzootic vs. reference as the main factor and year as the nested factor, Sokal and Rohlf, 1995). Based on these results, wetlands with annual avian cholera mortality ≥50 birds during 1997–98 were classified as outbreak wetlands. To confirm avian cholera mortality, waterfowl carcasses (≥5) were collected by SNWRC staff and evaluated using standard diagnostic pathology and culture methods at the National Wildlife Health Center (NWHC) (Madison, Wisconsin, USA).

TABLE 1. Observed avian cholera mortaility on study wetlands at the Sacramento National Wildlife Refuge Complex, California.

Wetland tract (ha)	Winters 1991–96		Winter 1997	Winter 1998
	Years with outbreaks[a]	Mean mortality (±SE, n = 6)		
Enzootic wetlands				
Delevan T2-1 (31.6)	2	113.8 (±79.7)	31	0
Delevan T5-2 (64.8)	3	88.8 (±55.8)	0	9
Delevan T16 (85.8)	3	72.8 (±30.5)	56	8
Colusa T13A (38.5)	2	71.5 (±43.1)	20	23
Colusa T15A (38.1)	3	63.0 (±21.6)	20	16
Reference wetlands				
Delevan T6 (35.6)	0	5.7 (±4.3)	0	1
Delevan T11 (52.2)	1	36.5 (±13.2)	125	7
Delevan T15 (53.0)	0	9.3 (±6.2)	0	1
Colusa T2 (38.1)	0	18.3 (±6.1)	7	0
Colusa T5 (39.7)	0	11.2 (±5.0)	13	29

[a] Number of years with confirmed avian cholera mortality, in which at least 50 dead birds were observed on the wetland.

Sample collection and testing

Ten randomly selected sites within each wetland were marked with wooden stakes and repeatedly sampled four times during each winter (every 2 to 3 wk during the winter of 1997 and every 3 to 4 wk during the winter of 1998). At each of these sites, we collected water and sediment samples for isolation of *P. multocida* using the methods of Samuel et al. (2003). We used the cryopreservation method and quality assurance procedures described by Samuel et al. (2003) to preserve water and sediment samples before attempting isolation of *P. multocida.* Isolation of *P. multocida* from water and sediment samples was performed at NWHC using the procedures described by Moore et al. (1994), and suspect colonies were identified and serotyped by the methods of Samuel et al. (1997). *Pasteurella multocida* isolates were serotyped by the gel diffusion precipitin test (Heddleston et al., 1972).

At each of the 10 sample sites within a wetland, we measured water temperature (C), pH, oxidation and reduction (redox) potential (mv), specific conductivity (μSiemens/cm), and dissolved oxygen (mg/l) using a Yellow Springs Instrument (YSI Incorporated, Yellow Springs, Ohio, USA) water quality meter (610 DM) and probe (600 XL); we also measured water depth (mm). Equal water samples from each sample site were combined for each wetland and were analyzed for turbidity, chemical ions, and protein content. Turbidity was measured in nephelometric turbidity units (NTU) using a Hach 2100P turbidimeter (Hach Company, Loveland, Colorado, USA). Concentrations (ppm) of aluminum, boron, calcium, chlorine, copper, iron, magnesium, manganese, nitrate, phosphate, phosphorous, potassium, sodium, sulfate, sulfur, and zinc were determined using an Applied Research Laboratories 34000 spectrometer (Thermo Jarrell Ash Corporation, Franklin, Massachusetts, USA). Total protein concentration (μg/ml) was determined using a DU-65 ultraviolet/visible spectrophotometer (Beckman Instruments, Inc., Fullerton, California) following a Sigma Chemical Company, Procedure #TPRO-562, a modification of the procedure from Smith et al. (1985).

Statistical analysis

We used Fisher's exact and chi-square tests (Conover, 1999) to determine whether enzootic wetlands were more likely to have detectable levels of *P. multocida* than reference wetlands and to determine whether the prevalence of *P. multocida* isolates was greater in enzootic wetlands than in reference wetlands, respectively. We used a split-plot ANOVA with repeated measures (Kirk, 1995) to evaluate associations between wetland water characteristics and avian cholera. Each water characteristic was analyzed separately for two different comparisons: 1) wetlands experiencing outbreaks vs. wetlands with little or no avian cholera mortality, and 2) wetlands with *P. multocida* and those wetlands with no detectable pasteurellae. In addition, we used multivariate ANOVA (MANOVA) to test water characteristics previously associated with persistence of *P. multocida* (calcium, magnesium, protein, and sodium; Rosen and Bischoff, 1949; Titche, 1979; Windingstad

TABLE 2. Recovery of *Pasteurella multocida* serotype 1 from study wetlands at the Sacramento National Wildlife Refuge Complex, California during winters 1997 and 1998.

Wetland tract	Winter 1997	Winter of 1998
Enzootic wetlands		
Delevan T2-1	0/40[a]	0/40
Delevan T5-2	0/40	0/40
Delevan T16	0/40	2/40
Colusa T13A	0/40	3/40
Colusa T15A	0/40	2/38
Reference wetlands		
Delevan T6	0/40	5/40[b]
Delvan T11	0/40	1/40
Delvan T15	0/30	1/40
Colusa T2	0/40	0/38
Colusa T5	0/40	0/40

[a] Number of water samples with *P. multocida*/number of water samples tested.

[b] One additional *P. multocida* isolate recovered from 40 sediment samples.

et al., 1984, 1988; Bredy and Botzler, 1989; Price et al., 1992). The SNWRC location was considered a nested factor within each analysis because Colusa and Delevan NWR had different water sources. Each sample collection was used as the repeated measure within each wetland. Water characteristics not conforming to assumptions of normality and homoscedasticity were transformed by $\log_{10}$ or square root.

RESULTS

Avian cholera mortality on the study wetlands fluctuated annually with 272 and 94 carcasses collected from the study wetlands in the winters of 1997 and 1998, respectively (Table 1). One of five enzootic wetlands and one of five reference wetlands experienced avian cholera outbreaks with mortality of ≥50 birds during the winter of 1997 (Table 1). Mortality ≥50 birds did not occur on any wetland in the winter of 1998. Although enzootic wetlands were not more likely to experience mortality events ≥50 birds than reference wetlands in winter 1997 (Fisher's exact test; $P=0.56$) or winter 1998 ($P=1.00$), enzootic wetlands experienced higher mean mortality (two-level nested ANOVA with mortality $\log_{10}$ transformed; $F_{1,8}=7.11$, $P<0.03$).

TABLE 3. Mean (standard error) concentrations of physical and chemical water characteristics of study wetlands at the Sacramento National Wildlife Refuge Complex, California during winters 1997 and 1998.

Water characteristic	Mean (SE)
Aluminum (ppm)	0.409 (0.11)
Boron (ppm)	0.166 (0.01)
Calcium (ppm)	24.598 (1.87)
Chlorine (ppm)	23.078 (2.19)
Dissolved oxygen (mg/l)	10.437 (0.44)
Iron (ppm)	0.784 (0.12)
Magnesium (ppm)	18.586 (1.34)
Manganese (ppm)	0.070 (0.01)
Nitrate (ppm)	0.119 (0.02)
pH	7.993 (0.06)
Phosphate (ppm)	0.275 (0.02)
Phosphorous (ppm)	0.159 (0.03)
Potassium (ppm)	3.174 (0.21)
Protein (μg/ml)	9.706 (0.47)
Redox	145.294 (12.41)
Sodium (ppm)	52.705 (4.41)
Specific conductivity (mV)	405.806 (26.62)
Sulfate (ppm)	9.311 (1.11)
Sulfur (ppm)	15.401 (2.14)
Temperature (C)	12.237 (0.72)
Turbidity (NTU)	64.414 (15.07)
Zinc (ppm)	0.011 (0.00)

We tested 786 water and 786 sediment samples for the presence of *P. multocida* (Table 2). During winter 1997, *P. multocida* was not detected in any of our study wetlands. During winter 1998, *P. multocida* was isolated from three enzootic and three reference wetlands. *Pasteurella multocida* serotype 1 was recovered from 14 of 396 (3.5%) water samples and one of 396 (0.3%) sediment samples. Enzootic wetlands were not more likely to have detectable levels of pasteurellae (Fisher's exact test; $P=0.48$), nor was the prevalence of positive samples greater on these wetlands (chi-square test; $\chi^2=0.00$, $P=0.99$).

Epizootic mortality in our study sites occurred only at Delevan NWR during winter 1997: therefore, comparisons of water characteristics between wetlands experiencing and not experiencing outbreaks was limited to wetland sites within this refuge (Table 3). Within Delevan NWR, pH levels were significantly greater (ANOVA, $F_{1,3}=63.68$, $P=0.004$) in wetlands experi-

encing outbreaks (pH mean±SE: 8.1±0.08) than in those wetlands experiencing little or no mortality from avian cholera (pH: 7.7±0.07). The MANOVA analysis to compare calcium, magnesium, protein, and sodium between outbreak and nonoutbreak wetlands could not be performed because of the small number of wetlands.

Because no *P. multocida* was detected during winter 1997, ANOVA and MANOVA comparisons of water chemistries based on wetlands with detection of pasteurellae were restricted to winter 1998. During winter 1998, aluminum concentration in wetlands with detectable levels of *P. multocida* (Al mean±SE: 1.14±0.4) were significantly greater than in wetlands where no pasteurellae were recovered (0.3 ppm±0.13) (square root transformed; ANOVA; $F_{1,5}$=9.56, P=0.03). Calcium, magnesium, protein, and sodium concentrations in combination did not differ between wetlands based on detection of *P. multocida* (MANOVA; Wilk's lambda 0.08, $F_{4,2}$=6.01, P=0.15).

DISCUSSION

Similar to previous avian cholera mortality patterns observed at SNWRC, mortality during our study fluctuated between years. Although enzootic wetlands tended to have higher mortality during 1991–98, avian cholera mortality exceeding 50 birds was not observed frequently in 1997 or 1998, nor were outbreaks more likely to occur in enzootic wetlands during these latter 2 yr.

The *Pasteurella multocida* isolated from the water samples and sediment samples all were serotype 1. Serotype 1 is primarily responsible for avian cholera mortality in the Pacific and Central and Mississippi flyways (Brogden and Rhoades, 1983; Windingstad et al., 1984; Hirsh et al., 1990; Wilson et al., 1995).

In our study, there was greater overall avian cholera mortality during the winter of 1997 than in 1998 and two wetlands experienced outbreak level mortality of ≥50 birds in 1997; however, we were unable to detect *P. multocida* in the water or sediment of these wetlands. In contrast, we recovered *P. multocida* from wetlands during the fall of 1997 (Lehr, 2000) and the winter of 1998 when annual mortality at SNWRC was lower and no outbreaks were observed in any study wetlands. These results are in contrast to higher occurrence of *P. multocida* and greater distribution of avian cholera outbreaks in California during 1998 (Samuel et al., 2003). Our results also are inconsistent with previous studies documenting the presence of *P. multocida* in wetlands that experienced avian cholera mortality (Rosen and Bischoff, 1949; Rosen, 1969; Titche, 1979; Price and Brand, 1984; Windingstad et al., 1984, 1988; Backstrand and Botzler, 1986).

There are several possible explanations for the lack of an association between avian cholera mortality and the detection of *P. multocida* in wetlands. Because our sampling was limited to 10 sites, it is possible that *P. multocida* was present in wetland water and sediment but at undetectable levels during winter of 1997. It also is possible that birds acquired the pasteurellae from different wetlands than where they died. Because the onset of clinical signs from avian cholera ranges from six to 48 hr after infection (Friend, 1999), highly mobile waterfowl could die some distance from the original site of infection. However, the assumption of infection and mortality in the same wetland seems common in field studies on avian cholera (Rosen and Bischoff, 1949; Rosen, 1969; Titche, 1979; Price and Brand, 1984; Windingstad et al., 1984, 1988; Backstrand and Botzler, 1986).

Lastly, a variation in virulence and survival of *P. multocida* might explain why avian cholera outbreaks were observed without detecting pasteurellae in the environment. Virulence among strains within the same *P. multocida* serotype may vary (Wobeser, 1997). For *P. multocida* isolates collected at our study wetlands, virulence ranged from 0 to 100% in Pekin ducks (Ta-

ble 1 in Samuel et al., 2003). In contrast, the virulence of isolates collected during the winter of 1997 from other wetlands within SNWRC (Sutter NWR; 39°04′N, 121°44′W) ranged from 67% to 100% (Table 1 in Samuel et al., 2003). If the survival of pasteurellae is inversely related to virulence, as suggested by Rosen and Bischoff (1949), we may have not detected *P. multocida* in 1997 because of lower survival in the wetland environment.

We found higher pH levels at Delevan NWR in wetlands experiencing outbreaks in the winter 1997. We also found higher aluminum concentrations for wetlands from which *P. multocida* was isolated in winter 1998. Aluminum and pH and have not been previously associated with avian cholera. Given the number of statistical tests performed in our analyses, it is possible that these differences are a result of a Type I statistical error. We did not find significant differences in calcium, magnesium, or total protein between wetlands experiencing avian cholera outbreaks and those with little or no mortality or between wetlands with and without detectable *P. multocida.* We also did not find higher levels of sulfate, chloride, and specific conductance as predicted by the Windingstad et al. (1984) profile of an avian cholera wetland. Thus, the patterns we detected in water characteristics during this study were not consistent in distinguishing wetlands with detectable *P. multocida* and wetlands without the bacterium. Overall, we did not find clear evidence to support the hypothesis that the physical and chemical characteristics of wetland water can facilitate the presence of *P. multocida* and thereby allow some wetlands to serve as long-term sources (reservoirs) for *P. multocida.*

Carcass collection to reduce bacterial contamination in wetlands is the current method used to control avian cholera in wildfowl. Other tools to manage avian cholera have been suggested, including manipulation of water chemistry to reduce the survival of *P. multocida* (Friend, 1999). However, our results are discordant with previous field and laboratory studies that have associated survival or occurrence of *P. multocida* with physical and chemical properties of water (Bredy and Botzler, 1989; Price et al., 1992; Windingstad et al., 1984). Although it would be advantageous to predict which wetlands are at risk for avian cholera epizootics, we found that water characteristics fluctuated seasonally and from year to year on the intensively managed wetlands at SNWRC and that correlations, both between water characteristics and mortality or presence of *P. multocida,* were not consistent from year to year. We found no consistent evidence that managing the physical properties of water would have a significant impact on the likelihood of an avian cholera epizootic. Furthermore, manipulation of wetland water characteristics would have no substantial impact on the presence of *P. multocida* in wetlands.

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