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ENVIRONMENTAL CHARACTERISTICS ASSOCIATED WITH THE OCCURRENCE OF AVIAN BOTULISM IN WETLANDS OF A NORTHERN CALIFORNIA REFUGE

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Abstract: Avian botulism is an important disease affecting many species of waterbirds in North America, but the environmental conditions that initiate outbreaks are poorly understood. To determine wetland attributes associated with outbreaks of avian botulism in waterbirds at the Sacramento National Wildlife Refuge (SNWR), California, we compared environmental characteristics between wetlands where outbreaks occurred (outbreak wetlands) and did not occur (nonoutbreak wetlands). In June through October 1987–89, we monitored the occurrence of avian botulism via observations for sick or dead sentinel mallards (*Anas platyrhynchos*) placed in 4 wetland enclosures. During this same time period, we collected environmental data from the water column and sediments of each wetland enclosure at 10–14-day sampling intervals. Multivariate analysis was used to reduce 22 environmental variables to 7 factors for inclusion in subsequent statistical analyses. We found that outbreak wetlands had significantly lower redox potential than nonoutbreak wetlands. The probability of botulism in sentinel mallards was associated with increasing temperature, increasing invertebrate abundance or biomass, and decreasing turbidity. However, because these factors were not consistently higher in outbreak wetlands compared to nonoutbreak wetlands, they may have a more proximate effect in initiating an outbreak.

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Since the early 1900s, Type C avian botulism has caused the largest disease losses known to occur in North American waterfowl, killing tens of thousands to millions of birds in a single outbreak (Locke and Friend 1987). During the last 5 years, losses from botulism outbreaks in several U.S. and Canadian wetlands have been estimated in the millions (National Wildlife Health Center [NWHC], unpublished data). Avian botulism is a paralytic disease of birds resulting from ingestion of neurotoxin produced by the bacterium, *Clostridium botulinum,* Type C. This bacterium is a strict anaerobe that forms dormant spores in aerobic or other adverse environmental conditions. Dormant spores are re-

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sistant to heating and drying, can remain viable for years, and are widely distributed in wetland sediments (Mitchell and Rosendal 1987). *C. botulinum* spores can also be found in the tissues of many wetland inhabitants (Jensen and Allen 1960) such as aquatic insects, mollusks and crustacea, birds, and other vertebrates (Reed and Rocke 1992). Botulinum toxin is produced only when environmental conditions are suitable for spore germination and cell growth.

Despite the widespread distribution of spores of *C. botulinum* Type C, outbreaks of avian botulism in waterbirds are sporadic and unpredictable, occurring annually in some wetlands, but not in adjacent wetlands. Several ecological factors are thought to play a critical role in the timing and location of outbreaks by favoring spore germination and bacterial replication, providing a suitable energy source or substrate for bacterial growth and toxin production, and a means to transfer the toxin from the substrate to birds. Toxin is presumably transferred to waterfowl via invertebrates consumed as food or incidentally while feeding on other food items. The most suitable conditions for bacterial growth and toxin production in wetlands and the ecological features that precipitate outbreaks are unclear. For many years, avian botulism was thought to occur in shallow, stagnant waters with low dissolved oxygen, alkaline wetlands, and when mud flats were flooded or drained during warm summer months (Kalmbach and Gunderson 1934, Quortrup and Holt 1941, Bell et al. 1955, Rosen 1971). However, these conditions do not adequately characterize the timing and location of many botulism outbreaks in wetlands with deep, well-oxygenated water and stable water levels, or outbreaks that occur in late winter or spring.

From 1987–89, we conducted studies on the occurrence of avian botulism in several wetlands at SNWR. Our objectives were (1) monitor the occurrence and severity of botulism mortality in captive-reared mallards placed as sentinels in wetland enclosures; (2) compare specific environmental characteristics, including invertebrate populations and sediment and water conditions, between wetland enclosures in outbreak wetlands and in nonoutbreak wetlands; (3) determine if botulism outbreaks in sentinel birds were associated with temporal changes in environmental characteristics in wetland enclosures; and (4) correlate environmental characteristics with botulism mortality rates in sentinel mallards.

STUDY AREA

Our study was conducted at SNWR, near Willows, California. The refuge comprises 2,995 ha of intensively managed wetlands and is an important area for many species of migratory birds, especially waterfowl. This site has a documented history of Type C avian botulism outbreaks in waterbirds since 1958, typically occurring during late July through October (J. G. Mensik, SNWR, personal communication), and Type C botulinum spores were found to be highly abundant in SNWR wetlands (Sandler et al. 1993). We selected 10 wetlands for our study, ranging from 4 to 75 ha in size: 4 wetlands were flooded year-round, and 6 were flooded seasonally (Sep–Apr). Vegetation varied among wetlands, but dominant plant species included hardstem bulrush (*Scirpus acutus*), cattail (*Typha* spp.), swamp timothy (*Crypsis schoenoides*), smartweed (*Polygonum* spp.), and cocklebur (*Xanthium strumarium*).

METHODS

All selected wetlands were drained in April–May 1986, and a 1.6-ha enclosure was built in each as previously reported (Rocke and Brand 1994). We placed 40–50 captive-reared mallards (males and females) in enclosures to act as sentinels for the occurrence of botulism in each wetland and to determine relative mortality rates from botulism (Rocke and Brand 1994). Sentinels were released in June or July (Sep for seasonally flooded wetlands) and maintained through October from 1987 to 1989. Free-flying birds had access to the uncovered enclosures at all times. All animal handling and husbandry protocols were approved by the NWHC Animal Care and Use Committee.

To recover any sick or dead sentinel birds, all enclosures were searched on foot or by canoe and with assistance of retrieving dogs, 3–4 times each week. If mortality was detected, wetland enclosures were searched daily. Healthy sentinel birds were counted during each search and trapped periodically to conduct an accurate inventory. Sick, dead, and missing sentinel birds were replaced with healthy birds to maintain a consistent number of bird-exposure-days.

All sick birds found within enclosures were removed; a blood sample was withdrawn from the jugular vein and tested for Type C botuli-

num toxin via the mouse neutralization test (Quortrup and Sudheimer 1943). Sick sentinel birds were then euthanized by cervical dislocation and necropsied. All intact fresh carcasses found within enclosures were also necropsied. Blood collected from the heart of each carcass was tested for botulinum toxin. Because botulinum toxin can form postmortem in carcasses, an enclosure was not confirmed to have had botulism mortality in birds if botulinum toxin was detected in only a single carcass. For enclosures where botulism outbreaks occurred, daily botulism mortality rates for specific time intervals (10–14 days) were calculated by dividing the number of sick and dead birds by the number of bird-exposure-days within the interval when mortality occurred (Samuel and Fuller 1994).

We collected environmental data from the water column and 2 strata in the sediments (S1, S2) of each wetland enclosure at 10–14-day sampling intervals. Water column samples were collected at 1 location along each of 5 transects equally spaced within each enclosure; sample locations were randomized for each sampling interval. At these collection sites, we pumped water from approximately 7.5 cm above the sediment–water interface via a hand pump operated from a small boat. Pumped water was allowed to run freely for a few minutes and then collected by running the flowing sample down the side of a collection jar to avoid aeration. Oxidation–reduction potential (redox potential) was measured immediately with a specific ion electrode (Orion Research, Boston, Massachusetts, USA). Standardized redox potential (redox potential corrected to pH 7.0 and a temperature of 25°C) was calculated according to the formula reported by Smoot and Pierson (1979). Specific conductance (Markson Science, Phoenix, Arizona, USA), pH (Model 51; Yellowsprings Instrument, Marion, Massachusetts, USA), and turbidity (Hach, Loveland, Colorado, USA) were measured via standard meters. After water column samples had been collected and processed from a small boat, we collected a core sample from the upper 2.5 cm of sediment via a 30.5- × 2.54-cm stainless steel core sampler. These core samples were submitted to a local laboratory (Monarch Laboratories, Chico, California, USA) for determination of percent organic matter (POM) via standard techniques (American Public Health Association 1985).

Samples of interstitial water from the sediments (S1, S2) were collected at 5 permanent sampling stations arranged in an X-configuration to minimize disturbance of sediments prior to sampling and to ensure a representative sample. We used an interstitial water collection device described by Euliss and Barnes (1992) to collect samples from 2 sediment strata, 2.5 cm below the sediment–water interface (S1) and 11.25 cm below the sediment–water interface (S2). Nitrogen gas was used to evacuate the samples to prevent contamination with atmospheric oxygen (Euliss and Barnes 1992). Water from the collecting device was slowly added down the sides of collection jars to avoid aeration, and redox potential, pH, and conductivity were measured with standard meters as described above.

We collected invertebrates from the water column and benthic level at each of the sites previously sampled for water column data via a modified 10-cm-diameter single-tube device described by Euliss et al. (1992) that was placed 5 cm into the substrate. Benthic samples were sectioned by hand as described by Mackay and Euliss (1994). Prior to storage in 80% ethanol, we used a modified self-cleaning screen to concentrate samples into residues (Euliss and Swanson 1989). Invertebrate samples were sorted by hand into taxonomic groups, enumerated, dried to a constant mass at 60°C, and weighed to the nearest milligram on an analytical balance. We used keys by Usinger (1956), Ward and Whipple (1959), Grodhaus (1967), Pennak (1978), and Merritt and Cummins (1984) to identify specimens.

Data Analysis

Means for each environmental variable measured within each enclosure were calculated for each 10–14-day sampling interval. We estimated missing values for several means (n = 10) via regression analysis with highly correlated subsets of variables (BMDP procedure AM; Dixon et al. 1988). Means of the environmental variables (environmental parameters) were inspected for outlying values and nonnormal data distributions. To stabilize the variance, samples of invertebrates (counts and biomass) were $\log_{10}$-transformed [e.g., $\log_{10}$(biomass + 1.0)] following recommendations of Elliott (1977).

We used principal component analysis (SAS Institute 1989) to determine the variance structure among the 22 environmental parameters so as to aggregate highly correlated environmental

measurements and to parsimoniously reduce the number of parameters for subsequent analyses (Morrison 1976). Principal components with eigenvalues >1.0 were selected from the 22 variables. We used varimax rotation to produce factors that maximized the contribution of each environmental parameter to a single factor (Cooley and Lohnes 1971), which provided enhanced biological interpretation of each rotated factor. The rotated environmental factors provided the basis for further analysis of the relation between environmental parameters and the occurrence of botulism in sentinel birds.

Using repeated-measures analysis of variance (ANOVA; Kirk 1982), we tested for environmental differences between outbreak and nonoutbreak wetlands during 20 sampling intervals (6 from 1987, 6 from 1988, 8 from 1989). Scores from environmental factors (response variable) were analyzed by separate ANOVA. Changes among sampling intervals (the repeated measure) and sampling interval interactions with outbreak (or nonoutbreak) classification were also analyzed by ANOVA. We conducted all analyses using SAS PROC GLM with Type III sums of squares (SAS Institute 1989). We conducted *F*-tests for differences between outbreak and nonoutbreak wetlands with the mean square (MS) and degrees of freedom (df) for the wetland enclosures within each outbreak classification. The *F*-tests for differences among sampling intervals and for sampling interval and outbreak classification interactions were conducted with the MS and df for the interaction of sampling intervals and wetland enclosures within each outbreak classification.

We evaluated associations between environmental factors and the occurrence of botulism outbreaks in sentinel mallards during the 10–14-day intervals surrounding (5–7 days before and after) the collection of environmental samples from each outbreak wetland. A binary classification was used for each sampling interval with >1 botulism mortality or ≤1 mortality. In addition to environmental factors, we also included covariates for wetland enclosure, year, and autocorrelation (Bonney 1987) in the occurrence of botulism outbreaks between sequential sampling intervals. Analyses were conducted with the BMDPLR (Dixon et al. 1988) backwards stepwise logistic regression models. We used the procedures of Hosmer and Lemeshow (1989) in developing our results and assessing the appropriateness of our logistic regression models.

Finally, we determined the association between environmental factors and daily rates of botulism mortality in sentinel birds in wetland enclosures during outbreaks. We conducted a Pearson product-moment correlation analysis (SAS Institute 1989) between environmental factors and daily botulism mortality rates of sentinel birds during the 10–14-day intervals surrounding collection of environmental samples from each wetland enclosure.

Little is known about the relation between botulism outbreaks and environmental conditions. Because we considered our analyses exploratory in nature and because sample size was generally small, which resulted in lower statistical power, our results were considered significant at $P \leq 0.10$.

RESULTS

Of the 10 wetlands originally included in the study, only the 4 wetlands flooded year-round were included in our analyses. Outbreaks in seasonally flooded wetlands were inconsistent, and the environmental data collected in these wetlands were insufficient to warrant statistical evaluation.

Botulism outbreaks occurred in sentinel mallards in 2 wetland enclosures (P-2, P-8) in both 1987 and 1989 (Fig. 1); no outbreaks were detected in 1988. During these botulism outbreaks, weekly mortality rates in sentinel mallards ranged from 1.0 to 8.8 deaths/100 birds. Although botulinum toxin was found in 2 carcasses in another enclosure (T-F) in 1988, these cases were widely separated in time (>2 months), and botulism was not detected in wild birds in this or any other wetland in 1988. Because our criteria for classifying outbreak wetlands for this study required the detection of >1 carcass with botulinum toxin, the cases in T-F in 1988 were not considered to constitute an outbreak.

The range and variability of wetland conditions in our 4 study sites (means, minimums, maximums) fell within expected ranges (Table 1). Water temperatures varied from 13.0 to 26.8°C, with sediment temperatures generally 1–2 degrees higher. The water was very fresh, with specific conductivity values in the range of 88–896 μmhos/cm, and water pH was neutral to moderately alkaline, within a range of 6.0–8.4. Invertebrates included in our statistical

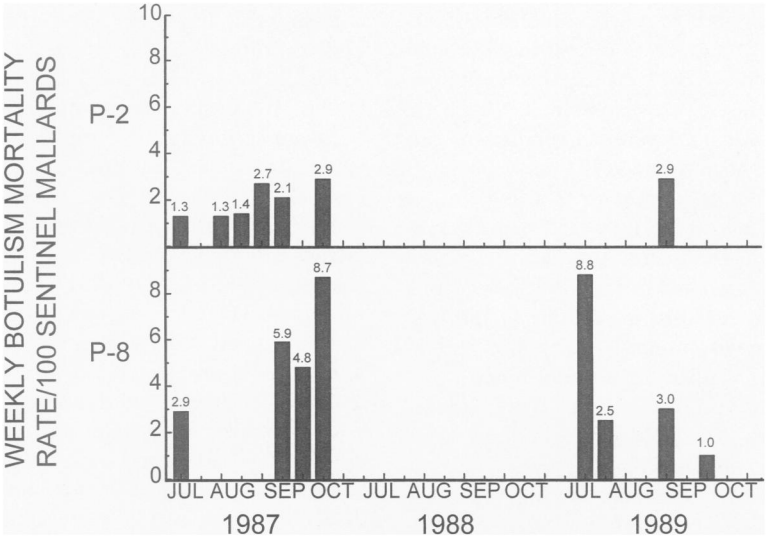

Fig. 1. Weekly rates of botulism mortality per 100 sentinel mallards in 2 1.6-ha wetland enclosures (P-2, P-8) where botulism outbreaks occurred at the Sacramento National Wildlife Refuge, California, 1987–89.

analysis (counts and benthic biomass) included gastropods, nematodes, oligochaetes, eubranchipods, lymnaeid snails, cyclopoid copepods, ostracods, unknown dipterans, ceratopogonids, chaoborids, culicids, tipulids, ephydrids, notonectids, muscids, stratiomyids, unknown chironomids, and chironomids of the genera *Goeldichironomus, Glyptotendipes, Tanypus,* and *Procladius.* Other invertebrates had very low biomass or counts and were only included in total biomass calculations.

Seven principal components identified by factor analysis accounted for 79% of the variation in the original 22 environmental parameters. Rotated factor loadings showed that environmental parameters in the soil and water were highly correlated (Table 2). The resulting 7 environmental factors had a relatively simple interpretation in relation to the original environmental parameters. Redox potential variables (REDOX) explained the largest portion of the variation (23%) found in the environmental measurements. Temperature variables (TEMP) and a factor consisting of specific conductivity and water pH (SC–pHW) accounted for an additional 26% of the variation. The fourth factor (pHSOIL) represented soil pH, and the fifth factor (INVERT) represented variables related to invertebrates. The sixth factor (DO–DEPTH–POM) was positively associated with dissolved oxygen and water depth but negatively associated with percent organic matter. Water turbidity was the only environmental variable strongly represented by the seventh factor (TURB).

Comparisons Between Outbreak and Nonoutbreak Wetlands

Repeated measures ANOVA indicated outbreak wetlands had lower REDOX factor scores ($F_{1,2}$ = 18.3, P = 0.05) than nonoutbreak wetlands during our study (Fig. 2). None of the remaining environmental factors showed a consistent difference (Ps > 0.20) between the outbreak and nonoutbreak wetlands. Significant changes in environmental factors among sampling intervals were found for REDOX ($F_{19,38}$ = 12.6, P < 0.01; Fig. 2), TEMP ($F_{19,38}$ = 24.8, P < 0.01; Fig. 3), pHSOIL ($F_{19,38}$ = 2.88; P < 0.01; Fig. 4), and SC–pHW ($F_{19,38}$ = 3.42, P < 0.01; Fig. 5). No sampling interval differences (Ps > 0.20) were found for INVERT, DO–DEPTH–POM, or TURB factors. The only significant interaction between botulism outbreaks and sampling intervals was found for SC–pHW ($F_{19,38}$ = 2.14; P = 0.02).

Comparisons Within Outbreak Wetlands

In outbreak wetlands (P-2, P-8), logistic regression analyses showed an association between the probability of botulism in sentinel mallards and TEMP (χ^2_1 = 8.95, P < 0.01), INVERT (χ^2_1 = 5.64, P = 0.02), and TURB (χ^2_1 = 12.78, P < 0.01) factors. No differences (Ps

Table 1. Environmental variables measured and averaged (no. of sampling intervals [*n*], mean [$\bar{x}$], minimum [min] and maximum [max]) in 1.6-ha wetland enclosures where botulism outbreaks occurred (outbreak wetlands) and did not occur (nonoutbreak wetlands) in sentinel mallards at the Sacramento National Wildlife Refuge, California, 1987–89. Outbreak wetlands were further separated into outbreak intervals (i.e., when botulism outbreaks occurred in sentinel mallards) and nonoutbreak intervals (i.e., when no outbreaks occurred). For each sampling interval, environmental measurements averaged from 5 locations distributed throughout the wetland enclosure included redox potential (redox) and standardized redox potential (stand. redox) measured in millivolts, temperature measured in °C, specific conductivity measured in μmhos/cm, pH, numbers of benthic invertebrates (benthic counts; log transformed), mass (g) of benthic invertebrates (benthic biomass; log transformed), mass (g) of total invertebrates (total biomass; log transformed), dissolved oxygen in the water measured in mg/L, percent organic matter in the sediments, water depth measured in centimeters, and water turbidity measured in nepholometer turbidity units.

	Outbreak wetlands (P-2, P-8)								Nonoutbreak wetlands (T-F, T-19)			
	Outbreak intervals				Nonoutbreak intervals							
Variable	*n*	$\bar{x}$	min	max	*n*	$\bar{x}$	min	max	*n*	$\bar{x}$	min	max
Redox W[a]	15	208	50	338	40	196	−26	467	56	219	8	477
Redox S1[b]	15	111	−24	303	40	19	−171	254	56	80	−80	337
Redox S2[c]	15	62	−40	256	39	47	−165	289	56	49	−174	375
Stand. redox W	15	251	86	367	40	227	16	505	56	248	17	485
Stand. redox S1	15	109	−43	252	40	28	−177	274	56	86	−65	317
Stand. redox S2	15	61	−43	252	39	47	−165	289	56	63	−160	359
Temperature W	15	22.4	18.3	26.5	41	20.6	13.0	26.6	57	20.4	12.1	26.8
Temperature S1	15	23.9	19.4	28.3	40	22.1	13.5	28.0	56	21.7	13.7	27.6
Temperature S2	15	23.8	19.5	27.6	39	22.1	13.9	28.0	56	21.8	14.4	27.3
Conductivity W	15	474	285	882	41	428	231	896	57	336	88	864
Conductivity S1	15	793	584	1086	40	733	530	1018	56	670	222	1186
Conductivity S2	15	1028	745	1275	39	1020	688	1510	56	1140	679	1819
pH W	15	7.73	7.09	8.80	41	7.54	7.00	8.77	57	7.51	6.38	8.75
pH S1	13	7.26	7.00	7.47	38	7.26	6.75	8.03	53	7.19	6.50	8.35
pH S2	13	7.14	6.94	7.53	37	7.30	6.73	8.02	53	7.28	5.99	8.87
Benthic counts	15	1.68	1.24	2.15	38	1.53	0.83	2.41	55	1.72	0.87	2.64
Benthic biomass	15	0.010	0.001	0.042	38	0.006	0.000	0.049	55	0.005	0.00	0.02
Total biomass	15	0.012	0.003	0.045	38	0.009	0.002	0.051	55	0.008	0.00	0.08
Dissolved oxygen	15	3.94	1.51	7.05	41	4.20	0.92	10.32	57	4.20	1.00	10.05
% organic matter	15	12.1	4.5	22.5	39	10.8	4.7	18.3	57	6.1	3.8	7.8
Depth	15	52.5	35.8	100.8	41	53.6	22.8	105.7	57	53.8	34.8	85.6
Turbidity	15	11.4	2.4	34.3	40	23.8	2.2	150.0	57	25.6	2.1	78.2

[a] W refers to water samples collected 7.5 cm above the sediment–water interface.
[b] S1 refers to interstitial water samples collected 2.5 cm below the sediment–water interface.
[c] S2 refers to interstitial water samples collected 11.25 cm below the sediment–water interface.

Table 2. Seven principal factor patterns after varimax rotation of environmental variables measured in wetland enclosures at the Sacramento National Wildlife Refuge, California, 1987–89. Standardized regression coefficients indicate sign and relative strength of environmental variables influencing each factor. Only important coefficients are included.

Factor	Environmental variable	Coefficient
REDOX	Redox potential W[a]	0.858
	Redox potential S1[b]	0.925
	Redox potential S2[c]	0.937
	Standardized redox potential W	0.863
	Standardized redox potential S1	0.924
	Standardized redox potential S2	0.923
TEMP	Temperature W	0.962
	Temperature S1	0.951
	Temperature S2	0.948
SC–pHW	Specific conductivity W	0.743
	Specific conductivity S1	0.850
	Specific conductivity S2	0.760
pHSOIL	pH W	0.558
	pH S1	0.926
	pH S2	0.947
INVERT	Benthic invertebrates	0.466
	Benthic invertebrate biomass	0.910
	Total invertebrate biomass	0.905
DO–DEPTH–POM	Dissolved oxygen	0.575
	Depth	0.556
	Percent organic matter	−0.647
TURB	Turbidity	0.906

[a] W refers to water samples collected 7.5 cm above the sediment–water interface.
[b] S1 refers to interstitial water samples collected 2.5 cm below the sediment–water interface.
[c] S2 refers to interstitial water samples collected 11.25 cm below the sediment–water interface.

> 0.10) were found for other environmental factors or serial correlation among sequential sampling intervals, although REDOX (χ^2_1 = 2.47, P = 0.12) and pHSOIL (χ^2_1 = 2.61, P = 0.11) approached our significance criteria. Coefficients from the logistic regression model indicated increasing values of TEMP and INVERT were correlated with a higher probability of botulism outbreaks. However, water turbidity appeared to have the opposite relation: decreasing levels of turbidity were correlated with a higher probability of botulism outbreaks. When REDOX and pHSOIL were included in the logistic regression model, they showed opposite effects. Botulism outbreaks were positively related to REDOX and negatively related to pHSOIL.

Preliminary examination of daily mortality rates during outbreaks indicated a larger range of weekly mortality rates in P-8 than in P-2 (Fig. 1). Therefore, correlations between mortality rates and the 7 principal factors were conducted separately for each enclosure. For P-2, daily

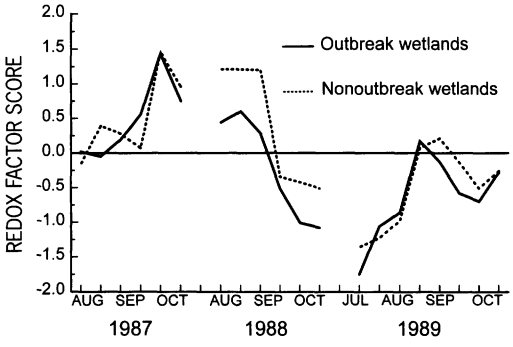

Fig. 2. Mean principal factor scores for redox potential (REDOX) in wetland enclosures where botulism outbreaks occurred and did not occur at the Sacramento National Wildlife Refuge, California, 1987–89.

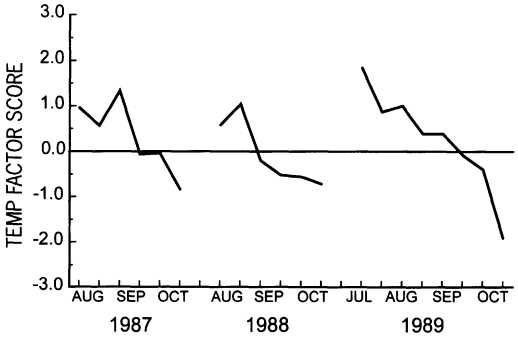

Fig. 3. Mean principal factor scores for temperature (TEMP) in wetland enclosures at the Sacramento National Wildlife Refuge, California, 1987–89.

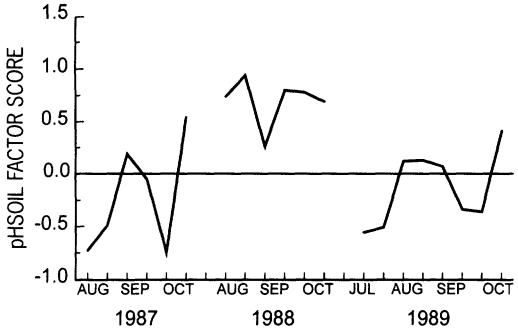

Fig. 4. Mean principal factor scores for sediment pH (pHSOIL) in wetland enclosures at the Sacramento National Wildlife Refuge, California, 1987–89.

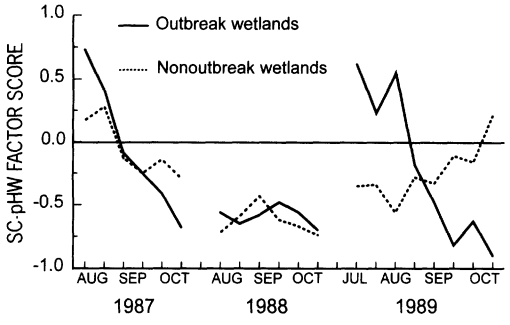

Fig. 5. Mean principal factor scores for specific conductivity and water pH (SC–pHW) in wetland enclosures where botulism outbreaks occurred and did not occur at the Sacramento National Wildlife Refuge, California, 1987–89.

mortality rates during botulism outbreaks (excluding Jul 1987 due to missing factor data) were negatively correlated with SC–pHW ($\rho = -0.87$, $P = 0.02$, $n = 6$) and INVERT ($\rho = -0.90$, $P = 0.01$, $n = 6$) factors. In contrast, daily botulism mortality rates for P-8 (excluding Jul 1987) were positively correlated with TURB ($\rho = 0.83$, $P = 0.02$, $n = 7$).

DISCUSSION

For most botulism outbreaks in wild waterfowl, the site of botulinum toxin ingestion and the population of birds that is actually at risk for contracting botulism are unknown, which makes comparisons to local environmental conditions difficult. In contrast, the use of sentinel mallards in our study provided a known and consistent population at risk, assured knowledge of the site of toxin ingestion, and permitted the calculation of botulism mortality rates. Botulism outbreaks in sentinel mallards occurred 4 times during our study, with weekly losses ranging from 1.0 to 8.8 birds/100 at risk. For a typical fall (Sep–Oct) mallard population of ≥25,000 at SNWR (J. G. Mensik, SNWR, personal communication), mortality rates of 1–9% could result in the death of 250–2,200 mallards/week, assuming the entire population was at equal risk of contracting botulism.

Comparisons Between Outbreak and Nonoutbreak Wetlands

Factor scores for REDOX were generally lower in outbreak wetlands than nonoutbreak wetlands. In biological systems, redox potential of the surrounding environment influences the direction of biochemical reactions, the operation of enzyme systems and electron acceptors, and thus the metabolic processes that yield energy for growth (Hewitt 1950). In soils or sediments, measured values of redox potential reflect the intensity of reduction (Gambrell and Patrick 1978) and the type of microbial activity that prevails (Watanabe and Furusaka 1980), but measurements of redox potential are often difficult to interpret. Because *Clostridium botulinum* is a strict anaerobe, one might expect more growth to occur at lower redox potential. However, laboratory studies suggest, in the absence of oxygen, that redox potential alone does not appear to be a limiting factor in the growth of *Clostridium botulinum* (Smoot and Pierson 1979) and may interact with other factors such as salinity and pH. The association between botulism outbreaks and redox potential may possibly be indirect and due to the competitive or even synergistic effects of other wetland microbes.

No other consistent differences were found between factor scores in outbreak and nonoutbreak wetlands, although significant temporal changes were detected for REDOX, TEMP, and pHSOIL factors (Figs. 2–4). The strong seasonal pattern for TEMP was expected, as soil and water temperature steadily declined from July to October. For REDOX and pHSOIL, however, yearly differences, as well as seasonal patterns, were evident. In 1988, pHSOIL and REDOX patterns appeared different than patterns observed in 1987 and 1989. Interestingly, in 1988, no botulism outbreaks were detected in sentinel or free-ranging wild birds anywhere on the refuge, which was fairly unusual for SNWR. We also found a significant interaction for SC–pHW between wetland classification and time (Fig. 5). In 1987 and 1989, when botulism outbreaks occurred, seasonal trends in

SC–pHW were similar in values and ranges in outbreak wetlands. For nonoutbreak wetlands, however, the seasonal trends differed: SC–pHW decreased during 1987 but increased during 1989. In 1988, when no botulism occurred, values of SC–pHW in both outbreak and nonoutbreak wetlands were lower than 1987 and 1989 and were without apparent seasonal trends.

Comparison Within Outbreak Wetlands

For outbreak wetlands, logistic regression analysis revealed that the probability of botulism in sentinel mallards increased with higher TEMP and INVERT factor scores and lower TURB factor scores. Botulism outbreaks occurred in the wetlands we studied only during sampling intervals with water temperatures ≥18.3°C (Table 1). Water temperature ranged from 13.0 to 26.6°C during nonoutbreak intervals and ranged from 9.6 to 26.8°C in nonoutbreak wetlands. The seasonal occurrence of botulism in waterbirds has long been recognized (Kalmbach and Gunderson 1934). Thus, an association between wetland temperature and the probability of botulism outbreaks was not very surprising. However, elevated temperature alone does not appear to initiate outbreaks.

Invertebrates are thought to be the primary source of toxin for birds, acting as either a substrate for toxin production or a means of transfer from the substrate to the birds. Previous investigators (Jensen and Allen 1960) presented empirical evidence suggesting botulism outbreaks at the Bear River Migratory Bird Refuge in Utah coincided with a sharp decline of the predominant benthic invertebrates following a population peak, presumably in response to decreasing oxygen. The authors postulated that dead invertebrates provided substrate that facilitated bacterial growth and production of botulinum toxin. In our study, the average biomass of invertebrates was higher in outbreak intervals (total: 0.010 g; benthic: 0.012 g) than either nonoutbreak intervals (total: 0.006 g; benthic: 0.009 g) or nonoutbreak wetlands (total: 0.005 g; benthic: 0.008 g; Table 1). Hence, our results suggested invertebrates may play a role in initiating botulism outbreaks by providing a means of toxin transfer.

Average turbidity during outbreak intervals (11.4 units) was less than half the values during nonoutbreak intervals (23.8 units) and in nonoutbreak wetlands (25.6 units; Table 1). Lower turbidity in a wetland would allow more light penetration, thereby increasing primary productivity, and we would not expect this factor to directly affect the growth of *C. botulinum*. More likely, the association between decreasing turbidity and botulism outbreaks is indirect and due to the influence of turbidity on invertebrates or other microbial populations, and hence their interactions with *C. botulinum*.

Botulism mortality rates were associated with different environmental factors in P-2 and P-8 (the 2 enclosures where outbreaks occurred), and these relations appeared to contradict the results of our previous analyses. In P-2, a higher botulism mortality rate was positively associated with lower INVERT and SC–pHW factor scores, but the range of weekly botulism mortality rates was limited in this enclosure (0.013–0.029). Perhaps the changes in invertebrate and SC–pHW factor scores were related to other wetland characteristics and were simply coincident to changes in the botulism mortality rate. In P-8, which experienced a wider range of botulism mortality (0.010–0.088), the daily rates were associated with higher turbidity, seemingly in contrast to our logistic regression analysis that indicated that higher turbidity decreased the probability of an outbreak. We suspect increased investigator activity within the enclosure upon discovery of a botulism outbreak may have inadvertently elevated the turbidity of the water and resulted in an artificial association.

Interestingly, dissolved oxygen, percent organic matter, and water depth (the DO–DEPTH–POM factor), variables previously believed to play a major role in the initiation of botulism outbreaks (Kalmbach and Gunderson 1934, Quortrup and Holt 1941, Rosen 1971), were not important in our study. We found no association between DO–DEPTH–POM factor scores and the probability of botulism outbreaks within an enclosure, nor were any differences in this factor detected between outbreak and nonoutbreak wetlands. Botulism outbreaks occurred in sentinel mallards in wetlands with low to moderate levels of dissolved oxygen in the water column (1.5–7.0 mg/L), a wide range of organic matter in the sediments (4.5–22.5%), and moderate water depth (36–101 cm; Table 1). Our study showed that shallow water is not a prerequisite to, nor is dissolved oxygen predictive of, a botulism outbreak in waterbirds.

MANAGEMENT IMPLICATIONS

The number of permanent wetlands (4) used in this study was small, and all were located

within a narrow geographic area (SNWR); therefore, the range of environmental conditions we measured was not very broad. We were able to analyze the data for simple associations between environmental parameters and botulism outbreaks, but not for complex associations that might be expected with variables like pH and redox potential, for which optimal ranges are likely to occur. For example, botulism outbreaks occurred in wetland enclosures with a sediment pH (pH S1) that ranged between 7.0 and 7.5 (Table 1), whereas a much broader range of pH was evident in enclosures where outbreaks did not occur (6.0–8.3). In spite of its limitations, the results of our study convey a preliminary indication of wetland characteristics that are associated with botulism outbreaks and provide a basis for future research and development of management strategies to reduce botulism losses. Most notably, we identified several environmental conditions associated with the occurrence of botulism outbreaks in high-risk wetlands at SNWR, including higher temperature and higher invertebrate abundance or biomass. However, because these factors were not consistently higher in outbreak wetlands compared to nonoutbreak wetlands, they may have a more proximate effect in initiating an outbreak. Other wetland characteristics, such as redox potential, appear to play a greater role in determining whether a wetland is at high or low risk for botulism outbreaks, and these risk factors apparently vary annually.

Because wetlands tend to be seasonally dynamic, evaluation of environmental conditions that confer risk is complicated. Our results suggest several factors warrant further examination, including temperature, redox potential, pH, and conductivity. Future investigations should consider if characteristics of individual wetlands predetermine their risk for botulism outbreaks, and if other proximate conditions are required to initiate mortality. Also, more work is needed to determine how mortality rates from botulism are related to environmental conditions. Knowledge of these factors could be used to develop wetland management strategies that reduce the risk of botulism outbreaks and reduce mortality of waterbirds.

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