Differences in the Vulnerability of Waterbird Species to Botulism Outbreaks in Mediterranean Wetlands: an Assessment of Ecological and Physiological Factors

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ABSTRACT

Avian botulism kills thousands of waterbirds every year, including endangered species, but information about the differences between species in vulnerability to botulism outbreaks and the capacity to act as carriers of *Clostridium botulinum* is still poorly known. Here, we estimated the vulnerability to botulism of 11 waterbird species from Mediterranean wetlands by comparing the number of affected birds with the census of individuals at risk. The capacity of different species to act as carriers was studied by detecting the presence of the *C. botulinum* type C/D botulinum neurotoxin (BoNT) gene in fecal samples and prey items of waterbirds in the wild and by the serial sampling of cloacal swabs of birds affected by botulism. We found differences among species in their vulnerabilities to botulism, probably related to feeding habits, season of arrival, turnover, and, possibly, phylogenetic resilience. The globally endangered white-headed duck (*Oxyura leucocephala*) showed mortality rates in the studied outbreaks of 7% and 17% of the maximum census, which highlights botulism as a risk factor for the conservation of the species. Invasive water snails, such as *Physa acuta*, may be important drivers in botulism epidemiology, because 30% of samples tested positive for the BoNT gene during outbreaks. Finally, our results show that birds may excrete the pathogen for up to 7 days, and some individuals can do it for longer periods. Rails and ducks excreted *C. botulinum* more often and for longer times than gulls, which could be related to their digestive physiology (i.e., cecum development).

IMPORTANCE

Botulism is an important cause of mortality in waterbirds, including some endangered species. The global climate change may have consequences in the ecology of wetlands that favor the occurrence of botulism outbreaks. Here, we offer some information to understand the ecology of this disease that can be useful to cope with these global changes in the future. We have found that some species (i.e., coots and dabbling ducks) are more vulnerable to botulism and have a more relevant role in the onset and amplification of the outbreaks than other species (i.e., flamingos and grebes). Feeding habits can explain these differences in part; in addition to the well-known role of necrophagous fly maggots, we found here that water snails are frequent carriers of *Clostridium botulinum*. This is relevant, because these water snails can thrive in eutrophic and polluted wetlands, exacerbating other changes driven by climate change in wetlands.

Avian botulism is an intoxication characterized by a severe flaccid paralysis of the muscles and final death by respiratory failure caused by the botulinum neurotoxins (BoNTs) produced by different types of *Clostridium botulinum* (1). There are 7 types of BoNT single toxins, plus their mosaics (with characteristics of two toxins), and each one affects different animal species and/or humans. In particular, avian botulism outbreaks in freshwater waterbirds are generally caused by the mosaic type C/D (2, 3, 4). Outbreaks mostly occur between summer and autumn, when high temperatures can favor the multiplication and toxigenesis of *C. botulinum* on bird carcasses. Eutrophic wetlands with a low potential redox offer anaerobic environments, and the repeated occurrence of botulism outbreaks can favor the presence of spores in the digestive tract of waterbirds. Under these conditions, any mortality of waterbirds (some of them containing *C. botulinum* spores in their digestive tracts) can start an exponential process known as the carcass maggot cycle. Briefly, during this cycle, maggots that develop on carcasses along with *C. botulinum* accumulate BoNT and act as a source of intoxication for healthy waterbirds, resulting in an exponentially increasing mortality rate that resembles that of an infectious disease (3, 5, 6).

Botulism stands as one of the major diseases of waterbirds, because it causes high waterbird losses, affects a broad spectrum of species, occurs annually, and seems to be increasing in its geographic area (7). Populations of species that are numerous and geographically widespread may cope with sporadic and sudden high losses (i.e., *Anas platyrhynchos* mallards or *Fulica atra* Eurasian coots), while populations of endangered or local species are more vulnerable to stochastic processes, and a single botulism outbreak may have catastrophic consequences for their viability (3). For example, in Taiwan, botulism killed 73 black-faced

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spoonbills (*Platalea minor*) out of a population of 1,600 mature individuals and, in Hawaii, it killed 183 Laysan ducks (*Anas layсанensis*) out of a population of 680 mature individuals (8, 9). Botulism has been also linked with the disappearance of the great black-backed gulls (*Larus marinus*) breeding in eastern Lake Ontario (10) and with declines in the population of pintail ducks (*Anas acuta*) in the United States (7). In Spain, 2 threatened species, the white-headed duck (*Oxyura leucocephala*) and the ferruginous duck (*Aythya nyroca*), regularly die during botulism outbreaks (11). In addition, white-headed ducks usually breed in highly eutrophic wetlands, such as the ones that receive effluent of wastewater treatment plants (12, 13), where the risk of botulism outbreaks is high (11, 14).

Vulnerability to botulism intoxication differs between bird species, and only vultures seem to be resistant (1). Foraging behavior is probably a significant factor influencing this, because dabbling waterfowl and surface-feeding shorebirds seem to be more prone to intoxication than diving ducks or probers (1, 11, 15). The relative vulnerability to botulism of waterbird species, however, has yet to be quantitatively evaluated, with mortality estimates from censuses completed during disease outbreaks. This is central to determining priority conservation measures against botulism, particularly in the case of endangered species vulnerable to botulism.

Another aspect to study about avian botulism ecology is the potential of birds as carriers or reservoirs. It is still unknown how long a bird may maintain spores/cells in its gastrointestinal tract or whether the disease is a toxico-infection, when the toxin is produced in the digestive tract because of the lack of competition of a robust microbiota, or is an intoxication, when the toxin is ingested from the environment (16). In previous studies, Reed and Locke (5) reported that 21 of 40 healthy mallards inhabiting wetlands with botulism history carried *C. botulinum* spores in their ceca. We also found that waterbirds can spread these bacteria with their feces (14). In fact, the similarity of strains of *C. botulinum* type C/D in both extremes of the Palearctic migratory flyway (Sweden and Spain) may reflect this role of waterbirds as carriers (2).

With this study, we aimed to (i) estimate differences in vulnerability to botulism of common waterbirds inhabiting Mediterranean wetlands, including the globally endangered white-headed duck, (ii) study potential carrier organisms of the bacterium that could explain differences in vulnerability related to diet, and (iii) investigate the fecal excretion of *C. botulinum* type C/D by healthy and intoxicated birds.

MATERIALS AND METHODS

Study area. The study area consisted of 3 wetlands in Castilla-La Mancha, a flat region situated in the central Spanish plateau mostly devoted to agriculture: Tablas de Daimiel floodplain, Navaseca Lake, and Prado Lake. Tablas de Daimiel is a national park (TDNP) that protects 1,675 ha of a floodplain wetland; it is a special protection area for birds and is included in the Ramsar List. Navaseca Lake (24.3 ha) is located near TDNP (about 6.5 km). It is a permanently flooded wetland that is highly eutrophic, because it receives the effluent of a wastewater treatment plant. Prado Lake (50.5 ha) is situated near TDNP (about 25 km); it is a temporary, endorheic, saline wetland that also receives inputs of treated wastewater and is highly eutrophic. These 3 wetlands are winter refuge and/or breeding sites of a variety of waterbird species, including endangered species, such as the crested coot (*Fulica cristata*), white-headed duck, ferruginous duck, or marbled teal (*Marmaronetta angustirostris*). Avian botulism is endemic in the region, where outbreaks have been attributed to the mo-

saic *C. botulinum* type C/D (2, 11). Botulism outbreaks occurred in summer to early autumn in Navaseca and Prado Lakes in 2011 and 2012 during the study period. Botulism type C/D was confirmed as the principal cause of death in both lakes by using the mouse bioassay test with plasma from diseased birds (17). The antitoxin type C used in the bioassay was purchased from the Centers for Disease Control (reference no. BS0611). The climate in this area is cold-temperate continental, with a pronounced dry season and annual rainfall of around 400 to 500 mm. All of the wetlands studied are between 603 and 670 m above sea level.

Estimates of living and dead waterbirds in Navaseca Lake. Waterbird censuses were performed in Navaseca Lake during outbreak periods in 2011 and 2012 in order to estimate the numbers of birds at risk in each moment. In this wetland, a protocol for the collection of sick waterbirds was established, which allowed us to compare the proportion of sick or dead birds removed every day with the number of live birds at risk in the previous census for the different species. Navaseca Lake was too large to be observed from one point, and there were islands in it, so we divided it in 6 sections to facilitate the census. A census of live birds of different species was performed from a peripheral road just after sunrise by using binoculars and a telescope at 6 observation points (one for each section). A tape recorder was used for counting the observed individuals of each species, and the record was afterward transcribed to a database. The censuses were performed during outbreak periods: in 2011, 4 censuses were performed between July and August at intervals of 15 days; in 2012, 9 censuses were performed between the second half of June and August with weekly intervals (except one missing count at the end of July). As part of the botulism control protocol, and to obtain data about affected waterbirds, 1 or 2 persons surveyed on foot the shore of Navaseca Lake every morning, collecting dead and sick birds. Once a week, the survey was also taken by boat in order to collect birds from several small islands located in the center of the lake. After identification of the species by experts, all of the dead birds were buried, and the sick ones were sent to the wildlife rehabilitation center of the regional government (Joint of Commonalities of Castilla-La Mancha [JCCM]). The numbers and species of all dead (and sick) birds were recorded. Although these counts tend to underestimate the mortality, they were used as an estimation index to be compared with the census of live birds. Finally, 23 sick birds that died in the wildlife rehabilitation center were necropsied, and samples of cecum, intestines, and liver were analyzed for the presence of *C. botulinum* by real-time PCR.

Sampling of aquatic invertebrates. During outbreak periods in Navaseca and Prado Lakes in 2011, we sampled aquatic invertebrates and small fishes to identify possible sources of intoxication for waterbirds. We captured them from the water surface of the shore using a 500- μ m-mesh sieve. In 2012, we made a greater sampling effort of snails in Navaseca Lake, because we observed that these invertebrates were very abundant and that they fed on carcasses, so we suspected that they could be an important source of BoNT for birds. Captured invertebrates were kept alive in sterile plastic containers until analysis during the following 24 h. In the laboratory, they were identified and pooled according to the taxonomic family. Samples of invertebrates and fishes included specimens of nonbiting midge larvae (Chironomidae, $n = 15$), water boatmen (Corixidae, $n = 24$), back swimmers (Notonectidae, $n = 4$), crustaceans (Ostracoda, Copepoda, and Cladocera, $n = 15$), freshwater snails (Physidae, $n = 53$), beetle larvae (Coleoptera, $n = 5$), mayfly larvae (Ephemeroptera, $n = 3$), soldier fly larvae (Stratiomyidae, $n = 2$), and mosquitofish (*Gambusia holbrooki*, $n = 8$).

Sampling of waterbird feces and healthy bird cloacal swabs. Waterbird fecal samples were collected during outbreak and nonoutbreak periods. Fecal sampling during outbreak periods was performed in 2011 between late July and October in Navaseca Lake (number of sampling visits [n_s] = 4) and Prado Lake ($n_s = 1$). Fecal sampling during nonoutbreak periods was performed between June and early July 2011 in Navaseca Lake ($n_s = 3$), between August and October 2011 in TDNP ($n_s = 3$), and between January and April 2012 in Navaseca Lake ($n_s = 5$) and in Tablas de Daimiel National Park ($n_s = 1$). In each sampling visit, around 30 fresh

fecal samples from Anatidae (ducks), Rallidae (rails), and Charadriiformes (shorebirds and gulls) were collected with sterile swabs and kept at 4°C in plastic bags with zip closures until processing during the next 24 h. In order to ensure the identity of the fecal samples from these birds, samplings were performed in the shores where flocks of birds of a single family or order were observed resting for long periods.

Healthy bird cloacal sampling was done at Navaseca Lake once per season in 2012. We principally captured common moorhens (*Gallinula chloropus*) by using funnel traps located on the shore of the lake, where groups of them usually fed. In previous studies, monitoring of feces showed the presence of *C. botulinum* in 2.9% of droppings of rails, including common moorhens, which suggested that healthy birds of this species could be carriers of the bacteria (14). We also tried trapping moorhens in TDNP, but we were not successful. Traps were baited with grain and checked every hour. Captured birds were removed and held in bags before banding and sample collection. Age (juvenile or adult) was determined using plumage criteria (18). Birds were marked with a metal ring, and a cloacal sample was taken with a sterile swab and kept in a sterile transport container (Aptaca, Copan) until analysis. After sampling, the birds were released. Cloacal swabs were also taken from recaptured birds.

Fecal excretion of *C. botulinum* type C/D by sick birds. In 2012, waterbirds showing botulism clinical signs were collected during the 2 outbreaks that occurred in Navaseca and Prado Lakes. They were monitored to study the chronology of fecal excretion of *C. botulinum* type C/D after admission at the regional wildlife rehabilitation center. Upon admission, birds were marked with color plastic bands, and a cloacal sample was taken from each with a sterile swab that was kept in a transport container (Aptaca, Copan). For the following 5 days, birds were maintained in nursing pens and received support treatment consisting of antibiotic therapy (marbofloxacin [Marbocyl 2%]; Vetoquinol), vitamin complex (Duphapharl Multi; Pfizer), intravenous fluids (Ringer's lactate), and tube feeding. During this period, another 2 cloacal swabs were taken on days 3 and 5. After this time, if birds recovered, they were taken to a bigger enclosure before being released. During this period, cloacal swabs were taken when birds needed to be handled and before they were released. The intended sampling scheme was to collect cloacal samples at 2-day intervals; however, due to the different times of recovery of each bird and to avoid interfering with the center's staff work, the sampling scheme was varied.

***C. botulinum* type C/D detection.** Cloacal swabs, bird feces, necropsy samples, and aquatic invertebrates were processed for detection of *C. botulinum* type C/D, as previously described (19). Briefly, samples were cultured in 9 ml of commercial cooked meat broth supplemented with vitamin K₁, glucose, and hemin (BD BBL cooked meat medium with glucose, hemin, and vitamin K) using an anaerobe container system (BD GasPak 129 EZ) over a period of 3 days at 37°C. DNA was extracted by boiling the pellet obtained from 1 ml of the culture broth in 300 µl of distilled water. The solution obtained after centrifugation was used as the PCR template. Real-time PCR was performed as described by Sánchez-Hernández et al. (20) for the detection of the genes encoding both type C and type C/D mosaic toxins (2). This protocol permitted detection of spores, vegetative cells, or DNA, so we could identify the birds harboring or excreting the bacteria.

Statistical analysis. The overall mortality rate during botulism outbreaks in Navaseca Lake was calculated as the percentage of dead and sick individuals of each species relative to the maximum number of individuals of the species recorded in that year. Mortality rates were compared among species in each study year with the χ^2 test. The differences in the vulnerabilities to botulism between species were studied using the recorded data of sick and dead birds of each species relative to the census of live individuals in the previous days (1 to 2 weeks). The relative vulnerability index of each species has been calculated as the means of the standardized residuals obtained after running a generalized linear model (GLM) with the number of sick or dead birds collected each day as the dependent variable and with the square root or the logarithm ($x + 1$) of

the previous census of live birds and the Julian date (count of days since 1 June) and the year as explanatory variables. This approach permitted us to use all of the data set at a daily scale (of sick and dead bird counts) and minimized the bias produced by low counts of some species. Therefore, this vulnerability index could be more appropriate than the mortality rate described before to rank the vulnerability of the species to botulism. Comparisons of this vulnerability index among species were performed with a one-way analysis of variance test followed by a *post hoc* comparison with the Tukey test. Only the most abundant species were considered in the study of the vulnerability index, i.e., Anatidae (5 species), including mallard, gadwall (*Anas strepera*), northern shoveler (*Anas clypeata*), common pochard (*Aythya ferina*), and white-headed duck; Rallidae (2 species), including common moorhen and Eurasian coot; and little grebe (*Tachybaptus ruficollis*), black-winged stilt (*Himantopus himantopus*), black-headed gull (*Chroicocephalus ridibundus*), and greater flamingo (*Phoenicopterus roseus*). The Fisher exact probability test was used to compare the frequencies of detection of *C. botulinum* type C/D in bird fecal samples collected during outbreak and nonoutbreak periods among species and between juveniles and adults. It was also used to compare the frequencies of the pathogen in cloacal swabs of different bird species at the wildlife rehabilitation center. Significance for the statistical analyses was set at a *P* value of <0.05 . The analyses were performed with IBM SPSS Statistics 22.

RESULTS

Vulnerability of waterbirds species to avian botulism. During the census performed in Navaseca Lake between 2011 and 2012, the most commonly observed species were Eurasian coot, greater flamingo, northern shoveler, white-headed duck, black-necked grebe, mallard, and moorhen, but differences in abundance of some species (i.e., black-headed gull, coot, and northern shoveler) were observed between years (Table 1). During the same period, 646 dead or sick birds with clinical signs of botulism were collected: 357 in 2011 and 289 in 2012 (Table 1). The presence of BoNT type C/D was confirmed with the mouse bioassay in the plasma of 5 birds collected during the outbreaks. The BoNT type C/D gene was detected in at least 1 sample of cecum, intestines, and liver of 8 of the 23 (35%) necropsied waterbirds during the outbreak in Navaseca Lake in 2012. In 2011, the first waterbird carcasses were observed in the third week of July, and the number of dead birds increased until mid-August; the last dead birds were collected at the beginning of October. In 2012, the first dead birds appeared in mid-June, and the number of dead or sick birds reached a peak at the end of June and then decreased until the end of August. The numbers of affected birds from certain species varied between years: shovelers, gadwalls, white-headed ducks, moorhens, and black-winged stilts were more affected in 2011, while mallards, gulls, grebes, and coots were more affected in 2012 (Table 1). Coots represented 39% of all the affected individuals, followed by mallards (14%), shovelers (10%), gulls (8%), white-headed ducks and gadwalls (7%), black-winged stilts and moorhens (5%), pochards (6%), and, finally, flamingos and grebes (0% to 1%). Regarding the percentage of dead and sick birds relative to the maximum census of live birds counted each year (percentages in 2011 to 2012), significant differences were observed among species (χ^2 tests, $P < 0.001$) (Table 1).

GLM analysis performed with the individual pairs of census and mortality data at different times showed a better fit with the square root transformation of the census (coefficient of determination [R^2] = 0.32) than with the log transformation (R^2 = 0.24). The best-fitted model showed a significant positive relationship between the census and the number of sick and dead birds for each species (Wald's χ^2 = 47.84; $P < 0.001$) (Fig. 1A), and it also

TABLE 1 Census and estimated mortality of waterbirds during the botulism outbreaks in Navaseca Lake in 2011 and 2012

Species common name	Species scientific name	Census ^a		Mortality ^a	
		Median no.	Maximum no.	No.	% ^b
Greater flamingo	<i>Phoenicopterus ruber</i>	150 and 180	150 and 294	0 and 0	0 and 0
Little grebe	<i>Tachybaptus ruficollis</i>	90 and 124	125 and 218	1 and 4	0.8 and 1.8
Gadwall	<i>Anas strepera</i>	27 and 36	28 and 69	25 and 17	89.3 and 24.6
Mallard	<i>Anas platyrhynchos</i>	55 and 108	233 and 226	27 and 56	11.6 and 24.8
Northern shoveler	<i>Anas clypeata</i>	336 and 18	721 and 295	61 and 1	8.5 and 0.3
Common pochard	<i>Aythya ferina</i>	41 and 14	64 and 97	11 and 11	17.2 and 11.3
White and headed duck	<i>Oxyura leucocephala</i>	130 and 134	173 and 211	29 and 15	16.8 and 7.1
Eurasian coot	<i>Fulica atra</i>	184 and 451	221 and 690	117 and 117	52.9 and 17.0
Common moorhen	<i>Gallinula chloropus</i>	107 and 50	231 and 166	25 and 3	10.8 and 1.8
Black-winged stilt	<i>Himantopus himantopus</i>	26 and 46	34 and 109	29 and 2	85.3 and 1.8
Black-headed gull	<i>Chroicocephalus ridibundus</i>	3 and 88	7 and 500	3 and 45	42.9 and 9.0

^a Values for 2011 and 2012, in this order.^b Calculated as percentage of dead animals divided by the maximum census.

FIG 1 Relationship between the mortality (sick and dead birds collected) and the census of live birds on previous days in Navaseca Lake during 2011 and 2012 outbreaks. (A) Comparison of the vulnerability index (mean $\pm$ standard error) to botulism of waterbird species based on the standardized residuals of the generalized linear model between mortality and census. (B) Mean results sharing a letter (a to d) are not significantly different.

showed that, in 2011, the number of affected birds was significantly higher than in 2012 (Wald's $\chi^2 = 22.09$; $P < 0.001$) (Table 1). The effect of the Julian date was not significant. The vulnerability index was calculated from the standardized residuals of the best-fitted model, and the comparison among species revealed that coots and mallards were the most vulnerable species to botulism intoxication, while greater flamingos and little grebes were less vulnerable ($F_{10,132} = 14.108$; $P < 0.001$) (Fig. 1B).

Potential determinants of *C. botulinum* carriage. The presence of aquatic invertebrates with toxigenic bacteria was evaluated as a potential factor for species that include these invertebrates in their diet. Regarding this, the *C. botulinum* BoNT type C/D gene was detected in 16 out of 53 (30%) samples of freshwater snails (*Physa acuta*) and in 2 out of 2 soldier fly larvae (Stratiomyidae). None of the other samples of aquatic invertebrates or fishes yielded *C. botulinum* BoNT type C/D genes.

The retention/excretion of the toxigenic bacteria through the gastrointestinal tract of waterbirds was considered another significant factor for the vulnerability and carriage of *C. botulinum* in waterbird species and, especially, for their contribution to the epidemiology of the outbreaks. A total of 89 moorhens, 1 water rail (*Rallus aquaticus*), and 1 mallard were captured in the funnel traps; from these, 46 individuals were adults, 38 were subadults, and 7 were chicks. Nineteen moorhens were recaptured once, 2 were recaptured twice, and 1 was recaptured 3 times. None of the 117 cloacal swabs from these healthy birds captured in the funnel traps in Navaseca Lake showed the presence of the BoNT type C/D gene (Table 2).

During nonoutbreak periods, 0.8% ($n = 365$) of waterbird fecal samples yielded BoNT type C/D genes, while during outbreak periods, 16.3% ($n = 153$) of the samples yielded it, with this difference being significant (Fisher's exact test, $P < 0.001$) (Table 2). Regarding excretion by the type of bird, 23% of fecal samples from Rallidae (coots and moorhens), 16% of samples from Laridae (gulls), and 9% of samples from Anatidae (ducks) yielded BoNT C/D genes when collected during botulism outbreaks; these percentages were not statistically different. Out of outbreak periods, 1.4% of fecal samples from Rallidae and 1.9% of samples from Laridae yielded BoNT C/D genes.

As for the sick birds admitted to the wildlife rehabilitation center with botulism signs, 68 individuals from 4 families and 9 species were sampled: 28 Anatidae, 21 Rallidae, 18 Laridae, and 1

TABLE 2 Comparison of the prevalences of BoNT type C/D gene detected by real-time PCR in samples of waterbird feces and cloacal swabs collected during botulism outbreak and nonoutbreak periods in 3 wetlands^a

		No. positive/no. total (%)	
Wetland period by season	Wetland	Cloacal swabs	Bird feces
Nonoutbreak period			
Early summer 2011	Navaseca		1/83 (1.2)
Summer 2011	Tablas		1/62 (1.6)
Autumn 2011	Tablas		0/34 (0)
Winter 2012	Navaseca	0/44 (0)	1/106 (0.9)
Spring 2012	Navaseca	0/34 (0)	1/56 (1.8)
	Tablas		0/24 (0)
Autumn 2012	Navaseca	0/28 (0)	
Total		0/106 (0)	4/365 (0.8)
Outbreak period			
Summer 2011	Navaseca		20/96 (20.8)
	Pozuelo		2/24 (8.3)
Early autumn 2011	Navaseca		2/27 (7.4)
Summer 2012	Navaseca	0/9 (0)	1/6 (16.6)
Total		0/9 (0)	25/153 (16.3) ^b

^a Cloacal swabs are from healthy waterbirds (mostly moorhens) trapped during the study.
^b Prevalence during outbreak was significantly higher than during nonoutbreak period (Fisher's exact test, $P < 0.001$).

Podicipedidae (grebe) (Table 3). In total, 32 of these individuals were finally released, and 37 died during the recovery process. Twenty-six (37.7%) of the birds excreted *C. botulinum* BoNT type C/D at some time during the period at the wildlife rehabilitation center. The excretion of BoNT type C/D decreased with the time at

the center, from 30% positive cloacal swabs on day 1 to 0% on day 7; however, 2 individuals, a coot and a gadwall, excreted it again after the 7th day, just before being released. Anatidae and Rallidae excreted BoNT type C/D more often ($P < 0.05$) and for a longer time than gulls (Table 3).

DISCUSSION

In this study, we have observed that vulnerability of different species of waterbirds to botulism intoxication varied, i.e., coots and mallards were more vulnerable than flamingos and grebes. Feeding behavior, season of arrival to the wetland, and turnover rates probably influenced this difference, but some genetic resilience could not be discarded, and some experimental trial is necessary to study these interspecific differences. Moreover, BoNT type C/D genes could be detected in waterbird feces year round, at a very low frequency during nonoutbreak periods and at a markedly higher frequency during outbreak periods, which shows that waterbirds may harbor *C. botulinum* type C/D in their gastrointestinal tracts and act as dispersers of the pathogen between wetlands. In addition, if these carrier birds die, their carcasses may become substrates for the growth of *C. botulinum* and contribute to the start and spreading of the outbreaks; but, do all waterbird species contribute equally to the epidemiology and dispersal of botulism?

Based on the GLM analysis, the most vulnerable species in our study were coots, followed by mallards, gadwalls, and black-headed gulls; the least vulnerable were greater flamingos and little grebes. Accordingly, the most affected waterbirds during avian botulism outbreaks previously reported in Spain were mallards, coots, common teals (*Anas crecca*), black-headed gulls, and shovellers (11, 21); however, neither of these studies took into account the number of healthy individuals of each species at risk of botulism intoxication. Interestingly, in a previous study carried out in our same study area, which involved 13 botulism outbreaks and

TABLE 3 Excretion of *C. botulinum* BoNT type C/D gene by different species of sick waterbirds during the recovery process^a at a wildlife rehabilitation center after a botulism outbreak

Species			No. positive/no. total (%) ^b by recovery time					
Family	Common name	Scientific name	Overall (1 to >7 days)	1 day	3 days	5 days	7 days	>7 days ^c
Podicipedidae	Black-necked grebe	<i>Podiceps nigricollis</i>	1/1 (100)	1/1 (100)	0/1			
	All species		1/1 (100) AB	1/1 (100) AB	0/1			
Anatidae	Mallard	<i>Anas platyrhynchos</i>	7/11 (63.6)	7/11 (63.6)	3/6 (50)	0/1	0/1	0/8
	Gadwall	<i>Anas strepera</i>	4/9 (44.4)	3/9 (33.3)	1/4 (25)	1/2	0/1	1/5 (20)
	Common pochard	<i>Aythya ferina</i>	2/3 (66.7)	1/3 (33.3)	0/2	0/1	0/1	0/1
	White-headed duck	<i>Oxyura leucocephala</i>	1/5 (20)	1/5 (20)	0/1	1/1		
	All species		14/28 (50) A	12/28 (43.3) A	4/13 (30.7)	2/5 (20)	0/3	1/14 (7.1)
Rallidae	Eurasian coot	<i>Fulica atra</i>	8/19 (42.1)	4/19 (21)	5/17 (29.4)	3/11 (27.2)	0/9	1/12 (8.3)
	Common moorhen	<i>Gallinula chloropus</i>	1/2 (50)	1/2 (50)				
	All species		9/21 (42.8) A	5/21 (23.8) A	5/17 (29.4)	3/11 (27.2)	0/9	1/12 (8.3)
Laridae	Black-headed gull	<i>Chroicocephalus ridibundus</i>	1/18 (5.5)	1/18 (5.5)	0/9	0/7	0/8	0/4
	Lesser black-backed gull	<i>Larus fuscus</i>	1/1 (100)	1/1 (100)				
	All species		2/19 (10.5) B	2/19 (10.5) B	0/9	0/7	0/8	0/4
All families			26/69 (37.7)	20/69 (30)	9/40 (22.5)	5/23 (21.7)	0/20	2/30 (6.7)

^a Recovery period, 1 to >7 days.
^b Percentages of the presence of *C. botulinum* were not significantly different between bird families where values share an uppercase letter.
^c One mallard, 1 gull, and 2 coots were sampled 2 times, and another coot was sampled 3 times. The total number of birds sampled was 24.

around 20,000 dead waterbirds, only 8 individual white-headed ducks were reportedly affected (11), which may have led to the belief that this species is not vulnerable to botulism. In contrast, in the present study, just during 2 outbreaks, a total of 43 dead white-headed ducks were collected, which represented 17% and 7% of the maximum census in Navaseca Lake during 2011 and 2012, respectively. This difference is probably due to the increase in the population of this species in the last decades in Castilla-La Mancha, and it suggests that botulism may slow down its recovery, especially taking into account the attraction of this species to wastewater wetlands (12, 13), such as Navaseca Lake, where the risk of botulism outbreak is high (14). Moreover, direct estimates of botulism mortality should be conservative because of the fast removal of carcasses by scavengers and the difficulty of finding sick or dead birds in the dense vegetation around the lake, which means that the number of affected white-headed ducks may be even higher.

Differences in feeding habits may explain variations in vulnerability to botulism (3). This fact also indicates that BoNT type C/D may concentrate in specific compartments (i.e., invertebrates) of the wetland. For example, based on a literature review and over 2,000 diagnostic records at the USGS National Wildlife Health Center, dabbling ducks are among the species at greater risk of botulism (3). Accordingly, in our study, diving ducks (white-headed duck and common pochards) seemed to be less affected than dabbling ducks (gadwall and mallard) and coots. The feeding habits of the majority of waterbirds are not accurately known, and they probably change with the seasons and the type of prey available in each wetland, which makes it very difficult to identify possible sources of toxin for each species. Mallards and gulls, which were among the most vulnerable species in this study, are omnivorous and opportunistic species (22) and may ingest the botulinum toxin from a variety of sources, such as maggots from carcasses, which are known to be a major source of the toxin (1, 23). Coots, the most vulnerable species, are mainly herbivorous, with some consumption of invertebrates (22); so, one source of toxin for them may be freshwater snails that are frequently observed on plants and on carcasses. In this study, 30% of freshwater snails (*Physa acuta*) collected during outbreaks yielded BoNT type C/D genes; this snail eats detritus from which it may acquire *C. botulinum* and its toxin. Previous studies also detected BoNT type C toxin in water snail tissues and reported an association between them and carcasses (23). Interestingly, this snail is characterized by a high reproductive rate, a high passive dispersal capacity, and high tolerance to polluted water (24). Previous studies have linked invasive species with the (re)emergence of avian botulism type E in several locations of the Great Lakes of North America, where the invasion of dreissenid mussels (*Dreissena bugensis* and *Dreissena polymorpha*) and round gobies (*Neogobius melanostomus*) presumably has produced habitat transformations and the development of anoxic conditions that favored *C. botulinum* (25). It is possible that the invasive freshwater snails also facilitate avian botulism outbreaks by serving as an important source of toxin for waterbirds due to their abundance and their necrophagous habits. This role may be especially important in wastewater wetlands, where botulism is more frequent (14), due to the tolerance of these snails to pollution. Further research about toxin concentration in water snails and other invertebrates is needed to determine their epidemiological importance. On their part, flamingos and grebes probably do not include prey that harbor the botulinum toxin in

their diets. Flamingos primarily feed on *Artemia* (not frequent in our study area) or alternatively on seeds, ostracods, and chironomid larvae (26, 27), and grebes actively prey on *Artemia*, other invertebrates, such as corixids, or small fishes (28). In previous studies, we analyzed a relevant number of samples of chironomids, corixids, and crustaceans, and they did not yield BoNT type C/D genes, even during outbreak periods (14). Alternatively, as flamingos and grebes are genetically closely related (29), we hypothesize that these species are more resistant to the toxin, as is the case of vultures (1). The absence of a well-developed cecum in flamingos and grebes compared to ducks and coots (30) may also explain these differences among species.

Another factor that can influence species vulnerability to botulism is the time of arrival of migratory species to the wetlands, as may have been the case for northern shovelers. In 2011, large flocks of shovelers arrived at Navaseca Lake at the end of the summer, coinciding with the peak of mortality and resulting in a high proportion of dead shovelers. Meanwhile, in 2012, the peak of mortality occurred at the end of June when they had not arrived yet, so the proportion of dead shovelers was smaller. This pattern explains the relatively low vulnerability index for this species, which is a dabbling duck frequently reported in botulism bird losses (11). Similarly, Rocke et al. (31) suggested that brown pelicans (*Pelecanus occidentalis*) were more affected by botulism than white pelicans in Salton Sea, because they arrived earlier in the season. Also, the turnover rates (32) of the different species may affect the number of affected individuals of a population; for example, species that spend only a few days in the wetland are less likely to be intoxicated than sedentary ones (i.e., coots). The species that have constant arrival and departure of new individuals may also result in a higher number of dead birds than average counts, as may be the case for teals (*Anas crecca*), not included in this study due to their low numbers in the studied period but frequently found in botulism losses elsewhere. Turnover rates might also vary between years, and this can influence the vulnerability and may explain the differences in mortality rate in different years, e.g., black-winged stilts in this study.

Very little has been published about excretion of *C. botulinum* by waterbirds. Our results indicate that it is a reflection of the abundance of the bacteria in the environment, because prevalence in feces (0.8% during nonoutbreak periods and 16.3% during outbreaks) was similar to the reported prevalence in sediments (0% to 2.4% during nonoutbreak periods and 30% during outbreak periods) (14). Interestingly, fecal samples of Rallidae (coots and moorhens) showed the highest presence of *C. botulinum* (23% during the outbreaks), which indicated that the carcasses of these species may have an important role in the initiation of botulism outbreaks. We did not find *C. botulinum* in cloacal swabs of healthy birds, indicating that they do not usually excrete it during nonoutbreak periods and that this bacterium does not form part of the normal microbiota of waterbirds, though we cannot discard the possibility that they harbored it in their ceca in small quantities that were undetectable with a cloacal swab. Similarly, Hardy and Kaldhusdal (33) did not find *C. botulinum* type C/D in cecum samples from 100 healthy broiler flocks and concluded that botulism is a sporadic and exogenously acquired event in broiler farms. Nevertheless, our findings suggest that *C. botulinum* passes through the intestinal tract of healthy waterbirds after they ingest it along with sediment or food items; therefore, waterbirds may disperse the toxigenic bacteria, especially during outbreak periods

when the abundance of the microorganism in the environment is higher. Green et al. (34) stated that ducks, shorebirds, and other waterbirds have great potential as dispersers of aquatic organisms and that different bird species can have very different roles in this dispersion. For example, the maximum retention time of propagules of freshwater invertebrates and algae ingested by waterbirds may reach 24 h (35), and it has been estimated that the gull's gut pass time for seeds is between 9 and 17 h and that they can fly between 300 and 700 km during this period, dispersing the seeds (36). In this sense, the pulsed-field gel electrophoresis (PFGE) characterization of *C. botulinum* type C/D strains isolated from botulism-intoxicated bird samples collected in Sweden and Spain showed that they were genetically very similar (2, 37), which supports the hypothesis that waterbirds may have spread *C. botulinum* type C/D during their migratory travel across Europe (4).

The results obtained at the wildlife rehabilitation center showed that up to 37.7% of sick birds may excrete *C. botulinum* type C/D and that, even under antibacterial treatment, a few birds (6%) still excreted *C. botulinum* after the 7th day, when they were already recovered. Based on this finding, we suggest that, after an outbreak, treated and recovered waterbirds should not be released before the 7th day of treatment. Moreover, Anatidae and Rallidae excreted *C. botulinum* type C/D more often and for a longer time than Laridae. One explanation for this is the different morphologies of their digestive systems. In particular, the functionality of the cecum influences the retention time, because it is blind-ended and retains contents for longer periods of time (38). In addition, anaerobic microbiota is predominant in this organ (30), where *C. botulinum* has already been detected (5, 19). Anatidae and Rallidae have larger ceca than Laridae; in general, herbivores and omnivores have larger ceca than piscivores and granivores (30). In fact, in this study and in a previous one, we found that the majority of positive fecal samples collected in the field belonged to Rallidae (14). This finding suggests that the waterbird species with larger ceca may harbor the pathogen for longer periods and, thus, may have a more important role in the dispersal of avian botulism.

The persistence of *C. botulinum* in the digestive tract of sick birds beyond 24 h indicates that it may remain for some time as part of the intestinal microbiota, but further information about intestinal colonization is necessary, because one may then expect to find cases of toxico-infectious botulism in wild waterbirds, as described in foals (39), children (16), and broilers (40, 41). If *C. botulinum* could replicate and produce BoNT in the ceca of birds, this may lead to the toxico-infectious form that could open new insights in the epidemiology of avian botulism. Here, it should be considered how the antibiotic therapy with marbofloxacin might have influenced these results, because changes in intestinal microbiota can lead to colonization by *C. botulinum* (16). On the other hand, *C. botulinum* can be sensitive to different types of antibiotics (42), but there are no specific data for the effect of quinolones.

Conclusions. Vulnerability to botulism intoxication is different for each waterbird species, and it depends on feeding behavior, turnover, and time of arrival to the wetland; also, some phylogenetic component may exist. This implies that concentrations of highly vulnerable species during high-risk botulism periods may increase the likelihood of an outbreak and the mortality rate. In this study, the threatened white-headed duck was not among the most vulnerable species, but botulism killed around 17% and 7% of the individuals inhabiting the studied lake in 2011 and 2012, respectively, so this disease should be considered in future conser-

vation programs. Among the sources of intoxication for some waterbird species, the invasive water snail *Physa acuta* may be particularly important, as it is very abundant in some wetlands and frequently harbors *C. botulinum*. Even though *C. botulinum* does not seem to form part of the normal microbiota of healthy waterbirds, the waterbirds may excrete and disperse it between wetlands after ingesting it from the environment. The persistence of *C. botulinum* in the intestinal tract of some birds may open the possibility of the development of toxico-infectious botulism if the intestinal microbiota has been altered, but further research is necessary to confirm this hypothesis.

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