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New insight in the epidemiology of avian botulism outbreaks: necrophagous flies as vectors of *Clostridium botulinum* type C/D

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Summary

Avian botulism outbreaks spread through the bird carcass–maggot cycle, in which *Clostridium botulinum* and blowflies interact to ensure their reproduction in a mutualistic relationship where neurotoxin/spore-bearing maggot is one of the keystones. Here we investigated the hypothesis that adult blowflies may also play a significant role in botulism outbreaks by carrying *C. botulinum* cells between carcasses. We carried out a field experiment placing bird carcasses free of *C. botulinum* type C/D in containers only accessible to necrophagous flying insects in wetlands where avian botulism outbreaks were occurring and in control sites. Additionally, we performed laboratory trials to evaluate if blowflies may carry *C. botulinum* type C/D and for how long. Maggots bearing *C. botulinum* type C/D developed in 27.5% of carcasses placed in wetlands during botulism outbreaks. Calliphoridae flies in laboratory trials were able to transfer *C. botulinum* between two points and excreted it in their spots for up to 24 h after an infective feeding. Our results confirm that adult necrophagous flies play a role in the spreading of botulism outbreaks, which have implications in the epidemiology of this disease.

Introduction

Avian botulism is an intoxication caused by the botulinum neurotoxin (BoNT), which causes a severe flaccid paralysis of the muscles and death in birds and mammals (Rocke and Bollinger, 2008; Defilippo *et al*., 2013). This neurotoxin is secreted by *Clostridium botulinum*, a strictly anaerobic and spore-forming Gram-positive bacterium. Several types of BoNT serotypes exist, the type C/D being the most common in avian botulism outbreaks in Europe (Woudstra *et al*., 2012; Anza *et al*., 2014a). In a global basis, botulism is one of the most important diseases for waterbirds (Rocke, 2006), and it is an emerging serious disease in poultry (Lindberg *et al*., 2010). Birds get intoxicated after ingesting the toxin along with their prey (invertebrates or fishes), and it has also been suggested that spores may germinate in their digestive tract, producing a toxico-infection (Rocke, 2006; Lindberg *et al*., 2010).

Outbreaks in waterbirds occur mostly between summer and autumn, when high temperatures along with increasing biomass and anaerobic conditions in wetlands allow the growth of *C. botulinum* in the environment (Rocke and Bollinger, 2008; Anza *et al*., 2014b). In wetlands from south-central Spain, outbreaks occur almost every year and usually last 1–2 months between June and October. Mortality rate varies between years and locations, and it has been positively correlated with temperature (Vidal *et al*., 2013). These outbreaks propagate and become self-perpetuating by a process known as the carcass–maggot cycle of avian botulism, during which toxin-loaded maggots amplify the number of intoxicated birds: first, a bird with *C. botulinum* in its digestive tract dies, and its carcass provides an anaerobic and protein-rich environment where the microorganism grows and produces toxin; simultaneously, maggots develop in the carcass accumulating the toxin (which does not affect invertebrates); and finally, healthy birds eat the toxin-loaded maggots, die and generate new substrate for the growth of *C. botulinum* and toxigenesis. This cycle continues until conditions become unsuitable (i.e. decreasing temperatures, carcass removal, bird disaggregation) for bacteria and/or larval growth (Wobeser, 1997; Soos and Wobeser, 2006). Percentages of carcasses that develop toxic maggots during botulism outbreaks vary between studies from 85–90% (Duncan and Jensen, 1976) to 12–74% (Soos and Wobeser, 2006). Environmental factors, such as temperatures and fly activity, may contribute to this variance. Although the highest concentrations of toxin have been

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described in carcass–maggots, other types of invertebrates, especially scavenger snails and beetles, may also carry spores and toxin, and serve as secondary sources of bacterium and/or its toxin for birds (Duncan and Jensen, 1976; Vidal *et al*., 2013; Anza *et al*., 2014b). Avian botulism outbreaks are difficult to prevent because *C. botulinum* spores are ubiquitous in wetlands, and environmental conditions that lead to spore germination and toxin production are diverse, unclear and complex to manage (Rocke and Bollinger, 2008). This is why outbreaks control relies on prompt detection and removal of bird carcasses, so as to stop the carcass–maggot cycle (Evelsizer *et al*., 2010b).

Flies are mechanical vectors of a variety of diseases and play an important role in spreading them. For example, Turell and Knudson (1987) demonstrated that stable flies can mechanically carry *Bacillus anthracis* from anthrax-infected guinea pigs to healthy ones, and Cohen and colleagues (1991) observed that the control of flies in a military base decreased the incidence of shigellosis by 85%. Different pathogens, such as *B. anthracis*, *Escherichia coli*, *Salmonella* spp. or *Staphylococcus* spp., have been isolated from flies' external surfaces and their spots (vomits and faeces) (Nazni *et al*., 2005; Fasanella *et al*., 2010; Lindsay *et al*., 2012). *Clostridium botulinum* type C or C/D cells and toxin have already been detected in adult Calliphoridae flies and Dermestidae beetles caught near bird carcasses during botulism outbreaks (Duncan and Jensen, 1976; Vidal *et al*., 2013). Blow and flesh flies (families Calliphoridae and Sarcophagidae) feed and oviposit on carrion (Norris, 1965), and the role of their maggots in the spreading of botulism outbreaks by the carcass–maggot cycle is well documented (Hubálek and Halouzka, 1991), but to our knowledge there are no studies focused on the potential role of adult flies as vectors of *C. botulinum* type C/D during the propagation of avian botulism outbreaks. Flies feed and lay eggs over different bird carcasses, so we hypothesize that, during this process, they may transfer *C. botulinum* cells and increase the number of carcasses with toxin loaded maggots, therefore spreading the outbreaks. Moreover, we propose that this relationship between necrophagous flies and *C. botulinum* may be studied as a mutualism, because both species ensure the substrate for their reproduction (bird carcasses) with their interaction. We tested this hypothesis by placing bird carcasses free of *C. botulinum* type C/D in containers only accessible to flying insects in wetlands where an outbreak was taking place so as to determine if the flies trapped in them and the maggots born from their eggs carried the pathogen. We also performed laboratory experiments to determine under controlled conditions if Calliphoridae flies can transport viable *C. botulinum* type C/D cells and for how long they can eliminate them in their spots.

Results and discussion

Field experiment: transport of C. botulinum *type C/D between carcasses by necrophagous insects*

The study was carried out in different wetlands and urban areas of the province of Ciudad Real, in south-central Spain, for two consecutive years, 2012 and 2013. Eighty-three bird carcasses free of *C. botulinum* type C/D inserted in containers only accessible to flying necrophagous invertebrates (Fig. S1) were hung on vegetation 150–200 cm above the ground until maggots infestation was observed (between 5 and 14 days). Fifty four were set in botulism endemic wetlands (43 during outbreak periods and 11 two months before an outbreak) and 29 in control sites (Table 1). Maggots developed in 78 carcasses and were analysed in pools of three to eight individuals from each carcass for the presence of *C. botulinum* type C/D by real-time polymerase chain reaction (PCR) after culture enrichment (Sánchez-Hernández *et al*., 2008; Vidal *et al*., 2011). This assay amplifies genes encoding both, type C and type C/D mosaic toxins, but as in the study area type C/D is predominant we assumed that we were detecting this mosaic type (Vidal *et al*., 2013; Anza *et al*., 2014a). Eighty-one individual necrophagous flies (43 from outbreak and 38 from control and pre-outbreak sites) and 55 beetles (37 from outbreak and 18 from control and pre-outbreak sites) randomly selected from 79 containers were analysed with the same PCR assay (Table 1). Based on the Fisher's test, there were significant differences ($P < 0.01$) in the frequency of detection of *C. botulinum* type C/D in maggots (27.5%) and in adult flies (18.6%) collected in outbreak sites compared with the frequency observed in control and pre-outbreak sites where the pathogen was absent (Table 1). The highest prevalence was detected on Sarcophagidae flies (27%), followed by Calliphoridae flies (19%), Muscidae flies (9%) and Dermestidae beetles (8.1%), although there were no significant differences between families (Table 2). In many wetlands, botulism is an endemic disease (birds intoxicate at a low rate) with periodic epidemics (birds intoxicate at a greater rate) when large outbreaks occur. The most critical factor for the spreading of large avian botulism outbreaks is the density of carcasses that contain *C. botulinum* spores which will germinate and produce toxin (Rocke and Bollinger, 2008; Evelsizer *et al*., 2010a). In this sense, Wobeser (1997) stated that the secondary poisoning is the major factor causing large outbreaks and proposed that these occur when the average number of birds dying of secondary poisoning attributable to a single carcass is > 1. This further depends on four factors: 'the probability of carcasses developing maggots', 'the probability of maggots accumulating toxin', 'the contact rate between suscep-

Table 1. Presence of *C. botulinum* type C/D detected with real-time PCR in samples of necrophagous invertebrates collected in carrion traps placed in wetlands and urban areas for the field experiment.

Site status	Site[a]	Date	Carcasses (parasitized/ total)	Positive maggot pools/total no. of parasitized carcasses (%)[b]	Positive flies/total no. of flies (%)[c]	Positive beetles/total no. of beetles (%)[c]
Control	Urban area	July–August 2012	3/5	0/3	0/0	0/0
	Urban area	September 2013	10/10	0/10	0/0	0/5
	Tablas Daimiel	June–July 2013	14/14	0/14	0/19	0/9
	All		**27/29**	**0/27**	**0/19**	**0/14**
Pre-outbreak	Navaseca	May 2013	11/11	0/11	0/19	0/4
Control + pre-outbreak			**38/40**	**0/38**	**0/38**	**0/18**
Outbreak	Navaseca	July–August 2012	22/24	4/22 (18.2)	4/27 (14.8)	1/8 (12.5)
	Navaseca	August 2013	10/10	3/10 (30)	1/8 (12.5)	0/11
	Pozuelo	July–August 2013	8/9	4/8 (50)	3/8 (37.5)	2/18 (11.1)
	All		**40/43**	**11/40 (27.5)[d,e]**	**8/43 (18.6)[e]**	**3/37 (8.1)**

a. Tablas Daimiel, Navaseca and Pozuelo are wetland areas.
b. Pool of maggots contain three to eight individuals.
c. Flies and beetles were analysed individually; they can proceed from the same trap.
d. Prevalence during 'outbreak' significantly higher than in its correspondent 'control' samples ($P < 0.01$).
e. Prevalence during 'outbreak' significantly higher than in its correspondent 'control + pre-outbreak' samples ($P < 0.01$).
Prior to the experiment, cloacal samples of the 83 bird carcasses used as baits (14 one day chickens, 4 adult quails or 55 adult partridges) were tested to confirm that they were free of *C. botulinum* type C or C/D. In bold are the sum of the previous values.

tible birds and toxic material', and 'the proportion of contacts resulting in intoxication and death' (Soos and Wobeser, 2006). The first factor largely depends on the activity of blowflies, and the second factor may also be influenced by flies because, as we have shown, they may act as vectors of *C. botulinum* type C/D cells and contribute to colonize with it up to 27.5% of previously uncontaminated carcasses. Taking into account that the presence of *C. botulinum* type C/D in the environment in the study area is generally low (6% in sediment samples, Vidal *et al*., 2013), the contribution of flies to the spreading of the outbreaks can be substantial. In this sense, Reed and Rocke (1992) found high levels of toxin in 16% of maggots developing on previously healthy euthanized bird carcasses and in 10% of maggots developing on botulism-intoxicated bird carcasses placed in adjacent pools; they suggested that the euthanized bird carcasses were already infected with spores, but another feasible hypothesis is that flying necrophagous invertebrates transported them, as observed here. Further support to our findings is that several studies have shown that adult flies are vectors of a variety of pathogens (Nazni *et al*., 2005; Fasanella *et al*., 2010; Lindsay *et al*., 2012). The absence of the pathogen in our controls ruled out that cross-contamination occurred from our traps themselves.

Transport of C. botulinum *type C/D by Calliphoridae flies*

Sixty Calliphoridae flies captured in urban sites with odour-baited traps (Fig. S1) were maintained inside two plastic framed cages (30 flies each) of 50×50 cm covered on all sides by mosquito net (Fig. S2). In one cage, 20 sterile cotton-wool swabs (CLASSIQSwabs™ Copan) saturated in a solution of cooked meat broth and glucose (BD BBL cooked meat medium with glucose, BD) were pierced in one of the walls, and another 10 swabs saturated in the same solution but containing

Table 2. Presence of *C. botulinum* type C/D in individuals of different families of necrophagous flies and beetles in sites where an avian botulism outbreak was taking place and in sites with no outbreak.

	Non-outbreak sites			Outbreak sites		
Family of fly or beetle	No. of individuals	No. of positive individuals	% positive individuals[a]	No. of individuals	No. of positive individuals	% positive individuals[a]
Calliphoridae	13	0	0	21	4	19
Muscidae	14	0	0	11	1	9.1
Sarcophagidae	11	0	0	11	3	27
Dermestidae	18	0	0	37	3	8.1

a. Percentages of detection of *C. botulinum* type C/D were not significantly different between families or sites.

25 spores μl^{-1} of *C. botulinum* type C/D were pierced in the opposite wall. The *C. botulinum* type C/D strain used was isolated from the gastric content of a black-headed gull (*Chroicocephalus ridibundus*) collected in the summer of 2005 in the province of Ciudad Real (internal reference IREC-B136) and confirmed as *C. botulinum* type C/D by PCR (Vidal *et al*., 2013). The second cage was placed beside and used as control with 20 swabs saturated in the same solution of cooked meat broth pierced between two opposite walls (Fig. S2). After 48 h, 3 of the 20 (15%) previously sterile cotton swabs and 1 of the 30 flies (3.3%) from the first cage tested positive to *C. botulinum* type C/D by real-time PCR (same assay as for the field experiment), while all the samples from the control cage (swabs and flies) were negative. This experiment performed under controlled conditions definitely show that Calliphoridae flies can transport *C. botulinum* cells and supports the field findings.

Excretion of C. botulinum *type C/D by Calliphoridae flies*

In order to study the excretion time of *C. botulinum* type C/D by flies, 12 Calliphoridae flies were captured in urban sites with odour-baited traps (Fig. S1), and their spots were tested by real-time PCR to confirm that they were negative to the pathogen. Then, they were placed in individual Petri dishes and fed with a solution of cooked meat broth and glucose containing 25 spores μl^{-1} of *C. botulinum* type C/D (Fig. S3). After, they were transferred to new sterile Petri dishes at 3, 6, 9, 12, 24 and 27 h, and the spots (vomitus and faeces) that remained in the dishes were collected with sterile cotton swabs and tested by real-time PCR (same assay as in previous experiments) for the presence of *C. botulinum* type C/D. In seven occasions, there were no spots in the Petri dishes, so the swabs were passed over their surface. Eleven of the 12 flies excreted *C. botulinum* type C/D at least once between 3 and 24 h post-infective feeding. After 3 h, *C. botulinum* could be detected in the spots of 7 of the 12 flies (58%), and this number decreased every 3 h; after 24 h, the pathogen was only detected in the spots of one fly (8%), and after 27 h it was absent. These results demonstrate that flies can excrete *C. botulinum* type C/D cells during the following 24 h after a single infective exposure. Moreover, *C. botulinum* was absent from the surface of the seven Petri dishes where the exposed flies landed but did not deposit spots, which suggests that flies transmit the pathogen faecal orally rather than by direct contamination with spores present on their body surfaces. These results are in agreement with Fasanella and colleagues (2010), who recovered anthrax organisms from fly spots mainly 2–12 h after an infective feeding, with a peak at 10 h, but no longer than 24 h. Calliphoridae flies can live up to 5 weeks so they can act as vectors for a substantial period of time. In addition, some species usually move up to 6 km and sometimes up to 32 km (Cragg and Hobart, 1955; Braack and De Vos, 1990), so its role as mechanical vectors of *C. botulinum* type C/D spores should not be underestimated.

Perspectives

These results highlight the importance of prompt removal and proper disposal, by burying or burning, of bird carcasses so as to avoid the propagation of the outbreaks by flies. Studies about the efficiency of fly control to prevent the spreading of botulism outbreaks would be necessary to determine the real importance of flies in the epidemiology of the outbreaks. For example, it has been demonstrated that fly control can reduce the incidence of human diseases, such as shigellosis, by 40–85% (Cohen *et al*., 1991; Farag *et al*., 2013), and the prevalence of *Campylobacter* spp.-positive poultry flocks by 77% (Bahrndorff *et al*., 2013). Moreover, new experiments are needed for understanding the real epidemiological potential of Calliphoridae as it would be crucial to know if flies only act as mechanical vectors of *C. botulinum* spores, or if they are bioenhancers of transmission following spore germination and replication in their intestinal tract as it has been demonstrated for other pathogens (Fasanella *et al*., 2010). Finally, from the ecological point of view, it is interesting to point out that the relationship between *C. botulinum* type C/D and blowflies has characteristics of a mutualism because both organisms take advantage of it (Herre *et al*., 1999; Landry, 2012). Carrion in nature is an unexpected, ephemeral and patchy nutrient-rich resource for a variety of living organisms. These ephemeral patches support high levels of diversity where direct and indirect interactions occur between vertebrates, insect species and microbes that compete for the same resource (Barton *et al*., 2013). In this framework, *C. botulinum* type C/D is a poor competitor, and its growth is limited by other bacteria in sediments (Sandler *et al*., 1998), so in turn it produces a lethal toxin that kills vertebrates generating carcasses where it multiplies more easily. Meanwhile, maggots also grow in those carcasses, accumulate the botulinum toxin – without suffering its effects – and carry the toxin and *C. botulinum* cells to new healthy birds, spreading the outbreaks and creating new organic resources for both, *C. botulinum* and flies. Moreover, flies can act as vectors of *C. botulinum* type C/D cells, thus increasing the probabilities of toxin production in the carcasses where they lay eggs. Without flies, the chances for *C. botulinum* type C/D to kill/colonize new birds (via maggots) or carcasses (via flies) in nature would be reduced or non-existent, and maybe some fly phenotypes could be dependent upon this relationship.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. A. Design of the carrion-fly traps used in the field experiment consisting of a 5 l water plastic bottle with two holes in the bottom that allowed the pass of carrion flies attracted by the odour of a bird carcass used as bait and where maggots develop. B. Design of the odour-baited fly traps used for capturing flies for the laboratory experiments: a. holes, b. liver pork on an agar plate base, c. opaque plastic pot, d. funnel, e. plastic bag, f. string.

Fig. S2. Design of the experimental cage. Twenty sterile cotton-wool swabs saturated in a solution of cooked meat broth and glucose placed in one wall and 10 swabs saturated in the same solution but containing 25 spores μl^{-1} of *C. botulinum* type C/D placed in the opposite wall.

Fig. S3. Calliphoridae fly feeding on a *C. botulinum* type C/D infective swab *prior* to excretion experiment. Fly spots can be observed on the surface of the Petri dish.