Local mortality events in migrating sandpipers (*Calidris*) at a staging site in southern Brazil

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In this paper we report two mortality events affecting three sandpiper species, Red Knot *Calidris canutus*, White-rumped Sandpiper *C. fuscicollis* and Sanderling *C. alba*, during northward migration through southern Brazil. Relative to local abundances of the three species studied, Red Knots were by far the most affected, with first year males and lighter birds suffering the highest proportion of mortality. A total of 54 birds (40 Red Knots, 11 White-rumped Sandpipers and three Sanderlings) were recovered for toxicological, parasitological and bacteriological tests. Tests for *Pasteurella* sp. and algal toxins were mostly negative, although the effects of other toxins, including domoic poisoning, could not be ruled out. Dissections disclosed the presence of acanthocephalan *Profilicollis* sp. and trematode (Cyclocoelidae) parasites. Microbial evaluations resulted in the isolation of bacteria (genera: *Aeromonas*, *Pseudomonas*, *Shewanella*, *Enterococcus*, *Escherichia, Proteus*, *Citrobacter*) in a subset of the dead birds, with highest prevalence in knots. These parasites and infections may have increased susceptibility to, or been exacerbated by, the fatal disease or poisoning, but were not likely the cause of death. Although we were not able to determine the definitive cause of death, studying these local mortalities allowed us to report the presence of helminths and bacteria carried by shorebirds and to investigate several possible causes of mortality.

INTRODUCTION

In recent years, a large body of scientific knowledge on wildlife disease has been established (reviewed in Deem *et al.* 2001). Although there is a large volume of routine diagnostic work involving small numbers of individuals, published articles reporting mortality in free-living animals tend to focus on massive events (>1,000 deaths) and the management of populations experiencing epizootics (Deem *et al.* 2001). However, mass mortality from a single cause may be the tip of the iceberg when it comes to the cost of disease on the health of free-living animals and the viability of wild populations. The cumulative effects of a broad spectrum of chronic diseases could represent the portion of the iceberg below water (Friend *et al.* 2001), yet little is known about chronic diseases in wild birds or about their contributions to mortality. This gap in our knowledge highlights the need for reports on a wider variety of mortality events, including local mortality events, involving smaller numbers of casualties, especially in lesser-studied parts of the world. These local mortality events provide opportunities to describe parasites and pathogens carried by casualties, even if these infections are not the definitive cause of death.

Shorebird species represent a group of highly migratory birds whose flyways span the globe (Piersma & Baker 2000). These birds are frequently found in close proximity to game species such as ducks and are exposed to epidemic diseases such as botulism and avian cholera (Adams *et al.* 2003).

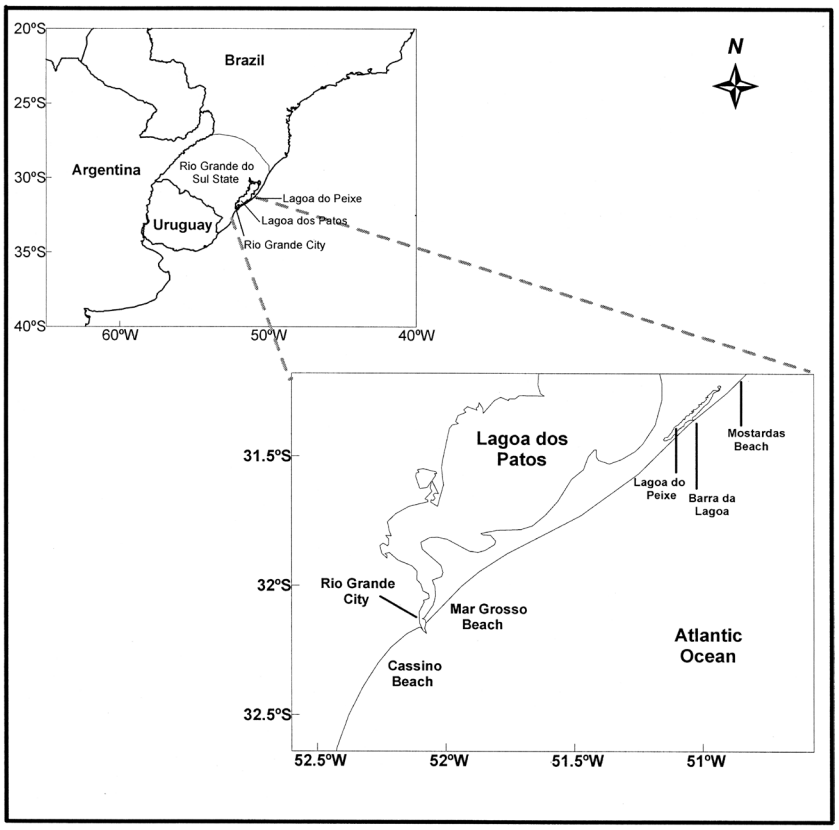

Fig. 1. Map of the study area showing localities mentioned in the text.

Furthermore, because their diet is rich in shellfish they may act as sentinels for algal or contaminant poisoning. In this paper we describe in detail two local mortality events occurring during northward migration through Brazil. We then offer recommendations on how more can be learned from mortality events in the future.

METHODS

Description of study sites and mortality events

Both mortality events occurred in the State of Rio Grande do Sul (RS), southern Brazil. The first event (briefly mentioned in Baker *et al.* 1999 and Piersma & Baker 2000), occurred at Lagoa do Peixe (between 31°00'S, 50°54'W and 31°20'S, 51°10'W; Fig. 1) and affected Red Knots *Calidris canutus*, White-rumped Sandpipers *Calidris fuscicollis* and Sanderlings *Calidris alba*. The event was witnessed during research activities between 2 and 8 April 1997, and the first casualty, a Red Knot, was found dead on the beach near Barra da Lagoa on the morning of 6 April. The following morning 26 Red Knots, 10 White-rumped Sandpipers and three Sanderlings were collected along a 10-km stretch of shore south of Mostardas Beach. About half of these birds were dead when found, the others were lethargic and hardly responded to any form of handling. The dead and dying birds were not emaciated and the only indication of gastrointestinal distress was the presence of green feces (an unusual color for knots) sticking to their cloacal feathers. Rescue was attempted for five Red Knots and the three Sanderlings by repeatedly administering sugar-rich water with a bit of salt, but birds either died within the subsequent 10 h or showed no improvement. Birds that had not died were killed by cervical dislocation, and all birds were stored at minus 20°C. On 8 April, an hour before departure from the study area, another seven Red Knots and one White-rumped Sandpiper were found alive, lethargic and unresponsive along the shoreline of the lagoon. Two of the Red Knots had been ringed in good health only the previous afternoon, indicating that the cause of death was acute. These birds were also killed and were frozen (after 5 h of transport). During the return trip from the field, along the 34 km of beach between the research station at Barra da Lagoa and Mostardas, another 13 sick or dead Red Knots (not collected) were noted among about 550 Red Knots and several hundred Sanderlings and White-rumped Sandpipers.

In May 2000, a similar die-off was witnessed by researchers based at Universidade Federal do Rio Grande (FURG), Rio Grande, while they carried out shorebird censuses from 2 May to 20 June. This event occurred about 200 km south of Lagoa do Peixe, at the Cassino and Mar Grosso Beaches (between 31°51'S, 51°43'W and 33°17'S, 52°25'W), on either side of the mouth of Lagoa dos Patos (Fig. 1). Between 2 and 12 May, six Red Knots were recovered after being found in a moribund state on the beach. Another bird in obvious distress was seen isolated from the small flocks feeding on the shoreline; however, it was not captured. The recovered knots were moved to a marine wildlife rehabilitation center (CRAM-FURG) at Rio Grande, and fed with minced fish and vitamin complex, but died within 4 to 73 h of capture. None showed any signs of recovery. Clinical signs were very similar to those observed in 1997: birds did not move their legs and had spread and motionless wings, they exhibited spasmodic and repetitive movements of neck and head, and had green feces attached to the cloacal feathers. Like the 1997 event, birds were not emaciated and body mass ranged from 134 to 175 g. During the period of the mortality event, Red

Knot flocks on the beach ranged from 100 to 500 birds, and healthy knots were seen feeding on the egg masses of large Mole Crabs *Emerita brasiliensis*.

Factors affecting mortality: age, sex, and biometric data from the 1997 event

In addition to recovering distressed birds from beaches (see above), unaffected birds were captured using both mist-nets and cannon-nets from 2 to 7 April 1997. All birds (affected and unaffected) were aged as second year (hatched in 1996) or adult based on an examination of contour feathers and the degree of wear of the primaries (Prater *et al.* 1977) and were weighed to the nearest 1 g. Bill length, total head length and stretched flattened wing chord length were taken to the nearest 1 mm, and the sex of each bird was determined molecularly (see Baker *et al.* 1999 for details).

Examining possible causes of mortality

Toxicological tests

Tests for paralytic shellfish poisoning (PSP) were performed in 2000 on the stomach contents of the six Red Knots collected that year as well as on samples of Wedge Clams *Donax hanleyanus*, Yellow Clams *Mesodesma mactroides*, and Mole Crabs *Emerita brasiliensis*. Extractions were performed using 0.1 M hydrochloric acid, according to the *Association of Official Analytical Chemists* protocol (AOAC 1990). Analysis was performed using the post-column oxidation method (Oshima 1995). The goniautoxins GTX1-GTX4 toxins were analyzed using a mobile phase solution composed of 2mM 1-heptanesulfonate in 10 mM ammonium phosphate buffer at pH 7.1. Saxitoxin STX and neoSTX toxins were analyzed using a mobile phase solution composed of 2 mM sodium 1-heptanesulfonate in 10 mM ammonium phosphate buffer at pH 7.1: acetonitrile (10:5, v/v). Chromatography was carried out using a Whatman 4.6 × 250 mm column, compacted with 10 µm PATISIL C-8 particles. After chromatography, post column derivatisation was carried out at 85°C with 7 mM of periodic acid in 50 mM buffered potassium phosphate. The reaction was stopped using 0.5 mM acetic acid and fluorescence was then read using a Fluorimeter (FR551 Shimadzu) with 330 nm excitation and 390 nm emission. Results were compared with standards of 6 toxins: GTX4, GTX1, GTX3, GTX2, saxitoxin and neosaxitoxin. Tests for other toxins, including amnesic shellfish poisoning (ASP; domoic acid), were not performed since the samples had been frozen, and freezing degrades domoic acid (LP pers. obs.).

Sea water samples were also collected from the coast adjacent to where casualties were found in 2000. Direct aliquots and filtered sea water samples (collected without preservatives) were observed under a transmission electron microscope for phytoplankton with the potential for toxin production by Drs Clarisse Odebrecht and Odete Moreira (FURG).

Parasitological and bacteriological tests

In 1997, 29 of the Red Knots recovered dead or dying, as well as three Sanderlings and three White-rumped Sandpipers, were shipped to the Royal Netherlands Institute for Sea Research (NIOZ) where the birds were dissected and examined for gross lesions by GMD and TP. These included the two birds that had been captured in good health only a day before they were found dying. Subsequently, 17 Red Knots and three Sanderlings were transported to the University of Utrecht where the worms in two of the Red Knots were identified. For the remaining 15 Red Knots and the three Sanderlings, bacteriological culturing on blood agar, brilliant green agar and serum bouillon was performed (at 37°C) using liver and intestinal samples (work performed as per standard laboratory procedures in the lab of GMD).

Dissections for gross lesions were also performed by JPJr and LB on five Red Knots from the 2000 event. Skins were prepared for use as museum specimens and deposited at the Bird Collection at FURG, the remaining carcass material was frozen or fixed in 4% formaldehyde for 24 h and then stored in 70% ethanol. The viscera of each individual were removed and the organs as well as their contents were washed in 60 µm sieves to strain for small parasites. The green feces were not examined since we felt that parasites present in feces would be detected in the gut contents. Washed content and the walls of the visceral organs were examined visually under a stereoscopic microscope and any worms detected were fixed in AFA (Alcohol, 70 GL, 93%; Formalin, 5%; Acetic acid, 2% (Humanson 1979)), stained with Semichon Carmine, clarified in beech wood creosote and mounted in Canada balsam for permanent glass slide preparation. Specimens were examined and measured using an optical microscope for identification (Petrochenko 1958) with nomenclature updates based on Nickol *et al.* (1999). Representative vouchers of acanthocephalans from the 2000 event were deposited at Helminthological Collection of Instituto Oswaldo Cruz (CHIOC), Rio de Janeiro (Brazil).

Statistical analysis

To examine factors affecting mortality in the 1997 event, we used generalized logistic regression (logit link function, binomial probability distribution) on data from both healthy birds sampled during mist- and cannon-netting (N = 116) and birds recovered in a moribund state (N = 27; total N = 143). We included age and sex as factors, and body mass or condition index (see below) as continuous covariates. The following biologically relevant interaction terms were included: age*sex, age*condition or body mass, sex*condition or body mass and age*sex*condition or body mass.

To obtain a measure of body size we performed a principal component analysis on total head length, bill length and wing length measurements. For this analysis we used the mean for each sex and age class for the 12 birds (11 juveniles, 1 adult) that were lacking wing length measurements, as this increased the sample sizes but not the variance.

This analysis extracted a single component that explained 67.3% of the total variability in body size. Regression analysis showed a significant relationship between body mass and the body size component ($R^2 = 0.19$, $F = 33.4$, $P < 0.001$), indicating that bigger birds were also heavier. To account for this relationship, we used the residuals of this regression as an index of body condition. All analyses were performed using the software package Statistica 6.1.

RESULTS

Factors affecting mortality

In 1997 second year male Red Knots suffered a 33% mortality (6/18 dead) compared with 18% mortality in second year females (2/11), 18% mortality in adult males (13/71),

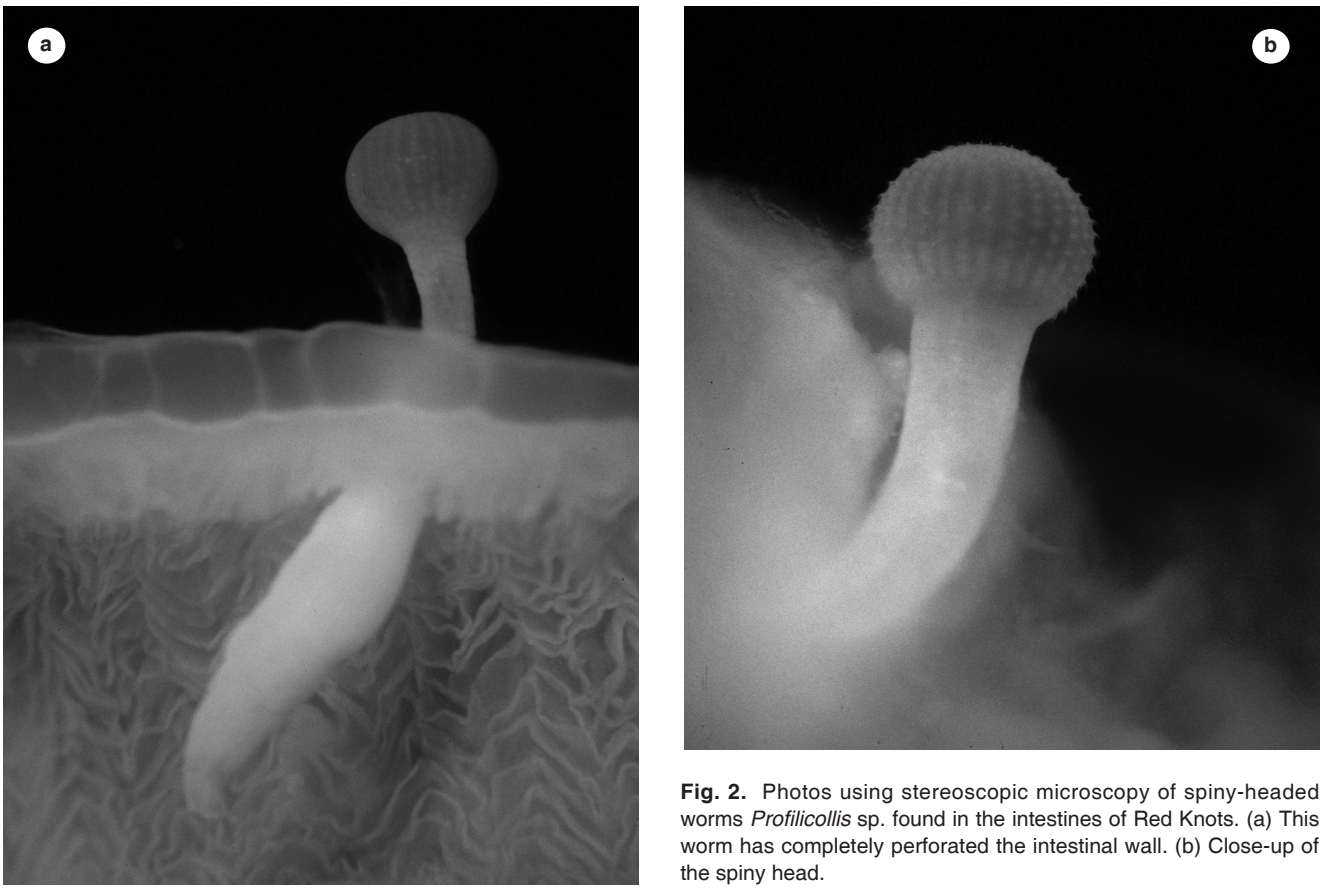

Fig. 2. Photos using stereoscopic microscopy of spiny-headed worms *Profilicollis* sp. found in the intestines of Red Knots. (a) This worm has completely perforated the intestinal wall. (b) Close-up of the spiny head.

and 14% mortality in adult females (6/43), but this pattern was not statistically significant (Fisher's exact test: juveniles P = 0.685, adults P = 0.798). Factorial ANOVA showed that the condition of males was significantly lower than that of females (P = 0.004), and condition of second year birds was significantly lower than in adults (P = 0.011). The same trends were found for body mass but with a significant age*sex interaction (P = 0.016), likely because these second year birds were not fattening for onward migration as adults were. Logistic regression revealed no statistically significant relationships between the likelihood to be dead and age, sex and condition (all P-values >0.05). However, there was a marginally significant trend for lower body mass birds to be among the dead, irrespective of age and sex (P = 0.054).

Possible contributors to mortality

Toxicological tests

The toxin causing paralytic shellfish poisoning (PSP) was not detected in the stomach contents of the six knots tested, and potential toxic microorganisms were not identified in sea water samples from the 2000 event. However, traces of PSP were found in the samples from Wedge Clams. Although the concentrations were very low, near the detection level (~100 fentomol of SXT), depuration of the toxin can be very rapid and animals analyzed could have been previously contaminated at a higher level.

Parasitological and bacteriological tests

Distended blood vessels and colons such as those reported by Woodard *et al.* (1977), were not found during investigations for gross lesions in either 1997 or 2000, although the presence of green feces adhering to the cloacal feathers was found in all cases. However, dissections revealed worm infections in the intestines and air sacs of Red Knot, but not White-rumped Sandpiper or Sanderling specimens. Worms were found in the intestines of all 29 of the Red Knots dissected from the 1997 mortality event. Intensities were roughly estimated as ranging from three to about 30 worms per individual; however, the detailed counting needed for mean intensity analyses was not performed. Detailed examinations in two Red Knots identified the intestinal worms as spiny-headed worms (Acanthocephala, *Profilicollis* sp. pictured in Fig. 2a) and indicated that many of these worms were in the process of perforating or had already perforated the intestinal wall (Fig. 2b). Trematodes (Cyclocoelidae) were also found in the air sacs of four of 29 Red Knots, at roughly estimated intensities of <30 worms per bird. In the five dissections performed in 2000, 14 acanthocephalans, *Profilicollis altmani,* were detected in three of the five Red Knots (CHIOC No. 37455; 37456; 37457 a–d, mean infection intensity = 6; mean abundance = 3.6; using terminology from Bush *et al.* 1997). Careful straining of viscera and gut contents through 60 μm sieves to detect smaller helminths (such as capillarids, tetramerids, trematodes and tapeworms) revealed no further worm infections.

In 2000, knots changed their diet from mainly molluscs *Donax* sp., to mainly egg masses of female Mole Crabs *Emerita brasiliensis,* probably due to a shortage of their preferred *Donax* prey (L. Bugoni pers. obs.). Mole Crabs are an intermediate host for acanthocephalans (Crompton & Nickol 1985), and at Cassino Beach, 84.4% of adult female Mole Crabs were found with cystacanths of *P. altmani* attached to egg masses (intensity 3.7, J.P.Jr unpubl. data, Fig. 3a). Cystacanths were in a developmental stage ready for

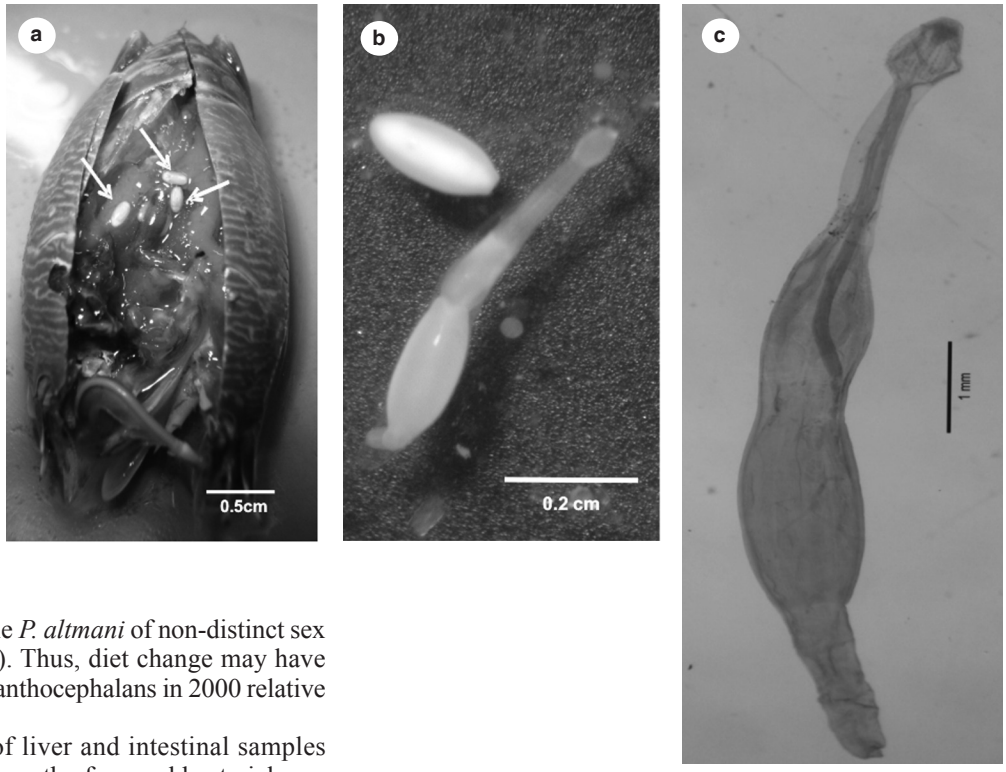

Fig. 3. (a) Mole Crab *Emerita brasiliensis* mature adult female. Macro photograph of a medial dorsal incision revealing encapsulated cystacanths (arrows). (b) Photo using a stereoscopic microscope of *Profilicollis altmani* cystacanths. (c) Photomicrography from the glass slide of *Profilicollis altmani* juvenile from a Red Knot *Calidris canutus.*

infection (Fig. 3b) and juvenile *P. altmani* of non-distinct sex were found in knots (Fig. 3c). Thus, diet change may have increased knot exposure to acanthocephalans in 2000 relative to other shorebird species.

Bacteriological cultures of liver and intestinal samples from 17 Red Knots showed growth of several bacterial species (see Table 1 for details): *Shewanella putrefaciens*, *Enterococcus faecalis*, and *Escherichia coli* (3 of 17), *Aeromonas hydrophila* (2 of 17)*, Pseudomonas putida, Proteus vulgaris* and *Citrobacter freundii* (1 of 17). In the three Sanderlings examined, bacteria were not isolated from the livers, but the intestines of all three birds were positive for *Enterococcus faecalis* and *Escherichia coli* and one was also positive for *Aeromonas hydrophila*. None of the isolated bacteria species are considered primary pathogens, and some are compatible with post-mortem contamination (*A. hydrophila*) or normal intestinal microflora (e.g. *E. faecalis*, *E. coli, C. freundii* and *P. vulgaris*; Liebl & Martin 2009). However, if present before death (which we were not able to test) all may have exacerbated existing problems. *Pasteurella multocida* (the causative agent for avian cholera) was not found in cultures from either species.

Table 1. Results from bacterial cultures of the liver and intestines of Red Knots *Calidris canutus* and Sanderling *Calidris alba*. Only infected individuals are shown. No bacteria were cultured from birds 1, 3, 4, 5, 7, 12, 13, 15 and 16.

Bird	Liver	Intestine
Red Knots		
2	*Aeromonas hydrophila*	*Shewanella putrefaciens*, *Enterococcus faecalis*
6		*Proteus vulgaris*, *Citrobacter freundii*
8		*Aeromonas hydrophila*
9		*Enterococcus faecalis*, *Escherichia coli*
10		*Shewanella putrefaciens*
11	*Pseudomonas putida*	*Shewanella putrefaciens*
14		*Enterococcus faecalis*, *Escherichia coli*
17		*Escherichia coli*
Sanderling		
1		*Enterococcus faecalis*, *Escherichia coli, Aeromonas hydrophila*
2		*Enterococcus faecalis*, *Escherichia coli*
3		*Enterococcus faecalis*, *Escherichia coli*

DISCUSSION

Factors affecting mortality

We found a marginally significant trend that lighter birds suffered the highest mortality. Body mass can decrease quickly when a bird is ill. Thus, lower mass in dead birds may have been a consequence of infection or poisoning rather than a cause. However, two of the Red Knots found dead had been ringed in good health only the previous afternoon and had not lost weight between ringing and death.

Possible contributors to mortality

Toxins

In both mortality events, birds exhibited spasmodic and repetitive movements of the neck and head suggesting the action of a neurotoxin. No PSP toxins were detected in Red Knot stomach contents, Mole Crabs, Yellow Clams or sea water samples collected in 2000. However, PSP toxin was detected at very low levels in Wedge Clam samples. PSP tends to either kill its victims quickly, within one to two hours, or if death does not occur, then the victims recover within two to three days (Lagos & Andrinolo 1989). Some birds in the 2000 event survived up to 73 hours after capture, but did not recover. This suggests that PSP poisoning is an unlikely cause of death, unless it was exacerbated by another as yet unidentified contributor to mortality. Nevertheless, algal poisoning (especially by domoic acid which was not tested) cannot be ruled out as a cause of death in either the 1997 or the 2000 event. Organisms which produce PSP and domoic

acid occur in the region and have been detected in water and mussel samples in the area in recent years (LP pers. obs.). Furthermore, *Emerita* sp. are known carriers of domoic acid (Ferdin *et al.* 2002) and *Emerita brasiliensis* were found in the stomach contents of affected shorebirds in both 1997 and 2000. We were unable to test for botulinum toxins or non-biological pollutants and contaminants. Therefore these cannot be ruled out as contributors to mortality.

The neurological clinical signs we report are similar to those described in relation to thiamine deficiency in Europe (Balk *et al.* 2009). However, whatever killed the birds in the 1997 and 2000 events was relatively acute, whereas thiamine deficiency as described by Balk *et al.* (2009) produces complete loss of appetite, emaciation, and death more than a week after the first clinical signs appear.

Acanthocephalan and trematode infection

Parasite infections were found in Red Knots in both the 1997 and the 2000 events. We found trematodes at a prevalence of 14% (4 of 29) somewhat higher than the 2.3% prevalence reported by Underhill *et al*. (1994). However, since not all affected birds (or species) were parasitized, these worms were not likely the sole cause of death. These data suggest the need for further evaluation of the importance of both trematode and acanthocephalan infections as contributors to mortality in wild birds. Parasitism may have synergistic effects, exacerbating, or being exacerbated by other contributors to mortality. For example, Red Knots suffered much higher mortality and parasitism, relative to local abundances, than Sanderlings or White-rumped Sandpipers. The acanthocephalan worms found in the knots in 1997 had perforated the intestinal wall (Fig. 2) causing damage. Similar, though more severe, acanthocephalan peritonitis caused by another species of *Profilicollis* (*P. chasmagnathi*) has been implicated in a large mortality of Olrog's Gulls *Larus atlanticus* in Argentina (see Fig. 2 by La Sala & Martorelli 2007, and note that their image is similar to the image we present in Fig. 2).

Recommendations for the future

This paper was written because several authors, none of whom were trained veterinarians or pathologists, had the opportunity to witness an important mortality event in a remote region with very few facilities. Under less than ideal circumstances, carcasses were stored frozen and export permits were obtained (not an easy task), with the hope that the cause of death might be determined. Veterinarians and pathologists were consulted for help and one (GMD) volunteered to examine the 1997 specimens. When a second, similar mortality occurred, carcasses were again preserved; however, despite obtaining all the necessary permits, the specimens did not make it to Europe. They were held for several days (during which time they thawed and were rendered useless) and were then returned to Brazil. Despite these constraints on infrastructure and veterinary/pathology personnel, we were able to report on infections of acanthocephalan and trematode parasites, to test for algal poisoning, and to screen for some bacterial infections (including avian cholera). We were not, however, able to perform histopathology on any carcasses/organs, nor to test for botulinum toxins, pesticide, pollutant or other contaminant poisoning, viral diseases, or protozoan parasites. This prevented us from determining the cause of the mortality and contributed to a struggle regarding publication (which has likely exacerbated the current lack of published reports detailing mortality events in shorebirds). Our experience represents a mismatch between the ideal versus the possible regarding complete pathological analysis in ornithological fieldwork. Below we suggest ways that this mismatch might be improved.

Diagnoses of future mortality events could be further improved if researchers witnessing mortality events followed a uniform sample collection and preparation protocol when submitting carcasses or samples for diagnostic study. Section 1 in the freely available Field Manual of Wildlife Diseases provides excellent guidelines for specimen collection and transport (Friend & Franson 1999). The preparation of samples for long-term storage (formalin-fixed, alcohol-fixed, frozen at –20°C and/or –80°C, serum banking and samples stored in DNA and RNA preservatives for PCR and RT-PCR assays) is particularly important since properly stored samples can be re-examined as new techniques become available or different questions are asked.

Furthermore, since our data were collected, new assays have been developed for viral (i.e. avian influenza, Fouchier *et al.* 2000; Ward *et al.* 2004, West Nile virus, Ziermann & Sánchez-Guerrero 2008, Newcastle disease, Roy & Venugopalan 1999); bacterial (i.e. avian cholera, Samuel *et al.* 2003); botulism (Franciosa *et al.* 1996, Grenda & Kwiatek 2009); and protozoan (i.e. avian malaria, Fallon *et al.* 2003) pathogens. These newer tests require less infrastructure and, coupled with the presence of dedicated wildlife disease programs in countries outside of Europe and North America, will hopefully reduce the need to transport samples away from their country of origin for diagnosis.

Finally, when searching the literature for reports on other local mortality events affecting shorebirds, we found an extensive review of die-offs affecting aquatics birds (seabirds, shorebirds, waders, and seaducks) in the USA and its territories (Newman *et al.* 2007). This report highlights the usefulness of such data for examining spatial and temporal trends in disease and toxin related mortality. However, we were unable to find a similar repository of mortality event information on a global scale. An informal electronic survey sent out to wader biologists world wide (in English, Portuguese and Spanish) revealed that mortality events in shorebirds at the global scale were rarely witnessed by the respondents. This highlights the importance of publishing and having a central repository for such mortality events when they are observed. Given the paucity of information on parasites and pathogens carried by shorebirds, ideally such a repository would include details of mortality events, as well as parasites and pathogens carried by the birds regardless of whether these diseases can be definitively implicated as the cause of death. Where possible, this central repository of information might also include health assessment data (i.e. complete blood count, serum biochemistry profiles) from integrated field programs. These data will help to establish what is "normal" for a given population and can then be compared to data taken from individuals affected during mortality events (Deem *et al.* 2001).

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