



REVIEW

# Health and Environmental Risk Assessment Project for bottlenose dolphins *Tursiops truncatus* from the southeastern USA. I. Infectious diseases

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**ABSTRACT:** From 2003 to 2015, 360 free-ranging Atlantic bottlenose dolphins *Tursiops truncatus* inhabiting the Indian River Lagoon (IRL, n = 246), Florida, and coastal waters of Charleston (CHS, n = 114), South Carolina, USA, were captured, given comprehensive health examinations, and released as part of a multidisciplinary and multi-institutional study of individual and population health. The aim of this review is to summarize the substantial health data generated by this study and to examine morbidity between capture sites and over time. The IRL and CHS dolphin populations are affected by complex infectious and neoplastic diseases often associated with immunologic disturbances. We found evidence of infection with cetacean morbillivirus, dolphin papilloma and herpes viruses, *Chlamydiaceae*, a novel uncultivated strain of *Paracoccidioides brasiliensis* (recently identified as the causal agent of dolphin lobomycosis/lacaziasis), and other pathogens. This is the first long-term study documenting the various types, progression, seroprevalence, and pathologic interrelationships of infectious diseases in dolphins from the southeastern USA. Additionally, the study has demonstrated that the bottlenose dolphin is a valuable sentinel animal that may reflect environmental health concerns and parallel emerging public health issues.

**KEY WORDS:** Cetacean · Bottlenose dolphin · Cetacean Morbillivirus · Papilloma · Lobomycosis · Paracoccidioidomycosis ceti · *Chlamydiaceae* · Arbovirus

## INTRODUCTION

The Atlantic Bottlenose Dolphin Health and Environmental Risk Assessment (HERA) Project was started in 2003 as a multidisciplinary and multi-institutional cooperative effort designed to evaluate individual and population health of bottlenose dolphins

*Tursiops truncatus* in 2 southeast US coastal regions: Charleston, South Carolina (CHS), and the Indian River Lagoon, Florida (IRL) (Bossart et al. 2006). Prior to 2003, both geographic areas had evidence of bottlenose dolphin stock depletion, unusual mortality events, epizootic disease, and/or anthropogenic habitat degradation. HERA Project goals were to estab-

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lish standardized methodology for measuring dolphin health and risk assessment, to investigate associations between health status and environmental stressors and, if indicated, to use the data to promote environmental improvement and conservation. As a typically long-lived apex predator which often exhibits a relatively high degree of site fidelity, bottlenose dolphins are emerging as an important sentinel species for monitoring environmental health and for evaluating the potential relationship between exposure to infectious agents and anthropogenic contaminants (Bossart 2011). In this paper, we review the health assessment and infectious disease data for the IRL and CHS dolphins from 2003 to 2015. This integrated summary indicates that both dolphin populations have unique health challenges that can impact individual and population health, and it also provides important new information on regional environmental and public health issues.

### DOLPHIN STUDY SITE LOCATIONS

The detailed environmental characteristics of the IRL and CHS study sites are provided elsewhere (Fair et al. 2006a, Reif et al. 2017 [this issue]). Briefly, the IRL is a shallow-water estuary that comprises 40% of Florida's central east coast (Bossart et al. 2003). The lagoon is an aggregate of 3 estuarine water bodies (Indian River, Banana River, and Mosquito Lagoon) and extends 250 km from Ponce De Leon Inlet in the north to Jupiter Inlet in the south (Fig. 1).

Previous studies of health in IRL dolphins were based largely on data from stranded animals (Hersh et al. 1990, Stolen 1998). Since the 1970s, up to 40% of dolphin strandings along the east coast of Florida have occurred in the IRL (Stolen et al. 2007). Pathologic findings in stranded dolphins from the IRL suggested that a substantial portion of the observed mortality was due to infectious diseases and that immunologic dysfunction was a likely component in disease pathogenesis (Bossart et al. 2003). Importantly, the IRL experienced 3 Unusual Mortality Events (UMEs) (i.e. strandings which occur at significantly elevated rates) in 2001, 2008, and 2013 (Marine Mammal Commission 2002, NOAA Fisheries 2015), for which the cause remained undetermined after investigation. Additionally, IRL dolphins were involved in the 2013–2015 bottlenose dolphin UME in the Mid-Atlantic caused by cetacean morbillivirus (CeMV; see below). The HERA Project was partially designed to provide long-term surveillance for health parameters in the event of a UME.

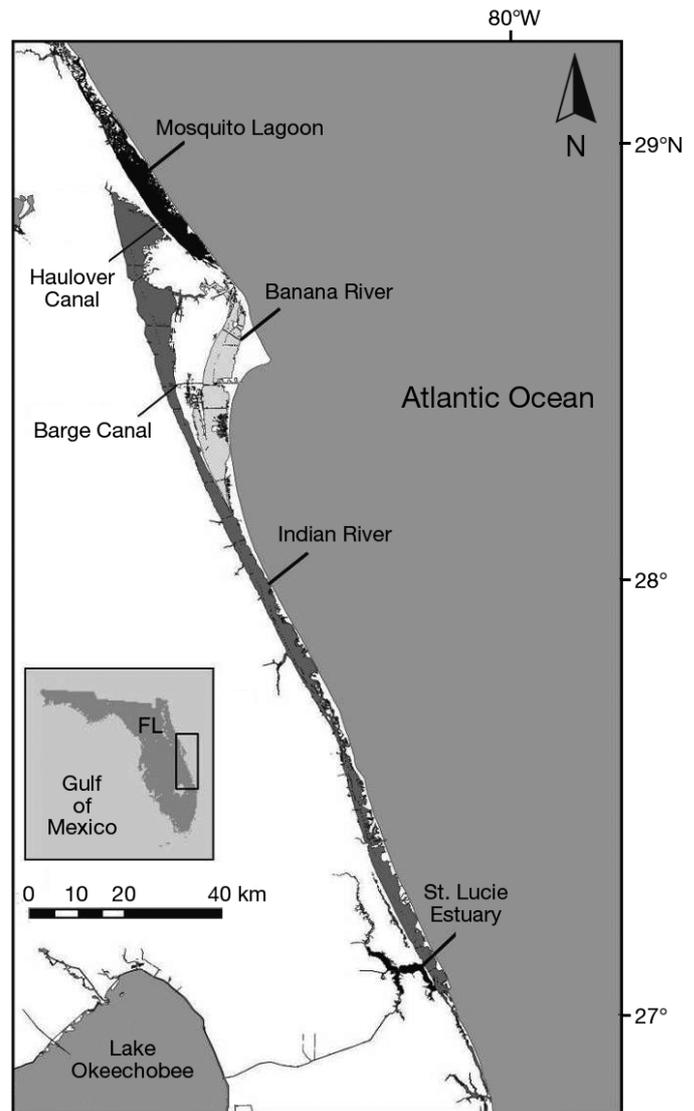


Fig. 1. Study site in the Indian River Lagoon, Florida (USA)

The CHS study site is situated in the central region of the state's coastal zone and consists of portions of the CHS Harbor estuary and the Stono River estuary (Fig. 2). Standardized stranding data have been accumulated for dolphins in the CHS area since 1992. From 1992 to 1996, bottlenose dolphins accounted for the majority (73%) of marine mammal strandings in South Carolina, with CHS representing the highest number (McFee & Hopkins-Murphy 2002). Although the cause of mortality for the majority of bottlenose dolphins stranded in South Carolina is unknown, during 1997 to 2003, human interaction accounted for 25% of dolphin strandings (McFee et al. 2006). Further analysis by McFee & Lipscomb (2009) showed that 31% died from infectious diseases and 47% from non-infectious diseases. Bacterial infec-

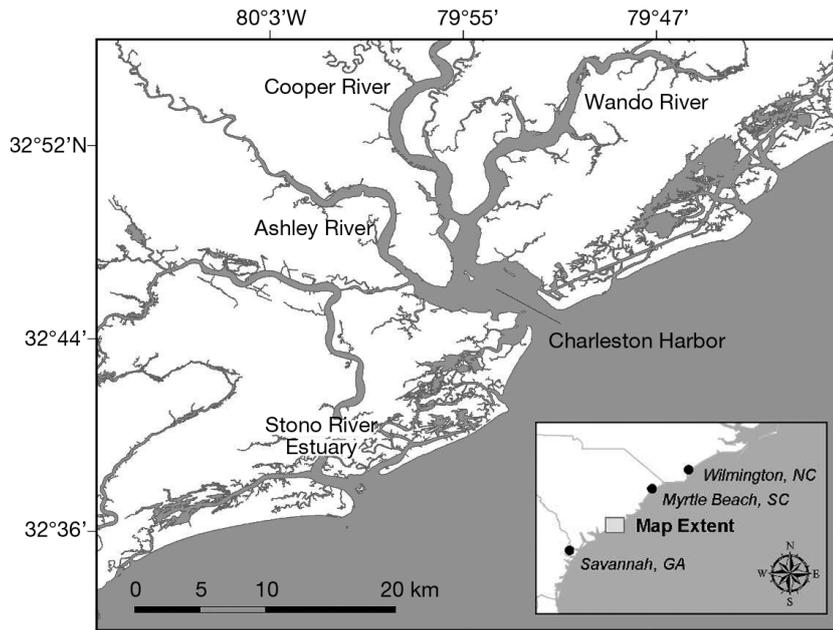


Fig. 2. Study site in Charleston, South Carolina (USA)

tions were the primary cause of infectious disease mortality, and emaciation was the leading cause of non-infectious mortality.

Photo-identification survey data indicate that both IRL and CHS dolphins display long-term residency patterns and site fidelity with an estimated population of 1000 dolphins for the IRL (Mazzoil et al. 2008) and ranging both seasonally and annually between 202 and 652 for the CHS estuarine dolphins (Speakman et al. 2010). IRL dolphins display site fidelity to relatively broad geographic areas, with most dolphins spending time in more than one IRL segment (Mazzoil et al. 2008). Because of their residency patterns, the populations of IRL and CHS dolphins are suitable for assessing risks associated with environmental factors. Differences in patterns of agriculture, urbanization, and concentrations of anthropogenic contaminants provide additional rationale for a comparative health assessment.

A recent study using microsatellite loci and mitochondrial DNA (mtDNA) control region sequences investigated patterns of genetic differentiation among the IRL and CHS dolphin populations. Additionally, the mtDNA was used to compare these populations with those from the Northwest Atlantic, Gulf of Mexico, and Caribbean. Results showed strong differentiation among inshore, alongshore, and offshore habitats. Both the mitochondrial and microsatellite data showed the CHS and IRL dolphin populations to be highly differentiated, and the microsatellite data for the first time revealed the

presence of 2 distinct populations (genetic clusters) within the IRL (Richards et al. 2013). The information regarding genetic clusters became available in 2013. Therefore, with the exception of the 2015 HERA capture and release in the northern IRL, genetic data were not available for consideration.

## DOLPHIN HEALTH ASSESSMENTS

Dolphins were captured, sampled, and safely released in the IRL during June of 2003–2007, 2010–2012, and 2015 and in the waters near CHS during August of 2003–2005 and 2013. Sampling strategy and standard operating procedures and techniques used for capture/release and sample collection are described in detail elsewhere (Fair et al. 2006a). Briefly, study sites included all portions of the IRL. CHS capture sites included CHS Harbor, the Stono estuary, and mouths of the Ashley, Wando, and Cooper Rivers where they empty into Charleston Harbor (Fig. 2).

Sampling was conducted opportunistically as dolphins were sighted. Not all areas were sampled in each capture year, primarily for logistical and economic reasons and to comply with limitations on capture criteria and numbers specified in the NOAA Fisheries research permit. The numbers of dolphins evaluated in CHS was smaller than that for the IRL with a single capture year after 2005 (2013), which precludes a meaningful comparison within the CHS population. Most analyses considered the IRL as a single population and CHS as a second population. An exception in the IRL was our analyses of paracoccidioidomycosis ceti where clinically we suspected the disease prevalence was higher in the southern IRL and we performed multiple analyses to confirm this observation (Murdoch et al. 2008).

Health assessment evaluation included measurement of body weight and morphometrics, monitoring of vital signs (heart rate, respiration rate, cutaneous body temperature), a physical examination by an experienced marine mammal veterinarian, and the collection of a suite of biologic samples including blood (complete blood count, serum chemistry analytes, serum protein electrophoresis, and a suite of immunologic parameters); gastric fluid, feces, and nasal sinus exudate (cytologic evaluation and micro-

biologic and antibiotic sensitivity testing); urine (standard urinalysis); and blubber and skin biopsies (contaminant and histopathologic analyses). Further details of additional parameters evaluated are provided in previous publications including reference values for hematologic parameters and thyroid hormones developed for both populations (Fair et al. 2006b, 2011, Goldstein et al. 2006, 2012, Bossart et al. 2008, 2011, 2014, 2015, Reif et al. 2008, 2009, Schaefer et al. 2009). Based on physical examination findings and the results of laboratory analyses, health status categories were determined by a panel of marine mammal veterinarians as previously described (Reif et al. 2008). Dolphins were classified as clinically healthy (no abnormalities noted), possibly diseased (1 or more clinical abnormalities noted requiring further diagnostics), and definitely diseased (1 or more clinical abnormalities noted which would require medical treatment if in a managed setting). Age was estimated by counting post-natal dentine layers in an extracted tooth (Hohn et al. 1989).

When a tooth was unobtainable, age was estimated from total body length ranges established for these 2 dolphin populations (McFee et al. 2012). Adults were defined as dolphins 6 yr of age or older and subadults from 3.5 to 5.9 yr, as described previously (Bossart et al. 2008). Calves and pregnant dolphins in the third trimester were not included due to permit restrictions. All methods were approved under US National Marine Fisheries Service Scientific Research Permit Nos. 998-1678 and 14352 issued to G. Bossart and Florida Atlantic University IACUC protocol number A10-13. Additional details for specific health-related findings described below are found in previous publications as cited.

**HEALTH STATUS CLASSIFICATION AND DISEASE PREVALENCE**

Health assessments were made on 360 dolphins (246 IRL/114 CHS) representing 224 males (154 IRL/70 CHS) and 136 females (92 IRL/44 CHS). Age categories included adult males (113 IRL/51 CHS), subadult males (41 IRL/19 CHS), adult females (68 IRL/26 CHS), and subadult females (24 IRL/18 CHS). The

Table 1. Prevalence of normal, possible, and definite disease in bottlenose dolphins *Tursiops truncatus*, by site, 2003 to 2007, all captures (adapted from Reif et al. 2008). CHS: Charleston, South Carolina; IRL: Indian River Lagoon, Florida; OR: odds ratio (calculated with CHS as the referent group)

Health status	Capture site			OR	95% CI	p
	CHS (Prevalence, %)	IRL (Prevalence, %)	Total			
Normal	44 (48.9)	55 (39.3)	99 (43.0)	1.0	–	
Possible disease	27 (30.0)	35 (25.0)	62 (27.0)	1.04	0.91–2.07	0.91
Definite disease	19 (21.1)	50 (35.7)	69 (27.7)	2.11	1.04–4.31	0.03
Total	90	140	230			

Table 2. Temporal trends in the prevalence of definite disease in bottlenose dolphins *Tursiops truncatus* among first captures, by capture year and site, 2003–2007

Year	Charleston			Indian River Lagoon		
	Diseased	Number tested	Prevalence (%)	Diseased	Number tested	Prevalence (%)
2003	4	47	8.5	12	42	28.6
2004	6	17	35.3	9	33	27.3
2005	6	18	33.3	7	14	50.0
2006	–	–	–	6	21	28.6
2007	–	–	–	5	10	50.0
Total	16	82	19.5	39	120	32.5

health status classification and prevalence of disease results were divided into 2 periods: 2003–2007 and 2010–2015, in part to determine whether the prevalence of disease and specific disorders had changed, and in part to reflect the 2 separate 5 yr research permits.

Health assessment categories for all captures during the 5 yr period 2003–2007 are shown in Table 1. During this period, the prevalence of definite disease was higher in the IRL (36%) than at CHS (21%;  $\chi^2$ -test,  $p = 0.03$ ).

Data for the 5 yr period 2003–2007 were analyzed by year of capture to evaluate temporal trends in disease prevalence (Table 2). The prevalence of definite disease among first captures increased at both sites between 2003 and 2005. At CHS, the prevalence of definite disease increased from 8.5% in 2003 to 33.3% in 2005, a 3.9-fold increase ( $p < 0.01$  for the trend). In the IRL, the prevalence of definite disease increased from 28.6 to 50.0% between 2003 and 2005 but stabilized somewhat in 2006 and 2007. For the entire 5 yr of observation, the prevalence of definite disease in the IRL was 32.5% on initial capture.

Two infectious diseases accounted for a substantial proportion of the observed morbidity between 2003 and 2007. Paracoccidiodomycosis ceti (PC; the re-

cently proposed name of the disease caused by a novel uncultivated strain of *Paracoccidioides brasiliensis* and historically named lacaziosis/lobomycosis; Vilela et al. 2016) and orogenital papillomatosis (OP) accounted for two-thirds (67%) of the diagnoses of definite disease among first captures. Eleven percent of dolphins captured in the IRL had PC, which was not identified among dolphins at the CHS site. OP was diagnosed in 12% from both capture sites. In addition, papillomas were detected in 10 of 28 repeat examinations, an incidence of 36%.

The remaining dolphins with indicators of definite disease were classified on the basis of other clinicopathologic findings (Reif et al. 2008). The most common abnormalities were in the complete blood counts in which leukocytosis with absolute neutrophilia, lymphocytosis, and/or eosinophilia was the most consistent finding. Other abnormalities included regenerative anemia, low serum alkaline phosphatase, low serum iron, abnormal immunoglobulin profiles, or abnormalities in other serum analytes, particularly elevation in hepatic transaminases. Cytologic evidence of severe neutrophilic gastric inflammation was also found (Reif et al. 2008).

The prevalence of disease in the IRL and CHS dolphins from 2010 to 2015 is shown in Table 3. There was no significant difference between the prevalence of definite disease between the IRL and CHS for 2010 to 2015, although the data for CHS were based on a single capture year.

With respect to temporal patterns, the prevalence of definite disease in dolphins from both sites in 2010 to 2015 was not significantly different from the earlier reporting period (37 vs. 20% for CHS, 29 vs. 33% for the IRL; Table 3). The data suggest that morbidity in both populations remains elevated, but is relatively stable. Several of the infectious diseases responsible for much of the morbidity are endemic, e.g. OP in both populations and PC in the IRL (Bossart et al.

2005, 2008, 2015, Reif et al. 2006). Interestingly, the results from assessments of health status from 2003 to 2015 did not show substantial differences between CHS and the IRL; much of the disease burden in both populations was due to infectious diseases.

The findings reported to date represent only the first phase of risk assessment employing standard methods of physical examination and clinicopathologic testing. An assessment of mortality is difficult due to the small number of HERA dolphins that died, were recovered, and were in a condition adequate to conduct a thorough post mortem examination. We suspect that many deceased dolphins are never recovered due to shark predation and other factors. A relatively high proportion of dolphins sampled had definite disease in both populations. This high prevalence may indicate that environmental stressors, albeit of differing origins, are impacting both populations.

### Cetacean morbillivirus (CeMV)

Sera from IRL and CHS dolphins captured between 2003 and 2007 were tested for antibodies to CeMV. Positive CeMV titers were found only in IRL dolphins (12%). Importantly, we found evidence for recurring cetacean CeMV infection in IRL HERA dolphins in the first post-CeMV epizootic (1987–1988) period, as well as evidence for chronic subclinical morbillivirus infection (Bossart et al. 2010). Two dolphins showed evidence of probable seroconversion to CeMV during the study, indicating that active infection had occurred between the sampling events. These dolphins were seronegative when sampled initially during 2006 and had positive titers to CeMV when re-sampled 1 yr later (Bossart et al. 2010). In addition, antibodies were detected against CeMV in IRL dolphins born after the 1987–1988 epizootic of morbillivirus, suggesting that CeMV infections were occurring in the absence of widespread mortality. Specifically, the prevalence of antibody to CeMV was 10% among dolphins born after 1988, providing evidence of subclinical infection during an inter-epidemic period (Bossart et al. 2010).

These data were consistent with the finding of CeMV infection in dolphins that stranded in the Gulf of Mexico from 1993 to 1994 (Lipscomb et al. 1996) and with serologic evidence of infection and recurrent epidemics in the Western Atlantic and Gulf of Mexico (Duignan et al.

Table 3. Temporal trends in the prevalence of definite disease in bottlenose dolphins *Tursiops truncatus* by capture year and site, 2010–2015

Year	Charleston			Indian River Lagoon		
	Cases	Number	Prevalence (%)	Cases	Number	Prevalence (%)
2010	–	–	–	8	26	30.8
2011	–	–	–	6	27	22.2
2012	–	–	–	6	18	33.3
2013	7	19	36.8	–	–	–
2015	–	–	–	6	18	33.3
Total	7	19	36.8	26	89	29.2

1996) through the mid-1990s. Interestingly, the first CeMV infections in stranded dolphins occurred in the Indian and Banana Rivers of the IRL in 1982 and preceded the massive die-off in 1987–1988 and further outbreaks in the Gulf of Mexico between 1992 and 1994. In retrospect, the 1982 increase in strandings of bottlenose dolphins in the IRL was associated with CeMV infection, based on the finding of antibodies in 5 of 6 dolphins sampled in that year and the absence of antibodies in 24 samples obtained from the same area in 1980 (Duignan et al. 1996).

A second, large epidemic of CeMV infection began along the eastern US seaboard in 2013 which was designated the 2013–2015 bottlenose dolphin UME in the Mid-Atlantic (NOAA Fisheries 2015). Increased numbers of stranded dolphins were observed along the coasts of New York, New Jersey, Delaware, Maryland, and Virginia, as the CeMV epidemic moved southward in a manner similar to that in 1987–1988. The total number of confirmed cases was 1374 through 30 June 2014 (Fauquier et al. 2015). Interestingly, between 2010 and 2012, 70 sera from IRL dolphins were tested for antibodies to CeMV and 6 sera were positive (9%). The prevalence of seropositive animals was similar to that in our earlier analysis from 2003 to 2007 (10%) (Bossart et al. 2010).

Cases of CeMV infection from the 2013–2015 UME were first diagnosed in stranded dolphins in the northern IRL in October 2013, suggesting transfer of CeMV from the coastal dolphin population to the relatively more isolated IRL dolphin population. CeMV cases continued to occur in Florida moving south until 2015 (NOAA Fisheries 2015). In June 2015, a HERA capture and release was undertaken in the same areas of the IRL where deaths from CeMV infection had occurred in 2014. Sera from 16 IRL dolphins were tested for antibodies against CeMV. All sera were negative. Additionally, no clinical abnormalities compatible with a morbillivirus infection were observed. All of the sera from CHS dolphins tested negative, thus this naïve population is vulnerable to CeMV outbreaks.

To further investigate CeMV infection in IRL dolphins, a suite of clinicoimmunopathologic variables was evaluated in dolphins seropositive for CeMV and seronegative healthy dolphins (Bossart et al. 2011). Several clinically significant differences were found. In CeMV seropositive dolphins, innate immunity appeared to be upregulated with significant increases in lysozyme concentration and marginally significant increases in monocytic phagocytosis. Adaptive immunity was also affected. Mitogen-induced T lymphocyte proliferation responses were significantly reduced in

dolphins with positive CeMV antibody titers and marginally significant decreases were found for absolute numbers of CD4 lymphocytes. The findings suggested impairment of cell-mediated adaptive immunity, similar to the immunologic pattern reported with acute morbillivirus infection in other species (e.g. human measles, canine distemper) (Bossart et al. 2011). These data indicated that exposure to CeMV may result in subclinical dolphin infection in IRL dolphins producing immunologic changes that could influence overall health. The cell-mediated immunosuppressive effects associated with subclinical CeMV infection could make this dolphin population highly susceptible to opportunistic diseases. However, since the observed immunologic effects are not specific for CeMV infection, other undetected etiologic factors may also be responsible for the immunologic findings.

### Chlamydiaceae

Chlamydiosis has not been reported in marine mammals. However, antibodies to *Chlamydophila psittaci* have been described in Steller sea lions *Eumetopias jubatus* (Burek et al. 2005) and in Hawaiian monk seals *Monachus schauinslandi* (Aguirre et al. 2007). In 2009, we described the first evidence of exposure to *C. psittaci*, or a closely related *Chlamydiaceae* in HERA dolphins (Schaefer et al. 2009). In this study, the seroprevalence of *C. psittaci* was 85 and 84% in dolphins from the IRL and CHS, respectively. Our follow-up study demonstrated that dolphins with a seropositive *Chlamydiaceae* antibody titer had several immunologic irregularities when compared to healthy dolphins (Bossart et al. 2014). Dolphins with positive *Chlamydiaceae* titers had significantly lower Con A-induced T lymphocyte proliferation, LPS-induced B lymphocyte proliferation, and granulocytic phagocytosis when compared to healthy dolphins. Dolphins with positive *Chlamydiaceae* titers also had significantly higher absolute numbers of CD21 mature B lymphocytes, NK activity, and lysozyme concentrations when compared to healthy dolphins. Additionally, dolphins with positive *Chlamydiaceae* titers had significantly lower total alpha globulin and alpha-2 globulin when compared with healthy dolphins. The results suggested partial up-regulation of innate immunity associated with *Chlamydiaceae* exposure. Adaptive immunity was also affected in dolphins with positive *Chlamydiaceae* antibody titers. The increased absolute numbers of CD 21 mature B cells in dolphins with positive *Chlamydiaceae* titers suggested a partial up-regulation of humoral immunity

resulting from *Chlamydiaceae* exposure. However, mitogen-induced B and T lymphocyte proliferation responses were markedly decreased in dolphins with positive *Chlamydiaceae* titers, suggesting functional impairment of both humoral and cell-mediated immunity.

Dolphins with positive *Chlamydiaceae* titers also had significant increases in *Erysipelothrix rhusiopathiae* titers (Bossart et al. 2014). *E. rhusiopathiae* is a common marine microorganism that may cause disease in cetaceans (Bossart & Eimstad 1988). Both *E. rhusiopathiae* and *C. psittaci* infections have been described in marine birds (Hubálek 2004). We speculated that the high seroprevalence of antibody to both *Chlamydiaceae* and *E. rhusiopathiae* in HERA dolphins might be caused by shedding of the agents among local bird reservoir populations which in turn may serve as potential sources of both chlamydial and *E. rhusiopathiae* infection (Bossart et al. 2014). The data suggest inapparent *Chlamydiaceae* infection impacts immunologic status which could make infected IRL and CHS dolphins susceptible to other diseases. Thus, both *Chlamydiaceae* and CeMV infections could impact the overall health of dolphins as described above.

### Arboviruses

A summary of the prevalence of antibodies to a variety of other bacterial, protozoal, and viral pathogens from HERA dolphins has been published (Schaefer et al. 2009). Antibodies to several arthropod-borne viruses including West Nile virus and Venezuelan, western, and eastern equine encephalitis viruses were detected in IRL dolphins, representing the first reports of these pathogens in wild cetacean populations. The arboviruses examined in this study are commonly found in Florida, and the public health risk posed by these arboviruses is well documented (Day et al. 1996, Schaefer et al. 2009). Evidence of exposure to these pathogens in the dolphin population may indicate mosquito bite transmission to dolphins from infected bird reservoirs in the same geographical regions as human case activity (Schaefer et al. 2009).

### Paracoccidioidomycosis ceti

Historically, lobomycosis (or lacaziosis) was considered a chronic fungal disease of the skin and subcutaneous tissues that occurred only in dolphins and

humans under natural conditions. The presumed etiologic agent, *Lacazia loboi*, was described as a yeast-like organism found abundantly within lesions but not able to be cultured *in vitro* (Taborda et al. 1999, Schaefer et al. 2016). As a result, the development of diagnostic tools to help elucidate disease pathogenesis and epidemiology was impeded. In both species, the disease had a similar appearance of cutaneous keloidiform, white raised nodules that grew slowly over many years (Reif et al. 2006) (Fig. 3). Histologically, dolphin and human lesions consist of multifocal dermal granulomas with inflammatory infiltrates containing histiocytes, multinucleated giant cells, and large numbers of yeast-like cells, 6 to 12  $\mu\text{m}$  in diameter, arranged singly or in chains connected by tube-like bridges (Migaki et al. 1971, Bossart 1984). While historically the lesions and causative organisms appeared morphologically similar, initial gene sequencing of the organism from dolphins suggested it was more closely related to *Paracoccidioides brasiliensis* than to *L. loboi* of human origin (Rotstein et al. 2009). More recent molecular data from HERA dolphins and others supported these preliminary findings and show that a novel uncultivated strain of *P. brasiliensis* is the cause of dolphin lacaziosis/lobomycosis, which has now been renamed PC (Vilela et al. 2016). Previously, lobomycosis was often referred to as a zoonotic disease because it affected delphinids and humans. The new findings explain why the endemicity and natural history of PC in dolphins and lobomycosis in humans are strongly contrasted with very weak evidence of disease transmission directly to humans from dolphins (Reif et al. 2013).

In our initial analyses of data from HERA 2003 to 2004, we documented 9 cases of PC among the 75 IRL dolphins examined, a prevalence of 12% (Reif et



Fig. 3. Bottlenose dolphin *Tursiops truncatus* with paracoccidioidomycosis ceti from the Indian River Lagoon, FL

al. 2006). This was the largest series of documented cases of PC reported to date and suggested that the disease may be emerging in the IRL. Further results from HERA showed that PC did not occur in the CHS environment, was rare in the northern IRL, and was endemic in the southern reaches of the IRL. Conditions in the IRL appear to favor PC, particularly in the southern segments of the estuary which are characterized by higher average water temperatures, runoff from agricultural watersheds, and freshwater intrusion (Woodward-Clyde Consultants 1994, Sime 2005). The prevalence of PC in IRL dolphins between 1996 and 2006 was also estimated from analysis of photo-identification images from 704 dolphins (Murdoch et al. 2008). The total prevalence was 7%, and the spatial concentration of cases in the southern IRL was confirmed in this larger series. Presumptive cases were counted in the first year during which a lesion was identified to estimate incidence which did not increase over the course of the study period.

In an effort to characterize the pathogenesis of PC, we evaluated markers of immune status in HERA dolphins (Reif et al. 2009). Dolphins with PC had multiple abnormalities in hematologic and immunologic parameters when compared to normal dolphins. The absolute number of circulating lymphocytes and serum albumin concentration were reduced; segmented neutrophils, total serum globulins, alpha 1, total beta, total gamma globulins, and total protein were increased. Increases in globulins suggested that affected dolphins had an acute-phase inflammatory reaction or hypersensitivity response. Innate immunity parameters were relatively intact; phagocytosis and natural killer cell activity were not significantly different, but plasma lysozyme concentrations were elevated in dolphins with PC. Adaptive immunity was depressed in affected dolphins; statistically significant decreases were found in the absolute numbers of CD4+ helper T cells and CD19+ and CD21+ B cells. The ratios of CD2+ T cells to CD4+ cells and CD2+ to CD21+ cells were increased and the numbers of lymphocytes expressing MHC class II molecules were decreased in affected dolphins. Lymphocyte proliferation was reduced in response to stimulation with lipopolysaccharide and concanavalin A and antibody responses to common marine microorganisms, e.g. *Erysipelothrix rhusiopathiae*, were decreased. Taken together, the findings indicated that dolphins with PC exhibited significant immunologic impairment in adaptive immunity (Reif et al. 2009). The immune dysfunction associated with PC may reflect the host response to infection with this novel strain of *P. brasiliensis*. Alternatively, an

acquired immunodeficiency may render dolphins susceptible to opportunistic *P. brasiliensis* infection as previously suspected (Bossart 1984). Specifically, an acquired immunodeficiency secondary to high concentrations of mercury is discussed elsewhere as a hypothesis for the pathogenesis of PC (Reif et al. 2017).

An alternative hypothesis is that infection with *P. brasiliensis* results in an altered immune system in which infection with the pathogen switches the host response from a Th1 to a Th2 response (Reif et al. 2009). Th1 cells, which produce interferon (IFN)- $\gamma$ , interleukin (IL)-2, and tumor necrosis factor (TNF)- $\beta$ , evoke cell-mediated immunity and phagocyte-dependent inflammation. Th2 cells, which produce IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13, evoke IgE antibody responses and eosinophil accumulation, but inhibit several functions of phagocytic cells (phagocyte-independent inflammation). The lesions found in dolphins with PC are characterized by granulomatous inflammation containing numerous histiocytes and multinucleated giant cells and abundant numbers of yeast-like organisms (Bossart 1984, Bossart et al. 2003, 2015, Reif et al. 2006). Therefore, the inflammatory response to *P. brasiliensis* in dolphins appears to be ineffective in destroying the organism and preventing progression of the lesions. Similarly, the nodular skin lesions in human patients with lobomycosis are characterized by granuloma formation and infiltration of CD68+ histiocytes, CD4+ T cells, multinucleated giant cells, and to a lesser extent B cells (Vilani-Moreno et al. 2005). The cytokines observed in human lobomycosis are consistent with a Th2 response (Vilani Moreno et al. 2004). Thus, a similar mechanism could facilitate the growth of the fungus and prevent an effective immune response by switching to a Th2 pattern in PC.

### Orogenital papillomatosis

We detected orogenital neoplasms in dolphins from the IRL and CHS during the initial phases of the HERA Project (Bossart et al. 2005). This was the first report of genital papillomas in free-ranging bottlenose dolphins from Atlantic coastal waters. Tumors were found primarily in adult dolphins of both sexes. Oral tumors occurred as single as well as multiple lesions located on the anterodorsal aspect of the tongue (Fig. 4). Genital lesions were found on the penis or external male genital mucosa or on the external female genital mucosa beneath the genital slit (Fig. 5). One dolphin had a genital



Fig. 4. Multiple lingual papillomas in an Indian River Lagoon bottlenose dolphin *Tursiops truncatus*



Fig. 5. Multiple genital papillomas on the penis of an Indian River Lagoon bottlenose dolphin *Tursiops truncatus*

sessile papilloma biopsied on the first examination that was not present on a re-examination 8 yr later. Microscopically, all oral and genital tumors were sessile papillomas.

Transmission electron microscopy revealed the presence of herpesvirus-like intranuclear particles and enveloped cytoplasmic virions in all papillomas (Bossart et al. 2005). In addition, a novel papillomavirus (PV) was isolated from a genital papilloma from a HERA dolphin and a complete nucleotide sequence of the virus designated as *Tursiops truncatus* papillomavirus type 2 (TtPV-2) was determined (Rehtanz et al. 2006). This virus represented the first identified North American cetacean PV. Polymerase chain reaction (PCR) analyses of genital papillomas revealed the presence of delphinid herpes virus-4 (DeHV-4) while serological screening using an antibody-based TtPV enzyme-linked immunosorbent assay (ELISA) demonstrated previous and/or current infection of the HV-positive dolphins with at least one TtPV type

(Rehtanz et al. 2009, 2012). The TtPV ELISA was also used to assess the extent of dolphin PV infection in the IRL and CHS (Rehtanz et al. 2010). Ninety percent of free-ranging dolphins sampled in the IRL and CHS were antibody positive. Higher ELISA reactivity was observed among males compared with females. Sexually immature dolphins appeared more likely to seroconvert with age, and the mean age of dolphins with tumors was 11 yr. A high PV antibody prevalence was not necessarily associated with tumor development as shown by serologic evidence of PV infection in 76% of IRL dolphins without genital tumors. The data indicate that PV infection in HERA bottlenose dolphins is common and readily transmitted horizontally between dolphins, most likely through sexual contact (Bossart et al. 2008, Rehtanz et al. 2010). The high level of sexual activity and its central role in play and social ordering of schools among cetaceans (Wells et al. 1987) may support the maintenance of viruses in those populations.

With respect to viral etiology of cancer, HERA research produced the first evidence of tumor-associated combined PV- and HV-infection and evaluated the controversial effects of viral co-infection in tumor pathogenesis, an important question of comparative human health significance (Rehtanz et al. 2012). Human PVs are the causative agents of cervical carcinoma, the most common cause of cancer mortality in women in developing countries and the second most frequent cancer in women worldwide. The role played by human HVs such as herpes simplex virus type 2 (HSV2) as co-factors in such cancers remains unclear, although HSV2 co-infection is regularly found in PV-induced cervical carcinomas. Eight species-specific viruses, 7 PVs, and 2 HVs have now been separately shown to be associated with genital tumors in Atlantic bottlenose dolphins: TtPV1-6, as well as HVs provisionally assigned the names DeHV4 and -5 (delphinid HVs) (Van Elk et al. 2009, Rehtanz et al. 2012). Therefore, it appears that the viral causality in tumor development is complex since 'hit and run' (i.e. no PV DNA detected after infection and neoplastic transformation) and other mechanisms have been described for members of both viral families (Rehtanz et al. 2012).

The prevalence of OP in both IRL and CHS populations increased dramatically during the first 3 yr of the study, supporting the HERA laboratory data that an infectious agent(s) was driving disease emergence. HERA data now indicate that OP is an endemic disease in both populations. From 2010 to 2015, several dolphins were reexamined that had genital papillomas when first examined. In all cases, the

lesions had resolved, indicating that in the wild, genital papillomatosis is probably a self-limiting disease. This is an important observation since it suggests that genital papillomas do not progress to carcinomas and therefore will not impact mortality. The regression of genital papillomas is consistent with what is commonly observed in domestic animals such as dogs and cows (Bossart et al. 2002, 2008). However, the biologic behavior of oral papillomas in free-ranging dolphins is less clear. While we have not observed mortality in free-ranging dolphins with oral papillomas, oral papillomas in dolphins under human care can undergo malignant transformation and progress to life-threatening metastatic cancer (Bossart et al. 2005, 2008, March et al. 2016). Additionally, malignant transformation of oral papillomas can occur after a prolonged time. Further monitoring is required to determine whether malignant progression of oral papillomas occurs in free-ranging dolphins. These studies show the importance of long-term follow up of disease conditions in marine mammal populations.

We also described the hematologic, biochemical, and immunologic findings in HERA dolphins with OP (Bossart et al. 2008). Compared to healthy dolphins without tumors, dolphins with OP demonstrated hypoferrremia, hyperglobulinemia, and hyperalbuminemia likely associated with an acute phase inflammatory response, upregulated innate immunity (i.e. significantly higher granulocytic and monocytic phagocytosis and superoxide respiratory burst), and upregulated humoral immunity (i.e. significantly higher B lymphocyte proliferation and antibody titers to the common marine microorganisms *Escherichia coli*, *Erysipelothrix rhusiopathiae*, *Mycobacterium marinum*, *Vibrio cholerae*, *V. carchariae*, *V. vulnificus*, and *V. parahaemolyticus*). Combined, these findings were possible responses to the tumors and/or the viruses associated with the tumors. Also, dolphins with OP may have enhanced innate and humoral adaptive immunity due to increased exposure to other pathogens (Bossart et al. 2008).

In a related study, we recently described mucocutaneous lesions identified in biopsies of HERA dolphins taken between 2003 and 2013 (Bossart et al. 2015). Pathologic diagnoses were OP (40%), PC (17%), tattoo skin disease (TSD) (15%), nonspecific chronic to chronic-active dermatitis (15%), and epidermal hyperplasia (13%). This was the first long-term study documenting the various types, combined prevalence, and progression of mucocutaneous lesions in dolphins from the southeastern USA. Environmental and anthropogenic factors were consid-

ered to play a role in the pathogenesis, diversity, and prevalence of many of these mucocutaneous lesions (Bossart et al. 2015).

## CONCLUSIONS

Several studies of bottlenose dolphin populations in coastal US waters have evaluated various aspects of health. There are both similarities and differences in the methods used and in the research questions addressed by the investigators who conducted capture–release studies at various sites. In one instance, data from capture–release studies of 255 dolphins from 4 southeastern US coastal populations (IRL, CHS, Sarasota Bay, Florida, and Beaufort, North Carolina) were analyzed to establish reference intervals for hematologic and serum chemistry variables (Schwacke et al. 2009). The establishment of these reference values was made possible by the use of a single laboratory for all analyses.

The longest duration and most well-known of these studies is the Sarasota Dolphin Research Program, Sarasota Bay, Florida, which was originally designed as a long-term study of social structure and life history (Wells et al. 2004). Health and photo-identification data from Sarasota Bay dolphins have been used for multiple purposes, including descriptions of infectious diseases, microbial colonization, exposure to algal biotoxins, contaminant burdens, reproductive success and survival, and a variety of clinical conditions.

The US Navy Marine Mammal Program has maintained a population of bottlenose dolphins under managed care for over 50 yr. An evaluation of population mortality was conducted using data collected from 103 dolphins between 2004 and 2013 (Venn-Watson et al. 2015). The indicators analyzed included population age structures, annual survival, and crude mortality rates and median age at death. The analysis of these data as a retrospective cohort study was based on the long-term observation of individual animals over time, which differs from the cross-sectional design used in HERA.

The oil spill caused by the explosion on the *Deepwater Horizon* in the Gulf of Mexico in 2010 prompted a series of studies to evaluate the health of bottlenose dolphins in the area. Health evaluations and pathologic examinations conducted on dolphins from Barataria Bay, Louisiana, demonstrated hypoadrenocorticism, lung disease, and an overall poor prognosis for survival, consistent with the effects of exposure to petroleum products (Schwacke

et al. 2014). Longer-term follow up has shown decreased reproductive success and survival when compared to the data from Sarasota Bay dolphins (Lane et al. 2015).

Over the past 14 yr, the dolphin HERA study has generated a voluminous amount of traditional and novel health data which have provided important new information on individual and population dolphin health from 2 distinctly different estuarine regions of the southeastern USA. Interestingly, despite documented differences in observed diseases and concentrations of anthropogenic contaminants in the environment, the results from the health assessment did not show substantial differences between health status in CHS and IRL. The scientific publications generated from the HERA study have documented that both dolphin populations are impacted by newly characterized infectious and neoplastic disease, often associated with immunologic disturbances. Importantly, this *in toto* review of HERA data demonstrates the intricate and dynamic interactions that occur between infectious disease, anthropogenic contaminants, and/or immunologic responses. These interactions highlight the complexity of evaluating health in dolphins and may further complicate our understanding of the pathogenesis of disease and the detection of contributing factors in morbidity and mortality events, since the effect of any single factor may be confounded by other contributors. Furthermore, HERA research and other sustained wild dolphin health assessment programs have demonstrated that the bottlenose dolphin is a valuable sentinel species for understanding emerging or re-emerging diseases that may impact environmental and human health.

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