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## PAPILLOMAVIRUS ANTIBODY PREVALENCE IN FREE-RANGING AND CAPTIVE BOTTLENOSE DOLPHINS (*TURSIOPS TRUNCATUS*)

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**ABSTRACT:** Genital epithelial tumors of Atlantic bottlenose dolphins (*Tursiops truncatus* [Tt]) and Burmeister's porpoises (*Phocoena spinipinnis*) were formerly shown to be associated with papillomavirus (PV) infection. Papillomaviruses are highly prevalent viruses involved in the development of various tumor types in a wide range of animals, and so-called high-risk PVs contribute to malignant progression. In marine mammals, the incidence and prevalence of PV infection, transmission pathways, and persistence of infection are largely unknown. Using virus-like particles of bottlenose dolphin PV type 1 (TtPV1) as the antigen, enzyme-linked immunosorbent assay (ELISA) studies were conducted to evaluate PV antibody prevalence in bottlenose dolphins. In total, sera obtained from 115 dolphins were examined. Fifty-one percent of captive dolphins ( $n=18$  of 35) and 90% of free-ranging dolphins ( $n=72$  of 80) were antibody positive. Higher ELISA reactivity was observed among males compared with females. Sexually immature dolphins appeared more likely to seroconvert with age. Besides determining their PV antibody prevalence, each animal was also assessed for the presence of orogenital tumors. Interestingly, the mean age of free-ranging dolphins with tumors ( $n=21$ ) was 11.2 yr compared with 29.9 yr in captive dolphins with tumors ( $n=9$ ). Results from the current study suggest PV infection in bottlenose dolphins is common, that the main route of PV transmission among them may be horizontal, and that orogenital neoplasia may develop in early life stages of certain free-ranging bottlenose dolphins.

**Key words:** Antibodies, bottlenose dolphin, infectious disease, neoplasia, papillomavirus, serology, TtPV, tumors, *Tursiops truncatus*.

### INTRODUCTION

Papillomaviruses (PVs) are widely distributed infectious agents in humans and animals. By interfering with tumor-suppressor pathways of host proteins, PVs are able to induce a hyperproliferation of suprabasal epithelial cells (Chow and Broker, 1994). Depending on the immune status of the specific host and cofactors (i.e., smoking, X-rays, ultraviolet light, diet, or concurrent infections with other agents), benign cutaneous or mucosal tumors primarily associated with high-risk PV types can undergo malignant progression.

Papillomavirus infection is a necessary

cause of invasive cervical carcinoma (Wal-boomers et al., 1999), the second most frequent cancer of women worldwide (Einstein and Goldberg, 2002). Because human PVs (HPVs) are the most frequently sexually transmitted viruses (Garland, 2002), transmission pathways and disease incidence are of growing concern in animals as well. More than 50 nonhuman, species-specific, distinct PVs have been described to date, and novel types are constantly being detected in epithelial tumors from many vertebrates, including domestic species and wild animals.

A high prevalence of orogenital papillomas in free-ranging porpoises and dol-

phins has been documented (Van Bressem et al., 1996; Bossart et al., 2005). Recently, squamous cell carcinoma, suggestive of malignant transformation of benign papillomatous lesions, was described in a captive dolphin (Bossart et al., 2005). Subsequently, four mucosotropic, species-specific, cetacean PVs have been completely characterized: Atlantic bottlenose dolphin PV types 1, 2, and 3 [*Tursiops truncatus* PVs (TtPV1, -2, -3); EU240894, NC\_008184, EU240895; Rehtanz et al., 2006; Rector et al., 2008) as well as the Burmeister's porpoise PV type 1 [*Phocoena spinipinnis* PV (PsPV1); NC\_003348], with a second PsPV type potentially existing (Van Bressem et al., 2007). Among these viruses, TtPV2 may represent a high-risk type based on DNA sequence investigations (Rehtanz et al., 2006). Although the association of these PVs with mucosal tumors was demonstrated in all cases and such tumors seem to be highly prevalent in at least two cetacean species, nothing is known about PV prevalence in marine mammals. Despite the existence of more than 50 nonhuman PVs, transmission pathways in animals have not been investigated thoroughly, and the extent and distribution of PV infection in marine mammals has not been established.

The humoral response to HPV is directed predominantly to native conformational epitopes on the whole virion (Steele and Gallimore, 1990). Immunologic studies of PV infection have been impeded by the lack of a cell culture system for PVs and, thus, the absence of adequate targets for serologic assays. The recent use of virus-like particles (VLPs) as antigenic targets has addressed this problem (Cubie et al., 1998). These VLPs consist of the major capsid protein L1 of the respective PV type. When expressed in an appropriate eukaryotic cell system, L1 self-assembles into PV VLPs that demonstrate conformational epitopes.

To shed light on PV prevalence and transmission patterns in bottlenose dol-

phins, VLPs of TtPV1 and TtPV2 were developed for serologic screening and the validity of the corresponding test was investigated and confirmed (Rehtanz et al., 2009). In pilot studies, bottlenose dolphin sera reacted positively or negatively, respectively, in both the TtPV1 and TtPV2 enzyme-linked immunosorbent assay (ELISA), indicating cross-reactivity between the two VLP types. The TtPV1 VLPs were subsequently chosen as the antigen for the test, based on higher yields of type 1 VLPs after protein expression and particle purification (Rehtanz et al., 2009). Herein, we report the use of the TtPV1 ELISA test for determination of PV antibody prevalence in four groups of bottlenose dolphins and corresponding statistical analyses.

## MATERIALS AND METHODS

### Dolphin populations sampled

Bottlenose dolphin sera ( $n=119$ , from 115 animals) were obtained from two free-ranging populations ( $n=80$ ) and two captive groups ( $n=35$ ). Sera from free-ranging dolphins were obtained from the Atlantic Bottlenose Dolphin Health and Risk Assessment (HERA) Project, a collaborative effort between Harbor Branch Oceanographic Institution (HBOI), and the National Oceanic and Atmospheric Administration (NOAA). Dolphins were captured, examined and released in the Indian River Lagoon (IRL), Florida, USA, in June of 2004, 2005, and 2006 ( $n=51$ ), and in the estuarine waters near Charleston (CHS), South Carolina, USA, in August of 2004 and 2005 ( $n=29$ ). Captive dolphins were sampled in 2005 and 2006 in Sea Life Park (SLP) by Dolphin Discovery in Waimanalo, Hawaii, USA ( $n=22$ ), and in 2001 and 2002 in the Mundo Aquático Zoomarine (MAZ) in Albufeira, Portugal ( $n=13$ ). Dolphins studied were Atlantic bottlenose dolphins (*Tursiops truncatus*) with the exception of two Pacific bottlenose dolphins (*Tursiops truncatus gilli*; SLP).

Ages of free-ranging dolphins were determined by tooth examination according to Hohn and colleagues (1989). Ages of captive dolphins were estimated by veterinarians from the overall condition of the animals and their total body length according to Geraci and Lounsbury (2005). Sexual maturity was determined according to Odell (1975) and Mead

and Potter (1990). Females were considered sexually mature when they had reached ~2.3 m in length, at about 5 to 12 yr, males, when they had reached ~2.4 to 2.6 m, typically between their 10th and 12th yr. All dolphins were also physically examined for the presence of oral and/or genital tumors.

#### PV antibody prevalence ELISA studies

The determination of antibody reactivity to TtPV1 VLPs was carried out as previously described (Rehtanz et al., 2009). Briefly, incubations were performed at 37 C for 1 hr with three to five intermediate phosphate-buffered saline (PBS) washing steps. Sera and antibodies were diluted in PBS/1% bovine serum albumin (BSA). The ELISA microplates (Dynatech Laboratories, Inc., Chantilly, Virginia, USA) were coated with 50 ng of intact VLPs in PBS, followed by blocking with 5% BSA. The coated VLPs were incubated with dolphin sera diluted 1:300, followed by rabbit anti-dolphin immunoglobulin G, diluted 1:3,000, and alkaline phosphatase-coupled goat anti-rabbit antibody, diluted 1:5,000 (Sigma, St. Louis, Missouri, USA). The reactivities were measured at 405 nm (Spectra MRTM, Dynex Technologies, Chantilly, Virginia, USA) using 1041 *p*-nitrophenyl phosphate (Sigma).

One dolphin (CHS) with a TtPV1 ELISA mean absorbance of 0.128 represented the positive control animal. At the time of serum collection, this dolphin had an active genital tumor, which was subsequently diagnosed as being TtPV2-associated (Rehtanz et al., 2006). Serum from this dolphin showed a similar mean absorbance of 0.141 in the TtPV2 ELISA. A disease-free, 4-mo-old, dolphin calf (SLP) served as the negative control (Rehtanz et al., 2009). The seropositive cutoff value was defined as an absorbance of 0.1 after background subtraction, representing a minimum twofold standard deviation (Rehtanz et al., 2009).

Three dolphins were sampled more than once. One of those (CHS) was sampled before and after the health assessment to verify that the examination had no effect on the PV antibody prevalence result. Another animal (SLP) was sampled monthly for three consecutive months, whereas a third dolphin (MAZ) was sampled twice at a 7-mo interval to monitor possible short-term changes in the ELISA reactivity.

#### Statistical analyses

Geometric means were compared between sample groups unless otherwise stated. Four sera resulting from repeated sampling of three

of the 115 dolphins were not included in the analyses to preserve sample independence. *F* tests were performed to determine whether sample variances were statistically different, whereas two-sided *t* tests were performed to compare sample reactivities. *P* values are reported for either equal or unequal variances based on the results of the *F* test. Statistical significance was defined as  $P < 0.05$ . Chi-square tests were used to compare the prevalence of PV antibody across population groups. Yates' correction was applied when the expected frequency in any cell was  $< 5$ . Fisher's exact test was used when a cell contained a frequency of  $< 5$ . In preliminary analyses, males were found to be older, on average, in both captive and free-ranging populations ( $P = 0.048$  and  $P = 0.095$ , respectively). Therefore, the seroprevalence data were adjusted for age in logistic regression (SAS software, version 9.1, SAS Institute, Inc., Cary, North Carolina, USA).

#### Animal use

The acquisition of serum from captive dolphins for the current study complied with all relevant federal guidelines and institutional policies; sera from free-ranging dolphins were collected under National Marine Fisheries Service Scientific Research Permit 998-1678 issued to Gregory D. Bossart.

## RESULTS

#### PV seroprevalence and analysis of ELISA reactivity

Sera from 115 dolphins representing four different groups were investigated. The prevalence of anti-PV antibodies in bottlenose dolphins was high across the population groups. Antibody prevalence was significantly ( $P < 0.001$ ) higher in free-ranging dolphins (72 of 80; 90%) compared with captive dolphins (18 of 35; 51%). Dolphins in the IRL were more likely to be seropositive (48 of 51; 94%) than dolphins in CHS (24 of 29; 83%), but the difference was not statistically significant. Fifty-nine percent of the dolphins tested from the SLP had anti-PV antibodies (13 of 22), whereas 38% of the sera obtained from dolphins maintained in the MAZ showed reactivity in the assays (5 of 13); this difference was also not statistically significant.

The magnitude of ELISA reactivity

against PV was also analyzed. The overall mean reactivity was 0.179, the highest absorbance, 0.83, and the lowest, 0.015. Free-ranging dolphins generally had higher ELISA values than captive animals. The mean of the ELISA absorbance for all free-ranging dolphins was 0.176, whereas that of all captive animals was 0.103 ( $P=0.0017$ ). Dolphins from the IRL population had significantly higher reactivity when compared with CHS animals with mean ELISA values of 0.197 and 0.144, respectively ( $P=0.0028$ ).

One of the three seronegative dolphins from the IRL and all seronegative animals from CHS were sexually immature. Likewise, all sexually immature captive dolphins were seronegative. There were no age-related significant differences in PV seropositivity among all sexually mature dolphins ( $P>0.05$ ). However, among all sexually immature dolphins, including males up to 10 yr and females up to 5 yr, the youngest dolphins of all groups were seronegative, and seropositivity increased with increasing age (Fig. 1A). Within this group of sexually immature animals, the mean PV ELISA absorbance for those between 0 and 5 yr ( $n=18$ ) was 0.092, whereas that of dolphins older than 5 yr, but younger than 10 yr ( $n=15$ ), was 0.208 ( $P=0.0108$ ). Ten of 14 (71%) offspring of captive dams were seronegative, whereas the four seropositive dolphins were no longer sexually immature when tested. Among the captive offspring, all sexually immature dolphins ( $n=6$ ) were seronegative, regardless of the status of their parents. These data suggest vertical transmission does not commonly occur and that the main route of PV transmission among bottlenose dolphins may be horizontal.

Eight of nine seronegative animals (89%) from the SLP were born in captivity, whereas four of eight (50%) of those testing negative from the MAZ were born in captivity.

No significant differences in antibody prevalence were found between males and females among free-ranging dolphins

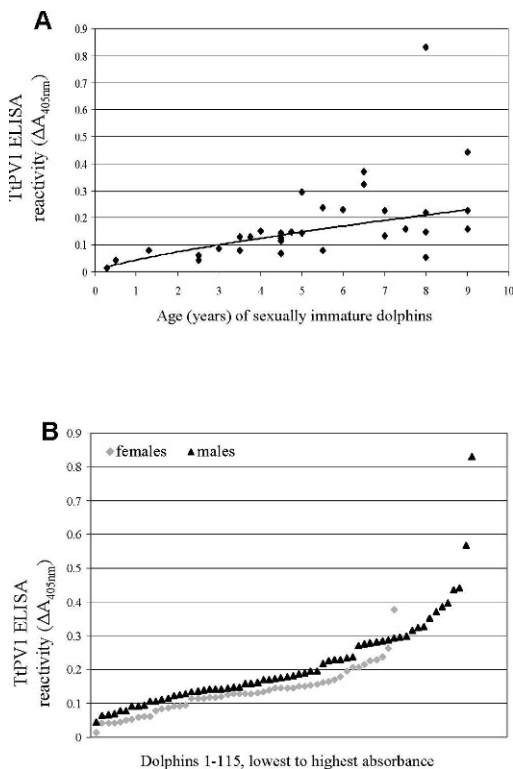


FIGURE 1. Gender and age in correlation with papillomavirus enzyme-linked immunosorbent assay (ELISA) reactivity in bottlenose dolphins. (A) A correlation of age with ELISA reactivity was noticed among sexually immature dolphins with an increasing reactivity mean in the papillomavirus (PV) ELISA with increasing age. Statistical significance was noted investigating sexually immature dolphins by comparing animals up to 5 yr of age with dolphins between 5 yr and 10 yr of age ( $P=0.0108$ ). (B) Females and male dolphins are listed from lowest to highest ELISA value on the x-axis. Males had slightly higher values compared with females ( $P<0.0001$ ).

(89% and 91%, respectively), but among captive dolphins, males were more likely to be seropositive than females (77% versus 28%,  $P=0.01$ ). Concerning ELISA value magnitude, male dolphin sera exhibited higher reactivity than sera from females (Fig. 1B). The means for males ( $n=64$ ) and females ( $n=51$ ) were 0.184 and 0.115, respectively ( $P<0.0001$ ). With the exception of one female, all values above 0.3 were obtained from males ( $n=11$ ). To determine the statistical influence of these higher values among males,

TABLE 1. Median, mean, and range for age of free-ranging versus captive dolphins with and without orogenital neoplasia (OGN).

Environment	Dolphins	<i>n</i>	Median age	Mean age	Age range
Free-ranging	No OGN	47	8.6	10.5	2.5–28
	With OGN	21	9.0	11.2	5–24
	Total	68	8.8	10.7	2.5–28
Captive	No OGN	26	15.5	17.9	0.3–47
	With OGN	9	36.0	29.9	10–39
	Total	35	21.0	20.9	0.3–47

the analyses were repeated excluding all animals with ELISA reactivity above 0.3. The difference between males ( $n=53$ ; 15 captive, 38 free-ranging) and females ( $n=50$ ; 17 captive, 33 free-ranging) remained statistically significant ( $P=0.0011$ ).

#### Orogenital papillomas and ELISA reactivity

A physical examination revealed that 21 of the free-ranging dolphins tested by ELISA (26%) had one or more genital or oral tumors. The TtPV2 was detected in a biopsy of the genital lesion from one dolphin (Rehtanz et al., 2006). Another dolphin had the highest ELISA reactivity with an absorbance of 0.83 and displayed an unusual bilateral tongue lesion (Bossart et al., 2005). Among captive dolphins, nine of 35 (26%) had a history of recurring oral and/or genital tumors after cryo-treatment. These lesions are currently being investigated to determine a possible causative agent. Of the 30 dolphins with orogenital neoplasia, 25 (83%) were seropositive in the PV ELISA, whereas two of the seronegative dolphins had values very close to the seropositive cutoff (0.092 and 0.096). No significant correlation between ELISA absorbance magnitude and the presence of an orogenital tumor was seen.

Age was determined for 67 of the 80 free-ranging dolphins (84%) tested (including all 21 with tumors) and for all captive animals. Mean, median, and range for age are shown in Table 1 for free-ranging versus captive dolphins and for dolphins with and without tumors. Mean and median ages of captive animals were approximately twice as high as those for

free-ranging animals were. There was no significant difference in age between free-ranging dolphins with or without tumors (mean ages: 11.2 yr versus 10.5 yr). However, the mean and median ages of captive dolphins with or without tumors differed. Captive dolphins with tumors ( $n=9$ ) were significantly older (mean age: 29.9 yr) than captive dolphins without tumors ( $n=26$ ; mean age: 17.9 yr;  $P=0.017$ ). Approximately 78% of the captive dolphins with tumors were older than the oldest free-ranging dolphins with tumors (Fig. 2A;  $P=0.0009$ ), whereas only 15% of captive dolphins without tumors were older than the oldest free-ranging dolphins without tumors (Fig. 2B;  $P=0.01$ ). These findings indicate that orogenital tumors may develop in early life stages in certain free-ranging dolphins.

#### DISCUSSION

Papillomavirus prevalence as well as transmission rates and pathways in cetaceans are currently unknown. In this first PV antibody prevalence study in marine mammals, some sera showed a low positive reactivity in the screening. After having immunized beagles in a dog-VLP vaccination study, a decrease of serum ELISA reactivity was observed a few weeks after challenge when no adjuvant was used, as opposed to an immunization with adjuvant (Suzich et al., 1995). This decrease may also occur in natural infection because low antibody titers against PV in positive individuals are not unusual (Shah and Howley, 1996). In the present

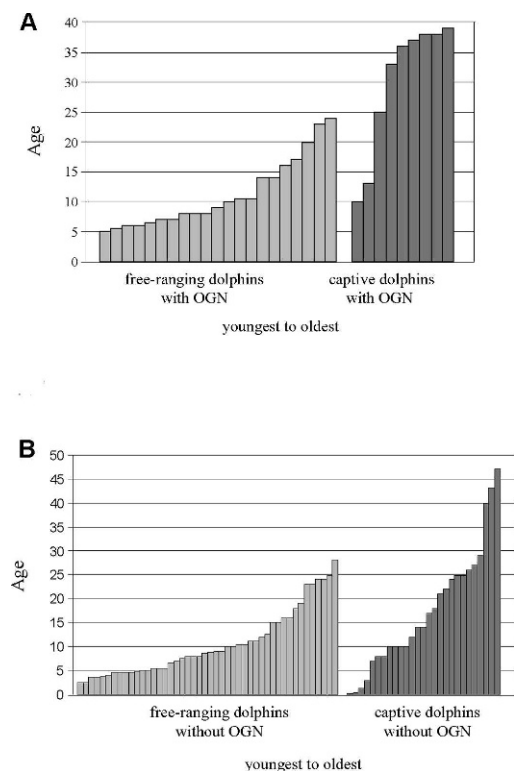


FIGURE 2. Age in correlation with orogenital neoplasia presence. Free-ranging dolphins with tumors were younger than captive dolphins with tumors. Dolphins are listed youngest to oldest, per group, on the x-axis. (A) Seventy-eight percent of the captive dolphins with tumors were older than the oldest free-ranging dolphins with tumors ( $P=0.0009$ ), whereas only 15% of captive dolphins without tumors were older than the oldest free-ranging dolphins without tumors ( $P=0.01$ ) (B). Orogenital neoplasia (OGN).

study with dolphins, some ELISA values were near the seropositive cutoff. For diagnostic purposes, values near this cutoff might be considered an appropriate, “indeterminate” reading. Future longitudinal measurements of ELISA reactivity will elucidate whether values slightly below the cutoff may represent early seroconversion from a very recent PV infection or recent clearance of infection.

Bottlenose dolphins live in fission-fusion societies, in which individuals travel in small groups that often change in composition with bonds developing according to their present social context.

Variation in social strategies within and between populations is common (Connor et al., 2000). Wandering individuals do not appear to live in stable groups and disperse from their natal group to join others for certain activities, including migration, feeding, and breeding (Connor, 2000). This behavior supports genetic diversity but, at the same time, facilitates pathogen transmission. Intense play and sexual activities both heterosexual and homosexual, including oral sex (Bagemihl, 2000), may account for a generally high PV antibody prevalence among dolphins. The observed ELISA reactivity across population groups suggests PV infection has generally been established in bottlenose dolphins. Antibody prevalence was higher among free-ranging than among captive dolphins. One explanation could be housing practices and/or the limited number of available mating partners for captive dolphins resulting in a lower probability of horizontal transmission. This hypothesis is supported by 89% of seronegative SLP dolphins being born in captivity. Conversely, half of the seronegative MAZ dolphins were born in captivity. The difference between facilities may be related to time of capture of the respective animal before horizontal transmission could take place and/or differences in housing organization.

Although horizontal transmission of HPVs infecting the genital mucosa is well-recognized, nonsexual transmission remains controversial. In humans, vertical PV transmission from mother to infant has not been investigated extensively. Some studies revealed evidence for vertical spread (Cason et al., 1995; Rice et al., 1999), whereas it was considered a rare event in other studies (Watts et al., 1998). Maternal viral load may be an important determinant for vertical transmission because mothers who transmitted HPV to their infants had significantly higher viral loads (Kaye et al., 1994). Data from a recent comprehensive report testing 100 mothers and their 111 children for HPV

infection failed to find evidence for mother to child transmission (Marais et al., 2007). All seronegative dolphins from CHS were sexually immature, which may indicate transmission among bottlenose dolphins most often occurs horizontally and that young dolphins are less likely to have PV antibody reactivity. This assertion is supported by all sexually immature captive dolphins being negative regardless of their parents' statuses. Furthermore, sexually immature dolphins were more likely to be seropositive with increasing age. This may be attributed to the onset of sexual behavior before sexual maturity (Booth, 1988). Thus, older sexually immature dolphins have had increased opportunities to become infected through beginning sexual play and other social activities than younger sexually immature dolphins. The seropositive captive offspring ( $n=4$ ) may have become infected after birth, because these dolphins were already sexually mature when tested.

Slightly higher PV reactivity was observed with serum of males compared with females. Genital mucosa surface sizes are approximately the same, which suggests that a larger mucosal area of possible exposure to viruses in males does not seem to be involved. The lives of male bottlenose dolphins in particular are characterized by bisexuality with frequent engagements in homosexual relationships (Bagemihl, 2000). As they exhibit very active sexual behaviors and play patterns with females and other males, higher exposure rates are plausible and may result in higher levels of humoral response.

In total, 30 of the 115 tested dolphins had a history of orogenital tumors. Five of these were seronegative in the PV screening, whereas two of those five had ELISA reactivities close to the seropositive cutoff. The observed tumors could be associated with a PV covered in the test as shown for the positive control animal (Rehtanz et al., 2006), but some of the tumors may also be associated with another unknown dolphin

PV not detected in the test or with another agent. On the other hand, some dolphins with tumors may have a weaker immune response to PV, resulting in low ELISA reactivity. In some cases, tumor development may also be the result of a low humoral response. In humans, VLPs used for immunization elicit antibody responses 14 to 40 times higher than those occurring during a natural infection, where they are often low (Ault et al., 2004; Fife et al., 2004; Harper et al., 2006). Papillomavirus infection seems to be highly prevalent in dolphins, as is the case in humans, and is not necessarily associated with clinically apparent disease in either species. The natural history of tumor progression or remission has not been established for cetaceans in the wild. Screening dolphins for antibodies will help to determine whether specific levels of anti-PV antibodies are protective against the development of tumors and which animals may be at greater risk.

Interestingly, whereas captive dolphins with tumors (26%) were mostly older animals, many of the free-ranging dolphins displaying tumors (26%) were considerably younger. The fact that the free-ranging dolphins assessed for health were, on average, younger than the dolphins tested from facilities does not affect the observation that orogenital tumors developed in earlier stages of life in both free-ranging dolphin populations investigated. In the present study, it was demonstrated that, among the populations tested, free-ranging dolphins not only had a significantly higher PV antibody prevalence but also had a magnitude of reactivity in the ELISA that was higher. This may be explained by their higher rates of exposure to one or more TtPVs through their active sexual lives and likely greater numbers of mating partners. Furthermore, being slowly reproducing, top-level predators, dolphins are sensitive to environmental disturbances. A number of marine mammal studies have recently documented that emerging or resurging infectious and



neoplastic diseases may reflect environmental distress (Bossart, 2006). Negative immunomodulatory effects of environmental contaminants, such as organochlorines and toxic heavy metals, have been described in several marine mammal species (De Guise et al., 1995; Beineke et al., 2005; Hammond et al., 2005; Levin et al., 2005; Kannan et al., 2006; Mori et al., 2006). Whether such environmental contaminants could have a similar effect on local free-ranging bottlenose dolphin populations remains to be investigated.

The analysis described here contributes to a greater understanding of the epidemiology of PV infection in marine mammals. In the future, repeated sampling will be necessary to elucidate transmission pathways and determine seroconversion and incidence rates. Research addressing other infectious agents in tumors, as well as fundamental research investigating cell-transforming capabilities of TtPVs, is needed to determine whether infection with PV is causal and whether PV acts alone or in conjunction with other agents. The potential for TtPVs to cause malignant transformation of benign tumors also remains to be investigated, as do other health risks possibly imposed by TtPV infection.

#### ACKNOWLEDGMENTS

This work was supported by a postdoctoral fellowship of HBOI to Manuela Rehtanz. Sera and lesions from free-ranging dolphins were collected under National Marine Fisheries Service Scientific Research Permit 998-1678 issued to G. Bossart as part of the HERA Project. Funding for this study was provided in part by the Florida Protect Wild Dolphins License Plate fund, Florida Ocean Initiatives Grant, NOAA's Fisheries Marine Mammal Health and Stranding Response Program, and the National Ocean Service. Sincere appreciation is extended to Z. Kurago, D. Malamud, and W. Abrams for their critical review of the manuscript and helpful discussions. Special thanks are extended to G. Fisch for further help with statistical analyses and to A. Marsh for his editing services. The authors also sincerely thank R. Lenzi, J. Pawloski, T. Goodreau, A. Scullion, P. Browning, A. Lopez,

and the Animal Training Staff from SLP, Waimanalo, Hawaii, as well as A. Salbany, from MAZ, Albufeira, Portugal, for animal handling and for providing dolphin samples as well as extensive animal background information.

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*Received for publication 20 November 2008.*