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Orthopoxvirus neutralising antibodies in small cetaceans from the Southeast Pacific

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Thousands of dusky dolphins (*Lagenorhynchus obscurus*), long-beaked common dolphins (*Delphinus capensis*), common bottlenose dolphins (*Tursiops truncatus*) of the inshore and offshore ecotypes (Van Waerebeek *et al*., 1990; Sanino *et al*., 2005) and Burmeister's porpoises (*Phocoena spinipinnis*) have been caught in fisheries off Peru, handled by fishmarket workers and commercialized for consumption by local people (Van Waerebeek and Reyes, 1994; Van Waerebeek *et al*., 1997, 1999) at least since the 1970s. In addition, traumas caused by fishing devices including gillnets, purse-seines and harpoons, probably cause the death of unknown numbers of injured small cetaceans that manage to escape (Van Bressem *et al*., 2006). These mammals are commonly infected by morbilliviruses, poxviruses, papillomaviruses, herpes-like viruses and *Brucella* sp. (Van Bressem and Van Waerebeek, 1996; Van Bressem *et al*., 1994; 1996, 1998, 2001a,b) and are infested by several species of helminth parasites (Reyes and Van Waerebeek, 1995; Van Waerebeek *et al*., 1990, 1993). Morbilliviruses, poxviruses and *Brucella* sp. as well as *Crassicauda* sp. nematodes have the potential for significant adverse impacts on population abundance by increasing natural mortality and/or by negatively affecting reproduction (Perrin and Powers, 1980; Miller *et al*., 1999; Van Bressem *et al*., 1999, 2006; González *et al*., 2002;). The question arises whether heavily exploited populations such as Peru's small odontocetes may suffer a leveraged, not simply additive, impact on its conservation status through an interaction of fisheries-related sources of mortality and morbidity with natural sources. This conservation concern flags these populations as priority subjects for research. In this paper we report on a serological survey for poxvirus exposure in Peruvian cetaceans.

A previous epidemiological survey indicated that poxviruses are endemic in Peruvian populations of *L. obscurus*, *D. capensis*, *T. truncatus* and *P. spinipinnis* (Van Bressem and Van Waerebeek, 1996). Infection is characterised by pathognomonic, irregular, gray, black or yellowish, stippled lesions known as "tattoos" (Figure 1) that may occur on any part of the body but which show a preferential corporal distribution depending on the species (Van Bressem and Van Waerebeek, 1996). The virus probably induces humoral immunity that may protect calves from the disease (Smith *et al*., 1983; Van Bressem and Van Waerebeek, 1996). The poxviruses affecting small cetaceans from the Southeast Pacific have not yet been isolated nor characterized. Other cetacean poxviruses were recently detected in both captive cetaceans, such as Indo-Pacific bottlenose dolphins (*Tursiops aduncus*), and in free-ranging rough-toothed dolphins (*Steno bredanensis*), striped dolphins (*Stenella coeruleoalba*) and bottlenose dolphins (*T. truncatus*) from Florida. A polymerase chain reaction assay was used, targeting the DNA polymerase and DNA topoisomerase genes of members of the subfamily *Chordopoxvirinae* (family Poxviridae) (Bracht *et al*., 2006). These cetacean poxviruses belong to a new genus of *Chordopoxvirinae*, but have a common, most immediate ancestor with terrestrial poxviruses of the genus *Orthopoxvirus* (Bracht *et al*., 2006). The genus *Orthopoxvirus* includes, among others, the smallpox virus (now eradicated in humans), vaccinia virus (the smallpox vaccine of unknown origin), and cowpox virus, endemic in European wild rodents and accidentally infecting humans, cats and cattle (Hazel *et al*., 2000; Esposito *et al*., 2004). The orthopoxviruses are closely related antigenically and genetically, and extensive cross-neutralisation and cross-protection occur between them (Moss, 1996).

The study reported here was carried out in 1993-1997 when very few data were available on cetacean poxviruses. On the basis of the brick-shaped morphology of cetacean poxviruses (Van Bressem *et al*., 1993) and the biology of *Chordopoxvirinae*, we hypothesized that the viruses infecting dolphins and porpoises from the Southeast Pacific may share antigens with members of the *Orthopoxvirus* genus.

Figure 1. Tattoo lesions on the throat of a dusky dolphin (*Lagenorhynchus obscurus*) from Peru (MFB-614).

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Thus, we conducted a serological survey for the presence of orthopoxvirus neutralising antibodies in 58 small cetaceans caught off Peru in 1993-1995. We tested for cowpox virus as a representative member of the genus *Orthopoxvirus* that infects mammals belonging to different orders, including Artiodactyla (even-toed ungulates), which are phylogenetically closely related to cetaceans (Milinkovitch *et al*., 1998; Gatesy *et al*., 1999).

Blood samples (hemolysed, except MFB-485) were collected from 6 *D. capensis* (all males), 27 *L. obscurus* (11 females, 16 males), 8 *T. truncatus* (2 inshore females, 1 offshore female, 5 offshore males) and 18 *P. spinipinnis* (8 females, 9 males, 1 male foetus) (Table 1). Most of these specimens were concurrently examined for tattoos in the context of an epidemiological study (Van Bressem and Van Waerebeek, 1996). All dolphins and porpoises had been dead for less than 48 hours, and were sampled in fisheries operating off Cerro Azul (13°00'S, 76°30'W) and Chancay (11°37'S, 77°16'W). *L. obscurus* and *P. spinipinnis* smaller than 160cm and 140cm, respectively, were considered calves. Sexual maturity was determined through macroscopic examination of gonads (semen in epididymides; minimum one corpus albicans or corpus luteum in one of the ovaries), from evidence of lactation, or was inferred from the standard body length. Van Waerebeek (1992) estimated that 50% of Peruvian *L. obscurus* in both sexes attain sexual maturity at 175cm. Female and male *P. spinipinnis* reach sexual maturity on average at 155cm and 160cm, respectively (Reyes and Van Waerebeek, 1995).

Stocks of cowpox virus (strain L97, Gaskell *et al*., 1983) were produced on chorioallantoic membranes (CAM) of 12 day-old chick embryos and passaged once on Vero cells. After freeze-thawing infected cells and removing cell debris by centrifugation, the stocks were titrated and aliquots stored at –80°C. Before applying virus neutralising (VN) tests, the virus suspension was thawed and ultrasonicated briefly on ice to avoid clumping of virions.

Serum samples were tested for the presence of cowpox virus neutralising antibodies essentially as described previously in other mammalian species (*e.g.* Bennett *et al*., 1985; Crouch *et al*., 1995) using Vero cells. Briefly, all sera were diluted 10-fold in phosphate buffer saline (PBS) with 1% foetal calf serum, complement-inactivated at 56°C for 20 minutes and centrifuged to pellet major debris. They were further diluted three- or five-fold for the VN tests. The thirty or fifty-fold dilutions of the sera were mixed with an equal volume (0.4ml) of a suspension containing approximately 300 plaque forming units (pfu)/ml of cowpox and incubated for 5 hours at 37°C. An aliquot of virus at 300pfu/ml diluted in PBS with 1% inactivated foetal calf serum was included in a well of each set of plates to control virus activity after the incubation period. Residual infectivity was detected by inoculation of the sera-virus mixtures on Vero cell cultures in 6-well plates (0.2ml/well). After 1 hour incubation at 37°C, the formation of secondary

Table 1. Distribution of maturity classes in male and female long-snouted common dolphins (*D. capensis*), dusky dolphins (*L. obscurus*), bottlenose dolphins (*T. truncatus*) and Burmeister's porpoises (*P. spinipinnis*) examined for orthopoxvirus neutralising antibodies.

Species	Maturity	♂			♀			Pooled sexes		
	Class	*N tested*	*N pos*	*% pos*	*N tested*	*N pos*	*% pos*	*N tested*	*N pos*	*% pos*
D. capensis	Immature	5	5	100	0	-	-	5	5	100
	Mature	1	1	100	0	-	-	1	1	100
L. obscurus	Calf	2	2	100	3	1	30	5	3	60
	Immature	2	2	100	4	2	50	6	4	66.7
	Mature	12	9	75	4	1	25	16	10	62.5
T. truncatus inshore	Immature	0	-	-	2	2	100	2	2	100
T. truncatus offshore	Immature	3	3	100	0	-	-	3	3	100
	Mature	2	2	100	1	1	100	3	3	100
P.spinipinnis	Calf	2	2	100	1	1	100	3	3	100
	Immature	4	2	50	2	2	100	6	4	66.7
	Mature	3	2	66.7	5	5	100	8	7	87.5

The number (N) of specimens tested and seropositive (pos) as well as the percentage of seropositive specimens (% pos) are given for each subsample.

plaques was prevented by an agarose overlay. Plaques were counted 48 hours after inoculation of the cell monolayer. Sera were considered positive when they reduced plaque formation by at least 50% at a dilution equal or higher than 1:30. The titre of 17 positive sera was further determined in the same VN test, using serial two-fold dilutions of the sera (starting at 1:20 [one serum], 1:50 [15 sera] or 1:100 [three sera]).

Cowpox virus neutralising antibodies were detected in the sera of all *D. capensis* and *T. truncatus* examined as well as in 17 of 27 (63.0%) *L. obscurus,* and in 14 of 17[3] (82.4%) *P. spinipinnis*. Among 12 positive dolphins and 7 *P. spinipinnis*, neutralising titres ranged from 40 to over 1600, and 50 to 200, respectively (Table 2). The high prevalence of orthopoxvirus neutralising antibodies with high titres indicates that Peruvian small cetaceans are commonly infected by poxviruses antigenically related to cowpox virus. To date, the only poxviruses microscopically encountered in these mammals are those causing the endemic and distinctive tattoo skin disease (Van Bressem and Van Waerebeek, 1996) and it is highly likely that infection by these viruses elicited the neutralising antibodies detected in this study. Furthermore, we suppose that the poxviruses infecting Peruvian small cetaceans are related to those recently detected by PCR in tattoo-like lesions in *S. bredanensis* and *S. coeruleoalba* from the North Atlantic (Bracht *et al*., 2006). Although these viruses probably belong to a new genus of *Chordopoxvirinae*, they share a common most immediate ancestor with terrestrial poxviruses (Bracht *et al*., 2006), which may explain the observed cross-neutralization with cowpox virus. Alternatively, a true orthopoxvirus may circulate in Peruvian small cetaceans. Further investigation including PCR using the primers described by Bracht *et al*. (2006) and serological studies should be undertaken in these and other cetacean populations from the Southeast Pacific.

The positive dolphins and *P. spinipinnis* included calves as well as sexually mature and immature individuals (Tables 1 and 2). Orthopoxvirus neutralizing antibodies were not detected in the serum of a near-term foetus from a seropositive *P. spinipinnis* (JAS-50) suggesting that no antibodies were transmitted during pregnancy. The same observation was made for dolphin morbillivirus antibodies (Van Bressem *et al*., 2001b). The cetacean placenta is of the epitheliochorial type (Harrison, 1969) and it is thought that maternal immunoglobulins are transmitted to the offspring through colostrum as in cattle and other even-toed ungulates (Macdonald and Bosma, 1985), congruent with phylogenetic evidence (Milinkovitch *et al*., 1998; Gatesy *et al*., 1999). Thus, the antibodies detected in the sera obtained from calves *L. obscurus* and *P. spinipinnis* (Table 2) were probably of maternal origin.

A high density of tattoos was observed in three immature *D. capensis* as well as in an immature and a mature *P. spinipinnis* that showed high VN titres against cowpox virus. This may reflect a yet incomplete immune response or poxvirus immune evasion (Johnston and McFadden, 2004; Liu *et al*., 2005). Very high prevalence levels (over 60%) of tattoo disease in the populations of *D. capensis* and *P. spinipinnis* studied (Van Bressem and Van Waerebeek, 1996) suggest that the poxvirus had evolved to counteract the immune response and to persist in the skin cells. The fact that tattoo skin lesions may last for months or even years and may grow very large (Geraci *et al*., 1979; Van Bressem and Van Waerebeek, 1996; Van Bressem *et al*., 2003) is also in favour of these hypotheses.

Significance of differences in prevalence of cowpox neutralising antibodies between sexes was verified for *L. obscurus* and *P. spinipinnis* with a two-tailed Fisher's exact test (Swinscow, 1981). Seroprevalence was significantly ($P= 0.04$) lower in females (36.4%, $n= 11$) than in males (81.25%, $n= 16$) *L. obscurus*. However, low sample sizes and in particular the low number of sera from adult females may account for this difference. There was no significant sexual variation ($P= 0.24$) in seroprevalence in *P. spinipinnis* (100% in 8 females, 66.7% in 9 males; Table 1). An epidemiological study conducted in these populations during the same period and at the same locations showed similar prevalences of tattoo lesions in male and female *L. obscurus* but twice as many affected males than females in *P. spinipinnis* (Van Bressem and Van Waerebeek, 1996).

Seropositivity rates were similar in both odontocete families (75.6% in 41 Delphinidae, 82.4% in 17 Phocoenidae). However, among the immature age class the titres of neutralising antibodies were higher in the dolphins (over 1600) than in the porpoises (200), suggesting a weaker humoral response in the latter. Alternatively, the porpoises may be infected by a strain or species of poxvirus antigenically less similar to cowpox virus than the virus infecting the dolphins. These hypotheses should be further explored.

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[3] The foetus is not considered in the statistics.

Table 2. Presence of orthopoxvirus-specific antibodies in the sera of small cetaceans caught off Peru in 1993-1995 as detected by a virus neutralisation (VN) test using cowpox virus.

SPECIES	CODE	SL (cm)	SEX	YEAR	LOCALITY	SM	PRESENCE OF TATTOOS	DENSITY OF TATTOOS	VN ANTIBODY TITRE AGAINST COWPOX
Delphinus capensis									
	MFB 113	188.5	♂	93	Cerro Azul	Imm	Yes	Medium	≥ 1600[a]
	MFB 675	200.5	♂	94	Cerro Azul	Imm	Yes	High	100[a]
	MFB 511	204.5	♂	94	Cerro Azul	Imm	No	-	50[a]
	MFB 510	207	♂	94	Cerro Azul	Imm	Yes	High	30
	MFB 508	210.5	♂	94	Cerro Azul	Imm	Yes	Medium	≥ 1600[a]
	MFB 529	240.5	♂	94	Cerro Azul	Mat	No	-	40[a]
Lagenorhynchus obscurus									
	MFB 535	116,5	♂	94	Cerro Azul	Calf	No	-	≥ 30
	MFB 514	130,5	♀	94	Cerro Azul	Calf	No	-	≥ 30
	MFB 542	132,5	♂	94	Cerro Azul	Calf	No	-	≥ 30
	MFB 72	173	♂	93	Cerro Azul	[Imm]	ne	ne	50[a]
	MFB 502	174	♂	94	Cerro Azul	[Imm]	No	-	≥ 50
	RBC 40	186	♀	94	Cerro Azul	Imm	No	-	50[a]
	MFB 503	190	♀	94	Cerro Azul	Imm	No	-	≥ 800[a]
	MFB 71	185,5	♂	93	Cerro Azul	Mat	No	-	≥ 30
	MFB 538	185,5	♂	94	Cerro Azul	Mat	No	-	≥ 30
	MFB 75	186	♂	93	Cerro Azul	[Mat]	ne	ne	≥ 30
	MFB 111	186	♂	93	Cerro Azul	[Mat]	Yes	Low	≥ 30
	MFB 506	187,5	♂	94	Cerro Azul	[Mat]	No	-	≥ 30
	MFB 509	191	♂	94	Cerro Azul	Mat	Yes	Low	≥ 50
	MFB 543	191	♀	94	Cerro Azul	Mat	No	-	≥ 30
	MFB 97	191	♂	93	Cerro Azul	Mat	ne	ne	≥ 30
	MFB 500	192	♂	94	Cerro Azul	Mat	Yes	ne	100[a]
	MFB 100	192	♂	93	Cerro Azul	Mat	ne	ne	≥ 30
Tursiops truncatus (inshore)									
	MFB 465	229	♀	93	Cerro Azul	Imm	Yes	Low	≥ 30
	MFB 485	253,5	♀	94	Cerro Azul	Imm	ne	ne	≥ 50
Tursiops truncatus (offshore)									
	MFB 533	262.5	♂	94	Cerro Azul	Imm	Yes	ne	≥ 30
	MFB 701	272.5	♂	94	Cerro Azul	Imm	Yes	Low	≥ 30
	MFB 616	295	♂	94	Cerro Azul	Imm	ne	ne	≥ 800[a]
	MFB 702	272	♀	94	Cerro Azul	Mat	No	-	≥ 800[a]
	MFB 532	294	♂	94	Cerro Azul	Mat	No	-	≥ 30
	MFB 608	303.5	♂	94	Cerro Azul	Mat	No	-	50[a]
Phocoena spinipinnis									
	JAS 46	130	♂	95	Chancay	Calf	ne	ne	≥50
	MFB 496	136.5	♀	94	Cerro Azul	Calf	ne	ne	100[a]
	JAS 44	135	♂	95	Chancay	Calf	No	-	50[a]
	MFB 749	145	♀	95	Chancay	Imm	No	-	≥ 30
	MFB 524	147	♀	94	Cerro Azul	Imm	Yes	Low	100[a]
	MFB 479	151.5	♂	94	Cerro Azul	[Imm]	Yes	High	200[a]
	MFB 494	157	♂	94	Cerro Azul	[Imm]	Yes	Low	≥ 50
	MFB 493	153.5	♀	94	Cerro Azul	Mat	No	-	≥ 30
	MFB 480	161.5	♂	94	Cerro Azul	[Mat]	Yes	High	100[a]
	JAS 43	163.5	♀	95	Chancay	Mat	ne	-	≥ 30
	MFB 526	164	♂	94	Cerro Azul	Mat	Yes	Low	50[a]
	JAS 50	169	♀	95	Chancay	Mat	Yes	ne	50[a]
	MFB 718	170	♀	94	Cerro Azul	Mat	Yes	Low	≥ 50
	JAS 48	MAT	♀	95	Chancay	Mat	Yes	ne	≥ 50

Acronyms: (SL) standard body length, (SM) sexual maturity, (Mat) mature, (Imm) immature, (ne) not examined. The square brackets indicate that sexual maturity was inferred from SL, 'a' in superscript indicates that the serum was titrated.

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