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Erysipelas vaccination protocols in dolphins *Tursiops truncatus* evaluated by antibody responses over twenty continuous years

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ABSTRACT: Erysipelas is an infection caused by *Erysipelothrix rhusiopathiae* that affects many different species around the world, including cetaceans. The acute septicemic form can rapidly cause death in bottlenose dolphins *Tursiops truncatus*. The ultimate goals of this long-term study were the development and identification of the most effective vaccination protocol against clinical erysipelas in *T. truncatus* using a commercially available swine vaccine, and to determine whether there is a need for a semi-annual vaccination versus an annual vaccination. The present study concentrated on the immunization of a dolphin population (7 wild-born and 22 captive-born individuals) with 2 swine vaccines, the European 'Eurovac Ery®' vaccine and the American 'ER Bac Plus®' vaccine, and immunological profile results over a 20-yr time period. The general protocol was a primo-vaccination (between 3 and 7 mo of age for calves) with or without a booster 1 mo post primo-vaccination and either annual or semi-annual vaccination thereafter. Sera were collected prior to vaccination, 2 wk post-vaccination and monthly. A dolphin-specific ELISA was developed to analyze the erysipelas-specific antibody response of vaccinated animals. The final ELISA results (n = 1362 samples from 29 animals at pre- and post-vaccination time) suggest that (1) there is a significant difference in antibody levels at the start of the vaccination between older and younger animals; (2) at least 3 vaccinations are necessary to obtain antibody levels above the levels at pre-vaccination; (3) thereafter, annual vaccinations seem sufficient to keep antibody levels above the levels at pre-vaccination; and (4) both vaccines induced similar responses. No case of erysipelas infection was observed in this population during the study.

KEY WORDS: Bottlenose dolphin · Vaccination · *Erysipelothrix rhusiopathiae* · Erysipelas · *Tursiops truncatus*.

1. INTRODUCTION

Erysipelas is an infection caused by *Erysipelothrix rhusiopathiae* that affects many different species around the world. It has been reported in mammals, birds, reptiles, amphibians and fish, with swine being economically the most important (Sweeney 1986, Gyles 1993, Opriessnig & Wood 2012). *E. rhusiopathiae* is a facultative pathogenic, facultative anaerobe, intracellular, non-motile, non-acid-fast, non-sporulating, Gram-positive rod-shaped bacterium (Wood 2000). The organism may be isolated from soil, food scraps and water contaminated by infected animals (Wood 1992). Carrier pigs can shed the

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organism in feces and can carry it in their tonsils. All pigs in a herd may be infected, but rarely do they all become clinically ill (Opriessnig & Wood 2012). The organism does not survive long in soil but is continuously being reintroduced via fecal fertilization or contamination. Commonly available disinfectants can inactivate *Erysipelothrix* spp. (Conklin & Steele 1979). However, when organic matter is involved, equipment and surroundings are more difficult to disinfect, especially without cleaning (Fidalgo et al. 2002). *Erysipelothrix* is resistant to salting and many other food preservation methods (Conklin & Steele 1979). It can survive for months in frozen or cooled meat, rotten carcasses, dry blood or fish meal (Cornelisse 1993), and will also survive for up to 6 mo in feces or fish outer slime if the temperature is cooler than 12°C (Wood 1992). The organism has been isolated from the gingiva of non-clinical swine and pinnipeds and is commensal on the cutaneous slime of both freshwater and saltwater fish (Hubbert et al. 1975, Suer & Vedros 1988, Bauwens et al. 1992). The route of transmission is primarily by mucosal contact or uptake via feed and water that can be contaminated with feces, but can also occur through infected wounds (Wood 1992).

In most mammals, the clinical disease can present itself as acute septicemia, endocarditis, arthritis or skin lesions (often called 'diamond skin disease' in swine), occurring either alone or in combination (Gyles 1993). The bacterium is zoonotic, with most cases in humans an occupational disease through scratches or puncture wounds in the skin and leading to a cutaneous infection (erysipeloid), but occasionally bacteremia and endocarditis have been described (Gyles 1993, Brooke & Riley 1999, Veraldi et al. 2009). The highest risk is found in people working with potentially infected animals, such as butchers, veterinarians, fishers or fish handlers (Reboli & Farrar 1989, Reboli & Farrar 2010).

Numerous recent, and less recent, scientific presentations and publications focus on the disease and vaccination trials in captive cetaceans since the first reported cases in Atlantic spotted dolphin *Stenella plagiodon* and bottlenose dolphin *Tursiops truncatus* by Seibold & Neal (1956). In human care, it has also been reported in Risso's dolphin *Grampus griseus* (Nakajima & Takikawa 1961), Pacific white-sided dolphin *Lagenorhynchus obliquidens* (Nakajima & Takikawa 1961, Kinsel et al. 1997), Amazon dolphin *Sotalia fluviatilis* (Grafton 1968), Indian Ocean bottlenose dolphin *Tursiops aduncus* (Thurman et al. 1983), white-beaked dolphin *Lagenorhynchus albirostris* (Buck & Spotte 1986), killer whale *Orcinus orca* (Bossart & Eimstad 1988) and beluga whale *Delphinapterus leucas* (Dunn 1990, Calle et al. 1993). In the wild, it has been reported in pilot whale *Globicephala melas* (Chastel et al. 1975), killer whale *Orcinus orca* (Young et al. 1997), harbour porpoise *Phocoena phocoena* (Bosseret et al. 2002), bottlenose dolphin *Tursiops truncatus* (Melero et al. 2011) and southern right whale *Eubalaena australis* calves (Fiorito et al. 2016). Most of the knowledge about erysipelas in cetaceans has been extrapolated from studies in swine, the most studied animal in relation to *E. rhusiopathiae*.

The infection in dolphins is exogenous and occurs mainly through ingestion of contaminated fish, with the organism gaining access to the bloodstream via a breach in the gastrointestinal mucous membranes; however, infection from other sources, through conjunctival, respiratory, parenteral and skin lesion routes, has been described (Wellmann 1955, Geraci et al. 1966, Wood & Shuman 1981, Sweeney 1986, Suer & Vedros 1988, Calle et al. 1993, Boehm et al. 2000, Greenwell et al. 2002, Walsh et al. 2005). The disease usually affects a single animal within a group sharing the same environment (Hoorens et al. 1988, Boehm et al. 2000, Gearhart et al. 2005). This could be explained by contamination of only a few feeder fishes in a particular lot and/or individual variation in, and leading to infection susceptibility (Calle et al. 1993, Kinsel et al. 1997).

Two major forms of the disease can be seen in dolphins: an acute septicemic form, often hyperacute, and leading to death without previous, or with very few, clinical signs, and a subacute form, with the appearance of typical grey diamond or rhomboid shaped skin lesions (Geraci et al. 1966, Sweeney & Ridgway 1975, Thurman et al. 1983, Hoorens et al. 1988, Kinsel et al. 1997). These lesions are a result of peripheral arterial thrombosis by bacterially produced neuraminidase, with subsequent cutaneous infarction, depigmentation and necrosis (Wood 1975). A less common vesicular glossitis form has also been described (Bossart & Eimstad 1988). Apart from the skin lesions, lethargy and loss of appetite accompanied with a strong neutrophilia might be seen (Geraci et al. 1966, Sweeney 1986, Calle et al. 1993).

If identified early enough, which is seldom the case for the acute form, recovery can be achieved by rapid treatment with antibiotics and general support (Thurman et al. 1983, Calle et al. 1993, Gearhart et al. 2005). Often, and with the purpose of minimizing possible resulting fatalities in cetaceans, antibiotics are immediately administered to suspected cases without waiting for a confirmed diagnosis. Managing

the environment, through the control of fish quality, has also been used to further help in the prevention of the disease. After a series of clinical erysipelas in their dolphin population and the identification of *E. rhusiopathiae* on the outer slime layer of the feeder fish, a facility started placing their fish in a 0.7 mg l^{-1} ozonated water bath for at least 4 min to destroy the bacteria without damaging the fish (Knowles & Ergler 2005). Another facility with previous fatal erysipelas cases tentatively added chlorine to their feeder fish thaw sinks, at 5 parts per million, and followed it with a fresh rinse. The chlorine level proved insufficient to kill *E. rhusiopathiae*; however, the necessary level would make the fish unpalatable (Walsh et al. 2005). Another method of prevention is induction of an active immunity through semi-annual vaccination of the cetaceans (Geraci et al. 1966, Sweeney 1986).

Modified live vaccines were initially utilized but discontinued after several cases of virulence reversion were reported, resulting in disease and subsequent death in animals post-vaccination (Gilmartin et al. 1971, Ridgway 1972, Colgrove 1975, Medway 1980, Dunn 1990). In the past, with the use of inactivated dead vaccines, people have experienced swelling at the injection site and even anaphylactic shock has been observed in dolphins, followed in some cases by the death of the animal very shortly after the booster or at later immunization. This led many facilities in the late 1980s to choose a one-time-only vaccination or even to cease the vaccination program completely (Sweeney 1986, Dunn 1990). However, there have been cases of animals vaccinated only once that succumbed a couple of years later to *E. rhusiopathiae* septicemia (Boehm et al. 2000). At the time, though anaphylactic shocks with or without subsequent death had been reported in the USA and Australia, none seemed to have been reported in Europe, leading scientists to believe the reaction may be related to different adjuvants in the American and European vaccines and/or from the local anesthetic formerly added to vaccines (Boehm et al. 2000, Wood 2000, A. Gaukler pers. comm.). When vaccinated nowadays, dolphins receive a commercial dead whole-agent swine vaccine.

The present study addresses the former vaccination concerns and analyzes the long-term effect of *E. rhusiopathiae* vaccination in a bottlenose dolphin population housed at Zoomarine, Albufeira, Portugal. We concentrate on the immunization of a *T. truncatus* population (a total of 7 wild-born and 22 captive-born animals) with 2 swine vaccines — the European 'Eurovac Ery®' vaccine (Eurovet Animal Health, previously called Aescovac Ery), used until May 2010, and then the American 'ER Bac Plus®' vaccine (Pfizer Animal Health) — and their erysipelas-specific antibody response (n = 1362 serum samples) through a dolphin-erysipelas-specific enzyme-linked immunosorbent assay (ELISA) of pre- and post-vaccination antibody levels over a time period of 20 yr. The institution of the study population, Zoomarine, after a couple of fatal *E. rhusiopathiae* cases in its population, approved of the study, namely the establishment of a vaccination protocol for its dolphins and the analysis of its effect on the antibody levels over time.

The ultimate goals were to obtain better insight into the most effective vaccination protocol with the use of a commercially available swine vaccine to protect *T. truncatus* against erysipelas infection, and to determine whether there is a need for a semi-annual vaccination versus an annual vaccination.

2. MATERIALS AND METHODS

2.1. Animals

The Atlantic bottlenose dolphins *Tursiops truncatus* in this research all reside at Zoomarine Portugal, apart from 2 animals that were sent to a sister facility (Zoomarine Italy) but stayed in the program, and 1 calf born at the Italian facility. The study comprised a total of 7 wild- and 22 captive-born animals (Table 1). Three captive-born animals came from another vaccinating European facility and 2 captive-born animals came unvaccinated from a different facility. Five animals died during the course of the study from causes unrelated to erysipelas.

The participating animals were divided in 4 different groups that had common age and origin properties (Table 1). Group 1 (WB-1) comprised 3 'older' wild-born animals that had been in the program since the beginning of the study in 1994. Group 2 (WB-2) comprised 4 'younger' wild-born animals that entered the program in 2000 and 2001. Group 3 (CB-3) comprised 9 'older' captive-born animals (born between 1989 and 1999) that entered the program at different ages and times. Group 4 (CB-4) comprised 13 'younger' captive-born animals (born after 2003).

2.2. Vaccines

Two different commercial swine inactivated vaccines were used in the study. A European vaccine,

Table 1. Gender, birth date and vaccination background of the dolphins allocated to the 4 study groups. Group 1 = WB-1, n = 3, older wild-born animals, in the vaccination program prior to 2000; Group 2 = WB-2, n = 4, younger wild-born animals, entering the vaccination program in 2000; Group 3 =CB-3,n = 9, older captive-born animals (between 1989 and 1999); Group 4 = CB-4, n = 13, younger captive-born animals (≥2003). F: female; M: male; WB: wild born; CB: captive born

Animal ID	Gender	Birth (death) date	Primo-vaccination age (yr/mo)	Vaccination schedule	Location
Group 1					
WB-1-1	F	1968	27 yr	Annual, then every 2.5 yr	Zoomarine Portugal
WB-1-2	F	1978	17 yr	Annual, then every 2.5 yr	Zoomarine Portugal
WB-1-3	F	1982	14 yr	Annual, then every 2.5 yr	Zoomarine Portugal
Group 2					
WB-2-5	M	1996 (27/6/2008)	6 yr	Annual then semi-annual	Zoomarine Portugal
WB-2-6	F	1996 (16/7/2005)	4 yr	Annual then semi-annual	Zoomarine Portugal
WB-2-4	M	1996	5 yr	Semi-annual	Zoomarine Portugal
WB-2-7	M	1996	5 yr	Semi-annual then annual	Zoomarine Portugal
Group 3					
CB-3-8	M	19/12/1989 (28/7/1998)	6.5 yr	Annual	Zoomarine Portugal
CB-3-10	M	14/08/1993 (5/4/2003)	3 mo	Annual	Zoomarine Portugal (born in Brugge, Belgium)
CB-3-13	F	1/10/1996	5 mo	Annual then semi-annual	Zoomarine Portugal
CB-3-9	M	25/10/1992	3 yr	Annual then semi-annual	Zoomarine Italy (born in Zoomarine Portugal)
CB-3-14	F	3/12/1996	6 mo	Annual then semi-annual	Zoomarine Portugal (born in Brugge, Belgium)
CB-3-15	M	28/07/1998	4.5 mo	Annual then semi-annual	Zoomarine Italy (born in Zoomarine Brugge)
CB-3-11	M	4/06/1995	8 yr	Annual then semi-annual	Zoomarine Portugal (born in Kolmarden, Sweden)
CB-3-12	M	11/09/1996	11 yr	Semi-annual (since 2013: placebo)	Zoomarine Portugal (born in Kolmarden, Sweden)
CB-3-16[b]	F	3/08/1999	4 mo	Semi-annual	Zoomarine Portugal
Group 4					
CB-4-17[b]	M	29/09/2003	4 mo	Semi-annual	Zoomarine Portugal
CB-4-18[b]	M	18/11/2003	1 yr	Semi-annual	Zoomarine Portugal
CB-4-19[a]	M	2/08/2004	3.5 mo[c]/8 mo[d]	Semi-annual	Zoomarine Portugal
CB-4-20[a]	F	22/11/2006	3 yr[c]/3.5 yr[d]	Semi-annual	Zoomarine Portugal
CB-4-21[b]	F	2/04/2007	3.5 mo	Semi-annual	Zoomarine Portugal
CB-4-22[b]	M	10/11/2008	8 mo	Semi-annual	Zoomarine Portugal
CB-4-23[b]	M	21/07/2009	4 mo	Semi-annual	Zoomarine Portugal
CB-4-24	M	25/10/2009 (2/9/2011)	8 mo	Semi-annual	Zoomarine Portugal
CB-4-25[a]	M	15/01/2010	7.5 mo[c]/11 mo[d]	Semi-annual	Zoomarine Portugal
CB-4-26[a]	F	9/11/2010	6.5 mo[c]/14 mo[e]/2 yr[f]	Semi-annual	Zoomarine Portugal
CB-4-27[b]	M	1/7/2012	9 mo	Semi-annual	Zoomarine Portugal
CB-4-28[b]	F	19/9/2012	7 mo	Semi-annual	Zoomarine Italy
CB-4-29[b]	M	24/9/2012	7 mo	Semi-annual	Zoomarine Portugal

[a]Calves with an adapted vaccination schedule
[b]First booster within 1 mo
[c]The first vaccination was not followed by a booster within 1 mo for medical reasons
[d]The second vaccination was followed by a booster within 1 mo
[e]The second vaccination was not followed by a booster within 1 mo for medical reasons
[f]The third vaccination was followed by a booster within 1 mo

manufactured by Hipra Laboratories (Gerona, Spain) and distributed first as Aescovac Ery® by Aesculaap Groothandel BV and then as Eurovac Ery® by Eurovet Animal Health BV, was used until May 2010 when, after being removed from the market for commercial reasons in 2006, the remaining stock ran out. It was a killed vaccine based on 4 serotype-2 immunogenic strains of *Erysipelothrix rhusiopathiae.* The bacteria were cultured in a serum-free medium, inactivated by formalin and adsorbed, together with their antigens, on aluminum hydroxide. The vaccine contained at least 200 IU per dose (2 ml). In May 2010,

following this market removal, the study switched to the American Er Bac Plus® vaccine (Zoetis Services), which an American institution had recently started to use. It is a bacterin vaccine, cultured in a serum-free medium, chemically inactivated and combined with Amphigen® (Pfizer), an oil-in-water based adjuvant.

2.3. Vaccination protocol

A complete blood analysis (complete blood count, serum biochemical profile, erythrocyte sedimentation rate, fibrinogen and serum protein electrophoresis) was performed prior to each vaccination (n = 416) in all animals, including in calves. If the blood results deviated from the animals' normal mean values, potentially indicating an infection and/or a medical problem, the vaccination was postponed until the blood values were back to normal.

Until 2012, 18 mg of the anti-histaminic dexchlorfeniramine maleate in coated tablets (Polaramine® Repetabs, Schering-Plough) was administered 1.5 h before vaccination (except for not-yet-eating calves) to potentially help in case of anaphylactic reaction. From 2012, dexchlorfeniramine maleate was switched to 240 mg fexofenadine (Mylan).

Animals were vaccinated by intra-muscular injection of 2 ml vaccine (for both Eurovac Ery and ER Bac Plus), with a 20 G × 2¾ inch (0.9 × 70 mm) or 21 G × 2 inch (0.8 × 50 mm) needle, depending on the size of the animal, inserted to the hub either into the left or right side of the animal, 10 cm below the mid insertion of the dorsal fin.

The general protocol was a primo-vaccination, with or without a booster 1 mo post primo-vaccination, and either annual or semi-annual vaccinations thereafter. Primo-vaccination was at 3 to 7 mo of age for captive-born animals within the study. Based on results obtained during another ongoing study (Lacave et al. 2001), new dolphins entering the program after 2000 followed the semi-annual vaccination schedule. Dolphins from Group 1 ('older' wild-born dolphins) continued with an annual vaccination, and eventually switched to a vaccination once every 2.5 yr towards the end of the study. All dolphins from Group 2 ('younger' wild-born dolphins) and Group 3 ('older' captive-born dolphins) switched from annual to semi-annual vaccination after 2003, but one dolphin (WB-2-7) that switched from semi-annual to annual (Table 1). During pregnancy, the vaccination program continued for WB-1-1 (calf CB-4-20) and CB-3-16 (calf CB-4-23) and was interrupted for CB-3-13 (calf CB-4-29) and CB-3-16 (calf CB-4-27).

Nine calves received their first booster within 1 mo of their primo-vaccination; 3 calves did not receive a booster within 1 mo of their primo-vaccination because of medical reasons, but did receive a booster within 1 mo of their second vaccination; and 1 calf (CB-4-26), also for medical reasons, received a booster within 1 mo of its 3rd vaccination (Table 1).

The vaccinated animals were monitored for 15 min by a veterinarian and kept in a pool with a lifting platform for 1 h post-vaccination, and emergency drugs (adrenaline, corticoids, atropine and doxapram) were syringe-prepared, ready to be used in case of adverse reaction. Trainers were requested to stay poolside for 1 h. Aside from calves and yet-to-be-trained animals that were handled on the lifting floor, all vaccinations were given under voluntary behaviour and without restraint poolside. No more than one animal was vaccinated on the same day.

2.4. Blood sampling

Aside from calves and yet-to-be-trained animals that were handled on the lifting floor, all blood samplings were performed under voluntary behavior. Blood samples were collected from the main vessel on the ventral part of the fluke into dry tubes, with no additive, with a 23 G (calves) or 21 G butterfly needle.

2.5. Serum samples

Blood samples were collected prior to vaccination, 2–4 wk post-vaccination and every month, as part of the ongoing husbandry program, and sera were kept at −20°C on site for banking. The statistics and graphical representations of the results shown in this publication are based on 1362 samples from the serum bank that were analyzed in 2 dolphin *Erysipelothrix*-specific ELISAs: one batch with sera from 1994 to 2011 and one batch with sera from 2011 to 2014. The graphical representation of the results is sometimes discontinuous. The reasons are multiple: (1) some samples were lost over time; (2) some samples were depleted during former analysis in the ongoing study; (3) animals that joined later on in the project did not have their sera banked in the previous facility; (4) some animals moved to another facility; and (5) there were, in some instances, deviations from the optimal vaccination schedule due to unsatisfactory blood results, clinical signs of other diseases or potential mechanical problems with the lift.

Sera collected from CB-3-14 (captive born and previously vaccinated at least 17 times) and from WB-2-7 (wild born and previously vaccinated at least 17 times) had consistent elevated antibody levels (represented through optical density [OD]) in the ELISA after vaccination and were pooled as a positive control. Eight sera from the captive-born calves CB-4-20 and CB-4-23, taken before their primo-vaccination, had low OD levels in the ELISA prior to vaccination and were pooled as a negative control. The samples from CB-4-20 were taken when the animal was 10 mo old. The samples from CB-4-23 were taken at 5, 6, 7 and 9 d of age. Upon depletion of this negative control, 13 sera from newer captive born calves CB-4-27 and CB-4-29 were analyzed before primo-vaccination. They had low OD levels in the ELISA and were pooled as a negative control. The samples from CB-4-27 were taken at 1, 2, 2.5, 3, 5.5, 6.5 and 8.5 mo of age. The samples from CB-4-29 were taken at 2, 3, 3.5 and 4.5 mo of age.

2.6. Serum processing

For practical reasons and for a better clot retraction, blood was kept for 1 h at room temperature after sampling and then overnight at 4°C to be centrifuged for 10 min at 1800 × *g* the next day, and then stored at −20°C. Purified (inactivated) sera were obtained by setting the samples for 30 min in a 56°C water bath, adding 1:4 kaolin (25% kaolin + phosphate buffered saline [PBS]) and rocking the mixture with a vortex. The samples were then incubated for 30 min at room temperature and then centrifuged at 5500 × *g* for 7 min. The purified sera were cooled for direct use or further banked at −20°C for later use.

2.7. Strains and antigen preparation

The coating antigens for the ELISA consist of 2 strains of *E. rhusiopathiae* (isolate 266/6611 and isolate Ery 5207) stocked in −80°C in 90% LYM medium (Lacave et al. 2001). Isolate 266/6611 was cultured from the liver of a 14-yr-old female dolphin that died from acute erysipelas septicemia in 1987 at the Bruges Dolphinarium, Belgium (Hoorens et al. 1988). This strain cannot be assigned to the classical serotypes. The second isolate came from different organs of a 5-yr-old male dolphin that succumbed from erysipelas at the Zoo of Antwerp, Belgium, in 1990. This isolate is partially identical to serotype 5 (G. Lacave & L. Schlater unpubl. data). The lyophilized bacteria were suspended in PBS and grown in brain–heart infusion broth for 72 h at 37°C, under constant rocking at 85 rpm, and then harvested through centrifugation at 3000 × *g* for 1 h. The resulting pellets were washed 3 times with a coating buffer (bicarbonate buffer 0.05 M, pH 9.4). The mixture was re-suspended in order to obtain a turbidity similar to the McFarland index 2, which was tested to be equivalent to 1×10^7 *E. rhusiopathiae* bacteria per milliliter solution. The suspensions were then frozen in 50 ml centrifuge tubes and stored at −20°C.

2.8. ELISA

A total of 100 µl of antigen suspension (a 1:1 mix of 266/661 and 5207 erysipelas strains) was added to each well of 96-well Polysorb® (Nunc) ELISA plates and incubated overnight at 4°C. The plates were subsequently treated with 200 µl blocking buffer (0.05 M bicarbonate coating buffer [pH 9.4] and 5% glycine) at 37°C for 4 h. After each incubation, the plates were washed 3 times with 300 ml washing solution (PBS supplemented with 0.05% Tween 20). Then 100 µl of a 1/20 dilution (in PBS + 0.05% Tween 20 + 5% non-fat dry milk) of each serum sample was added into duplicate wells and the plates were incubated at 37°C for 1 h. The same was done for the negative and positive controls. Subsequently, 100 µl of mouse anti-dolphin IgG monoclonal (1/40) (Mab) (ICBR, Hybridoma Core Laboratory, University of Florida, USA) (Nollens et al. 2007) and 100 µl of horseradish peroxidase-conjugated goat anti-dog IgG (1/100) (Kirkegaard and Perry Laboratories) were each added to one of the wells and the plates were incubated at 37°C for 1 h. These antisera were diluted in PBS supplemented with 0.05% Tween®20 and 3% bovine serum albumin (dilution buffer). To the wells with mouse anti-dolphin Mab, a 1/1000 in dilution buffer of horseradish peroxidase (HRP) conjugated rabbit anti-mouse IgG (H+L) was added (Dako P0260, Agilent). Lastly, 50 µl of substrate solution (ABTS [2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)] tablet in 5 ml ABTS buffer and 45 ml ultra-pure water) was added to each well at 37°C. The OD was measured at 405 nm.

The mean ± SD OD of the negative controls was 0.122 ± 0.027 when tested with the anti-dog IgG and 0.082 ± 0.017 when tested with the anti-dolphin IgG. The intra-assay coefficient of variability was 5.7% for anti-dog IgG and 8.2% for anti-dolphin IgG. The inter-assay coefficients of variability were 29.4 and 27.24, respectively. Therefore, to correct for this variability, the serum antibody levels were calculated as

percentage of the positive control, as described by Wilson et al. (2009), using the formula:

$$\frac{(\text{OD sample} - \text{OD negative control})}{\text{OD positive control} - \text{OD negative control}} \times 100$$

2.9. Statistical analyses

Statistical analyses were performed using GraphPad Prism 6. Data were first tested for a normal distribution with the D'Agostino-Pearson normality test. When data did not show a normal distribution, nonparametric tests were used. The Mann-Whitney *U*-test was used to compare whether 2 independent antibody level sets were significantly different. The Wilcoxon signed-rank test was used to compare increases in antibody level as a result of vaccination. A *t*-test was used to compare antibody responses following vaccination with Eurovac Ery® with the responses following vaccination with the ER Bac Plus® vaccine, because there was a normal distribution within both groups.

3. RESULTS

3.1. Clinical response to vaccination

During the time frame of this study, up to 29 vaccinations were administered to one dolphin (CB-3-9). No adverse reactions were reported following any vaccination or booster when using the Eurovac Ery® vaccine between 1993 and 2010. CB-3-12 presented a behavioral reaction (elevated breathing frequency and quitting the trainers' control) on its 9th and 10th vaccinations (the 3rd and 4th vaccination with the ER Bac Plus® vaccine, respectively) and vaccination was subsequently temporarily interrupted in this animal. No erysipelas case was reported in this dolphin population in the 20 yr of research, though the organism had been present on the premises before.

3.2. Graphical representation of the results

Dolphin anti-*Erysipelothrix* antibody levels at pre- and post-vaccination are represented in graphs as percentage of the positive control at the dates of blood sampling for each animal. Furthermore gender, birth date, age at primo-vaccination and vaccination dates are indicated. Only some representative antibody profiles of animals are shown to illustrate the results. As can be seen in Fig. 1, the profile of the antibody response determined with the monoclonal anti-dolphin IgG is quite similar to the one with the anti-dog IgG. This was so in all analysis, indicating that the anti-dog antibodies cross-reacted with dolphin IgG, as previously described (Lacave et al. 1997b). For clarity, only results with either the anti-dog IgG or the monoclonal anti-dolphin IgG are presented in the other figures, though both antisera have been used in parallel. Sometimes dolphins did not show an antibody response upon vaccination. The antibody profile of WB-1-1 (between 1994 and 2011) shows an absence of clear responses following some of the vaccinations, as indicated by the black arrows (Fig. 2). The *Erysipelothrix*-specific response in a dolphin (CB-3-9) that was switched from annual to a semi-annual vaccination in 2005 is shown in Fig. 3. This animal did not always react to its vaccinations (black arrow) and the response to semi-annual vaccination was low. Both vaccines induced dolphin anti-*Erysipelothrix* antibodies.

3.3. Results of statistical analysis

3.3.1. Comparison of antibody levels before primo-vaccination between wild- and captive-born animals. Statistical analysis showed a significant difference between wild- and captive-born animals ($p = 0.0190$), with higher values in wild-born animals. Mean antibody levels were 30.3 and 10.2 and median values were 15.5 and 4.3, respectively.

3.3.2. Comparison of antibody levels between younger and older dolphins before primo-vaccination. Antibody levels of sera before primo-vaccination were significantly higher in dolphins at 3 yr of age and older than in the younger dolphins vaccinated during their first year of life. The difference was highly significant ($p < 0.0001$), with a mean antibody level for younger animals (≤1 yr) of 4.8 and for older animals (≥3 yr) of 28.9; median values were 2 and 15, respectively.

3.3.3. Comparison of antibody levels between wild-born and captive-born older dolphins before primo-vaccination. No significant difference was seen between the antibody levels for wild- and captive-born dolphins of ≥3 yr of age at the moment of their first vaccination ($p = 0.9444$). The mean for wild-born animals was 30.3 and for captive-born animals 27.5, and the median values were 15.5 and 14.6, respectively.

3.3.4. Seroconversion following the number of vaccinations. Antibody responses following vaccination were analyzed comparing paired sera. After the 1st vaccination, there was no significant increase in

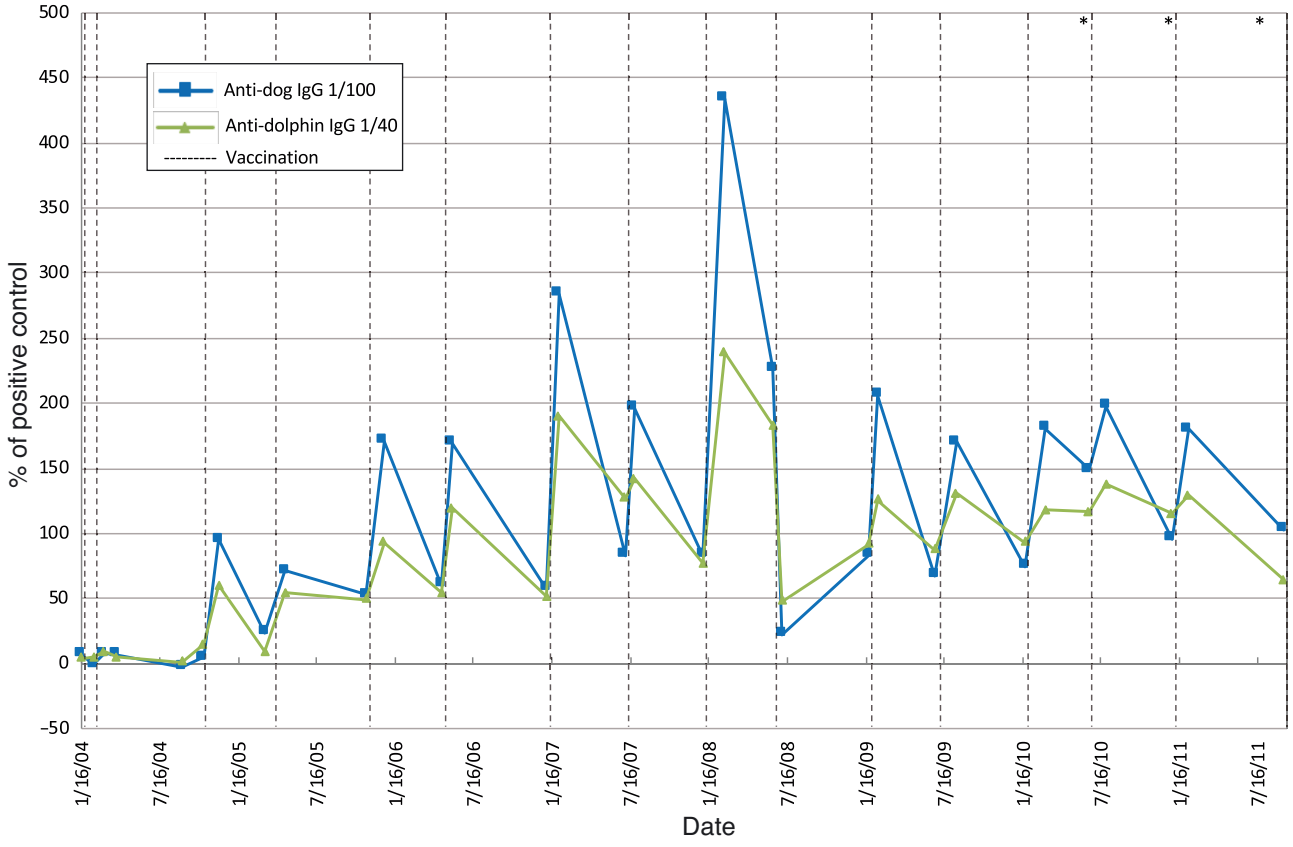

Fig. 1. Kinetics of *Erysipelothrix*-specific antibody levels following repeated vaccinations of captive-born dolphin CB-4-17 (male, born 29 September 2003, 4 mo at primo-vaccination). Time points of vaccination are represented by the dashed lines. The profile of the antibody response with anti-dog IgG (Anti-dog IgG 1/100) is nearly always parallel to the profile of the anti-dolphin IgG (Anti-D IgG 1/40). Asterisks (*) represent the usage of ER Bac Plus® instead of Eurovac Ery® as from June 2010. Dates given as mo/d/yr

mean antibody levels ($p = 0.5879$), but a significant increase was seen after the 2nd vaccination ($p < 0.0024$; Fig. 4). However, just before the 3rd vaccination, antibody levels were not significantly higher than the levels before the 1st vaccination ($p = 0.4623$), and were significantly higher again after the 3rd vaccination ($p < 0.0001$). From this vaccination on, the levels remained significantly higher in subsequent vaccinations. The statistics until vaccination 16 are presented in Fig. 4; however, the number of animals which received >16 vaccinations was insufficient for meaningful statistical analysis.

3.3.5. Comparison between annual and semi-annual vaccinations — all dolphins. Only sera taken after the 3rd and subsequent vaccinations were used to compare annual with semi-annual vaccinations, as the antibody levels were then significantly higher than before vaccination. More specifically, the sera taken just before a vaccination were analyzed: in the case of annual vaccinations, these are the sera taken at the end of a 12-mo period, and in the case of semi-annual vaccinations, the sera taken at the end of a 6-mo period after the previous vaccination. This was seen as representative for that period. As no animals had received an equal number of vaccinations and this could affect the results, the mean antibody levels for annual and/or semi-annual vaccinations were calculated for each of the animals. As such, 12 mean values were obtained for annual vaccinations and 20 mean values for semi-annual vaccinations. Groups were not significantly different ($p = 0.3815$), with a mean antibody level of 67.6 for the annually vaccinated animals and 55.6 for the semi-annually vaccinated, and a median of 64.55 and 45.7, respectively. The graphical representation of mean antibody levels from the annually and semi-annually vaccinated animals can be found in Figs. 5 & 6, respectively.

3.3.6. Comparison between wild-born and captive-born for annual vaccinations. The same calculation was used to compare wild-born (n = 6) and

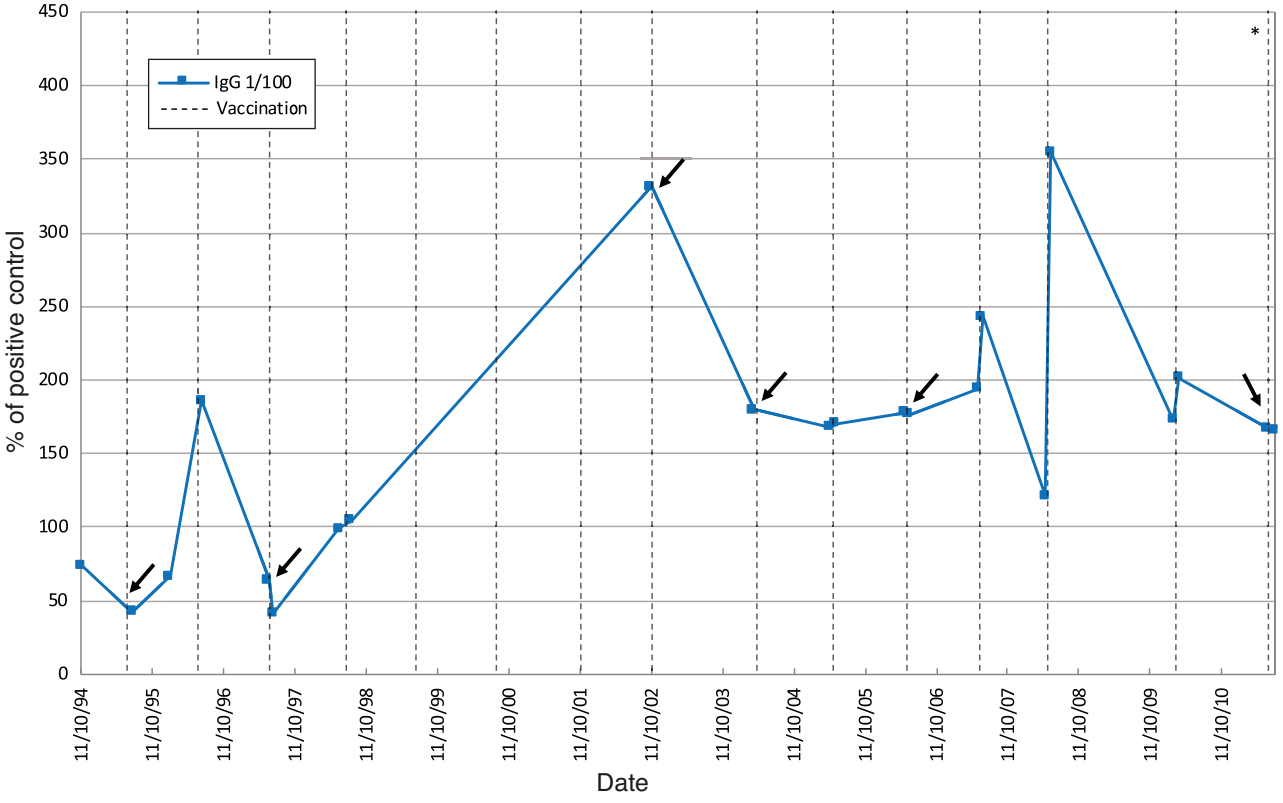

Fig. 2. Sometimes dolphins did not show an antibody response upon vaccination. The antibody profile of wild-born dolphin WB-1-1 (female, born ~1968, ~27 yr at primo-vaccination), represented between 1994 and 2011, shows the absence of clear responses following some of the vaccinations, as indicated by the black arrows. Time points of vaccination are represented by the dashed lines, and the switch from Eurovac Ery® (May 2010) to ER Bac Plus® is indicated by an asterisk (*). Only the response determined with anti-dog IgG antibodies is presented. Dates given as mo/d/yr

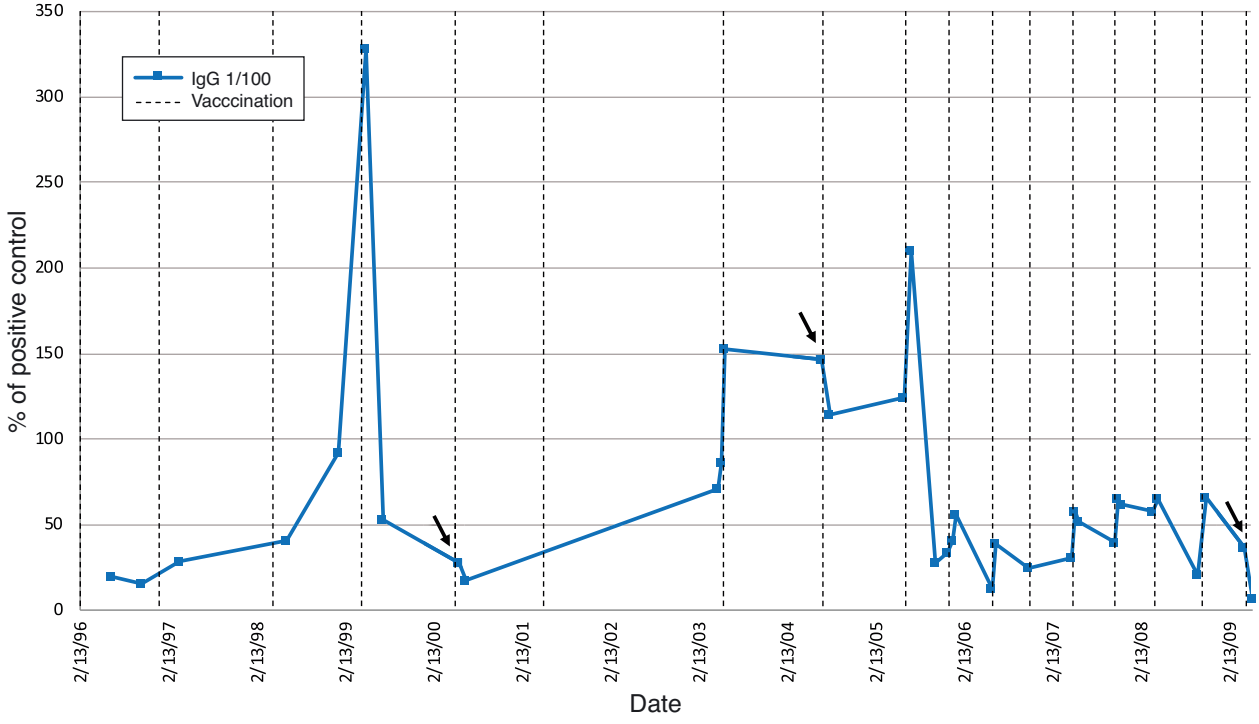

Fig. 3. The *Erysipelothrix*-specific response in captive-born dolphin CB-3-9 (male, born 25 October 1992, 3 yr at primo-vaccination), that was switched from annual to a semi-annual vaccination in 2005. This animal did not always react to its vaccinations (black arrow) and the response on semi-annual vaccination is low. Time points of vaccination are represented by the dashed lines. Only the response determined with anti-dog IgG antibodies is presented. Dates given as mo/d/yr

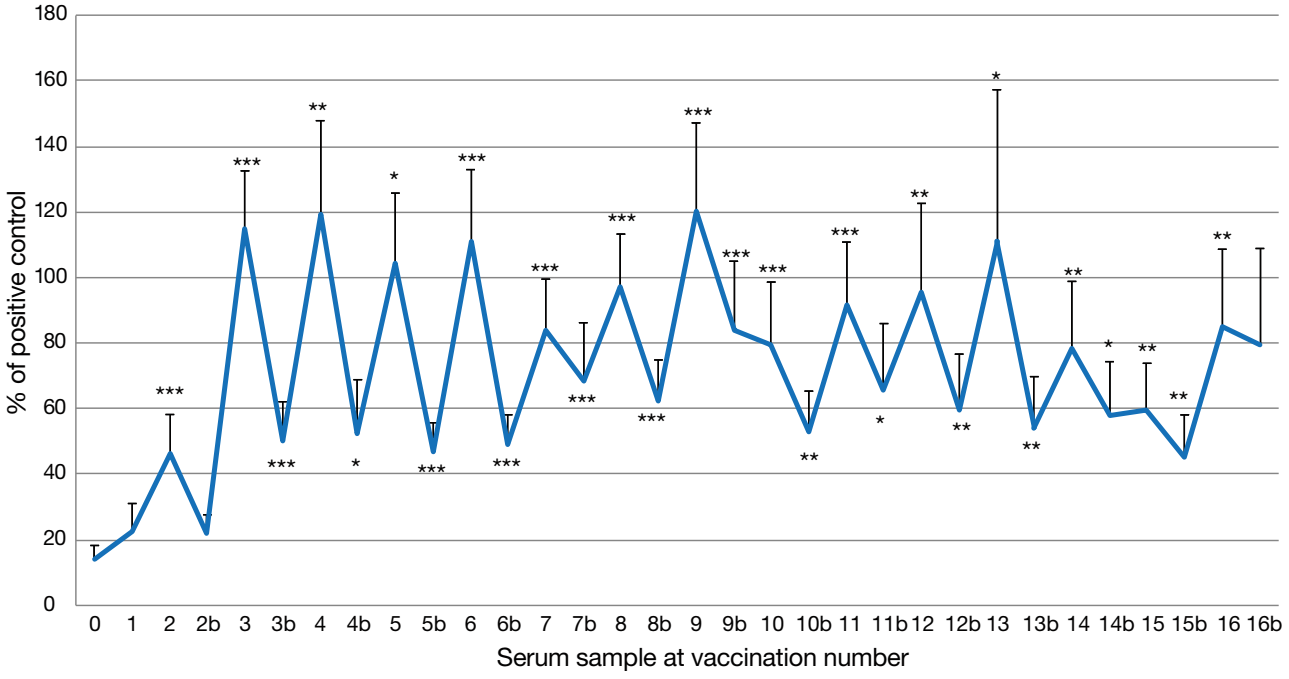

Fig. 4. Kinetics of the mean *Erysipelothrix*-specific antibody levels of all vaccinated dolphins shortly after vaccination (1 to 16) and just before revaccination (2b to 16 b). For annual vaccinations, the latter sample was taken at the end of a 12-mo period, and for semi-annual vaccinations at the end of a 6-mo period. Data are presented as means ± SEM. After the 16th vaccination, too few samples were available to allow statistical analysis. Asterisks indicate a significant difference compared with levels before the first vaccination (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

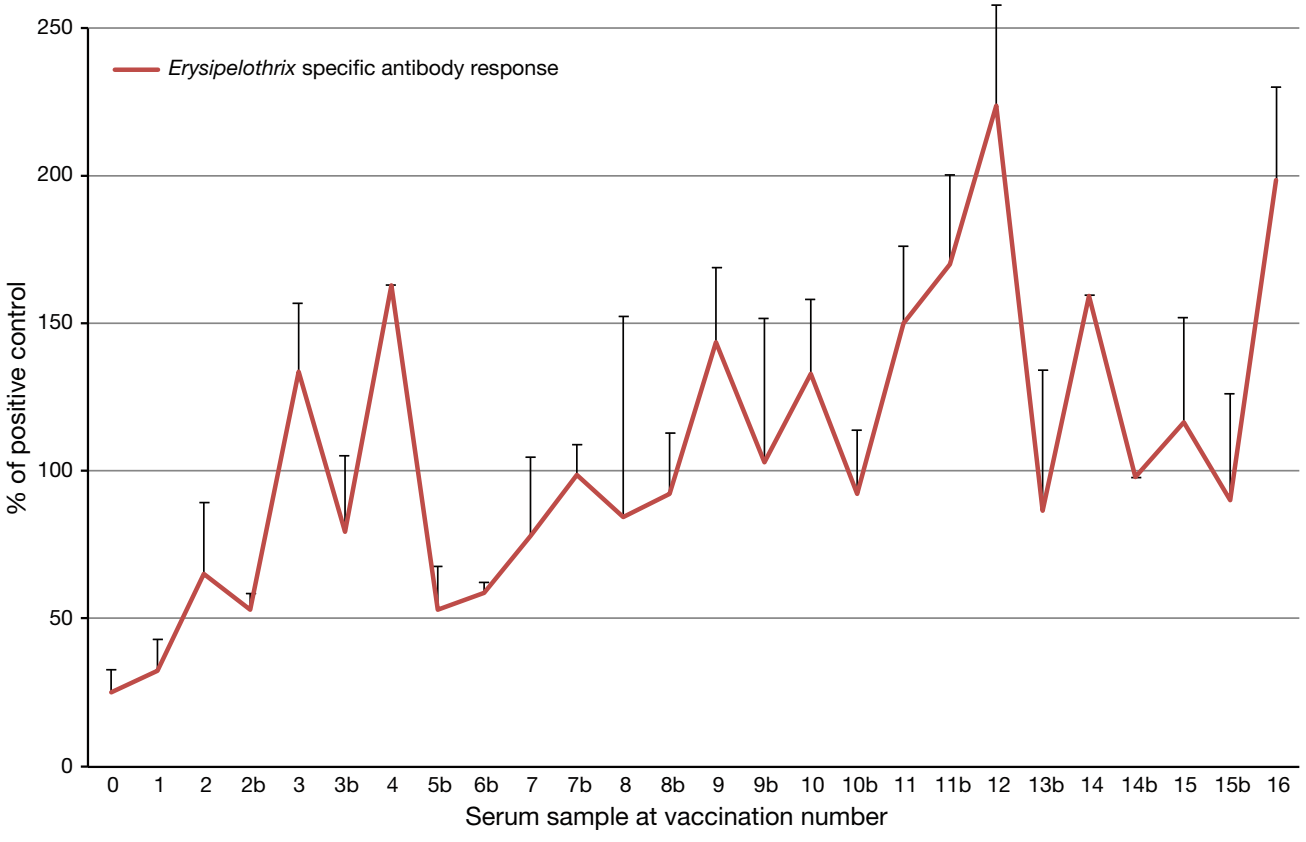

Fig. 5. Mean *Erysipelothrix*-specific antibody levels for all annually vaccinated dolphins shortly before and 2 wk after each vaccination; for vaccination numbers, 'b' represents the samples taken shortly before the next vaccination. Data are presented as mean antibody levels (percentage of positive control), with error bars representing ±SEM

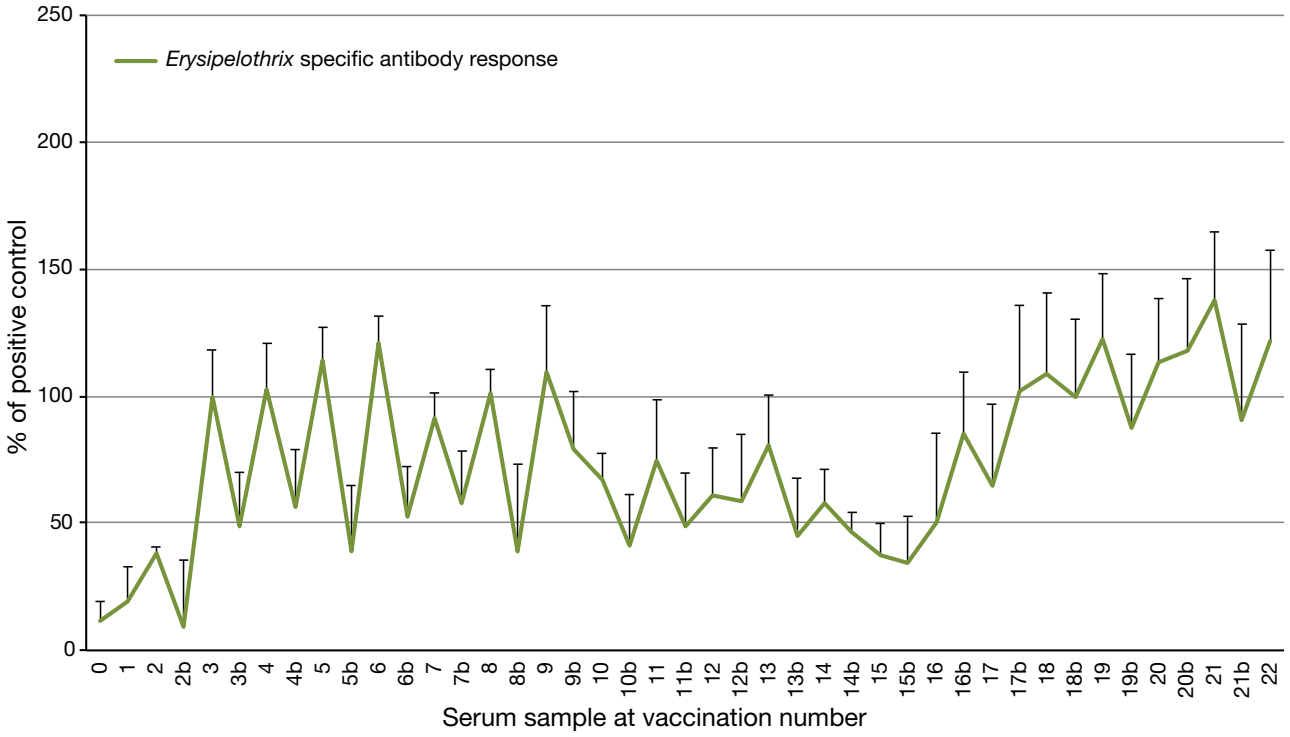

Fig. 6. Mean *Erysipelothrix*-specific antibody levels for all semi-annually vaccinated dolphins shortly before and 2 wk after each vaccination; for vaccination numbers, 'b' represents the samples taken shortly before the next vaccination. Data are presented as mean antibody levels (percentage of positive control), with error bars representing ±SEM

captive-born animals (n = 6) within the group of annually vaccinated animals. No statistically significant difference (p = 0.2381) was seen, with means for wild- and captive-born animals of 85.7 and 49.6 and medians of 69.85 and 44, respectively.

3.3.7. Comparison between wild-born and captive-born for semi-annual vaccinations. For the data calculated in Section 3.3.6, the mean antibody levels of semi-annually vaccinated wild-born animals (n = 3) were compared with those of the semi-annually vaccinated captive-born animals (n = 17). The results showed no statistical difference (p = 0.9096), with mean antibody levels of 52.2 and 56.2, and medians of 46 and 45.4 for wild- and captive-born animals, respectively.

3.3.8. Comparison between wild-born and captive-born post 3rd vaccination. Comparison of the mean responses in all wild-born animals (n = 7) with those of captive-born animals (n = 20) for post 3rd vaccination antibody levels, irrespective of whether they were vaccinated annually or semi-annually, revealed no significant difference (p = 0.1587). The mean antibody levels for the wild- and captive-born animals were 78.0 and 52.7, and median values were 66.8 and 38.2, respectively.

3.3.9. Comparison between older and younger animals post 3rd vaccination. When mean antibody levels post 3rd vaccination in the animals that were ≥3 yr old at the beginning of their vaccination regime (n = 12) were compared with the responses in animals ≤1 yr old at the beginning of their vaccination regime (n = 15), irrespective of whether they were vaccinated annually or semi-annually, the p-value decreased to 0.0729. Although not significant, this shows the tendency of the older animals to manifest an increased response in comparison with the younger animals. This is reflected in the mean antibody levels of 75.2 and 46.5, and median levels of 63.75 and 35.8 in wild- and captive-born animals, respectively.

3.3.10. Comparison of antibody levels post-vaccination between the Eurovac Ery and ER Bac Plus vaccines. For 14 animals, the mean antibody levels per animal 14 d to 2 mo post-vaccination were compared for both vaccines. There was no significant difference between the antibody levels induced by both vaccines (p = 0.4703).

3.4. Older wild-born dolphins after a 2.5 yr vaccination gap

The 3 oldest wild-born dolphins, which were vaccinated annually between 1994 and 2012, underwent a 2.5 yr gap before their last vaccination in this study.

All 3 reacted to the vaccination after a 2.5 yr gap (data not shown), but the statistical significance was not determined.

3.5. Primo-vaccination in Group 4 (younger CB dolphins)

Calves that were primo-vaccinated very early (3–4 mo) did not react as strongly to the primo-vaccination and first booster as calves vaccinated later on (7–8 mo) (Fig. 7), but the statistical significance was not determined.

4. DISCUSSION

The *Erysipelothrix* bacteria are ubiquitous, and it is difficult for disinfectants to fully remove the organism from the environment in the presence of organic material (Fidalgo et al. 2000, Wood 2000). They are commensal on the cutaneous slime of fish and growth occurs between 4 and 44°C (Opriessnig & Wood 2012). Furthermore, freezing does not kill *Erysipelothrix*, but merely results in a 2 log reduction in the number of organisms, leaving enough for infection (Suer & Vedros 1988). Therefore, a multiple approach for protection in delphinids needs to be implemented. High-quality housing, modern husbandry handling or the control of fish quality by stricter surveillance of the cold chain, such as fish transport, freezing storage parameters or handling, have proven very beneficial in the prevention of the disease (Boehm et al. 2000). In addition to the above, active immunity through vaccination is a very important part in the protection against the organism.

The commonly practiced vaccination schedule against *Erysipelothrix rhusiopathiae* in swine, which is the most and best studied animal regarding this organism, consists of vaccinating piglets at the age of 3 mo (priming), followed by a boost 2 to 3 wk later. Afterwards, they need to be re-boosted every 6 mo. In contrast to swine, there were many different vaccination protocols for dolphins in the literature (Geraci et al. 1966, Gilmartin et al. 1971, Ridgway 1972, Gray & Klontz 1974, Colgrove 1975, Sweeney & Ridgway 1975, Medway 1980, Sweeney 1986), until vaccination had ceased in many facilities in the late 1980s after several cases of anaphylactic hypersensitivity caused the death of animals very shortly after immunization or upon re-vaccination (Dunn 1990). When vaccinated currently, dolphins receive a commercial dead whole-agent, and recently, a study showed that the American ER Bac Plus® vaccine produced a rise in anti-*E. rhusiopathiae* antibody levels in vaccinated dolphins (Nollens et al. 2016).

During the present study, the schedule of vaccination of the sub-groups was adapted taking into account the preliminary results obtained during the course of the study (Lacave et al. 1997a, Boehm et al. 2000). The original protocol of vaccination, where adults would be vaccinated once a year and calves

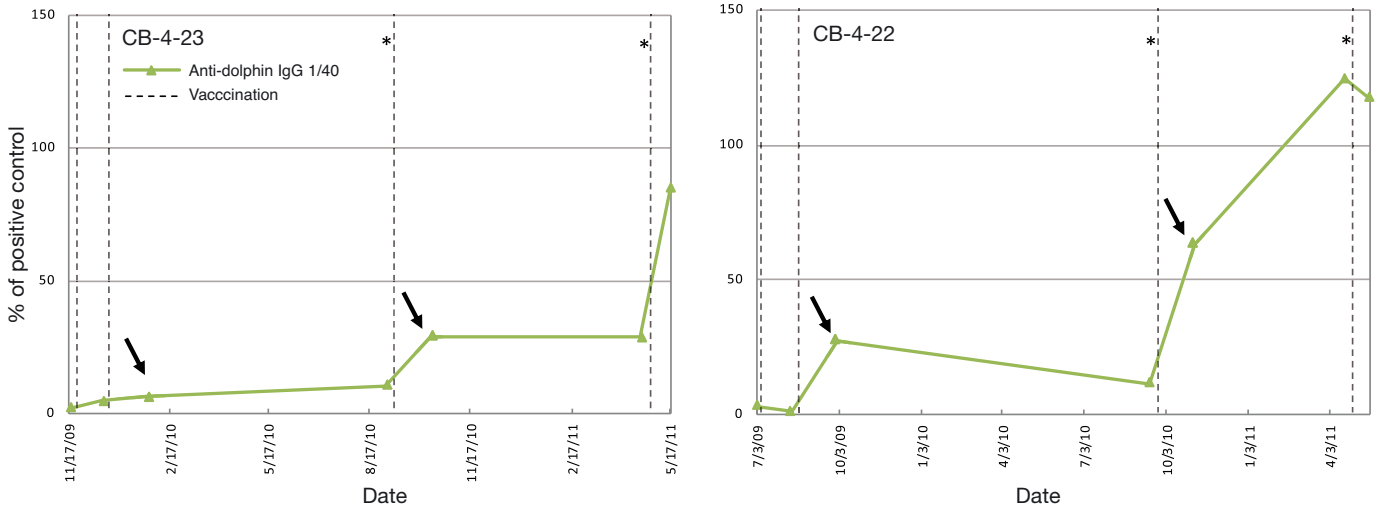

Fig. 7. The *Erysipelothrix*-specific response in captive-born dolphins CB-4-23 (male, born 21 July 2009, 4 mo at primo-vaccination) and CB-4-22 (male, born 10 November 2008, 8 mo at primo-vaccination), which show a different response at primo-vaccination. The calf primo-vaccinated at 4 mo (CB-4-23) did not react as strongly as the calf primo-vaccinated at 8 mo (CB-4-22) during its early vaccinations (arrows). Time points of vaccination are represented by the dashed lines. Asterisks (*) represent the usage of ER Bac Plus® instead of Eurovac Ery® as from June 2010. Dates given as mo/d/yr. Only the response determined with monoclonal anti-dolphin IgG antibodies is represented.

before the age of 6 mo, was based on available publications at the time (Geraci et al. 1966, Gilmartin et al. 1971, Ridgway 1972, Colgrove 1975, Sweeney 1986, Dunn 1990, Van Poucke 1994). Indeed, calves were described to start eating fish between 6 and 12 mo of age, when they would become susceptible to the disease through the ingestion of fish potentially carrying the bacteria. With the identification of fatal cases in calves younger than 6 mo, the primo-vaccination for newborn calves entering the study was set at 3 to 4 mo of age (Lacave 1994, 1999).

The results of a cross-protection study in mice, vaccinated with the commercially available swine Eurovac Ery® vaccine, showed protection against challenge with different *Erysipelothrix* strains isolated from dolphins. The duration of the protection, depending on the challenging isolate, was between 8 and ≥23 wk. However, the prolonged disease in vaccinated mice after challenge suggested that a booster vaccination is required post primo-vaccination for complete protection and booster vaccination at 6 mo for further protection (Lacave et al. 2001). These conclusions had led to the decision to adapt the protocol. New animals entering the study, whether young or adult, would undergo a booster vaccination 1 mo post primo-vaccination. The schedule would also switch from annual to semi-annual vaccination in younger animals already in the program and for any new animal entering the study. These were actually the recommendations from the 2 manufacturers of the vaccination for swine. Older animals were kept under the annual vaccination regimen to enable further comparisons, over a longer time period, of the effect of annual versus semi-annual vaccination on antibody levels.

The ultimate goal of this study was to obtain better insight into the most effective and safest vaccination protocol with the use of a commercially available swine vaccine to protect *Tursiops truncatus* against erysipelas infection, and to determine whether there is a need for a semi-annual vaccination versus an annual vaccination. To assess the efficacy of the protocol, antibody responses were followed using dolphin antigen-specific ELISAs. Cross-reacting anti-dog isotype specific IgGs were used in this study. These antibodies do specifically recognize the heavy chains of dolphin IgG and, in an ELISA, can give a good picture of the antibodies present in the serum of dolphins (Van Poucke 1994, Lacave et al. 1997b). That the profile of the anti-dolphin IgG responses nearly always paralleled the profile of the anti-dog IgG responses indicated that the polyclonal anti-dog IgG can be used as an alternative to the monoclonal anti-dolphin IgG (Fig. 1). The small differences seen could be due to differences in affinity or recognition of different IgG isotypes. The concentration of specific antibodies is presented here as a percentage of the positive control. This allows elimination of variation between different ELISAs. This method provides no information about the affinity of the antibodies for the antigens. This can only be done through titration; however, we opted not to do this in the present study because of the high number of samples analyzed. Nevertheless, measuring OD values allows us to study the kinetics of the antibody response following vaccination and to compare responses between the different animals. It is important to note that, although dolphin antibodies are protective, as demonstrated previously in preliminary mouse challenge studies (Hermans 1997, Lacave et al. 1997a), the OD values cannot be correlated with a protection level. This could only be done through a live-pathogen challenge study, which, due to the economical, operational and emotional value of captive cetaceans, is not realistic in dolphins. However, the efficacy of the vaccination protocol could be deduced as no case has been reported during the course of the study, though the organism had been present in the population previously.

The analysis of the results showed the following:

(1) There is a significantly higher antibody level in wild- than in captive-born animals before primo-vaccination. However, most wild-born animals were older at the time of primo-vaccination, and their immune system would have been completely mature and developed compared to the younger captive-born animals in the program. They might also previously have been exposed to a much wider variety of pathogens in the wild. Differences between captive and wild animals with respect to immune activation, duration and intensity can be related to regular cleaning of captive facilities, implementation of pathogen control programs, reduced energy expenditure in captive animals and a more stable environment in captivity than in the wild (Flies et al. 2016).

(2) The age at primo-vaccination is also an important factor with older animals having sigificantly higher antibody levels than younger ones.

(3) When we then compared antibody levels, before vaccination, between older (≥3 yr) wild-born and older (≥3 yr) captive-born dolphins, we found no significant difference. Their respective mean and median values are similar, and so the significant difference described in point 1 (see above) between wild-born and captive-born animals is indeed due to the difference in age: the wild-born dolphins were older when entering the vaccination program.

(4) The next question was to determine whether vaccination induced a rise in antibody levels and how this evolved with time and number of vaccinations. The results of the *Erysipelothrix*-specific antibody response over time indicate that at least 3 vaccinations are needed to obtain antibody levels above the pre-vaccination level.

(5) Another purpose of the study was to determine whether there was a difference between an annual and semi-annual vaccination regime and if this would affect the previous results. Based on the results obtained in point 4 (see above), only sera taken after the 3rd and subsequent vaccinations were used to compare annual and semi-annual vaccinations. The 2 groups were not significantly different, suggesting that annual vaccination would be sufficient. Interestingly, the mean antibody level was higher for the annual vaccinated animals (67.6) than for the semi-annually vaccinated animals (55.6) (Figs. 5 & 6). This could, however, be due to the fact that most of the wild-born animals, with higher starting antibody levels, belonged to the annually vaccinated group and that the wild-born animals were older animals with a stronger immune response due to previous contact with the pathogen or due to a more mature immune system.

Therefore, we analysed whether being wild born or captive born affected antibody levels in groups of (6) annually or (7) semi-annually vaccinated animals and found no statistical difference for either the annually or semi-annually vaccinated groups.

(8) We then also analyzed whether being wild born, with either annual or semi-annual vaccination, would yield, statistically, different post 3rd vaccination levels and again found no significant difference between both groups. However, as was the case for antibody levels before vaccination, wild-born animals showed higher antibody levels than captive-born animals after vaccination, with previous analyses suggesting that this occurs irrespective of vaccination schedule (i.e. annually or semi-annually).

And finally (9), we showed that there was no statistical difference whether an animal entered the program at a younger age(≤1 yr) or at an older age (≥3 yr) when controlling the antibody levels after the 3rd vaccination, but the p-value reflects the tendency of a higher response in the older animals, very likely related to longer exposure to the environment and a more mature and developed immune system.

These results differ from the preliminary results obtained in a cross-protection study in mice (Lacave et al. 2001), where the duration of protection, depending on the challenging isolate, reached a maximum at ≥23 wk, suggesting that a booster vaccination was required post primo-vaccination for complete protection and again at 6 mo for further protection. However, as in most cross-protection studies, only short-term protection was evaluated. The mice had not received 3 vaccinations, which has been shown during the present study, at least in *T. truncatus*, to be the minimum number of vaccinations necessary to obtain antibody levels above pre primo-vaccination levels, and the conclusion may not be applicable for long-term protection. Also, the general protocol for swine vaccination, after the original booster 2–3 wk post priming, is re-vaccination every 6 mo. Though pigs can live up to 20 yr, most vaccination studies have been done on livestock, where fattening pigs are slaughtered for meat at 6–7 mo of age and sows are kept for <3 yr (https://vetmed.iastate.edu/vdpam/FSVD/swine/index-diseases/erysipelas), a time span very different than the median life span of 34.3 yr of dolphins kept under human care (L. Willis unpubl. data).

The graphical representations of the antibody level showed that older wild-born dolphins, vaccinated annually, did not always react to the vaccine, sometimes showing no rise in their antibodies or even a drop after vaccination (Fig. 2). The same was seen for older dolphins born in captivity and vaccinated semi-annually (Fig. 3). It could be that residual antibodies, either from a former vaccination or from a former exposure to subclinical infection, were still present and thus 'neutralized' the effect of the vaccine upon re-vaccination. Nollens et al. (2016) reported that vaccine-induced antibodies were much longer-lived than antibodies generated from a natural *E. rhusiopathiae* infection. In contrast, younger dolphins born in captivity seem to react to every vaccination (Fig. 1). This could suggest that their immune system is not as well trained or developed as that of the older animals, so that they present a shorter immunity post-vaccination. In the last 2 decades, dolphins may have been overprotected in captive environments. They live in pristine, nearly sterile water, which may affect their immune system, which would no longer be naturally challenged by the environment (Van Bonn et al. 2008), and are treated the moment they present the slightest clinical sign of disease or change in blood parameters. However, in all cases in the present study, the antibody levels were always significantly higher post 3rd vaccination than the levels before primo-vaccination, suggesting that an annual vaccination is sufficient.

Older wild-born dolphins that did not react upon vaccination did react, however, in one of the consec-

utive ones. It was hypothesized that these animals very probably needed to be re-vaccinated, but that the interval between vaccinations could be increased, potentially by even several years. Therefore, in the last part of the study, a preliminary test with a 2.5 yr vaccination gap for the 3 older wild-born dolphins (group 1) was performed, though without statistical analysis, the sample size being very small (n = 3). They all reacted to the next vaccination, but their antibody levels were still always significantly higher than the levels before primo-vaccination (data not shown). Though the interval between vaccinations could probably still be increased for Group 1 animals, further results and more years of sampling are necessary to confirm this, and the vaccination schedule recommendation remains an annual booster for the moment.

We demonstrated previously that dolphin calves should preferably be vaccinated between the ages of 3 and 7 mo as they start to be offered fish around that age, which makes them much more susceptible to oral infection (Lacave 1994, 1999, Lacave et al. 2005). Several animals in the study had a primo-vaccination comprising a prime vaccination and a 1-mo booster, but not all these animals reacted equally. The calves receiving their primo-vaccination at 3.5 to 4 mo of age showed a weak reaction in comparison with calves that were primo-vaccinated at a later age (7 and 8 mo old; Fig. 7). Although maternal immunity could decrease the immune response upon vaccination, the young animals did not show higher antibody levels, so it is more likely that their immune system was still quite immature at the time of the first vaccination. Indeed, in the course of this study, 170 serum samples from 19 calves, between 1 d and 18 mo of age, have been analyzed for the evolution of their serum gammaglobulin concentration and their levels were much lower than in adult animals (Lacave & Cox 2000, Lacave et al. 2005, G. Lacave et al. unpubl. data). The disappearance of maternal immunity occurs around 1 mo of age and this supports the observation that OD values from ELISA were low in the young animals vaccinated in the present study. More blood samples during the first weeks of life have to be collected for a better analysis of transfer of passive immunity; however, in the absence of a medical problem, handling of dolphin calves at such a young age is not a common practice in most facilities, and the information is still very limited. The present study offers a limited, though substantial, view of dolphin calves' immune responses, and further research is still necessary. Though younger calves react less to vaccination compared to older calves at primo-vaccination, they are still exposed to *Erysipelothrix* infection, as has been indicated previously, and as such, it is advised to start vaccination early on.

In regard to both commercial swine vaccines used in this study, the statistical results (*t*-test, p = 0.4703) showed that the switch from the European Eurovac Ery® to the American ER Bac Plus® in May 2010 did not have a significant effect on the induced antibody response in dolphins, and indicated that they are both effective in *T. truncatus*.

Though vaccine allergy is generally rare (Bohlke et al. 2003), the risk of an anaphylactic reaction in dolphins was a critical concern in the vaccination protocol of the present study, mainly at booster time, as a potentially allergic animal would have become sensitized to the allergen the first time and would be at higher risk in subsequent vaccinations. In humans, these allergic sensitivities are generally believed to be related to some particular adjuvant, stabilizer or preservative in the vaccine (Grabenstein 1997, Leventhal et al. 2012, Petrovsky 2015). In the past, deaths of dolphins following such a reaction post-vaccination, albeit with inactivated dead vaccine and though never with a European vaccine, had prompted many marine mammal facilities to stop all vaccination of their dolphin populations. However, identification of several fatal erysipelas cases among the different dolphin collections supervised at the time by G. Lacave convinced her to resume vaccination, using a commercially available dead vaccine (Boehm et al. 2000). As security, and based on the guidelines on the management of anaphylaxis from the Resuscitation Council UK (2008), all emergency medications recommended in the case of an anaphylactic reaction (adrenaline, anti-histaminic, corticoids, atropine and doxapram) were prepared and available 'syringe-ready' on-site together with endotracheal tubes. The animals were vaccinated in a pool with a lifting floor, so as to enable staff to intervene rapidly in case of shock. A pre-medication with an anti-histaminic was also administered in the hours before vaccination, as recommended for people with vaccine allergies (Grabenstein 1997). Originally, dexchlorfeniramine maleate in coated tablets (Polaramine® Repetabs) was used. It is a first-generation histamine H1-receptor antagonist in blood vessels, respiratory tract and gastrointestinal tract that has a high antihistaminic activity and moderate anticholinergic activity that is often used in human medicine during allergic reactions (Huang et al. 1982). The coated tablets allowed for slow-release timing and longer protection. When the product was no longer available in oral form, the anti-histaminic

was changed to fexofenadine (Mylan), a second-generation peripheral H1-blocker, also recommended in human medicine for the prevention and reduction of allergic reaction (Smith & Gums 2009). The human recommended dosages were used in both cases. However, no studies were done during the course of this erysipelas study to analyze the presence of histamine receptors in dolphins or the specific effect of these 2 anti-histaminics in this species, but no ill effects were observed over the course of the study.

During this study, only one animal (CB-3-12) seemed to have reacted to vaccination. When given a placebo instead of its 11th vaccination, it showed no reaction, and vaccination was suspended for the following 2 years. In contrast, Nollens et al. (2016) reported some reactions in the first hours following administration of the 4th (n = 1), the 7th (n = 1), the 8th (n = 2) and the 11th (n = 1) vaccination with Er Bac Plus®. The reactions consisted of transient lethargy in all 5 animals, additional nausea in 3, with steroids and antihistamines administered in one case upon reaction on the 8th vaccination (H. Nollens pers. comm.). All animals stabilized and ate a couple of hours later. They were not pre-medicated. The ER Bac Plus® vaccine differs in strains and adjuvant compared with Eurovac Ery®. Eurovac Ery® contains aluminum hydroxide as the adjuvant, and is produced as a water-based suspension, whereas ER Bac Plus® uses a widely used commercial veterinary adjuvant, Amphigen®, a lecithin and mineral oil blend-based adjuvant, produced in an oil-in-water emulsion form (Wegmann et al. 2015). The relatively quick release of antigen from oil-in-water emulsion adjuvant vaccines might lead to a short duration of immune stimulation as such, a strong oil adjuvant is needed to achieve long-term protection.

Both humoral and cellular responses of the immune system seem to be associated with protection against *Erysipelothrix* infection (Timoney et al. 1988), the humoral response having been, in general, the most studied (Sawada et al. 1987, Galán & Timoney 1990, Shimoji et al. 1996, Shimoji et al. 1999, Imada et al. 1999, Shimoji 2000, Imada et al. 2003) compared to cellular immunity (Sitt et al. 2010). Furthermore, it was reported that in the absence of specific antibodies, the organism evades phagocytosis by phagocytic cells (Shimoji 2000). Nevertheless, the role of cellular immunity in the defense mechanism is less clear. Shimoji et al. (1998) demonstrated that acapsular *E. rhusiopathiae* strains, although unable to persist *in vivo*, elicited a complete long-lasting protection in mice. However, the bacterial antigen(s) involved in inducing this cell-mediated immunity remain(s) unidentified (Shimoji et al. 1998). It is not known whether protection can be obtained without immunoglobulins, whereas the opposite is true, as shown in swine with the use of hyperimmune serum for treatment and the protective effect of colostral immunity (Shimoji 2000, Opriessnig & Wood 2012). Furthermore, most swine vaccines use inactivated bacteria with an aluminum hydroxide-based adjuvant. This group of adjuvants only poorly induces classical cell-mediated immunity, as measured by delayed-type hypersensitivity (DTH) responses and CD8+ CTL responses. However, proliferative responses of CD4+ T cells as well as Th2 cytokine production have been found to be enhanced in murine and human studies, suggesting that alum boosts humoral immunity via help from Th2 cells. This type of vaccine is highly effective in preventing disease, but frequent re-vaccinations are needed as well (Lindblad 2004, Herbach 2005). As the choice of vaccine is of the utmost importance, both for its efficacy and safety, further research on the use of the different vaccines in dolphins and whether to continue with ER Bac Plus® is in progress (G. Lacave unpubl. data).

4.1. Conclusions

In the wild, dolphins are exposed to a much wider variety of pathogens and have very likely undergone more subclinical infections than their captive counterparts. Their immune system is more stimulated and better developed than the system of overprotected animals in human care, where the different environmental parameters are highly controlled, the food is prepared and stored with very strong protocols, and the animals are often immediately preventively treated with antibiotics when their blood values or clinical presentation is somewhat unsatisfactory. However, wild-born animals are becoming a minority in human care, the majority of them in Europe being in the eldest age group. It can also be expected that with age, the basal immunity level will rise and the immune response upon vaccination will become stronger. It was shown, through this 20-yr erysipelas vaccination study in a controlled dolphin population, that at least 3 vaccinations are necessary to obtain antibody levels maintained above the level present before primo-vaccination. The recommended protocol is a primo-vaccination followed by a 1st booster 1 mo later and the 3rd vaccination at 6 mo (due to the short life-span of the induced antibodies), followed by annual boosters. The present study showed that a primo-vaccination at a very young age (4–5 mo) often

triggers a lower antibody reaction than at a later age (7–8 mo). However, vaccination at a young age is still recommended, as very young calves, during the time frame of this study, have died from erysipelas in facilities that do not vaccinate. Preliminary ELISA results suggest that older animals born in the wild do not need annual vaccinations and may have enough protection with a vaccination every 2.5 yr. Both the European Eurovac Ery® and the American ER Bac Plus® vaccines induced antibody responses in *T. truncatus*. No erysipelas case was reported in this dolphin population in the 20 yr of the study, though the organism had been present on the premises before, suggesting the efficacy of the vaccination protocol.

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