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Evaluation of anti- *Erysipelothrix rhusiopathiae* IgG response in bottlenose dolphins (*Tursiops truncatus*) to a commercial pig vaccine

Running page head: **Bottlenose dolphin erysipelas vaccine**

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ABSTRACT: *Erysipelothrix rhusiopathiae* is the causative agent of erysipeloid in humans and of erysipelas in various animals, including bottlenose dolphins (*Tursiops truncatus*) in which an infection has the potential to cause peracute septicemia and death. The purpose of this study was to evaluate the efficacy of using an off label porcine (ER BAC PLUS®, Zoetis Inc.) *Erysipelothrix rhusiopathiae* bactrin in a bottlenose dolphin vaccination program by determining the anti-*E. rhusiopathiae* antibody levels in vaccinated dolphins over a 10 year period. Serum samples (n = 88) were analyzed using a modified fluorescent microbead immunoassay from 54 dolphins, including three with no history of vaccination, 51 dolphins with an average of five vaccinations, three of which had previously recovered from a natural *E.rhusiopathiae* infection. A mean 311-fold increase in IgG antibody index was measured in a subsample of ten dolphins 14 d after the first booster vaccination. Serum IgG antibodies titers were influenced by number of vaccines received ($r^2 = 0.47$, $p < 0.05$), but not by age, gender, history of natural infection, adverse vaccine reaction, vaccination interval or time since last vaccination. The commercial pig bacterin was deemed effective in generating humoral immunity against *E.rhusiopathiae* in dolphins. However, since the probability of an adverse reaction toward the vaccine was moderately correlated ($p = 0.07$, $r^2 = 0.1$) with number of vaccines administered, more research is needed to determine the optimal vaccination interval.

KEY WORDS: Dolphin, *Tursiops truncatus*, Erysipelas, *Erysipelothrix rhusiopathiae*, Vaccine, Prophylaxis

INTRODUCTION

The bacterial genus *Erysipelothrix* consists of three species, the type species *E. rhusiopathiae*, *E. tonsillarum* and *E. inopinata* (Walker 2004). *Erysipelothrix rhusiopathiae* can be isolated from the environment and from a variety of animal tissues. Infections with *E. rhusiopathiae* are common in pigs and turkeys and have also been reported in sheep, emus, chickens, penguins and other species (Swan & Lindsey 1998, Boerner et al. 2004, Walker 2004, Eamens et al. 2006, Kurian et al. 2012). The clinical manifestation of *E. rhusiopathiae* infection is commonly referred to as erysipelas in domestic animals and as erysipeloid in humans. In pigs, there are three main clinical forms (Brooke & Riley 1999, Walker 2004). The acute septicemic form is usually fatal when left untreated. Clinical signs can include any combination of sudden death, fever, lethargy, depression, stiff gait, reluctance to move, inappetence and characteristic pink, red or purple raised firm rhomboid skin lesions sometimes also called “diamond skin lesions”. This second subacute form is also associated with bacteremia but is clinically less severe than the acute form with lower mortality rates and quicker recovery of affected pigs. The third chronic form in pigs is often a consequence of acute, subacute or even subclinical *E. rhusiopathiae* infection with localized lesions in the heart (endocarditis) or joints (arthritis) (Brooke & Riley 1999, Walker 2004).

Disease caused by *Erysipelothrix* has been recognized and confirmed in several species of dolphins and whales, both in human care and in the wild (Young et al. 1997, Dunn et al. 2001, Melero et al. 2016). Two presentations of erysipelas have been reported in dolphins. A cutaneous form, characterized by raised rhomboidal or diamond shaped skin lesions, and a septicemic form (Dunn et al. 2001). While the septicemic form can be treated successfully by the prompt

administration of appropriate antibiotics, this condition often leads to death, since it is usually only preceded by very brief (hours) non-specific clinical signs such as decreased activity levels and appetite. The bacteremia is consequently often only recognized on necropsy (Dunn et al. 2001). *Erysipelothrix rhusiopathiae* causes no known disease in fish but can survive for long periods of time on the mucoid exterior slime coat of fish (Wood 1975). Human erysipeloid is frequently contracted following infection of superficial injuries sustained during swimming, fishing or handling seafood (Finkelstein & Oren 2011). The exact port of entry of the bacteria is unknown, but dolphins, like humans are presumed to contract *E. rhusiopathiae* from the slime coat of their food fish. Superficial cutaneous injuries could make this exposure route more likely. In swine and poultry, the prevention of erysipelas has largely relied on vaccination using attenuated live or inactivated bacteria or more recently recombinant antigens (Swan & Lindsey 1998, Eamens et al. 2006, Kurian et al. 2012). In these species, challenge studies have shown that vaccination conveys effective protection against all clinical manifestations, including death (Swan & Lindsey 1998, Imada et al. 2003, Eamens et al. 2006). Because of the bacteria's potential to cause death without obvious premonitory signs in dolphins, prevention of *Erysipelothrix rhusiopathiae* infection by vaccination has been of interest to marine mammal health professionals (Nollens et al. 2005, Walsh et al. 2005). Since no bottlenose dolphin-specific vaccine is available, the use of commercial swine erysipelas vaccines has been explored (Lacave et al. 2001, Nollens et al. 2005). Initial vaccination programs in cetaceans with commercial bacterins were abandoned because of adverse reactions consisting of both site reactions and anaphylaxis associated with the immunizations (Dunn et al. 2001). More recently, a commercial inactivated swine *Erysipelothrix* vaccine (Eurovac Ery, Eurovet) developed in Europe was found to provide safe and effective crossprotection in a mice experimentally infected

with E. *rhusiopathiae* isolates from dolphins (Lacave et al. 2001), however, the production of this vaccine has since been discontinued. Efforts to develop a DNA-based vaccine encoding the immunogenic 65 kDa *E. rhusiopathiae* surface protein proved ineffective and have been abandoned (Dunn et al., 2001). Earlier work has demonstrated that the recombinant p64 surface protein of *E. rhusiopathiae* that is employed in a commercial erysipelas vaccine for swine (ER BAC PLUS®, Zoetis Inc) is immunogenic to bottlenose dolphins (Nollens et al. 2007, Bernal-Guadarrama et al. 2014). Since 2003, bottlenose dolphins housed at the various SeaWorld parks have received this vaccine as part of the routine preventative medicine program (Walsh et al. 2005). The purpose of this study was to evaluate the effectiveness of the vaccination program by quantifying the IgG antibody levels developed in response to vaccination, and exploring biological factors influencing antibody levels in dolphins post vaccination.

MATERIALS AND METHODS

Animals

Fifty-four bottlenose dolphins (*Tursiops truncatus*) (22 male and 32 female) were group housed in habitats at either SeaWorld Florida or SeaWorld California. Animals were fed a diet of frozen-thawed whole fish, which contained some or all of the following fish species: Pacific herring (*Clupea harengus*), Columbia river smelt (*Thaleichthys pacificus*), Pacific sardines (*Sardinops sagax*), Atka mackerel (*Pleurogrammus azonus*), and squid (*Loligo sp*.) at approximately 3% of their body weight per day. All food fish was graded for human consumption. Animals were supplemented with Vita-Zu Marine Mammal tablets (Mazuri) which contain vitamins and folic acid.

Immunizations

A total of 298 immunizations were delivered to 51 bottlenose dolphins (2 to 11 for each dolphin) between 10 March 2003 and 19 February 2013 following the manufacturer's directions for pigs. Each dolphin received 2 ml of a commercial *Erysipelothrix rhusiopathiae* bacterin (ER BAC PLUS®) in the dorsal musculature lateral and cranial of the dorsal fin. All 51 dolphins received a primer vaccination, followed by a first booster vaccination 29 (± 18) days after the initial immunization, followed by either semi-annual (n = 10 dolphins) or annual (n = 41 dolphins) booster vaccinations. After each immunization, all animals were monitored for adverse reactions (listlessness, nausea or vomiting) for 60 min. Three dolphins were never immunized and were included as negative controls.

Sample collection, processing and storage

Fasting blood samples (n = 88) were collected between 29 October 1992 and 19 February 2013 from the dolphins at the discretion of the attending veterinarian either as part of the routine preventative medicine program or as part of the clinical management of a natural *E. rhusiopathiae* infection. For venipuncture, the dolphins were trained to present their fluke to the attending veterinarian for sampling using 3- 4" 21 gauge Surflo winged infusion sets (Terumo Medical Corporation). Blood was collected into BD Vacutainers (Becton Dickinson) containing activated thrombin for analysis in the on-site diagnostic laboratories. The thrombin-coagulated blood was centrifuged at 1,500 rpm for 10 min, and the serum was decanted and frozen at -80 °C for further testing.

Seroconversion following primovaccination

An initial blood sample was collected from a subsample of ten dolphins immediately before the first immunization with the vaccine (ER BAC PLUS®). The first booster immunizations were

administered 21 days later. Post-vaccination blood samples were collected 14 (± 1) days following the first booster from all ten dolphins.

IgG response after natural infection

Natural *E. rhusiopathiae* infections were confirmed between 15 March 1993 and 30 September 2002 in three dolphins by culturing *E. rhusiopathiae* from a blood sample (n = 2) or by observation of the pathognomonic diamond skin lesions with concurrent highly inflammatory blood profile (n = 1). For blood culture, 1.5 ml whole blood was added to a 1.5 ml Wampole Isolator tube (Alere Inc) pool-side after disinfecting the stopper with 10% Povidone-iodine. Upon arrival in the lab, the isolator tube was vortexed for at least 10 sec, and 0.3 ml of the content was withdrawn and inoculated onto a chocolate agar plate. The agar plates were incubated at 37°C until colonies appeared. Bacterial colonies were subsequently selected and identified using a ViTek automated bacterial identification system (BioMerieux Inc). From each dolphin, serum samples collected prior to the infection (n = 3), on the day of bacteremia or the first day clinical signs were observed (n = 3), and at varying intervals in the convalescent period (n = 7).

Biological variables influencing anti-*Erysipelothrix* titers

A single serum sample was collected from each of 49 immunized dolphins after an average of five immunizations (median = 6, min = 2, max = 11). In addition, a single serum sample was included from each of the three dolphins that were never immunized. For each dolphin, the gender (female = 0, male = 1), age (days), number of immunizations, mean vaccination interval (defined as the sum of the number of days between subsequent immunization divided by the

number of immunizations received), history of natural infection (no = 0, yes = 1), history of adverse vaccine reaction (no = 0, yes = 1) and time (days) since last immunization was recorded.

Serology

A Fluorescent microbead-based immunoassay (FMIA) developed for pigs was modified for use in dolphins as described in Melero et al (2016). The immunogenic recombinant fragment of 415 amino acids corresponded to the N-terminal half domain of SpaA protein called rSpaA415 was used as antigen for the FMIA (Giménez-Lirola et al. 2012a). Conjugation of the antigen to the magnetic beads was performed as previously described (Giménez-Lirola et al. 2012b). The assay was performed at room temperature using flat bottom FMIA plates (Bio-Plex Pro™ Bio-Rad). Coupled beads were mixed under constant vortexing at 500 rpm and diluted in storage buffer (0.1 M PBS, 10% goat serum (Gibco®, Life Technologies), 0.05% Tween 20, pH 7.2) to a final concentration of 2,500 beads/well (50 beads/µl). All serum samples were diluted 1:50 in assay buffer (0.1 M PBS, 10% goat serum (Gibco®, Life Technologies), 0.05% Tween 20, pH 7.2). Then, 50 µl of the bead suspension and 50 µl of the diluted sample were added to each well. Plates were incubated on a shaker for 60 min at 500 rpm and washed three times with PBS containing 0.05% Tween 20 (PBST). Next, 50 µl of a 1:300 dilution of biotin-conjugated anti-bottlenose dolphin IgG (Nollens et al. 2007) in assay buffer was added to each well and the plate was incubated on a shaker for 30 min. After three washing steps, 50 µl of a 1:100 dilution of streptavidin R-phycoerythrin conjugate (Moss) in assay buffer was added to each well. Finally, after 30 min of incubation on a shaker and three additional washing steps, the beads were resuspended in 100 µl of assay buffer and were analyzed using a flow cytometer (Luminex-200, Luminex Corp) at default settings set by the manufacturer for routine applications. Events were gated to exclude doublets and other aggregates. Median fluorescence intensity of the reporter

signal estimated from at least 50 beads was used for the data analysis. A well incubated with serum diluent served as a control for nonspecific serum reactivity. The Median fluorescence intensity data was corrected for background levels by subtracting the negative antigen signal from the positive antigen signal. All the samples were analyzed in duplicate in two separate independent runs by using the plate reader software (Bio-Plex ManagerTM version 6.0, Bio-Rad). Inconclusive samples were re-tested. Results were reported as a ratio of the Median fluorescence intensity of each sample to the Median fluorescence intensity of a randomly selected reference sample.

Statistical analysis

Data for the analysis were obtained from 49 immunized bottlenose dolphins, and three negative control dolphins without history of disease or vaccination. For the combined data set, a correlation between each independent variable (gender, age, number of immunizations, history of natural infection, history of adverse reaction, days since last immunization and mean number of days between immunizations) on the antibody index was determined using a linear regression to look for significance and predictability (r^2). Any variable that had a significance of $p < 0.1$ and $r^2 > 0.05$ was considered for inclusion into a regression model. The independent variables matching the criteria for inclusion were then analyzed using a multiple linear regression to determine the significance of each variable's contribution. Final variable inclusion or exclusion within the model was determined by a backward stepwise regression using the likelihood-ratio test between models with and without variables in question. Assumptions (normality and homoscedasticity of residuals) of the regression model were visually assessed with quantile normal plots of residuals and the Cook-Weisberg test. To determine the predicted probabilities for an animal having an adverse reaction as the number of vaccines increased were determined

by logistic regression of dependent variable adverse reactions (0 = no, 1 = yes) by the number of vaccines. If the model was significant ($p < 0.1$), then the predicted probabilities of experiencing a reaction were determined by using the "margins" command (Stata, 14, StataCorp). All statistical analysis were performed with a commercial software (Stata, 14, StataCorp) and values of $p < 0.05$ were considered significant.

RESULTS

Seroconversion following primovaccination

An increase in antibody levels to the bacterin (ER BAC PLUS®) was detected in all ten dolphins (Fig. 1). The mean antibody index of the initial blood samples of the ten dolphins was 0.5 (± 0.8). The mean antibody index of post-vaccination blood samples was 17.3 (± 3.1). On average a 311-fold rise in antibody index (SD = 301, median = 313, min = 7, max = 859) was detected. The mean antibody index of the three unvaccinated negative control dolphins was 0.05 (± 0.05).

Seroconversion following natural infection

An antibody response following natural *E. rhusiopathiae* infection was detected in all three dolphins (Fig. 2). The mean antibody index of the initial blood samples of the three dolphins was 0.09 (± 0.08) and the mean antibody index of blood samples collected at the time of bacteremia (n = 2) or when skin lesions were first noted (n = 1) was 0.02 (± 0.03). A peak antibody index level of 20.91 was detected in one of these dolphins 45 days post bacteremia. By day 167 following bacteremia the antibody index of this dolphin had decreased to 1.76. The

highest measured antibody index in the other two dolphins were 3.38 (day 62) and 1.17 (day 75), however, no prior collected sample was available from either animal.

Adverse reactions

Adverse reactions were identified in five dolphins following administration of vaccination four (n = 1), seven (n = 1), eight (n = 2) and 11 (n = 1). The adverse reactions consisted of transient lethargy in all five dolphins with additional nausea in three dolphins without deleterious effects beyond the first hour following immunization. Animals in which an adverse reaction was recognized were not immunized in subsequent years.

Biological variables influencing anti-*Erysipelothrix* titers

The surveyed population consisted of 22 male and 30 female bottlenose dolphins with a mean age of 4,786 (± 3,844) and 6,253 (± 3,073) days respectively. The immunized dolphins (n = 49) had received on average five immunizations (median = 6, min = 2, max = 11). Of the vaccinated dolphins, three dolphins had previously survived a natural infection, and an adverse vaccine reaction had been identified in five dolphins. The shortest vaccination interval of 35 days was implemented in a one-year-old young dolphin that had only received the primer and one booster. The mean vaccination interval for the other dolphins (n = 48) ranged between 123 and 759 days (mean = 341 ± 157 days). The dolphins had not been immunized between 23 and 2,920 days (mean = 464 ± 570 days, median = 353 days) at the time of sampling.

Only adverse reaction (AR: $F_{1,48} = 3.26$, $p = 0.08$, $r^2 = 0.05$) and number of vaccinations (Vaccine number, VN: $F_{1,48} = 32.01$, $p < 0.001$, $r^2 = 0.41$) were considered for inclusion in a regression model (Table 1). A regression model that included VN and AR (AR contribution: t =

243 1.06, p = 0.29; Model r^2 = 0.43) or VN, AR and AR*VN (t = -0.85, p = 0.4) was not improved
244 over a regression model with just VN (χ^2 = 0.94, p = 0.33, Table 1). Therefore, only VN was
245 used to predict index as follows: Index = 5.58 + 1.446*VN (Table 1). However, the model did
246 not appear to adequately describe the initial (< 3 vaccines) and late (greater than 7 vaccines) X,Y
247 relationship or slope. Therefore, a negative exponential regression equation was evaluated and
248 determined to produce the best fit (r^2 = 0.47, p < 0.0001) for the data (Table 1, Fig. 3).
249 Further, the logistic regression of AR verses VN exhibited an approximately significant
250 positive correlation (Log(p/1-p) = -4.6515 + 0.3940*VN, p = 0.07, r^2 = 0.1), and based on this
251 relationship, the predicted probabilities for an adverse reaction at the median number of vaccines
252 administered of six was 9.2 ± 4.6%. At eleven vaccines, the maximum number administered,
253 the probability of an AR occurring increased to 42.1 ± 27.0% (Fig. 4).
254

255 **DISCUSSION**

256 The results presented here suggest that the ER BAC PLUS® vaccine is effective in conferring
257 protection against natural *E. rhusiopathiae* infections in bottlenose dolphins. Firstly, the vaccine
258 was shown to be immunogenic to bottlenose dolphins, confirming earlier results (Nollens et al.
259 2007, Bernal-Guadarrama et al. 2014). Secondly, the ability to detect antibodies generated
260 following both natural and vaccine-induced immunizations using a for bottlenose dolphins
261 modified FMIA based on the major surface protein A indicated the presence of shared epitopes
262 in this region between the ER BAC PLUS® 65 kDa protein antigen and the *E. rhusiopathiae*
263 strains to which bottlenose dolphins are exposed. This cross-reactivity is key to cross-protection.
264 Thirdly, the antibody indices of the vaccinated bottlenose dolphins were within the same order of
265 magnitude of the peak levels measured following natural infection. Until the agglutinating or

complement fixating activity of both natural and artificial induced antibodies have been determined, comparable antibody indices can be presumed to confer comparable degrees of protection. Finally, where *E. rhusiopathiae* infections have historically occurred in the bottlenose dolphin populations housed at the two study sites in regular intervals (Sitt et al, 2010), erysipelas has not been diagnosed either ante-mortem or post-mortem in vaccinated bottlenose dolphins in the 10 years since the start of the vaccination program (unpublished data). A challenge study during which vaccinated and unvaccinated bottlenose dolphins are exposed to *E. rhusiopathiae* would be required to unequivocally confirm that the vaccine confers protection against *E. rhusiopathiae* and to determine which antibody index level is protective. However, such a study is impossible using bottlenose dolphins as subjects.

Vaccine-induced induced antibodies were much longer-lived than antibodies generated following a natural *E. rhusiopathiae* infection. Even though some bottlenose dolphins had not been vaccinated for a prolonged period of time (464 ± 570 days), the number of days since the last vaccination did not influence the animals' antibody index. Antibodies generated following a natural infection were shorter-lived and consequently having survived a natural *E. rhusiopathiae* infection did not influence the animals' antibody index. This difference in antibody half-life could be attributed to either the highly effective adjuvants admixed in the ER BAC PLUS® bacterin or due to the repeated exposure to the vaccine antigen.

Because of the longevity of the vaccine- induced antibodies, the number of vaccinations had the highest impact on antibody levels. However, this relationship between number of vaccinations received and antibody level is not linear, and the protective benefit gained from each additional vaccination appears to taper between 5 and 7 vaccinations. No other factors, including age, gender and ultimately also history of adverse reaction, significantly altered the

289 antibody levels in the studied bottlenose dolphin population. In addition, an obvious benefit of a
290 shorter vaccination interval on antibody levels was not identified. In contrast, an earlier study
291 investigating the cellular immune response following vaccination with the bacterin indicated
292 superior numbers of T-cells in bottlenose dolphins receiving six-monthly compared to annual
293 booster vaccinations (Sitt et al. 2010). The authors did however acknowledge that this superior
294 T-cell memory did not translate in an improved anamnestic response and recommended the
295 longer 12-month vaccination interval (Sitt et al. 2010).

296 Our results support the hypothesis that the commercial porcine ER BAC PLUS® vaccine is
297 effective in generating long-lived antibodies against *E. rhusiopathiae* in bottlenose dolphins and
298 is therefore likely to confer protection against erysipelas. Considering the longevity of vaccine-
299 induced antibodies and the lower benefit but increasing risk of adverse reactions with each
300 additional immunization, the vaccination interval could likely be prolonged beyond one year
301 once multiple vaccinations have been received. More research is needed to define the longevity
302 of antibodies after repeated vaccination and in order to determine the optimal vaccination
303 interval.

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Table 1**.** Regression model development for prediction of anti-*Erysipelothrix* antibody titers (Index) in response to vaccinations and the potential influence of biologic variables.

Independent Variables	Regression Parameters ($F_{1,48}$, p value, r^2)
Age of Animal (Age d)	0.18, 0.67, 0.004
Sex (Female = 0, Male = 1)	0.01, 0.93, 0.000
Erysipelothrix Bacteremia (No =0, Yes = 1)	0.20, 0.66, 0.004
Adverse Reaction (AR; No = 0, Yes = 1)	3.26, 0.08, 0.045
Number of Vaccines (VN)	32.01, <0.01, 0.41
Days since last vaccine (d)	0.01, 0.94, 0.000
Multiple regression analysis	
Index = 5.716 + (1.381*VN) + (2.13* AR) + (-1.067*VN*AR)	9.71, <0.001, 0.35
Independent Variables	
VN	t = 4.92, p = <0.001
AR	t = 1.06, p = 0.296
Interactions: AR by VN	t = -0.85, p = 0.4
Final Linear Model	
Index = 5.58 + 1.446*VN	27.8, <0.001, 0.37
Negative Exponential Model	
Index = 18.6819 * [1 – exp(-0.2795*vaccines)]	40.92, <0.0001, 0.47

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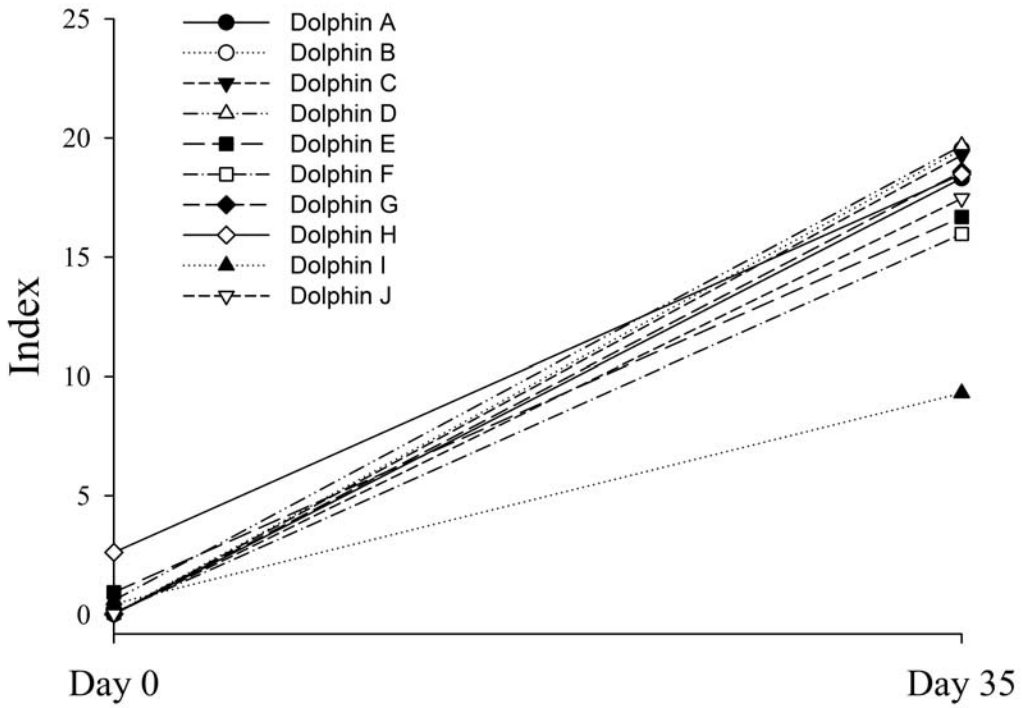

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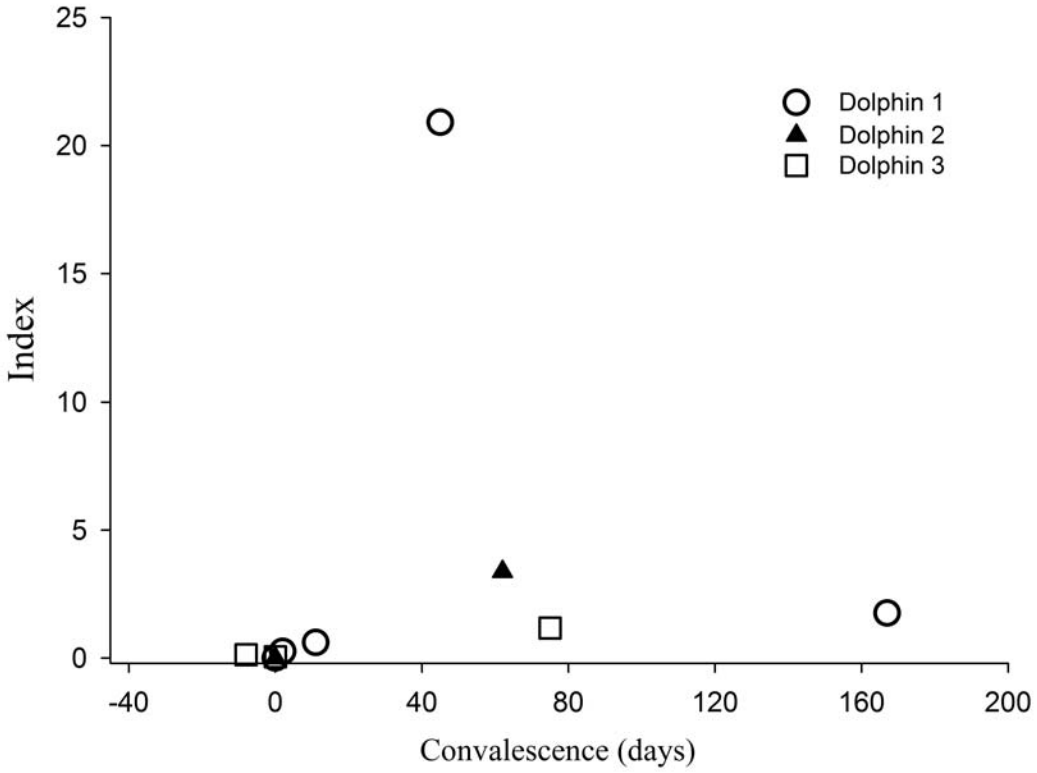
400 Fig. 1. The mean antibody index (± SD) of the initial (0.5 ± 0.8, n = 10) and post-vaccination
401 samples (17.3 ± 3.1, n = 10) collected 14 (± 1) days following the booster immunization from 10
402 bottlenose dolphins. On average a 311-fold rise in antibody index (SD = 301, median = 313, min
403 = 7, max = 859) was detected.



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405 Fig. 2. The mean antibody index of samples collected from naturally infected bottlenose dolphins
406 (n = 3) prior to infection ("initial"), at the time of acute infection ("infection") and in the
407 convalescent period ("convalescence"). The highest antibody index level of 20.91 was detected
408 in Dolphin #1 at 45 days post infection.

409

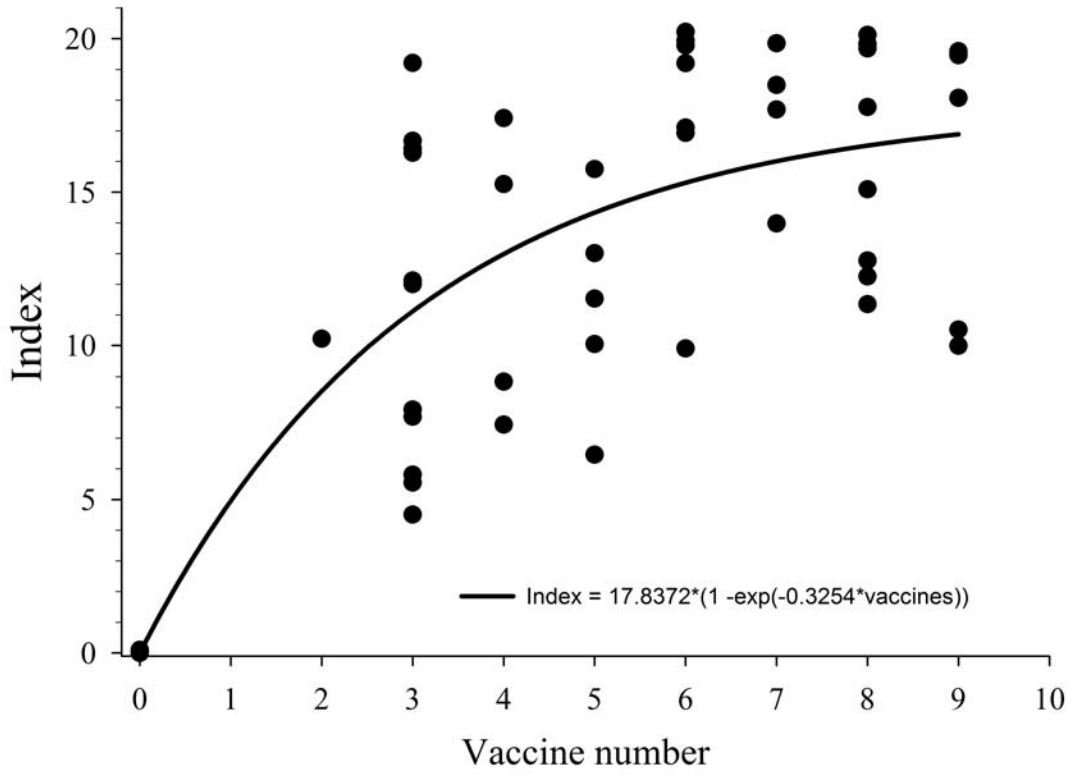
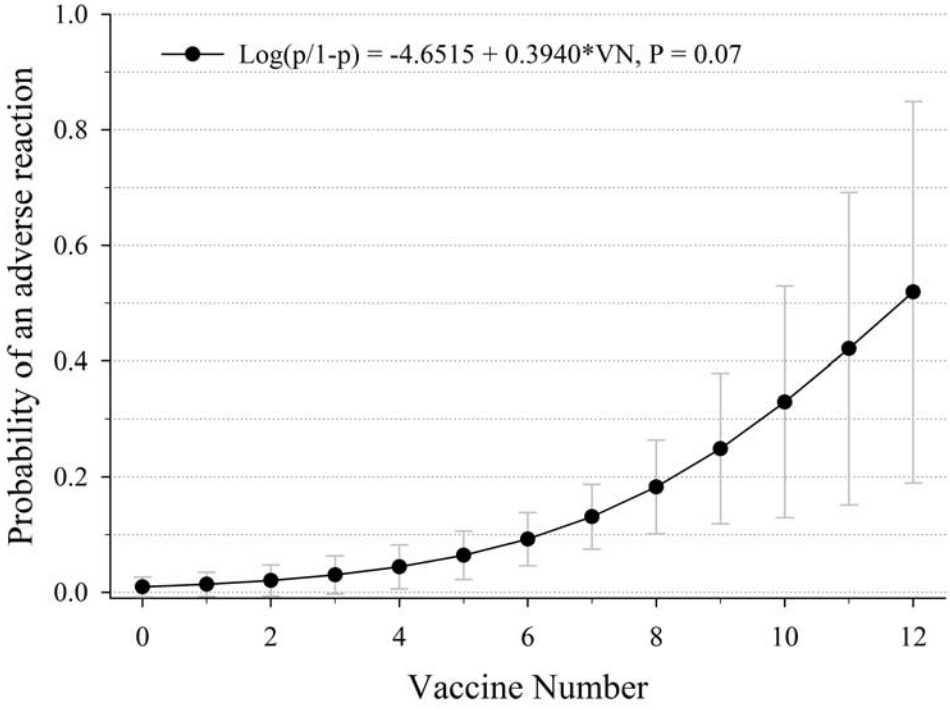


Fig. 3. A negative exponential regression of antibody index verse vaccination number ($r^2 = 0.47$, $p = 0.001$). The negative exponential regression defines an exponential rise to a maximum, which visually occurs from 5 to 7 vaccinations. Thus, the effectiveness of the vaccines at creating an antibody response appears to be leveling off with additional vaccines being of questionable value.



418

419 Fig. 4. The logistic regression of adverse reaction versus number of vaccination (VN) was
420 approaching significance (Log(p/1-p) = -4.6515 + 0.3940*VN, p = 0.07, r^2 = 0.1). Based on this
421 relationship, an increased probability of adverse reaction with increasing number of
422 immunizations received was detected.