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Developing an Assay to Quantify Antigen-Specific IgY in a Long-Lived Seabird

Anna Louise McCarter

DEVELOPING AN ASSAY TO QUANTIFY ANTIGEN-SPECIFIC IgY
IN A LONG-LIVED SEABIRD

by

Anna Louise McCarter, B.S.

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We hereby recommend that the thesis prepared under our supervision by <u>Anna</u>

<u>Louise McCarter</u> entitled <u>Developing an Assay to Quantify Antigen-Specific IgY</u>

<u>in a Long-Lived Seabird</u> be accepted in partial fulfillment of the requirements for

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_____
Supervisor of Thesis Research

_____
Head of Department

<u>   School of Biological Sciences   </u>
Department

Recommendation concurred in:

_____

_____   Advisory Committee

_____

Approved: **Approved:**

_____   _____
Director of Graduate Studies   Dean of the Graduate School

_____
Dean of the College

ABSTRACT

To maximize parental fitness, life history theory predicts that short-lived species will prioritize reproductive efforts over self-maintenance functions. The opposite is predicted for long-lived species, which ought to invest heavily in immune defenses, particularly those of the acquired immune system. Yet, most of our current understanding of immune system function has been from investigations of short-lived model species. Little is known about the induction and maintenance of immunological memory in wild species, and in particular, long-lived, wild species. This is due in part to limited availability of species-specific secondary antibodies for use in serological studies of non-model species. Moreover, commercially available ELISA kits designed for use with these secondary antibodies are expensive and limit sample size. This study presents a method to quantify antigen-specific IgY in the Nazca Booby (*Sula granti*), a long-lived seabird found on the Galápagos Islands, Ecuador. To identify antigen-specific IgY, blood samples were collected before and after birds were injected with keyhole limpet hemocyanin (KLH), a harmless molecule known to stimulate a specific immune response in vertebrates. Using an indirect ELISA, secondary antibodies produced against avian IgY were tested for cross-reaction with Nazca Booby anti-KLH IgY. Specific antibodies produced to KLH were quantifiable with the use of Bethyl Laboratories® anti-bird IgY as well as Sigma-Aldrich® anti-chicken IgY. Additionally, an indirect ELISA protocol was developed at a fraction of the cost of commercially available kits. Development of this method will

expand our understanding of immune function by allowing investigation of the induction

and maintenance of immune memory.

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INTRODUCTION

Processes such as self-maintenance and reproduction are important to an individual's fitness, but also costly in terms of the energy required to carry out these functions (Stearns 1992, Ardia et al. 2003, Lee et al. 2008). Since most resources are limited, organisms must optimize energetic allocation to these important, and often competing, processes to maximize reproductive success (Ardia et al. 2003, Lee et al. 2008). According to life-history theory, short-lived species should invest relatively more energy into reproductive effort, while long-lived species should shunt costs of reproduction to offspring in favor of self-maintenance functions (Stearns 1992). In the past, ecologists have relied on morphological measurements such as mass loss during a breeding attempt (Rands et al. 2006) or survival probabilities after a breeding attempt (Ghalambor and Martin 2001) to assess the costs of reproduction of individual breeders. This approach ignores underlying physiology that could be affected by reproductive effort, and researchers have begun to explore other avenues to assess the trade-off between reproduction and self-maintenance. One of these avenues is assessment of immune function, and the emerging field of investigation is called ecoimmunology (Martin et al. 2006b, 2011; Martin 2009). Ecoimmunology incorporates the perspectives of ecology, biology, physiology, and evolution into the field of immunology.

Immune systems provide defense against injury and disease. In vertebrates, responses of the immune system can be categorized according to the immune-defense-component model, IDCM, a model proposed in order to include a greater appreciation of the complexity of immune responses (Martin et al. 2006b). This model suggests that regardless of species, immune responses can be either nonspecific or specific while also being either constitutive or inducible (Martin et al. 2006b). Nonspecific, or innate, immune responses provide individuals with defense against a broad spectrum of pathogens. These defenses include barriers from the outside environment as well as nonspecific responses mounted by cellular and molecular components of the innate system including macrophages, natural killer cells, and complement proteins. Specific immune responses, which require previous exposure to a pathogen to then "remember" (immune memory) and clear it from the body more efficiently, are characterized by the action of T- and B-lymphocytes (Martin et al. 2006a). The cellular components of constitutive immune responses are continuously expressed and act to maintain immunological threats at any given moment, providing organisms with a first-line of defense (Lee 2006). Inducible immune responses are not continuously expressed, but instead are activated with the introduction of a recognizable pathogen (Martin et al. 2006b). Both nonspecific and specific arms of the immune system have constitutive and inducible elements.

Trade-offs also exist within the IDCM model. As the energy allocation to one arm of the immune system is increased, then energy allocation to the other arm may need to be reduced to create an optimum environment to fight infection or to limit overall

energetic investment to immune defense (Martin et al. 2006b). Generally, constitutive

and non-specific immune defenses are thought to impose small costs, while induced cell-

mediated responses impose high energetic costs (Fig. 1; Lee 2006).
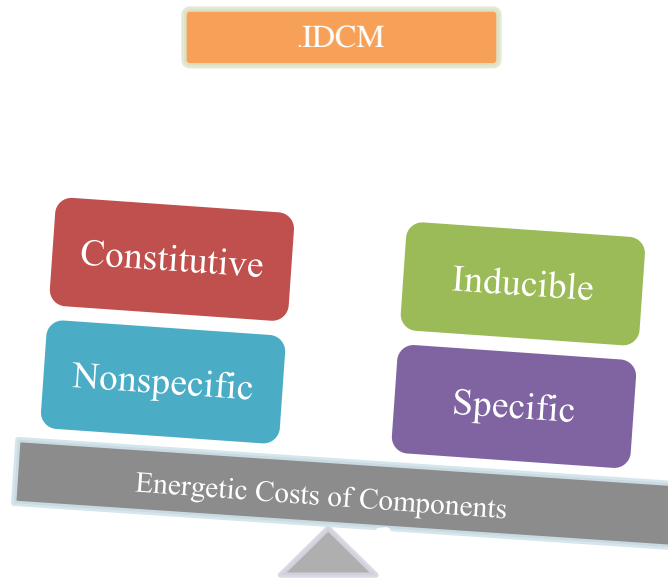
Figure 1: The immune-defense-component-model (IDCM) breaks immune responses into four categories: constitutive, nonspecific, inducible, and specific. Generally, inducible and specific immune responses have higher energetic costs than do constitutive and nonspecific responses (Martin et al. 2003, 2006b; Lee 2006b, Brace et al. 2017).

Energy allocation to different components of the immune system is expected to

vary between short-and long-lived species (Martin et al. 2006b). Organisms are predicted

to invest in different types of immune responses depending on the best way to combat a

particular immunological threat in addition to the costs and benefits each type of response

provides (Martin et al. 2006b, Graham et al. 2010). Short-lived species are expected to

invest primarily in less expensive immune functions since they should preferentially invest in reproduction over self-maintenance (Martin et al. 2006a, 2007). In addition, investment in immune memory function may be futile if the organism does not live long enough to face repeated exposures to a pathogen. Inducible defenses, which require a significant allocation of resources to develop and are beneficial when facing recurring exposures, particularly immune memory responses of B- and T-cells, should be more common among long-lived species (Lee 2006). Long-lived species are expected to invest in specific responses to pathogens and are known to have increased metabolism with an increase in the production of pathogen-specific antibodies (Lee 2006, Martin et al. 2006a).

Specific antibody-driven immune responses of the acquired immune system can be broken down into primary and secondary responses to a pathogenic exposure (Fig. 2). When introduced to a new pathogen, naïve B-cells that recognize antigens on the pathogen are activated and some of them differentiate into short-lived plasma cells, which produce and release antibodies. The first waves of antibodies produced by plasma cells to clear the infection during a primary response are IgM antibodies. Other activated B-cells travel to follicular germinal centers of lymphoid organs where they interact with follicular helper T-cells (T_{FH} cells). B-cells that have interacted with T_{FH} cells follow one of three pathways: 1) they differentiate into plasma cells that have greater affinity toward the pathogen; 2) they become memory B-cells that have greater affinity toward the pathogen; or 3) they undergo programmed cell death. The process of creating B-cells whose antibodies have better recognition of the pathogen is called affinity maturation. During the affinity maturation process, isotype switching also occurs, where the constant

region of the antibody is exchanged to create an antibody that can be directed more quickly to the appropriate site of infection. While mammals have five different classes of antibodies, which have evolved with different features to better target and bind to antigens, avian species have three classes of antibodies, including IgM, IgA, and IgY. Pathogens encountered in the blood and extracellular fluid usually trigger a switch to IgG (in mammals) or IgY (in birds). IgG or IgY antibodies are produced and released during the late stages of a primary response, allowing them to better target the pathogen and more effectively neutralize it or mark it for phagocytosis (Murphy and Weaver 2016). Antibodies produced during the primary response mark the pathogen for phagocytosis or neutralize the pathogen via binding (Lee 2006). Acquired immune responses not only produce antibodies in response to a variety of pathogens upon initial encounter, but can also store recognition of previously encountered pathogens to retain immunological memory in the form of memory B- and T-cells. Upon re-exposure to a pathogen, a subset of T-cells can now more efficiently recognize the pathogen and signal the production of specific antibodies much more quickly than was seen with the initial exposure, creating a secondary immune response (Fig. 2; Lee 2006). A subset of memory B-cells can differentiate into plasma cells that produce vast quantities of high affinity IgG or IgY antibodies (assuming a humoral antigen). The secondary response is therefore characterized by faster and greater production of high affinity antibodies (Fig. 2). Each exposure to the antigen is thought to create longer lived memory cells (Murphy and Weaver 2016). These primary and secondary responses make up the specific component of the IDCM (Fig. 1).
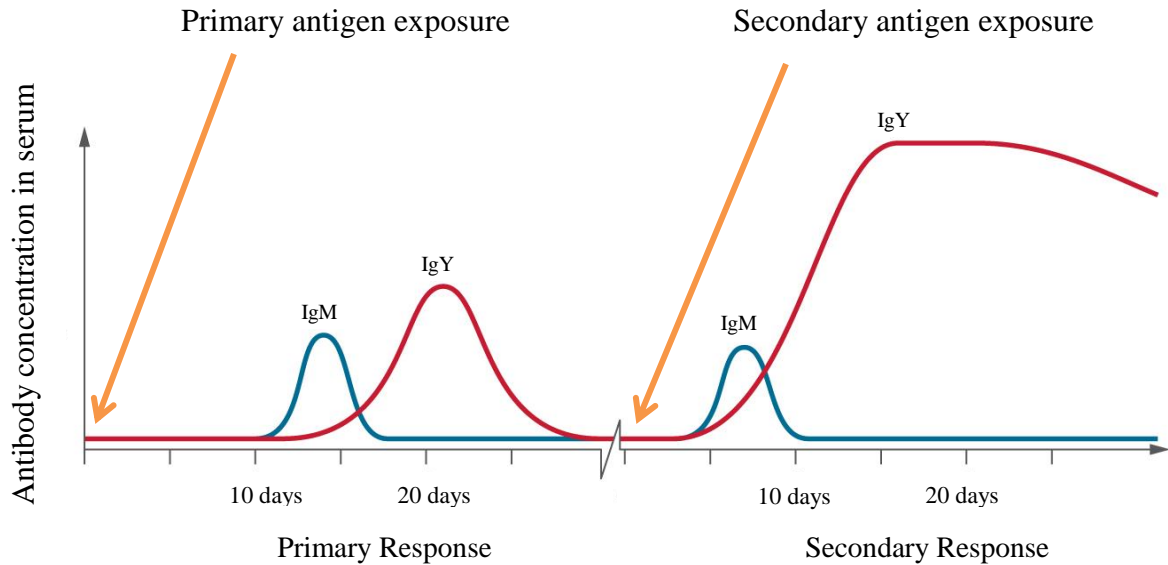
Figure 2: Antibody production for primary and secondary immune responses. Figure modified from OpenStax, Lumen Learning (https://courses.lumenlearning.com/microbiology/chapter/b-lymphocytes-and-humoral-immunity/).

Most of our understanding of immune function comes from laboratory studies of model organisms, particularly rodent species. Model organisms are convenient to use and do provide a wealth of information about the mechanisms involved in physiological processes, especially through the use of knockout mutants (Mak et al. 2001). Laboratory studies give us information about what animals are capable of doing, but cannot let us know what they actually do under natural conditions. This is because laboratory animals usually do not face the same ecological demands and natural selection processes that wild animals do, such as finding food, avoiding predators, thermoregulation, and exposure to multiple potential pathogens by living in non-sterile conditions. Also, laboratory studies of vertebrates are biased toward mammalian species. The recent development of models such as the IDCM has expanded the potential to understand the relationship between

ecological and evolutionary components influencing immune responses and other physiological processes competing for allocation of resources (Martin et al. 2006b, Lee 2006).

Most of our understanding of immune function in birds are from studies of poultry species highlighting their importance in agriculture (Schat et al. 2014). Poultry are short-lived, precocial species (Starck and Ricklefs 1998); as such, their allocation to different components of the immune system are expected to be different from long-lived, altricial species (Martin et al. 2006b). Although the basic premise of this expectation is appealing, evidence of this relationship between life history and immune defense remains equivocal. An investigation of interspecific variability in cell-mediated immune response (B- and T-cell response) and life history characteristics of 50 different bird species found that patterns were obscured by highly correlated predictor variables (longevity, body size, developmental rate, etc. (Tella et al. 2002)). In addition, this study used previously published works that employed a mitogen to induce an immediate lymphocyte response (Tella et al. 2002); as such, none of these studies investigated immunological memory function. Studies within species have been more fruitful, though few in number. A study of house sparrow (*Passer domesticus;* a short-lived species) immune function in populations that differ in pace of life characteristics found no differences in low cost constitutive defenses between fast- and slow-living sparrows (Martin et al. 2006a). Slow-living sparrows did have greater antibody proliferation and energy expenditure on immune responses, but they had lower investment in T-cell memory responses than fast-living sparrows (Martin et al. 2006a). Tree swallows (*Tachycineta bicolor*), a species with an intermediate lifespan, were the subjects of a study that examined the effects of

reproductive effort on immunity (Ardia et al. 2003). This study found that by increasing

brood sizes, females were less likely to produce secondary antibodies to a specific

antigen and ultimately their survivability decreased (Ardia et al. 2003). One study of a

long-lived colonial bird, the black-legged kittiwake (*Rissa tridactyla*), investigated the

variation in temporal antibody levels in response to a common pathogenic infection by

Borrelia burgdorferi (Staszewski et al. 2007). These birds are repeatedly exposed to

Borrelia on an annual basis at their breeding colonies. Anti-*Borrelia* antibody titers were

repeatable from year to year and also correlated with exposure to *Borrelia* the previous

year (Staszewski et al. 2007).

Still, few studies have been conducted on wild, long-lived species in an extended

time frame. Previous studies of long-lived seabirds have shown that they possess high

levels of circulating IgY, increasing with size of body as well as life span (TJ Maness,

unpublished data). Nazca boobies (*Sula granti*), a long-lived colonial seabird, show

double the amount of circulating IgY in comparison to short-lived birds (Apanius 1998).

IgY indicates antibody production for use by specific defenses (Warr et al. 1995), as well

as constitutively expressed natural antibodies (Lee et al. 2008, Sun et al. 2011). We

understand that long-lived seabirds are capable of maintaining circulating IgY, but there

is still much to be determined about the induction and extent of long-term memory in

long-lived and/or wild species. This information offers a better understanding of the

underlying factors influencing optimality of an immune response with other, competing,

physiological needs, as well as intricacies of the immune system including primary and

secondary anti-body driven responses and resistance to diseases (Martin et al. 2006b).

The quantification of antibodies in serum has led to a better understanding of humoral immune responses. Pioneering techniques such as native electrophoresis were used to identify the γ-globulin portion of the antibodies in serum, while more recent technique developments such as agglutination and Western Blot make identifying whole antibody concentration in serum possible (Martínez et al. 2003). These techniques do not, however, identify specific antibodies produced in response to an antigen, which is why enzyme-linked immunoabsorbent assays (ELISA) have gained popularity. The more recent use of ELISAs for specific antibody quantification can provide useful information regarding the induction and maintenance in wild, non-model species (Martínez et al. 2003). Several ELISA techniques could be used to quantify antigen-specific antibodies, such as sandwich, competitive, or indirect ELISAs.

Sandwich ELISAs require the use of matched capture and detection antibodies that bind different epitopes on an antigen, which makes this technique more or less exclusive to model species where matched sets of antibodies are available. Competitive and indirect ELISAs are the most commonly used ELISA techniques in wild bird studies (Fassbinder-Orth et al. 2017). Indirect and competitive ELISAs offer more flexibility allowing cross-reactivity between target antibodies with non-specific model antibody options as well as the use of signal amplification. Competitive ELISAs involve binding competition between a target antibody and a conjugated antigen-specific antibody for binding sites on an antigen. When more target antigen-specific antibody is bound to antigen, fewer binding sites are available for the detection antibody. The sample output of a competitive ELISA is therefore, inversely related to the amount of target antibody bound to the antigen. A disadvantage of this technique is that all antibodies produced

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against the antigen can bind, which makes study of induction of immune memory involving isotype switching difficult. Indirect ELISAs require the addition of a target primary antibody, which binds to the immobilized antigen, followed by a conjugated secondary antibody, which then binds to the target primary antibody-antigen complex. The sample output of an indirect ELISA has a proportional relationship to the amount of primary antibody in a sample, providing a useful method to determine specific antibody production in response to a particular antigen. An advantage of this technique is that secondary antibodies can be used to detect specific antibody isotypes allowing study of immune memory function. However, this technique is limited in non-model species due to the availability of commercially produced species-specific secondary antibodies, which is largely restricted to few avian orders, and the ability of these antibodies to cross-react with primary antibodies of other species varies and in many cases is unknown (Fassbinder-Orth et al. 2017). However, the indirect ELISA technique is only method that permits the detection of particular antibody isotypes produced against an antigen.

When using an indirect ELISA technique, the secondary antibody must effectively cross-react with the primary antibody of interest. Currently available secondary antibodies are largely restricted to antibodies produced by commonly studied domestic chickens, passerines, and more recently, a mix antibodies produced in four species (dove, duck, sparrow, and chicken) of non-wild birds (Martínez et al. 2003). Cross-reactivity between anti-chicken secondary antibodies with the primary IgY of wild birds has been confirmed through the combination of ELISA and Western Blot testing (Martínez et al. 2003, Cray and Villar 2008). Although cross-reactivity is possible due to somewhat conserved coding regions for antibodies across avian species, including IgY,

pilot studies are necessary to determine the level of cross-reactivity of primary and secondary antibodies as well as the optimum dilutions of serum and secondary antibodies in order for results to be quantifiable (Martínez et al. 2003). Additionally, commercially produced ELISA kits for use with these secondary antibodies in birds are expensive (~$500 a plate).

The goals of this study were:

1. To develop a more cost effective protocol for indirect ELISA than expensive commercially available kits.

2. To determine a secondary antibody that will cross-react with Nazca Booby anti-KLH IgY.

In order to ensure the production of antigen-specific IgY, birds were immunized with keyhole limpet hemocyanin (KLH). KLH is a large respiratory molecule known to be a harmless antigen to vertebrates, stimulating a robust specific immune response (Harris and Markl 1999). Information on the cross-reactivity of various secondary antibodies, with antigen specific IgY produced by Nazca Boobies could expand acquired immune studies to a wild, non-model species. The immune function of Nazca Boobies could provide insight into the energetic allocation of life history traits with implications for conservation of endangered long-lived species, such as the implementation of vaccination programs.

METHODS

Sample Collection

All samples were collected from a colony of Nazca boobies inhabiting Española Island, Galápagos Islands, Ecuador (Fig. 3) in August of 2003, and three months later in November of 2003. Blood samples were collected via brachial venipuncture from adult birds before being injected intraperitoneally with 100ng of KLH conjugated with dinitrophenol (DNP-KLH; USBiological) suspended in 100μL of sterile water (experimental and primary response control groups; Fig. 4) or 100μL of sterile deionized water (control group; Fig. 4). In order to assess antibody-driven immune responses to the antigen, additional blood samples were taken 4-12 days after injection. Samples were centrifuged and serum was preserved in a propane-powered freezer (-60°C) in the field. Samples were transferred on dry ice to a -80°C freezer in the laboratory and maintained there until analysis.

Experimental Groups

Treatment groups of birds consisted of three different groups: experimental (EXP), control, and primary response control (PRC) groups. The EXP received a primary injection with KLH in August 2003, while the control group of birds received primary injection with deionized water. Blood samples were collected before injection and again 4-12 days later to assess primary immune response (Fig. 4). In order to determine if

Nazca boobies have immune memory as would be indicated by a secondary immune

response, EXP and control birds were re-injected with KLH or water three months after

the initial injection and resampled as before, in November 2003. The PRC group was

injected with KLH at this time (Fig. 4). The PRC group controls for potential

environmental factors that could influence immune responses in birds not expected to

have a robust secondary antibody-driven response to immunization with KLH. At this

time, the EXP group should have a secondary immune response, the PRC group should

have a primary immune response, and the control group should have little to no

detectable anti-KLH IgY.
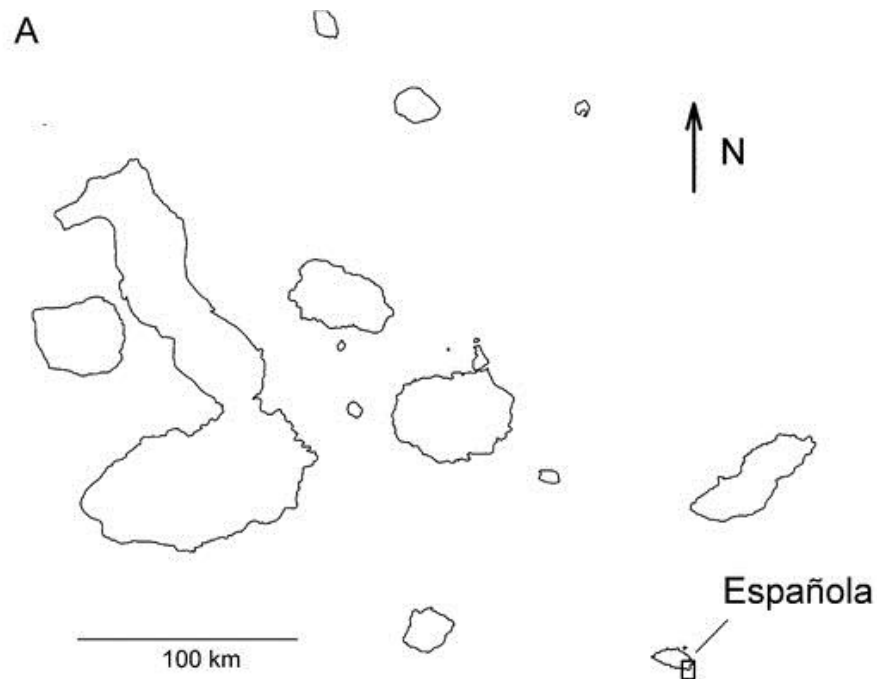
Figure 3: The Galápagos Islands, Ecuador. The box indicates the study site on Española
Island. Map is modified from Figure 2 in Apanius et al. 2008.
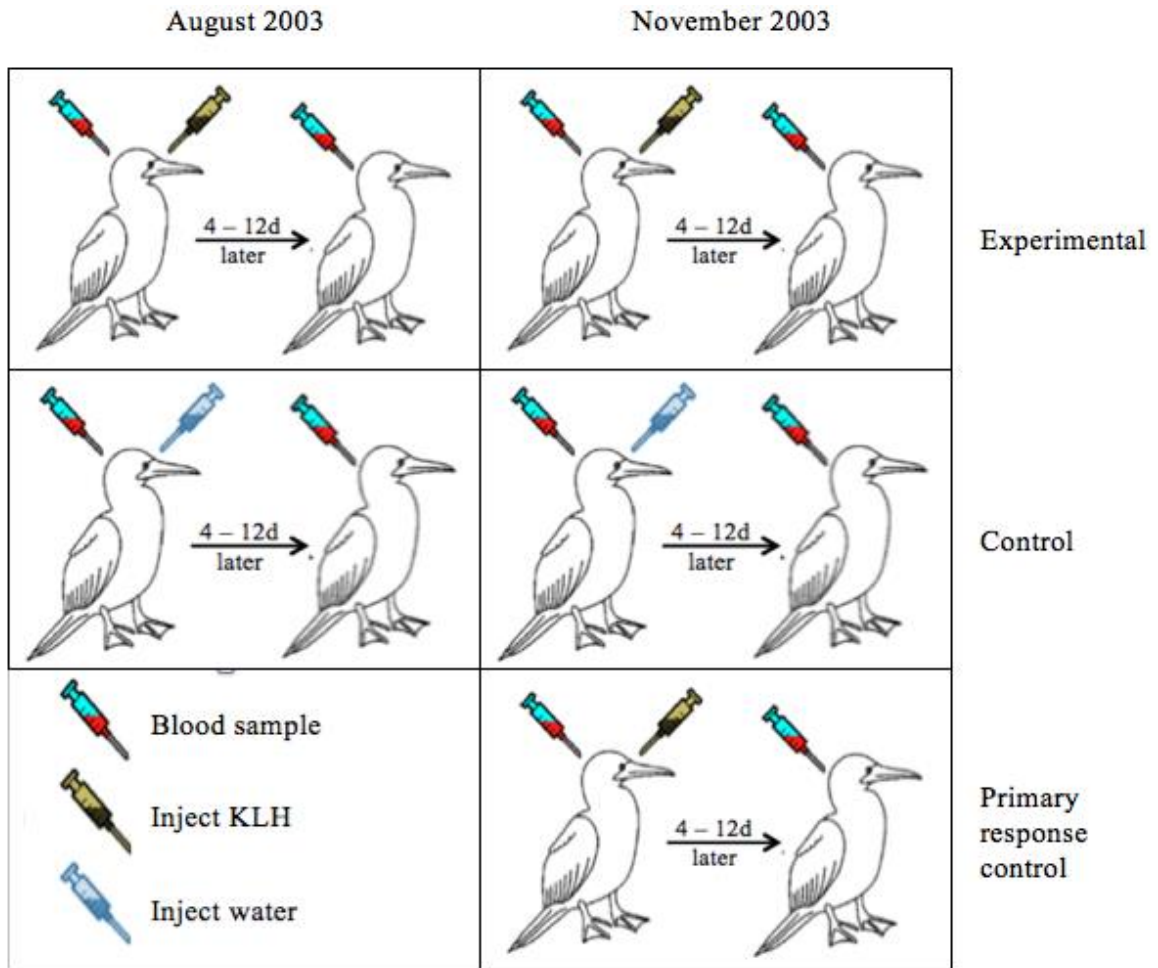
Figure 4: Sampling schematic showing primary and secondary injections of KLH (or water for control group) and blood sample collection pre and post-injection.

Assay Development

Secondary Antibodies Tested

KLH-specific antibody titres were measured with indirect ELISA analysis in 96-well plates, using different commercially available secondary antibodies for avian species. All secondary antibodies tested were polyclonal anti-IgY conjugated with horseradish peroxidase (HRP) in order to enzymatically react with tetramethylbenzidine

(TMB) to produce a color change that can be quantified using absorbance spectroscopy. IgY was isolated from different bird species and injected into different species to produce secondary antibodies (anti-IgY). Analyses of quantifiable KLH-specific antibodies were investigated using the following secondary antibodies: donkey anti-chicken IgY-HRP (H+L chain) by Gallus Immunotech®, rabbit anti-chicken IgY-HRP (whole molecule), by Sigma-Aldrich®, goat anti-bird IgY-HRP (H+L chain) by Bethyl Laboratories®, and rabbit anti-chicken anti-KLH IgG-HRP - (an ELISA kit) by Genemed Synthesis, Inc. Secondary antibodies were diluted to manufacturers' recommendation for ELISAs as well as to a titer twice as concentrated, in most cases, to test different working dilutions for influence on cross-reactivity. For example, Gallus anti-chicken IgY was diluted 1:15,000 and 1:7,500, while Bethyl anti-bird IgY and Sigma anti-chicken IgY were diluted 1:30,000 and 1:15,000. Each secondary antibody was tested on an equivalent mix of samples (N ~20) collected either before primary KLH injection (pre 1° KLH), after primary KLH injection (post 1° KLH) before secondary KLH injection (pre 2° KLH), after secondary KLH injection (post 2° KLH), before and after primary injections with water (pre 1° H_2O, post 1° H_2O, respectively), or before secondary injection with water (pre 2° H_2O). If possible, each secondary antibody was tested with the same sample; however, some Nazca booby serum samples did not have enough volume to be tested against all secondary antibodies. In this case, another sample from the same experimental group was substituted.

Indirect ELISA Protocol

The following protocol was developed based on the methods of Addison et al. (2010) with modifications. All solutions and samples were brought to room temperature

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before use. 100μL of KLH (suspended in 0.05 M carbonate-bicarbonate buffer) was incubated in Nunc MaxiSorp™ 96- well plates (ThermoFisher Scientific) at 4°C overnight. Unbound KLH was washed from the wells the next day using 300μL phosphate buffered saline with 0.05 % Tween 20 (PBS-tween 20) solution four times. To control for nonspecific binding, wells were then incubated at 4°C overnight with 200μL of Superblock™ (ThermoFisher). After blocking overnight, wells were washed again with PBS-tween 20. A maximum of 23 Nazca booby serum samples (100μL) were loaded in triplicates diluted 1:25 in Superblock, except for the Genemed assay where serum samples were diluted 1:20 in 20X in sample diluent concentrate provided with the kit. An internal standard (IS) – chicken reference serum (Bethyl Labs, Inc.) diluted 1:15,000 in Superblock – loaded as a pair of triplicates (100μL), was used to control for inter-assay differences (Fig.5). Five standards (std 1 – 5) were created via serial dilution from a stock solution of chicken anti-KLH IgY (2mg/ml; Gallus Immunotech®) in Superblock™ and also loaded in 100μL triplicates (Fig. 5). The greatest dilution (std 5) was a 6.25 million-fold dilution of the stock, with each previous standard (stds 4 – 1) concentration being half as dilute (or twice as concentrated as the previous standard). Standards of chicken anti-KLH antibodies with known concentrations were used to produce a standard concentration curve to determine sample concentrations (serving as a positive control). Blanks and non-specific binding controls served as negative controls and were also loaded in 100μL triplicates (Fig.5). Once loaded with all samples, standards, and controls, the plates were incubated overnight at 4°C and washed as above again the next day.

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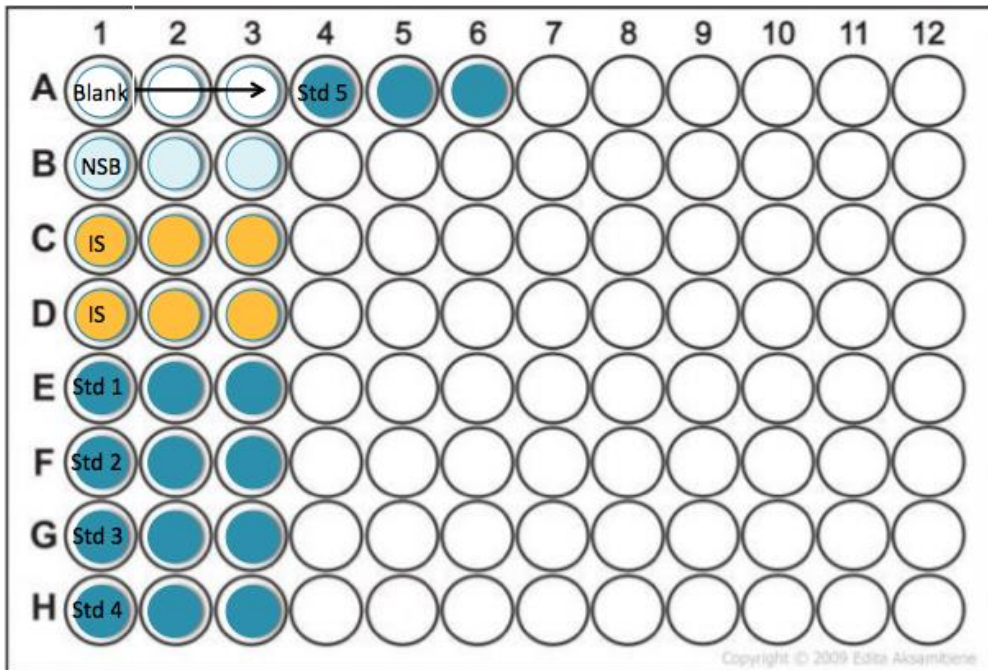
Figure 5: Plate layout for a single assay. Each plate included positive controls, standards made of serial chicken anti-KLH dilutions (std 1 – 5), as well as negative controls, blanks and nonspecific binding (NSB), internal standard (IS) chicken reference serum, controlling for inter-assay differences, and samples (remaining wells, maximum of 23), all loaded in triplicate.

In order to detect anti-KLH IgY antibodies in the Nazca booby serum as well as standards, 100μL of the secondary antibody, diluted as stated above for each manufacturer ELISA dilution in Superblock™ was added to all wells except for blanks. Plates were incubated at 37°C for one hour. After the incubation period, wells were washed and a 3,3',5,5'-tetramethylbenzidine (TMB; two 1mg tablets; Sigma Aldrich) and phosphate-citrate (20mL of a 0.05M) solution, which was created in the dark with sufficient time for tablets to dissolve completely. Four microliters of 30% hydrogen peroxide (Sigma Aldrich) was added to the solution just before pipetting 100μL to all wells containing samples, standards, and NSB for color production. 125μL Superblock™

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was added to blank wells at this time. 25μL of stop solution (ThermoFisher Sci.) was added to samples, standards, and NSB after 20 minutes of color development and the plate was read with a plate-reader (Multiskan FC Microplate Photometer, Thermo Scientific) measuring absorbance at 450nm. Greater optical density readings at 450nm indicated greater concentration of anti-KLH specific IgY antibodies. The Genemed secondary antibody was tested following the manufacturer's protocol for the ELISA kit.

Statistical Analysis

Coefficient of variation (CV) was used to assess intra-assay variability in loading of samples and standards with a cutoff a CV = 10%. If the CV was greater than 10%, then the sample loading that was most different from the other two was removed and the CV was recalculated. Sample loadings that could not be adjusted to a CV below 10% were assayed again. Inter-assay variability was assessed via CV of the IS. Standard curves were created for each secondary antibody tested from the known concentrations of chicken anti-KLH standards included in each plate to determine sample concentration. Standard curves were standardized to the greatest standard dilution, which set the lowest concentration to a value of one. Other dilutions were doubles of the previous concentration (e.g., 1, 2, 4, 8, 16). Absorbance was also standardized to the reading of the greatest dilution of the standard curve, so that the value of the lowest standard equaled one. Linear regression was used to determine a line of best fit between the natural log of the standardized anti-KLH IgY concentration and its corresponding standardized absorbance. This curve was used to determine the natural log of the Nazca booby anti-KLH IgY titer in a sample (Fig. 6). The minimal acceptable coefficient of determination

(R^2) of the linear regression for each standard curve was ≥ 0.95. A secondary antibody

had to have a standardized absorbance greater than 1.0 to fall on the standard curve (Fig.

6). Therefore, a positive cross-reaction of a secondary antibody with Nazca booby IgY

had to have a standardized absorbance ≥ 1.0. All statistical analyses were performed with

Microsoft ® Excel ® 2013 (© 2012 Microsoft Corporation).

RESULTS

All secondary antibodies tested produced standard curves with R^2 above 0.95 and

most were $\geq$ 0.98 (see Fig. 6 for an example). Standardized ELISA absorbances using

secondary antibodies from Genemed and Gallus were consistently below the 1.0 cut-off

value for positive cross-reactivity with Nazca booby IgY (Table 1, Fig. 7). This was true

for the 1:15,000 and 1:7,500 dilutions of the Gallus antibody. The Genemed secondary

antibody was not tested at different dilutions due to the cost of the assay kit and the fact

that it had the least cross-reactivity with the booby IgY of all secondary antibodies tested

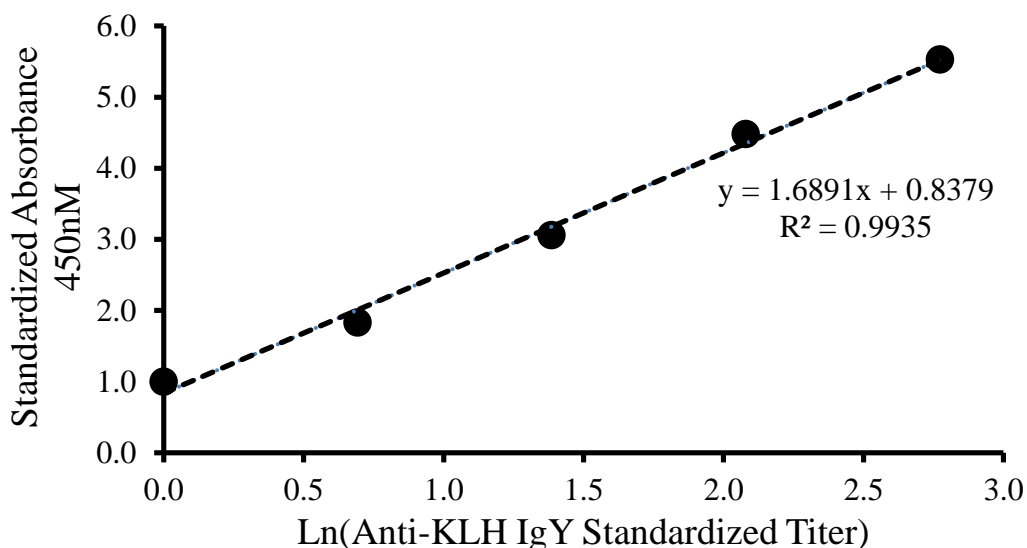(Table 1, Fig.7).

y = 1.6891x + 0.8379
R^2 = 0.9935

Figure 6: Linear regression of the natural log of standardized chicken anti-KLH IgY titer
and standardized absorbance at 450nM using Sigma anti-chicken IgY-HRP secondary
antibody diluted 1:30.000 averaged across ~20 plates.

Each secondary antibody was tested against an equivalent mix of birds from different treatment groups (Table 1), which should have varying results based on their expected immune responses to injection (primary, secondary, or no response).

Table 1: Anti-KLH IgY standardized titers for each secondary antibody (Genemed (1:100 dilution), Gallus (1:15000 dilution), Sigma (1:30000 dilution), and Bethyl (1:30000 dilution)) tested against a mix of serum samples taken before or after primary (1°) or secondary (2°) injection from birds (sample ID) of different treatments groups (i.e. experimental (KLH) or control (H_2O). Nazca booby serum samples were diluted 1:25 in Superblock, except for the Genemed assay where booby samples were diluted 1:20 in 20X in sample diluent concentrate provided with the kit.

Group	Sample ID	Genemed	Gallus	Sigma	Bethyl
Pre 1° KLH	5138	0.191	0.344	1.470	4.065
Pre 1° KLH	5147	0.064	0.321	0.952	3.457
Pre 1° KLH	5153	0.138	0.322	1.087	3.081
Post 1° KLH	5247	0.031	0.341	2.652	3.869
Post 1° KLH	5251	0.048	0.353	0.957	6.738
Post 1° KLH	5254	0.083	0.362	1.176	0.902
Pre 2° KLH	5337	0.116	0.424	1.293	3.833
Pre 2° KLH	5397	0.285	0.435	3.596	1.105
Pre 2° KLH	5408	0.120		0.944	2.403
Post 2° KLH	5451	0.050		1.200	4.763
Post 2° KLH	5461	0.119		2.066	
Post 2° KLH	5464	0.195			3.573
Post 2° KLH	5467	0.008			2.462
Post 2° KLH	5533	0.227			2.983
Post 2° KLH	5553	0.028		1.721	
Pre 1° H_2O	5162	0.144	0.322	1.240	1.137
Pre 1° H_2O	5163	0.059	0.337	1.358	0.677
Post 1° H_2O	5304	0.111	0.368	1.864	6.216
Post 1° H_2O	5309	0.185	0.399	1.428	1.739
Post 1° H_2O	5421	0.094			1.266
Post 1° H_2O	5422	0.164			1.758
Pre 2° H_2O	5565	0.016		0.913	3.733
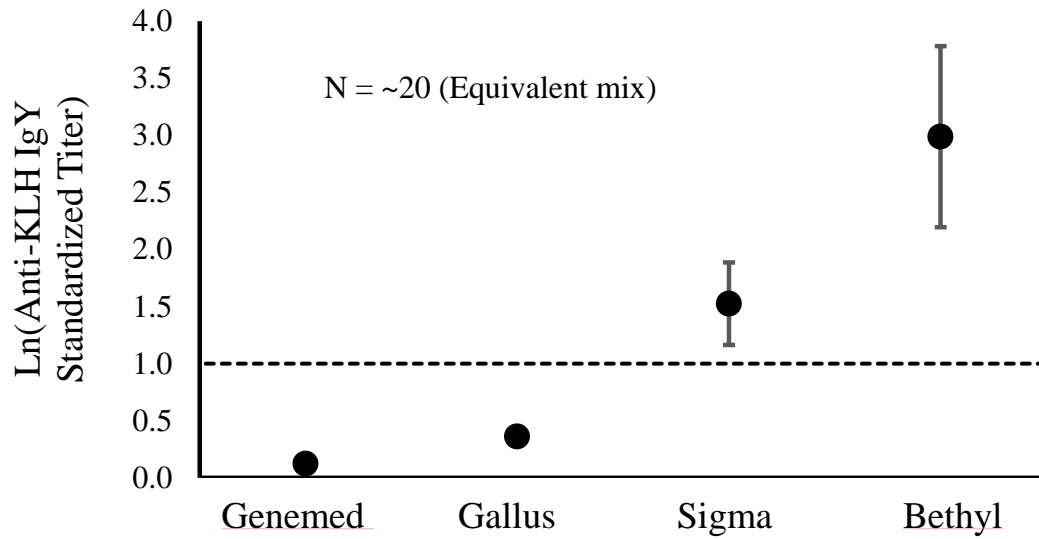
Figure 7: Secondary antibodies were tested against an equivalent mix of ~20 birds from different treatment groups. Error bars represent 95% CIs (these are contained within the data points for the Genemed and Gallus antibodies). Concentrations above the value of one (dotted reference line) were considered detectable.

Figure 8: Sigma and Bethyl secondary antibodies tested against experimental birds sampled before (Pre) or after (Post) primary (1°) or secondary (2°) injection with KLH. Error bars indicate standard error.

Sigma anti-chicken IgY (diluted 1:30,000) produced both detectable and varying results for birds from different treatment groups and sampling periods (Table 1, Fig. 7, Fig. 8). Bethyl anti-bird IgY (diluted 1:30,000) produced results with the greatest level of detection via ELISA as well as the most variability (Table 1, Fig. 7, Fig. 8). More concentrated dilutions of the Sigma and Bethyl secondary antibodies did not increase the cross-reactivity with Nazca booby IgY as indicated by their standardized absorbances at 450nm (data not shown). Bethyl secondary antibodies also produced results consistent with predictions for an increased immune response after 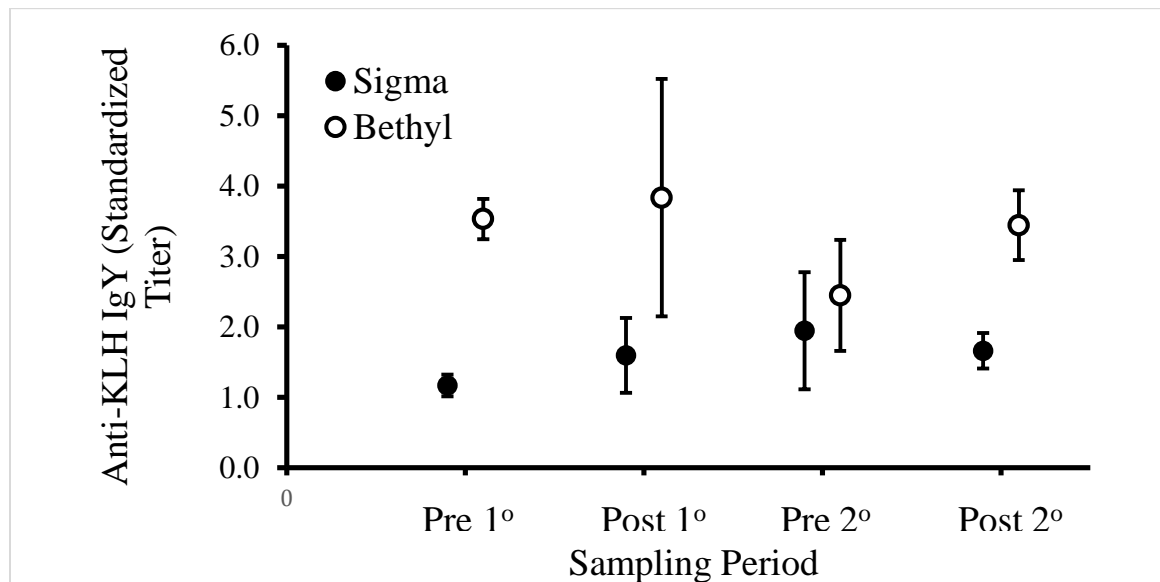primary and secondary injection with KLH (Fig. 8), although this was from a small number of samples that were not from the same individual (not a repeated sample).

Inter-assay variability was calculated for the Sigma and Bethyl secondary antibodies only because these were the only antibodies that cross-reacted with the booby IgY. Inter-assay variability of the Sigma secondary antibody assays was CV = 46.7%, while the variability of the Bethyl secondary antibody assays was CV = 63.7%.

These positive results were gathered using an indirect ELISA protocol, which permitted bulk ordering of all plates, reagents, and solutions. Assays were effectively run using this protocol at a fraction of the cost of using pre-coated plates and reagents included in expensive, commercially available kits. This protocol produced quantifiable results when secondary antibodies capable of cross-reactivity with Nazca booby IgY were identified. This method costs a total of ~$60 per plate, while the aforementioned ELISA kit costs $500 per plate.

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DISCUSSION

This study presents the first comparison of commercially available secondary antibodies for cross-reactivity with antigen-specific IgY produced by a long-lived, wild species of bird, the Nazca booby. Of the secondary antibodies tested, only Sigma-Aldrich® rabbit anti-chicken IgY and Bethyl Laboratories® goat anti-bird IgY cross-reacted with Nazca booby anti-KLH IgY to produce detectable results (Fig. 7, Fig. 8). Bethyl's goat anti-bird cross-reacted the best of the two, showing greater antibody concentration and variability, which was expected due to samples being collected from birds experiencing different immune challenge based on the treatment group they were assigned to (experimental or control), as well as the time of sample collection (pre or post injection). The variability in anti-KLH IgY titers measured using Bethyl's anti-bird IgY was more consistent with expected results per sampling period, showing greater antibody detection in birds which should be having primary and secondary responses to injection with KLH, while Sigma's anti-chicken IgY showed a decrease in the post-secondary injection with KLH (Fig.8). However this was tested on a small sample size coming from different birds and should be investigated further on a larger sample size using repeated samples from individual birds to confirm trends. Bethyl's antibody was also more affordable than the Sigma option ($152 vs. $180). The aforementioned indirect ELISA protocol in combination with use of Bethyl's anti-bird IgY costs approximately $60 per plate, a significant cost reduction from commercially available kits, which cost $500 per plate. The Bethyl secondary antibody had greater inter-assay variability (CV = 63.7%)

than did the Sigma secondary antibody (CV = 46.7%), which was corrected for before analyzing sample anti-KLH titers from various plates together. The internal standard (IS) used to assess inter-assay variability was a known concentration of chicken reference serum, so it could be that the Sigma anti-chicken IgY secondary antibody consistently bound to antibodies in the chicken reference serum better than Bethyl's mix of anti-bird IgY due to no cross-reactivity being required for Sigma's secondary antibody, producing less variability in IS reads between assays.

Bethyl's anti-bird IgY is made against heavy and light chain IgY isolated from dove, duck, sparrow, and chicken serum. Light chains are the same for all bird antibody isotypes, so it is possible some antibodies detected were of different isotypes (IgA or IgM; Fassbinder-Orth et al. 2017). Sigma's anti-chicken IgY is made against whole molecule IgY (although the manufacture does not say whether IgY is isolated from serum or yolk). Which could have aided in the slight variation in Nazca booby anti-KLH IgY detection. Additionally, the mix of species that IgY was isolated from for the anti-bird IgY, included species with more recent common phylogenetic ancestors than chickens, which could mean more genetically similar options for antibody binding to Nazca booby IgY.

The current selection of commercially produced test options for immunological studies has limited the focus of these studies to lab-reared, model organisms. With recent advances such as the emerging field of ecoimmunology and models such as the IDCM, it is important for these studies to include novel subjects which frame immune responses in the context of the organism's ecology and life-history under natural (wild) conditions. Furthermore, lab-reared, model organisms, tend to be short-lived species, which are

predicted to have different abilities to fight-off infection than their long-lived counterparts, due to energetic priorities being shifted to a focus on reproduction to maximize fitness. Insight into the immunological processes of wild, long-lived species has the potential to expand our understanding of the underpinnings of these competing processes and how they differ from those of short-lived species. ELISAs have gained more popularity as a test standard in this field for their ability to detect antigen-specific antibodies. This allows researchers to better understand the acquired immune systems and immunological memory of their subjects.

The expansion of these tests to non-model organisms is vital to our understanding of these processes. Some studies have relied on developing secondary antibodies for non-model organisms in order to perform studies looking at life history trade-offs as they pertain to the induction and maintenance of acquired immunity. For example, one study looked at KLH-specific antibodies produced by red-winged blackbirds (*Agelaius phoenicius*) in response to different times during the breeding season using rabbit anti-redwing black bird secondary antibodies produced for the study (Hasselquist et al. 1999). Rather than developing secondary antibodies to a species of interest directly, which requires a tremendous amount of time and resources, many researchers have turned to non-specific secondary antibodies, for cross-reaction with target antibodies (Martínez et al. 2003). Previous studies have reported use of commercially available secondary antibodies to detect antigen-specific antibodies in various avian orders using ELISA. The ELISA protocol and secondary antibody combination developed for use with red-winged blackbirds was also used in a study investigating the breeding success and clutch size of female pied flycatchers (*Ficedula hypoleuca*) whilst experiencing an immune challenge

to immunization with diphtheria-tetanus vaccination, with successful cross-reaction of the antibodies (Ilmonen et al. 2000, 2002). A study looking energetic trade-offs that accompany immunocompetence detected specific antibodies produced by Blue Tits (*Parus caeruleus*) to immunization with diphtheria-tetanus, used anti-chicken antibodies to detect the antigen-antibody complex via indirect ELISA (Svensson et al. 1998).

However, each of the studies mentioned above used short-lived, passerine test subjects. A recent study compared the use of multiple commercially available secondary antibodies (anti-passerine, anti-bird, and anti-chicken all from Bethyl) for cross-reactivity with multiple wild bird species across several orders (Fassbinder-Orth et al. 2017). This study found poor to no cross-reactivity for all secondary antibodies tested with the closest relative of the Nazca booby tested, the double-crested cormorant (*Phalacrocorax auritus*), also a member of the order Suliformes (Fassbinder-Orth et al. 2017). Therefore, my study is the first to identify a secondary antibody that will cross-react with a Suliform species.

My study will serve as a pilot study to investigate short and long-term immune memory in Nazca boobies. With a better understanding of the induction and maintenance of the acquired immune system, this study has potential to advance our understanding of the trade-offs that exist within the branches of the immune system as well as trade-offs between life-history traits competing for energy allocation, such as self-maintenance and reproduction, and how these trade-offs differ between short and long-lived species. With implications such as conservation applications potentially utilizing vaccination programs in wild, long-lived vertebrates, the expansion of this area of study could be vital to many wild species.

FUTURE DIRECTION

The information provided by this study, including the indirect ELISA protocol and the effective cross-reactivity of Bethyl anti-bird IgY with Nazca Booby anti-KLH IgY, will serve as the basis of an extended study looking at the extent of immunological memory of Nazca Boobies. Experimental (EXP) birds injected with KLH in August 2003, were re-injected three months later in November of 2003 (Fig. 4) and again nine years later in October 2012 to assess immune memory responses in short- and long-term time frames.

The ELISA protocol developed in my study should be used to analyze all samples using Bethyl's anti-bird IgY secondary antibody diluted to manufacturer's recommendation (1:30,000) to ensure uniformity of sample analysis. It is my recommendation that a fresh stock of this secondary antibody, as well as other reagents (TMB, Superblock, PBS-tween 20), are used going forward and test plates run for samples from all groups. Nazca booby samples should be analyzed in triplicate at varying dilutions (1:100, 1:50, 1:25) where existing sample volume allows, in order to detect differences in anti-KLH IgY titers between groups. Approaching this project going forward with a goal of perfecting the protocol for this particular secondary antibody will provide cohesiveness to sample analysis and set it apart from this thesis, which was aimed at comparing cross-reactivity of secondary antibodies using a more affordable approach than ELISA kits.

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