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Interpreting ELISA analyses from wild animal samples: some recurrent issues and solutions

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Summary

1. Many studies in disease and immunological ecology rely on the use of assays that quantify the amount of specific antibodies (immunoglobulin) in samples. Enzyme-Linked Immuno Sorbent Assays (ELISAs) are increasingly used in ecology due to their availability for a broad array of antigens and the limited amount of sampling material they require. Two recurrent methodological issues are nevertheless faced by researchers: (i) the limited availability of immunological assays and reagents developed for non-model species, and (ii) the statistical determination of the cut-off threshold used to distinguish individual samples that are likely to have or not to have antibodies against a specific antigen.

2. Here, we outline two solutions to deal with these issues. First, we show that implementing two assays with differing detection methods can help validate the use of reagents, such as antibodies, in species different from their intended target. We illustrate this by comparing the quantification of specific vaccinal antibodies against Newcastle Disease Virus (NDV) using two ELISA approaches in four seabird species (Cory's shearwater, European shag, European storm petrel, and Southern rockhopper penguin).

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3. Second, we provide a simple way to determine from the distribution of ELISA values whether the assayed samples are likely to be made of a single group of individuals (likely negative) or of two groups of individuals (negative and positive). We illustrate the use of this approach with two independent datasets: NDV antibody levels following vaccination and anti-*Borrelia* antibody levels following natural exposure.

4. The practical implementation of these methodological approaches could provide a way to efficiently apply ELISAs and other immune-based assays to address questions in the growing fields of ecological immunology and disease ecology.

Key-words: disease ecology, ELISA, ecological immunology, immuno-ecology, vaccination, Borrelia, Newcastle disease virus, seabirds

Introduction

Many studies in disease ecology rely on the use of assays that allow the quantification of specific antibodies in blood samples, but their application to non-model species and in natural settings raises specific methodological issues (Gardner, Hietala & Boyce 1996; Gilbert *et al.* 2013; Garnier & Graham 2014). In field eco-epidemiology surveys, determining the proportion of individuals of a given species that have circulating antibodies against specific infectious agents (seroprevalence) is notably valuable as a basic description of the proportion of the study population that has been exposed by the agent, but also to understand the dynamics of exposure and response of hosts to infection. This can be crucial

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when suspecting some species to play the role of reservoirs (Haydon *et al.* 2002; Mandl *et al.* 2015). When repeated sampling is possible, it allows the estimation of the incidence (the number of newly seropositive individuals in the population) and access to the history of infection of individuals, which can be very useful for inferring the dynamics of the agents (Keeling & Rohani 2008; Smith *et al.* 2009; Borremans *et al.* 2016). In many cases, the infectious agents of interest are pathogens suspected to circulate in the sampled populations and to be potentially responsible for disease outbreaks and/or mass mortality events (e.g., Atkinson & Samuel 2010; Ward *et al.* 2010; Peel *et al.* 2012; Duignan *et al.* 2014; Gorsich *et al.* 2015). However, even when pathogens do not appear to have a direct effect in wild animals, serological studies may prove crucial to understand the dynamics of pathogens that may spillover to humans such as plague (Stenseth *et al.* 2006) or bat-borne viruses (e.g., Hayman *et al.* 2012; Peel *et al.* 2012; Ogawa *et al.* 2015).

In ecological immunology studies, the plasma concentration of specific antibodies is often quantified in the context of experimental challenges of the immune system, which are done using purified proteins, such as Keyhole Limpet Hemocyanine (KLH), or vaccines (Staszewski & Boulinier 2004). These approaches aim at exploring possible trade-offs between the humoral immune response and other traits (Sorci *et al.* 2009; Demas & Nelson 2011). In addition, such studies are generally carried out in the wild and involve non-model species (i.e. not mice or chickens; Pedersen & Babayan 2011). The availability of specific reagents may, in that case, impose some limitations on the immunological analyses to be carried out as well as on the analyses of the results of the available assays.

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Most assays indeed rely on the use of conjugated antibodies that may be expected to bind to either antibodies of the considered species or to free antigens (Boughton, Joop & Armitage 2011). Most techniques require the use of enzyme-conjugated secondary antibodies. These secondary antibodies, when given the correct substrate, produce a colour whose intensity at a specific wavelength is correlated to the amount of antibodies in the original samples (Wild 2013). There are however significant differences in the specificity of antibodies required by different Enzyme-Linked Immuno-Sorbent Assay (ELISA) techniques. Competition assays rely on secondary antibodies specifically targeting the antigen of interest, for which they are competing with the antibodies of the species of interest present in the sample. These antibodies then saturate unused antigens, and higher colour intensities indicate lower antibody levels. In other words, the more colour is inhibited (referred to as 'percentage of inhibition', PI) the higher the concentration of antibodies in the original samples. The main limitation to set up competitive ELISAs is the lack of antigen-specific antibodies. These are not generally available, because ELISAs are rarely developed with wildlife infectious diseases as their main target. In indirect ELISAs (and in Western blots), the secondary antibodies target the antibodies of the species of interest that are bound to the antigen of interest. The intensity of the coloured reaction is then directly proportional to the amount of antibodies in the original sample. Indirect ELISAs are generally more widely commercially available than competitive ELISAs or easier to set up in the laboratory as they do not require antigen-specific secondary antibodies. However, ideally, the secondary antibody used should be specific of the species from which the sample originated to maximize the detection of antigen-antibody complexes.

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This is unfortunately rarely the case in ecological immunology studies for which species-specific reagents are largely lacking. In some cases, reagents developed for other species have been used successfully (reviewed in Garnier & Graham 2014). In the case of antibodies, their ability to bind to non-target species is likely variable and makes their use for interspecies comparative studies challenging or the estimation of seroprevalence difficult in large scale serological surveys spanning a wide range of species. A way to circumvent this problem is to use non-specific binders such as protein M (Grover *et al.* 2014) or protein A (e.g., Baker *et al.* 2014), but again the determination of positivity thresholds is non-trivial and thus prevalence estimates may be difficult to obtain or compare between studies.

Two recurrent methodological issues are thus faced by researchers in disease and immune-ecology: (i) the limited availability of assays measuring antigen-specific immunoglobulins that have been developed for non-model species, and (ii) the difficulty of statistically determining the proportion of individuals in a sample that have (or conversely do not have) detectable levels of antibodies against a specific antigen. Here, we investigate two simple practical solutions to deal with those issues. First, we show that the implementation of competitive ELISAs in a controlled setting can be used to validate the use of indirect ELISAs relying on conjugate antibodies that have been developed for a species phylogenetically related to the one studied, e.g., anti-chicken antibodies for wild bird species. We illustrate this by comparing the quantification of specific immunoglobulin Y (IgY; the avian functional equivalent of the mammalian immunoglobulin G) using two ELISA approaches in four seabird species. To control exposure, we vaccinated Cory's shearwaters (*Calonectris borealis*), European shags (*Phalacrocrorax aristotelis*), Mediterranean storm petrels

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(*Hydrobates pelagicus melitensis*), and Southern rockhopper penguins (*Eudyptes chrysocome*) against Newcastle Disease Virus (NDV). We expected strong positive correlations between the two ELISAs to validate the use of the cross-reactive antibody. Second, in line with previous considerations (Hens *et al.* 2012), we provide a simple way to determine from the distribution of ELISA values whether the assayed samples are likely to be made of a single group of (likely negative) individuals or of two groups of (negative and positive) individuals. We use two independent serological datasets to illustrate the usefulness of this approach, one produced using vaccination of Cory's shearwaters against NDV and another tracking natural exposure of Indian yellow-nosed albatrosses (*Thalassarche carteri*) to *Borrelia burdgorferi sensu lato*. We believe that the practical implementation of these methodological approaches could provide an efficient way to improve the interpretation of ELISAs and other immunological assays in ecology and evolution.

MATERIALS & METHODS

Validating indirect ELISAs using competitive ELISAs

ELISAs are probably the most common immunological assay used in non-model species. Two main types of ELISAs exist, differing only in the way the level of antibodies in the serum (or plasma) is quantified: the secondary antibody binds either to antigen-antibody complexes (indirect ELISA) or to the remaining "free" antigen (competitive ELISA). We proposed to run a competitive and an indirect ELISAs on the same set of samples in order to validate the use of conjugated antibodies in wild species: if the non-species-specific secondary antibody can

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be used in a given species, then the results from the indirect ELISA should correlate with the results of a species-independent (but less widely available) competitive ELISA. For illustration, we used seabird species and an indirect ELISA assay using an anti-chicken conjugate, which we predicted would effectively quantify specific antibodies in plasma samples. An anti-bird secondary antibody is also available (e.g. Seidowski *et al.* 2010), but because it was only developed with a small set of wild birds, it would only represent another, less widely available, cross-binding secondary antibody.

Samples were obtained from control birds and from birds vaccinated using an inactivated vaccine commonly used in ecological immunology targeting NDV (Nobilis Paramyxo P201, Intervet), a pathogen thought to be *a priori* absent in the populations we considered, and for which long term persistence of specific antibody level had been established in seabird species before (Staszewski *et al.* 2007). As part of a comparative study on maternal antibody decay in seabirds (Garnier et al. 2012, Ramos et al. unpublished), birds were vaccinated with a single subcutaneous injection of 0.25 ml of the NDV vaccine (0.10 ml on Mediterranean storm petrels) about three months to a year prior to blood sampling and the competitive ELISA was used to estimate the levels of anti-NDV antibodies. The work was conducted on Cory's shearwaters in the Canary Islands (Spain), on European shags on the Isle of May (Scotland), on Mediterranean storm petrels on Benidorm Island (Spain), and on Southern rockhopper penguins in the Falkland/Malvinas Islands. Blood samples were taken using heparinized syringes. After centrifugation, the plasma was stored at -20°C until analyses. A subset of samples was chosen for analysis with both the indirect and competitive ELISAs (Southern rockhopper penguin: n = 20; Cory's shearwater: n = 28; European shag: n =20; European storm petrel: n = 20) so that they would encompass the range of values of the

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competitive ELISA. They were run by the same person over a single day. Optical Densities (OD) at 450 nm (in both cases) were read on a spectrophotometer. For the competitive ELISAs (ID Screen® Newcastle Disease Competition kit, IDVet, Montpellier, France), the Percentage of Inhibition (PI) values are used, while for the indirect ELISAs (ID Screen® Newcastle Disease Indirect kit, IDVet, Montpellier, France), the $\log_{10}[(S/P) + 3.520]$ are used following the manufacturer's instructions. PI and S/P ratios were calculated using the OD of the sample and the mean OD of the negative and positive controls (NC and PC, respectively) of respective kits as follows: $PI= [(OD_{NC} - OD_{sample})/OD_{NC}] \times 100$ for the competition ELISAs and $S/P=[(OD_{sample}-OD_{NC})/(OD_{PC} - OD_{NC})]$ for the indirect ELISAs.

Determining the proportion of samples that are seropositive

In many cases when assays are adapted to wild species and ecological settings, it is difficult to set an *a priori* seropositivity threshold because of the lack of positive controls. Here, similar to approaches used for the Luminex quantification of fruit bat antibodies against Hendra virus (Burroughs *et al.* 2016) or in wild rodents exposed to *Coxiella burnetii* (Meredith *et al.* 2015), we propose to apply a simple statistical approach to (i) determine whether there are likely seropositive and seronegative samples in a dataset coming from an ELISA conducted in samples from a species, and (ii) set a positivity threshold. The approach assumes that the OD/PI values of positive and negative individuals would be both normally distributed around two different mean ODs (indirect ELISA) or PIs (competitive ELISA) with different standard deviations. When using ODs from indirect ELISAs or PI for competitive ELISAs, the mean of negative individuals would be the lowest one; this would be reversed when using OD values from a competitive ELISA. Using the packages 'MASS' (Venables &

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Ripley 2002) and 'mixtools' (Benaglia *et al.* 2009) in R, we propose to fit respectively one normal or a mixture of two normal distributions for the observed OD values. We then calculate the Akaike Information Criterion (AIC) for each fit and select the best fit accordingly (Burnham & Anderson 2002). Because the mixture model would capture either a bimodal population (if there is a sizeable part of the population positive) or a long tail due to several positive individuals, we expect the mixture model to provide a better fit than the single distribution model when there are positive individuals in the dataset. When the mixture model is statistically significantly different from the model assuming a single normal distribution (i.e. when the difference is AIC is over 2 points), we consider that positive individuals are detected above a threshold calculated as mean[negative normal] + 3 SD[negative normal], which corresponds to a 99% confidence interval. If negative individuals have higher ODs (in the case of OD values of competitive ELISA), the standard deviation should be subtracted rather than added to their mean to obtain a cut-off value. For ease of understanding, we however recommend the use of PI with competitive ELISAs. Otherwise, we consider that there is no positive individual detected. It should be noted that the distributions may be influenced by other factors such as age or time since infection (especially if the pathogens are epizootics and exposure may be limited in time). However, such information is rarely available in the wild. It is thus important to demonstrate the usefulness of the method without such information being taken into account.

As an illustration of the approach, we apply it to data (i) on NDV antibody levels following vaccination or sham injection in adult female Cory's shearwaters (see details of this dataset in Garnier et al. (2012); n = 94) and (ii) on anti-*Borrelia burdgorferi sensu lato* (*Bbsl*) antibody levels in breeding Indian yellow-nosed albatrosses (*Thalassarche carteri*; n = 49), sampled in

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2013 on Amsterdam Island (French Subantarctic territories) as part of an eco-epidemiology monitoring program. Lyme disease *Borrelia* transmitted by the seabird tick *Ixodes uriae* has been reported to circulate naturally in many seabird colonies (e.g., Olsen *et al.* 1995; Staszewski, McCoy & Boulinier 2008). Because of the large geographical range of this bacteria, we expected some level of exposure in the sampled population of yellow-nosed albatrosses. The anti-*BbsI* antibodies kit (Borrelia + VIsE IgG ELISA, IBL International, Hamburg, Germany) was manufactured for human use and designed to recognize mammalian antibodies: we thus replaced anti-human IgG antibodies by an anti-chicken IgY antibodies conjugated with peroxidase (A9046, Sigma-Aldrich, St. Louis, Missouri, USA), as done in previous studies (e.g., Staszewski, McCoy & Boulinier 2008; Lobato *et al.* 2011). We then followed the kit manufacturer instructions and ODs were obtained at 450nm.

RESULTS

Validating indirect ELISAs using competitive ELISAs

Comparisons between indirect and competitive ELISAs were performed in four seabird species, each with distinct distributions of OD values (Figure 1). These values ranged from relatively continuous (in the Cory's shearwater) to almost bimodal with a group of high values and a group of low values (in the European storm petrel). The results of both ELISAs were nevertheless highly positively correlated for all of the four species considered with R^2 values ranging from 0.73 up to 0.88.

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Determining the proportion of samples that are seropositive

We illustrate the statistical approach to determining cut-off values for indirect or direct ELISAs by presenting two contrasted situations (Figure 2). We first focus on the Cory's shearwaters - in which some females were vaccinated and others were sham injected as negative controls. The left panel of Figure 2 presents the distribution of PI values following this experiment. The statistical approach clearly selects the two normal distributions over the single normal fit (ΔAIC = -119), and the probability density functions of these distributions are clearly non-overlapping (Figure 2, Shearwater panel). As a result, the predicted cut-off value (PI% = 37.6%) properly classifies all control females as negative and all vaccinated females as positive. It is of note that this classification is in full agreement with that provided by the kit's positive cut-off (PI% > 40%).

However, this ideal situation is unlikely to arise with natural infections and only serves as a proof of concept. The right panel of Figure 2 presents serological data from natural infection of 49 breeding Indian yellow-nosed albatross with *Bbsl* and because the technique used in this case is an indirect ELISA developed for humans, it is impossible to use the manufacturer's instructions to determine a cut-off value. In that case, such a statistical approach again favours the mixture of normal over the single normal distribution (ΔAIC = -40). The two distributions are overlapping, but the one corresponding to the negative samples has a lower standard deviation and allows for the calculation of a cut-off value (broken line on figure 2, Yellow-nosed albatross panel). The predicted seroprevalence for *Bbsl* in Indian yellow-nosed albatrosses on Amsterdam Island is thus 12/49 individuals (24.5%). It should be noted that this prevalence estimate is conservative as the cut-off has been set to mean+3 SD, which corresponds to a 99% confidence interval. Reducing this cut-

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off to 2 SD of the mean (which would be a usual 95% confidence interval) would increase the seroprevalence to 15/49 (30.6%).

DISCUSSION

Given the current interest in, and arguably need for, serological assays to detect specific antibodies in disease ecology (Gilbert et al. 2013) and ecological immunology (Garnier & Graham 2014), it is important that practical methodological approaches are made available. Here, we addressed two simple practical issues that are commonly met by ecologists attempting to use such assays. We proposed a simple way to validate the use of commercially available conjugate polyclonal antibodies developed against model species (such as anti-chicken antibodies for wild birds) and we illustrated this with the quantification of antibodies against NDV in four seabird species. We also outlined the use of an AIC-based maximum likelihood approach to discriminate seronegative and seropositive samples when no *a priori* threshold of positivity or positive controls are available.

The results we obtained regarding the validation of the NDV indirect ELISA using a competitive ELISA are in line with previous reports of the use of commercial anti-chicken antibody for quantification of IgY of various wild species (e.g., Martinez *et al.* 2003). However, variability can exist depending on the polyclonal set of antibodies used and the wild species considered, highlighting the necessity of a practical validation that can be implemented in a wide array of species. In addition to the validation of the technique, this result has specific implications for the Cory's shearwater in which maternal antibodies can

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last much longer in nestlings than was known for chickens (Garnier *et al.* 2012). Interestingly, our study sheds light on the mechanism behind this long persistence. It was indeed hypothesized by Garnier et al. (2012) that specific characteristics of the IgY in this species could have interfered with the binding to anti-chicken antibodies. This does not appear to be the case given the congruent results between the competitive and the indirect ELISA approaches. This would indicate some level of conservation in the structure of IgY between seabird species and chickens. Further analysis, particularly at the gene/genome level, would help shed more light on these evolutionary aspects. This is also important because commercially available conjugated antibodies(such as the anti-chicken IgY) may be more widely available and can be used to adapt a variety of immune-based assays that have been developed for non-closely related species (e.g. ELISA and Western Blot kits developed to analyse human samples to detect antibodies against Lyme disease bacteria; Staszewski, McCoy & Boulinier 2008).

If reagents can be validated using the process we describe, the criteria for positivity associated with the serological technique may be used directly. However, this is not always possible as it requires the existence (or the possibility to develop) both a competitive and an indirect assay. If no gold standard is available, the *ad hoc* method outlined to determine the proportion of samples that are positive and the likelihood that a given sample is positive may be especially useful. This may be important in several cases. ELISA developed for domestic or model species can be used in wild species which puts the validity of the kit's positive controls into question. Similarly, when individuals have unknown natural history of exposure to the infectious agent, the distribution of ODs may be insufficient to determine

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the positivity in the absence of known positive individuals. This may be particularly important when several species have been sampled in a community and inter-species comparisons are made (e.g. Lobato *et al.* 2011; Miguel *et al.* 2013). Although it provides an objective way to assign individuals to a positive or negative status, it does not address the issue of false positives or false negatives (McClintock *et al.* 2010; Hens *et al.* 2012). The exact manner in which the cut-off is determined may also be of importance (Peel *et al.* 2013). For instance, Burroughs et al. (2016) use the intersection point of the distributions while we propose to use the mean + 3 SD as a cut-off point. We argue that our choice is more conservative as it ensures that 99% of possibly negative samples are placed below this cut-off (i.e. we minimize the risk of false positives). However, using the intersection point maximizes the correct assignment of individuals to either distribution and thus minimizes the risk of false negatives. This flexibility may be interesting depending on the context of the study. For instance, when screening wildlife for a possible emerging infectious disease, minimizing false negatives may be crucial. For prospective serological survey of endemic diseases, the reverse may be true. Another approach to setting the cut-off, particularly with bigger datasets, would rely on a bootstrap analysis to obtain confidence intervals around the cut-off value. Such issues can, and whenever possible should, be addressed via the analysis of samples using different assays and through modelling to account for potentially false negatives (for instance in a multistate occupancy framework; Elmore *et al.* 2014). When individuals can be recaptured over a series of sampling occasions, transition between immunological states can also be estimated together with their survival (Chambert *et al.* 2012; Choquet *et al.* 2013).

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One issue that we have not dealt with here regards the specificity of the ELISAs, which can be problematic if it is not acknowledged (Peel *et al.* 2014). In some cases, cross-reactive antibodies may result in false positive results and bias the estimates of seroprevalence. Assays that are more specific for a group of closely related infectious agents than for very specific pathogens may nevertheless be very useful as a first screening purpose or when it is independently confirmed that only one particular agent or strain of a group of closely related pathogens is circulating in the studied population. One example is that of serological studies on Avian Influenza viruses. An ELISA targeting the nucleoprotein, an antigen known to be shared by all the subtypes of the virus, is generally used to first screen efficiently all the samples. In a second step, the ELISA positive samples can be analysed using inhibition of haemagglutination assays specific to each viral subtype (Lebarbenchon *et al.* 2015) or increasingly available multiplex assays (Freidl *et al.* 2014). Another example deals with the circulation of flaviviruses in bird populations: ELISA screening using an assay specific for a broad range of flaviviruses has been efficiently implemented to detect populations with positive samples that have then been analysed using more specific virus neutralization assays (Arnal *et al.* 2014).

Serological assays that allow the detection of antibodies in sera or plasma (or egg yolks; Hammouda *et al.* 2014) have the potential to provide key information regarding the temporal dynamics of ecological exposure to infectious agents. They can represent complementary, and potentially more powerful, sources of information than direct detection of pathogens, as the latter can only be positive while the pathogen is being directly present in the blood or swab samples whereas the ability to detect antibodies can

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last for months or years after exposure for many infectious agents. Provided careful sampling and experimental frameworks are followed (Garnier & Graham 2014), these methodological approaches will likely still remain useful tools in disease and immune-ecology for many years, in complement to increasingly available molecular methods.

Authors contributions

RG, RR and TB conceived the experiments. RR, ASA, MP, HW, SB and JT performed the fieldwork. RG and RR performed the laboratory experiments. RG and RR analysed the data. RG and TB wrote the first draft of the manuscript. All authors contributed to the final version of the manuscript.

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Data and code availability

The datasets used in both the validation of the ELISA technique and the determination of the cut-off are available on Dryad: doi:10.5061/dryad.0qk1h (Garnier et al. 2017).

The annotated R code for the determination of the cut-off (in text format) is provided as a supplementary file.

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Figure legends

Figure 1. Correlation (and p-value) between the logarithm of the antibody level against NDV measured using an indirect ELISA with anti-chicken conjugated antibodies (X axis) and a competitive ELISA (Y axis) in four seabird species.

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Figure 2. Distribution of Percentage of Inhibition (PI) for Cory's shearwaters females vaccinated against NDV (left panel) and of Optical Density (OD) values for Indian yellow-nosed albatrosses used to quantify natural exposure to *Borrelia burdgorferi* sensu lato (right panel). The histograms present the normalized counts of individuals and the curves correspond to the probability density function of the two normal distributions (negative: green curve; positive: red curve). The broken line corresponds to the cut-off value estimated by the statistical model (mean of negative normal + 3 standard deviations).

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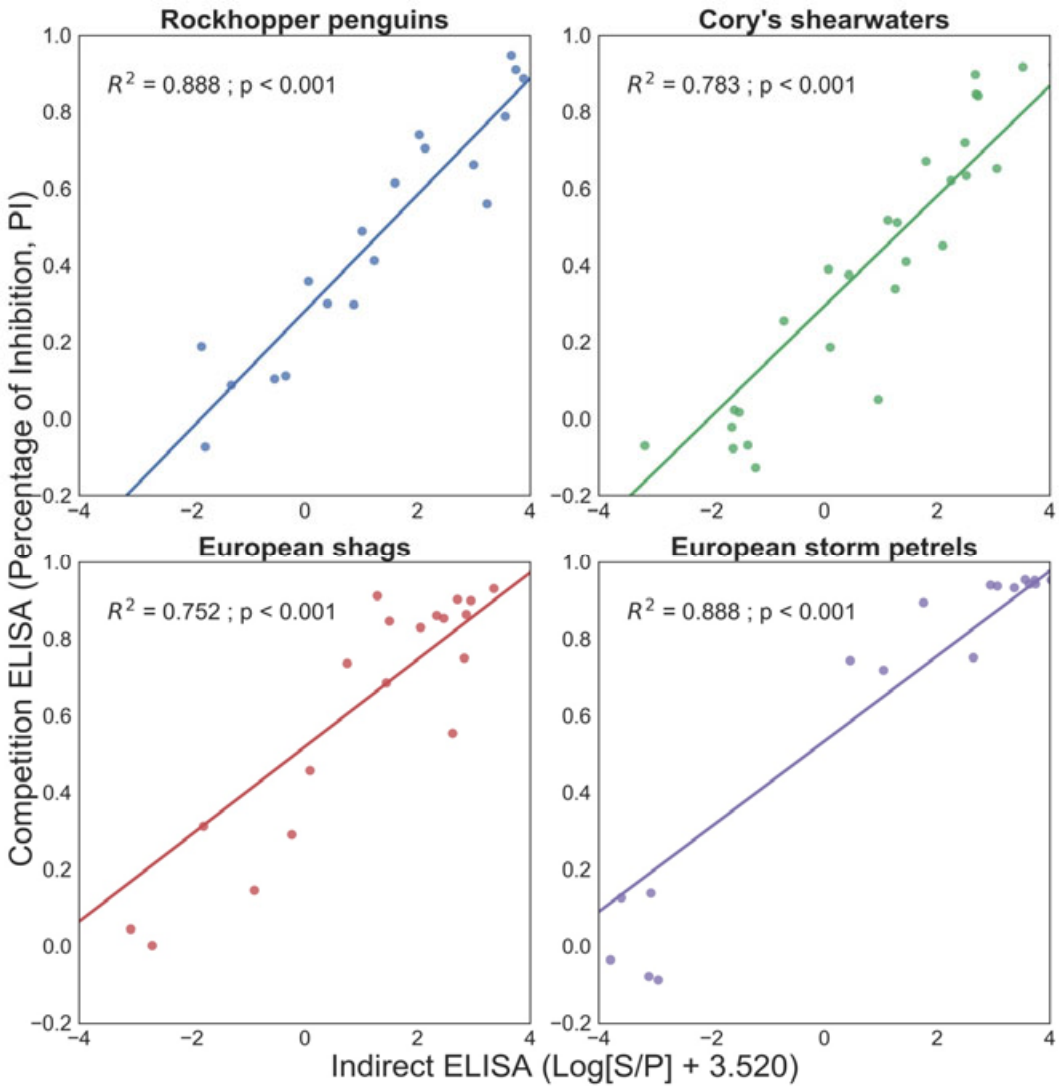

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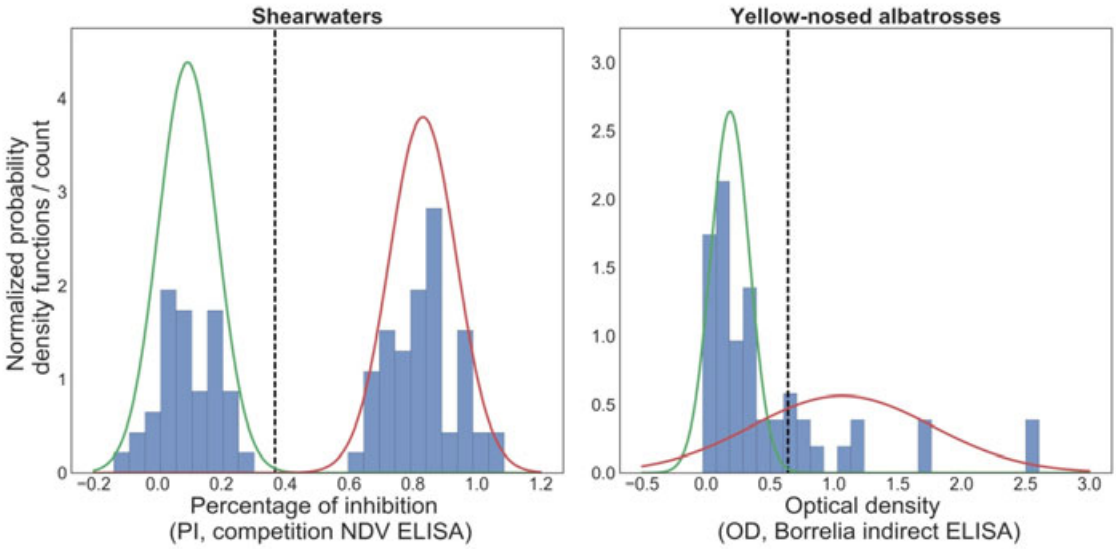

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