

Simplifying the phytohaemagglutinin skin-testing technique in studies of avian immunocompetence

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Summary

1. Researchers involved in ecology and toxicology, as well as many other aspects of avian biology, use phytohaemagglutinin (PHA) skin testing as a means of evaluating the immune status of individuals.
2. Immune function, one measure of individual quality, can be used as a sensitive, non-lethal variable that may be negatively affected in animals exposed to degraded, contaminated or otherwise disturbed ecological zones.
3. Typically this test has been applied by challenging one wing web with the immunostimulant PHA, while the other 'control' wing is injected with phosphate buffered saline (PBS). Injection sites on the wing web are measured before and 24 h after injection with PHA or PBS. The immune response is considered to be the difference between the two wings.
4. Results from PHA skin tests conducted on 608 birds in seven studies representing passerines, waterfowl, upland game birds and raptors are examined.
5. Numerous advantages to eliminating the PBS injection as the experimental control are: (i) decrease by half, the time required for testing; (ii) decrease handling-related stress on the birds (proportional to handling time); (iii) reduce the probability of errors at injection time; (iv) spare the other wing for different tests or uses (e.g. tuberculin DTH testing); and (v) decrease the coefficient of variation that is due to measurement inaccuracies.
6. The only disadvantage identified is that hypersensitive individuals (outliers) could be missed, which in this case represents 2 of 608 individuals.

Key-words: Avian immune response, phytohaemagglutinin skin test, T-lymphocyte immune response, wildlife bioindicators

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Introduction

Measures of immune function in birds, while routine in traditional areas of pathology and physiology of domestic fowl (e.g. Goto *et al.* 1978; van der Zijpp 1983; Laudert, Sivanandan & Halvorson 1993; Chu *et al.* 1995), have now been recognized as valuable tools to study behaviour, ecology and toxicology of wild species (e.g. Lochmiller, Vestey & Boran 1993; Dufva & Allander 1995; Saino, Calza & Møller 1997; Svensson & Skarstein 1997; Moreno *et al.* 1998). Variables related to immune function such as differential and total white blood cell counts, and production of antibody-producing lymphocytes against sheep red blood cells (SRBC) are commonly used but require specialized training or laboratory facilities. An inexpensive, easily applied skin test using phytohaemagglutinin (PHA) as a mitogen, has become a popular,

effective test that can be applied with minimal training and without specialized equipment.

The skin test provides a measure of the proliferative response potential of circulating T lymphocytes to an injected mitogen. Phytohaemagglutinin has long been recognized for its mitogenic and blastogenic properties (Hungerford *et al.* 1959). This test has been routinely applied, with some variation in the body sites used, according to a long-established protocol developed in poultry science (Goto *et al.* 1978). In the seminal work of Goto *et al.* (1978), histological assessment of skin that had been injected with 75 µg PHA revealed an infiltration by heterophils within 3 h. The transient heterophilia, a non-specific inflammatory reaction to mild antigenic stimulus, was no longer evident 6 h after injection. The main cellular response, observed at 6–12 h after injection, consisted of a prominent perivascular accumulation of

small lymphocytes. Macrophage infiltration was evident by 24 h, and the PHA stimulated hypercellularity had disappeared by 48 h postinjection. The lymphocytes, now referred to as T lymphocytes, were proven to be of thymic origin, since they did not appear in thymectomized chickens challenged with intradermal PHA.

The PHA skin test is being routinely applied in many avian studies, according to a long-established, standard protocol, which we believe may be conducted more efficiently, and without compromising precision of the test. Briefly (see Methods), the immune response is considered to be the difference in swelling in wing webs (patagia) injected with PHA and phosphate buffered saline (PBS) as a control. We believe that simplifications can be made to this test which have several advantages. In this paper we compare the traditional and a simplified approach on a phylogenetically diverse array of birds in seven different studies with variable objectives. By presenting such a diversity of results and including birds exposed to a variety of treatments, our proposal is compatible with the objectives of most ecological research projects.

Materials and methods

STANDARD PHA SKIN TEST OF CELL-MEDIATED IMMUNITY

The proliferative response of T lymphocytes was tested using the standard protocol for avian species (Goto *et al.* 1978; McCorkle, Simmons & Luginbuhl 1982; Kean & Lamont 1994). Briefly, in birds with feather cover, a 1-cm patch of skin on the mid-patagium was either plucked or trimmed clean of feathers and down on both left and right wings. For nestlings, no feather removal was necessary. The bare skin was swabbed with alcohol just prior to injection. In the left wing web, 20–40 µg PHA (see Table 1 for details on dosing details) in 20 µl of sterile PBS was injected subcutaneously with a 27-g needle, in small passerine nestlings. In the larger species, different PHA dosages

and injection volumes were used in an attempt to accommodate the size of the species. The same volume of sterile PBS as was used in the right wing was injected into the left patagium. Patagium thickness was measured to 0.01 mm, using either a digital micrometer (Mitutoyo 0–1 inch, Tokyo, Japan), or a gauge micrometer (Dyer OD gage 0.01 mm, The Dyer Company, Lancaster, PA) (study two only). Two to four measurements of patagium thickness were taken immediately prior to the injection, and again 24 h after injection.

AVIAN STUDIES PROVIDING DATA FOR ANALYSIS

We draw upon our experience in PHA skin testing from seven different studies of both wild and captive birds (see Table 1 for details). In study one at Simon Fraser University, Burnaby, British Columbia, the toxicology of Oil Sands consolidated tailing water was examined in nestling Zebra Finch (*Taeniopygia guttata*). Study two was an ecotoxicology study including wild Tree Swallows in the Oil Sands of Fort McMurray, Alberta. The immunotoxicity of mercury to captive Northern Pintail Ducks (*Anas acuta*), study three, was conducted at the University of Saskatchewan, Saskatoon, Saskatchewan. Studies four and five on captive American Kestrels (*Falco sparverius*) at McGill University, Montreal, Québec, dealt with behavioural, reproductive and immunological aspects of PCB exposure. Behavioural ecology was the primary focus of study six on wild American Kestrels on Besnard Lake, Saskatchewan. The seventh study involved the nutrition and ecology of captive Red-Legged Partridges (*Alectoris rufa*) in Jaén, Spain.

These studies covered a wide range of environmental and experimental conditions. The size of the experimental subjects was greatly variable among the studies (body mass ranged from 9 g to 800 g), the birds being investigated ranged in age from 8-day-old altricial nestlings to mature adults in both the wild and captive species. The situations under which the measurements were made were equally variable, from sin-

Table 1. Seven avian studies in which the PHA skin tests of immune function were analysed. The doses (µg) of phytohaemagglutinin (PHA) dissolved in sterile phosphate buffered saline (PBS) (injection volume µl) were injected into the right patagium. The same volume of PBS alone was injected into the left (control), patagium. Experimental conditions dictated the time span (sampling duration) required to test all the animals

Study	Species	Body mass (g)	Age	Locale	Dose (µg)	Injection vol. (µl)	Sampling duration (days)	Treatment	Investigator ^a
1	Zebra Finch	9	9 days	Captivity	20	20	10	Mine tailings and dexamethasone	J.E.S.
2	Tree Swallow	15	8 days	Wild	40	20	21	Mine tailings	J.E.S.
3	Northern Pintail	800	> 1 year	Captivity	100	100	1	Mercury	J.E.S.
4	American Kestrel	120	19–30 days	Captivity	50	50	5	PCBs	J.E.S.
5	American Kestrel	120	> 1 years	Captivity	50	50	1	PCBs	J.E.S.
6	American Kestrel	120	22 days	Wild	50	50	22	Cross-fostering	J.L.T.
7	Red-Legged Partridge	450	> 2 years	Captivity	100	100	2	Diet	J.L.T.

^aPerson injecting and measuring the wings.

gle days in a controlled laboratory environment, to many days in the field (Table 1). Such variation within and between studies was valuable in allowing the evaluation of as wide an array of immune responses as possible.

THE MEASUREMENT CHALLENGE

The major challenge in applying the PHA skin test is deriving the actual measurements of wing web thickness. This is believed to be the most error-prone part of the test. Measuring can be tedious, imprecise and fraught with complicating factors such as a minute bit of down caught under the micrometer contacts, or a developing, intradermal pin feather, or eye fatigue resulting in focusing difficulty while determining the exact time of contact between the measuring instrument and the skin. Details of our techniques are as follows: the bird is restrained by an assistant while the person who is measuring places the micrometer over the injection site. In small species the contact points of the micrometer may actually be larger than the entire patagium. When working with birds with very delicate, thin skin, the question of how to define adequate contact between the instrument and skin surface must be determined. In some cases, micrometers are spring loaded to a constant light pressure. However, in most of the species described here, it was not possible to use the manufacturer-adjusted pressure without distorting the wing thickness through compression. To

avoid this artefact which would lead to inaccuracies, as soon as micrometer contact caused the skin to begin twisting, the contact was gently released just until the skin orientation went back to normal. Then the reading was made. Each wing was measured at least in duplicate and the mean of the values was recorded.

Early on in the use of skin testing, researchers should establish the repeatability of their measuring technique, which becomes quite high with training and practice. The repeatability of measurements (Lessels & Boag 1987) taken in quadruplicate on fledgling American Kestrels (study six, $n = 142$) was high: $r = 0.99$, $F = 652.3$, $P < 0.001$, two-tailed.

The 'PHA response' of each bird (i.e. the measure of interest in this skin test) was determined using two different methods, to compare the traditional method with our simplified version. First the difference between the responses to PHA and PBS (i.e. each wing independently) was calculated. The change in thickness (mm) of the PBS (left) wing web (24 h postinjection thickness minus preinjection thickness), was then subtracted from the change of the PHA (right) wing thickness (i.e. variable called 'PHA-PBS' in Table 2). An alternate variable, 'PHA only', was used to express the T-cell response by using only the increase in thickness of the PHA-injected wing web (i.e. post – preinjection) ('PHA only' in Table 2).

Results

Comparisons between 'PHA-PBS' and the 'PHA only' responses of a total of 608 birds are made in Table 2. Pearson product moment correlation between these variables within each study revealed a very close association (see also Fig. 1). In studies three and five, there are two sets of analyses, one including and one excluding the data from the one bird in each study that showed an anomalous reaction (Fig. 2). Not surprisingly, in both cases the correlation became stronger when the outliers were omitted, while the SD and CV decreased slightly (Table 2). Overall, the response to PHA was so much greater than the (non)response to PBS, that the PBS control may be considered an effort ill-spent. Examination of the skin reactions to PBS injection in 142 American Kestrels and 271 Tree Swallow nestlings (Fig. 3a,b) revealed that in 30–35% of the birds, there was either no change or decreased thickness 24 h after injection. Those that did react positively had wing thickness increases of $\leq 250 \mu\text{m}$ in both species, which would barely differ from measurement error.

However, two individual birds had aberrant reactions to the test (Fig. 2). The birds in which the PHA test signalled an unusual, exuberant immune response in both wings (studies three and five), notes in the field manuals had described the bilateral injection sites as being hyperemic and remarkably enlarged. When an unexpected reaction occurs after PHA testing, there are two plausible explanations. The

Table 2. Comparisons of summary statistical parameters, mean (mm), standard deviation (SD), coefficient of variation (CV) and correlation coefficient (r) for two different methods of evaluating the T lymphocyte-mediated immune response in birds. Variable 'PHA-PBS' is the difference between the changes due to PHA and PBS treatments of the left and right wings, while 'PHA only' is the increase in wing web thickness before, and 24 h after injection with PHA

Study no. ^a	Variable	N	Mean	SD	CV	r^b
1	PHA-PBS	26	0.48	0.46		0.845
	PHA only	26	0.57	0.44	76	
2	PHA-PBS	271	0.86	0.35	41	0.949
	PHA only	271	0.93	0.35	37	
3	PHA-PBS	14	1.44	0.70	49	0.896
	PHA only	14	1.67	0.51	31	
	PHA-PBS ^c	13	1.57	0.53	34	0.980
	PHA only	13	1.71	0.51	30	
4	PHA-PBS	46	1.65	0.54	32	0.945
	PHA only	46	1.69	0.55	33	
5	PHA-PBS	50	1.68	0.49	29	0.887
	PHA only	50	1.75	0.64	36	
	PHA-PBS ^c	49	1.66	0.48	29	0.956
	PHA only	49	1.70	0.50	30	
6	PHA-PBS	142	2.73	0.74	27	0.996
	PHA only	142	2.76	0.74	27	
7	PHA-PBS	59	1.78	0.62	35	0.982
	PHA only	59	1.84	0.61	34	

^aSee Table 1.

^bPearson product moment correlation coefficient, all $P < 0.001$.

^cWith outlier removed.

researcher must consider the possibility of mistakenly injecting PHA in the wrong wing, but the contralateral swelling should help detect such an error if the bird's immune response is fairly normal. This possibility could be ruled out in favour of a hypersensitivity reaction in study three, based upon earlier tests in which the same individual duck had responded abnormally to a similar skin test.

Discussion

Because all the PHA skin tests within each study were conducted by one of two investigators, the experimental error due to individual injection and measuring techniques was minimized, thus enhancing the repeatability, and hence precision, of the measurements.

The advantages of conducting PHA skin tests using one wing only, with the preinjection measurement as

the control for the PHA mediated response, are multi-fold. First, the time required to conduct the test will be decreased by half, allowing the testing of higher numbers of animals, if that is a practical option. Alternatively, with the decrease in time required for testing, investigator fatigue can be reduced, which will, in turn, decrease the probability of error. With shorter handling times, the birds will experience less psychological stress, and reduced physiological stress, which should result in lower circulating corticosterone levels. This, in turn, will produce responses that are less affected by corticosterone-induced immunosuppression. Using only one test on one wing eliminates the opportunity for error that can readily arise during the actual physical application of two different, but very similar, treatments of the left and right wings. Subsequently, with fewer data to enter, once again, less time is required, and fewer opportunities for errors in data input exist. Another advantage for working with birds in which immunological function constituted part of the studies is that the other wing is spared for other types of testing, such as delayed type hypersensitivity tests to tuberculin.

In rare circumstances, the difference between left and right side responses, provided both the PHA-PBS and PHA only variables are examined, could alert the investigator to a hypersensitive individual, or one in which the immune response is otherwise abnormal. Without the comparative wing test, oddly responding individuals may go undetected, or their responses may be incorrectly interpreted as hyperreactivity of T lymphocytes. The most plausible explanation for the outlier birds in two of our studies would be that the excessive bilateral swelling seen in these cases was due to a hypersensitivity reaction, which denotes a severe reaction to a normally harmless material (Tizzard 1996). Instead of a specific T lymphocyte-mediated immune response (upon which the PHA skin test is predicated), a local, non-specific inflammatory response has been incited either by the PBS buffer or by a minute amount of skin-associated antigen introduced during injection.

Because two different people, five different avian species and seven different experimental conditions existed in the studies, the PHA skin test holds up as a robust technique for measuring one aspect of cell-mediated immunity in birds. Results, therefore, could be expected to be similar for other wildlife workers who use these techniques. We conclude that based upon our analyses of 608 individuals, this technique would be best applied using as the control value, the preinjection thickness of the test wing web. The changes to the testing protocol proposed here may reduce inaccuracies inherent in this technique.

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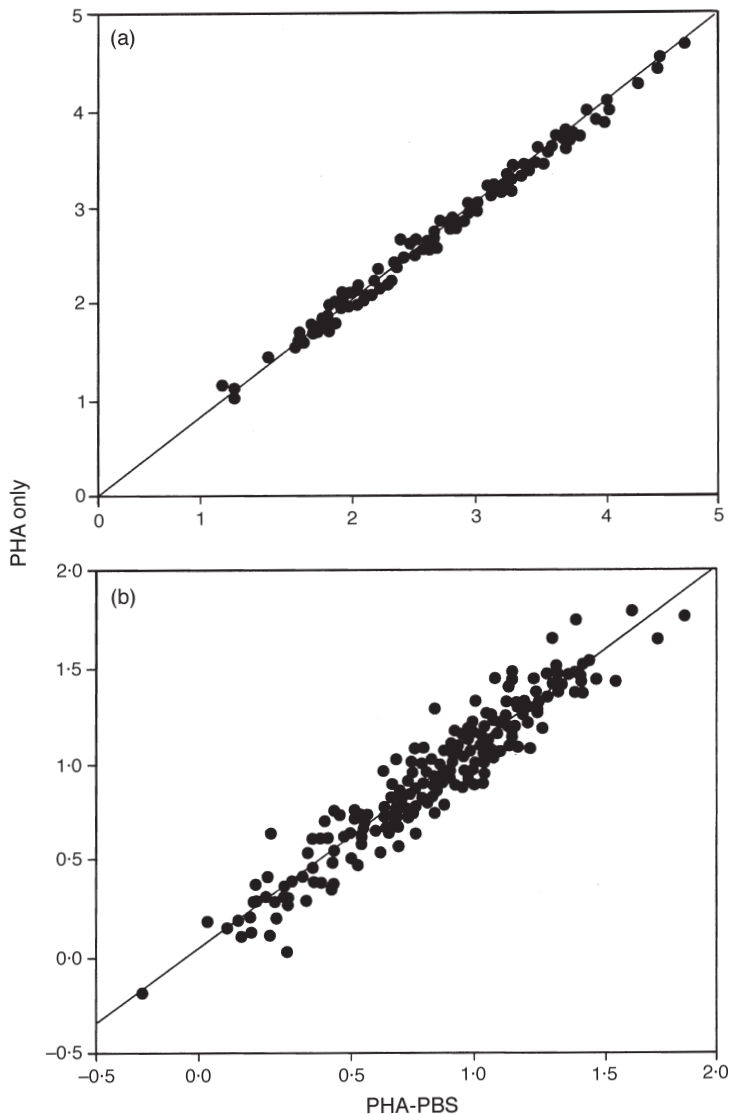


Fig. 1. Scatterplot of the skin test response measured as PHA-PBS compared with PHA only, for (a) 142 nestling American Kestrels and (b) 271 nestling Tree Swallows

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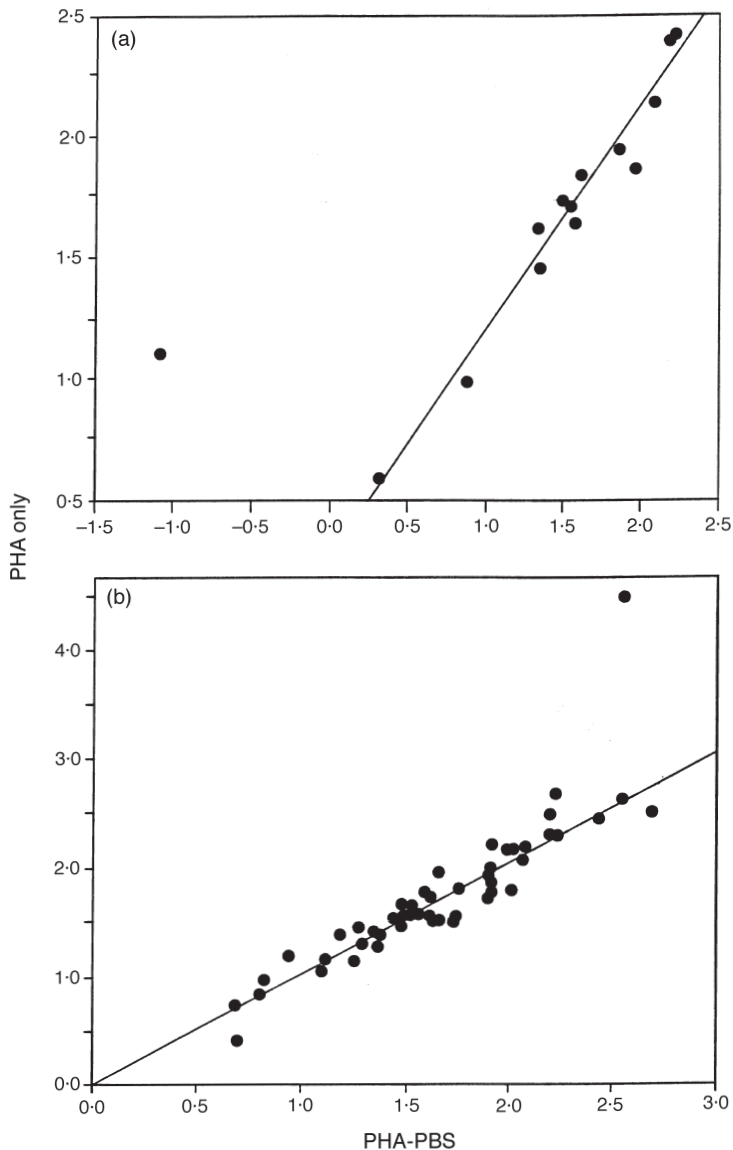


Fig. 2. Scatterplot of the correlation between two different variables (PHA-PBS & PHA only) used to measure the PHA skin response in (a) 14 adult Northern Pintail Ducks and (b) 50 adult American Kestrels. In both studies, there was one bird with an aberrant reaction. By considering the PHA only response, the outlier in (a) would have been missed, while conversely, the outlier in (b) would not be evident using the traditional method of measurement. The line of fit does not take into account the outliers.

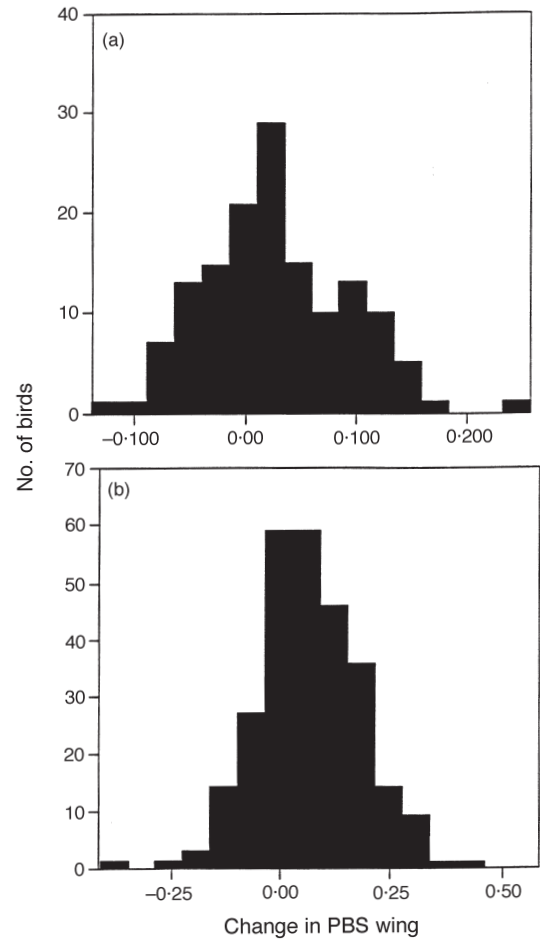


Fig. 3. Histograms of the change in skin thickness of the control, PBS-injected wing for (a) American Kestrels and (b) Tree Swallows depicted in Fig. 1. The mean change (mm) of the control wings in both Tree Swallows (0.07 ± 0.12 SD) and American Kestrels (0.03 ± 0.06) was negligible.

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