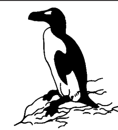
Commentary



The Auk 123(2):575–586, 2006
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TECHNIQUES FOR STUDYING INTEGRATED IMMUNE FUNCTION IN BIRDS

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Evolutionary physiologists and ecologists seek to understand the mechanisms that underlie trade-offs involving life-history traits. These trade-offs arise when resources are limited, and allocation of resources to certain traits limits the amount of resources available for other traits (Williams 1966, Stearns 1992). Recent studies have found that maintaining or activating immune function can be resource-dependent (Tsiagbe et al. 1987; Saino et al. 1997b, 2003; Alonso-Alvarez and Tella 2001) and metabolically costly (Demas et al. 1997, Lochmiller and Deerenberg 2000, Ots et al. 2001, Martin et al. 2002) and, therefore, may compete with life-history traits for nutrient or energetic resources (for reviews see Sheldon and Verhulst 1996, Lochmiller and Deerenberg 2000). Therefore, the study of immunocompetence (i.e. the ability of a host to prevent or control infection by pathogens and parasites) has become the focus of many studies on fitness-related trade-offs in free-living birds (Saino et al. 1997a; Hõrak et al. 2000; Norris and Evans 2000; Hanssen et al. 2003, 2004).

In their seminal review, Norris and Evans (2000) contrasted the techniques currently used by ecologists to measure immunocompetence with the techniques used by immunologists. Reviewing examples of trade-offs between immune-system maintenance and resource allocation to life-history traits, they concluded that future studies need to (1) assess multiple components of the immune system to make conclusions about how these components interact and (2) manipulate immunocompetence to measure the potential fitness consequences. Although many authors have since stressed the importance of examining integrated immunity (i.e. how the various components of the immune system work together; Keil et al. 2001, Sandland and Minchella 2003, Adamo 2004), few studies on free-living birds have fully appreciated the complexity of this system. Here I discuss alternative immunological techniques, currently used in studies of fish, mammals, and domestic poultry, that can be used to examine integrated immune function in free-living birds. I emphasize the importance of an integrated approach when examining the relationship between immune defense and fitness.

Measuring Immunocompetence

The immune system can be divided into three components: innate, cell-mediated, and humoral immunity (Roitt et al. 1998). The innate immune response involves nonspecific recognition, binding, internalization, and destruction of foreign material by phagocytotic cells (Roitt et al. 1998). Cell-mediated immune responses are mediated by T-lymphocytes (T-cells) that either regulate the function of B-lymphocytes (B-cells) and phagocytes or destroy infected host cells through interactions with antigens present on the surface of these cells (Roitt et al. 1998). Finally, the humoral, or adaptive, immune response involves production of specific antibodies (i.e. immunoglobulins [Ig]) by B-cells against antigens associated with pathogen infection; the response improves with repeated exposure to specific pathogens (Roitt

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et al. 1998). Although a multitude of assays is available for assessing the activation of each of these components of the immune system (mammals: Luster et al. 1988, 1992, 1993; poultry: Norris and Evans 2000; fish: Rice and Arkoosh 2002), evolutionary ecologists and physiologists studying free-living birds have chosen to focus on a small proportion of these immunological tests (Table 1). These tests were likely chosen on the basis of the ease of sample collection, the nondestructive nature of sampling, and the ease of sample analysis. However, results from some of these techniques, especially those that assess innate immunity, are ambiguous. For example, elevated numbers of leukocytes (i.e. white blood cells [WBC]) or a large leukocrit value could indicate an individual in good condition, with a healthy immune system, or an individual currently fighting infection. There is also evidence that WBC number does not necessarily correlate with WBC activity (Ladics et al. 1998, Wilson et al. 2001). Therefore, techniques that assess WBC concentration (e.g. WBC counts or Ig enzyme-linked immunosorbent assays [ELISA]) may not be reliable measures of immune function. By contrast, studies on immune function in other taxa use a variety of techniques, most of which are less ambiguous, are as easy to perform as those used in avian studies, and are equally nondestructive (i.e. many assays require ~150 μL of blood, and a combination of assays can be performed on only one 150-μL blood sample; Table 2).

Integrated Immunity

Many authors have cautioned against making generalizations about host immunity and disease resistance on the basis of results from a single immunocompetence assay that examines a single component of the immune system (Lochmiller 1995, Sheldon and Verhulst 1996, Zuk and Johnsen 1998, Norris and Evans 2000, Keil et al. 2001, Sandland and Minchella 2003, Adamo 2004). For example, a decline in the measured component of the immune system may be offset by up-regulation of the unmeasured components of the immune system or other aspects of the measured component (i.e. different cell types within the same arm of the immune system could compensate for declines in the measured cell type; Keil et al. 2001). Norris and Evans (2000) pointed out that, at the time of their review, studies on free-living birds that assessed more than one component of the immune system were nonexistent (but see Spinu et al. 1999, Szép and Møller 1999). Since 2000, only a handful of studies on wild birds have simultaneously examined more than one component of the immune system (Hõrak et al. 2000, Saino et al. 2003, Müller et al. 2004, Matson et al. 2005).

To assess integrated immunity, studies on laboratory mammals have examined hyperthermia (Nava et al. 1997, Raghavendra et al. 1999, Bilbo and Nelson 2002) and wound healing (Rojas et al. 2002, Kinsey et al. 2003). However, these techniques are not realistically applicable to field studies on birds because of repeated-measurement requirements, lethal endpoints, or effects on other aspects of physiology or behavior, such as sickness-related anorexia. By contrast, other studies on humans and laboratory mammals have measured circulating cytokine levels following lipopolysaccharide (LPS) challenge as a measure of integrated immunity (Lee et al. 1992, Sacco et al. 1998, Baykal et al. 2000). Cytokines (e.g. interferons, interleukins, tumor necrosis factor) function as signals between different components of the immune system; they are synthesized *de novo* by a variety of leukocytes in response to an immune stimulus to mediate inflammation by regulating leukocyte proliferation and differentiation (Roitt et al. 1998). Incorporation of this and other techniques (see Table 2) into the repertoire of immunological studies on free-living birds would simplify the simultaneous measurement of two, or even all three, components of the immune system.

Immunocompetence, Life-history Trade-offs, and Fitness

Studies examining the role of immunocompetence in fitness-related trade-offs assume that immune function is energetically costly and may "trade off" with other energetically demanding, fitness-related traits (e.g. growth, reproduction) during periods of limited resources. However, because of the complexity of the immune system, trade-offs can arise both within and between components of the immune system. Consequently, examining a trade-off between life-history traits and only one component of the immune system can be misleading. Therefore, studies examining immunology in

Table 1. Techniques used to measure immunocompetence in wild birds (adapted from Norris and Evans 2000).

Immune system component	Technique	Description	Interpretation
Cell-mediated immunity	WBC counts	Total number of WBC counted from a blood smear	Many WBC: ability to maintain many WBC in preparation for fighting infection or currently fighting infection
	Leukocrit	Height of the buffy layer in a centrifuged capillary tube as a proportion of the total sample height, an index of WBC abundance	Many WBC: ability to maintain many WBC in preparation for fighting infection or currently fighting infection
	Serum proteins	Quantification of serum proteins by gel electrophoresis, an index of natural, nonadaptive Ig concentration	Many Ig: ability to maintain many Ig in preparation for fighting infection or currently fighting infection
	PHA skin test [a]	Quantification of the inflammatory response to phytohemagglutinin (PHA)	Large inflammation: ability to mobilize T-cells to combat foreign antigens
Humoral immunity	Hemagglutination [b]	Quantification of agglutination of Ig and foreign erythrocytes	More agglutination: many Ig that recognize and bind to foreign antigen
	Ig ELISA [c]	Quantification of Ig against foreign antigens	Many Ig: ability to recognize foreign antigen and produce Ig to destroy the antigen
Integrated immunity	Hemolysis [d]	Quantification of lysis of foreign erythrocytes by complement and Ig	More lysis: more complement (innate) and Ig (innate or acquired) working together to recognize, bind, and destroy foreign antigens
	H:L ratio	Ratio of heterophil (H) to lymphocyte (L) numbers counted from a blood smear	Increase in the ratio between phagocytes (innate) and T- and B-cells (acquired) is an immune stress response

[a] Goto et al. 1978, Smits et al. 1999.
[b] Campbell et al. 1970, Cochet et al. 1998.
[c] Martínez et al. 2003.
[d] Demey et al. 1993, Parmentier et al. 2004, Matson et al. 2005.

Table 2. Alternative techniques to measure immunocompetence in wild birds.

Immune system component	Technique	Description	Interpretation	Sample requirements	Materials needed	References
Innate immunity	*In vivo* carbon clearance assay for phagocytotic activity.	Quantification of the carbon remaining in circulation at various time points after IV injection of a carbon source.	The clearance rate of carbon is an index of phagocytotic activity. High activity: ability to destroy foreign material.	Single capture with multiple handling: (1) preinjection blood sampling and injection, (2) postinjection blood sampling at selected intervals (e.g. 3 and 15 min). Nondestructive sample: blood (~100 μL per sample).	India ink, spectrophotometer or 96-well plate reader.	Cheng and Lamont 1988, Heller et al. 1992, Spinu and Degen 1993, Spinu et al. 1999.
	Migratory activity of leukocytes through agarose (chemotaxis assay).	Quantification of the movement of granulocytes through a gel toward a chemoattractant.	Larger distance traveled: ability of leukocytes to recognize a foreign antigen and move toward the infected area to fight the pathogen.	Single capture and handling: sampling done at time of capture. Destructive sample: head kidney cells. Nondestructive sample: blood (~150 μL).	Leukocyte isolation: tissue homogenizer (if using tissue samples), density gradient medium (e.g. Percoll or Ficoll-Hypaque), centrifuge, Trypan blue, hemocytometer. Chemotaxis assay: humidified incubator, agarose-coated slides, chemoattractant (e.g. casein), haematological stain, microscope.	Nelson et al. 1975, Kortet et al. 2003.

Table 2. Continued.

Immune system component	Technique	Description	Interpretation	Sample requirements	Materials needed	References
Innate immunity	*In vitro* respiratory burst assay (superoxide assay).	Quantification of reduction reactions by superoxide (O_2^-) released by activated phagocytotic leukocytes.	More reduction products: more superoxide produced, and thus greater phagocytotic activity of leukocytes.	Single capture and handling: sampling done at time of capture. Destructive sample: head kidney cells. Nondestructive sample: blood (~150 µL).	Leukocyte isolation: tissue homogenizer (if using tissue samples), density gradient medium, centrifuge, Trypan blue, hemocytometer. Superoxide assay: reduction substrate (e.g. nitroblue tetrazolium or phorbol 12-myristate 13-acatate), 96-well plate reader or luminometer.	Babior et al. 1973, Siwicki et al. 1994, Yada et al. 2001, Yada and Azuma 2002, Kortet et al. 2003.
Cell-mediated immunity	*In vitro* incorporation of [^{3}H]thymidine into proliferating T-lymphocytes.	Quantification of [^{3}H]thymidine incorporation into cells during DNA synthesis, as an index of T-lymphocyte proliferation.	More [^{3}H]thymidine uptake: greater number of viable, proliferating T-lymphocytes (index of T-lymphocyte growth and survival).	Single capture and handling: sampling done at time of capture. Destructive sample: spleen cells. Nondestructive sample: blood (~150 µL).	T-lymphocyte isolation: tissue homogenizer (if using tissue samples), density gradient medium, centrifuge, Trypan blue, hemocytometer. [^{3}H]thymidine incorporation: laminar flow hood, mitogen (e.g. Concavalin A, PHA), 96-well culture plates, [^{3}H]thymidine, incubator, microcell harvester, scintillation counter.	Strong et al. 1973, Kreukniet et al. 1994, Simms and Ellis 1996.

TABLE 2. Continued.

Immune system component	Technique	Description	Interpretation	Sample requirements	Materials needed	References
Cell-mediated immunity	*In vitro* metabolism of tetrazolium salts (MTT assay).	Quantification of color change of tetrazolium salts (MTT) due to cleavage of the tetrazolium ring in active mitochondria by mitochondrial dehydrogenase enzymes of living T-lymphocytes.	More color change: greater number of viable T-lymphocytes (index of T-lymphocyte growth and survival).	Single capture and handling: sampling done at time of capture. Destructive sample: spleen cells.	T-lymphocyte isolation: tissue homogenizer, density gradient medium, centrifuge, Trypan blue, hemocytometer. MTT assay: laminar flow hood, MTT, 96-well culture plates (option: MultiScreen filtration plates), 96-well plate reader.	Mosmann 1983, Sladowski et al. 1993, Lochmiller et al. 1994, Prendergast et al. 2002.
Humoral immunity	*In vitro* hemolytic plaque-forming cell (PFC) assay.	Quantification of plaques (clear areas produced by the lysis of erythrocytes) formed by plaque-forming cells (i.e. antibody-secreting cells) (modified Jerne plaque assay).	Many active antibody-forming cells post-challenge: ability to recognize foreign antigen and produce Ig to destroy the antigen.	Multiple capture and handling: (1) antigen challenge, (2) post-challenge (5–10 days) sampling. Destructive sample: spleen or kidney cells. Nondestructive sample: blood (~150 µl).	PFC isolation: tissue homogenizer (if using tissue samples), density gradient medium, centrifuge, Trypan blue, hemocytometer. PFC assay: antigen, antigen linked to erythrocytes (if using a non-erythrocyte antigen), cell culture incubator, microscope.	Jerne and Nordin 1963, Cunningham and Szenberg 1968, McCorkle and Leslie 1983, Kaattari et al. 1986, Denno et al. 1994, Lochmiller et al. 1994, Jacobson et al. 2003.

Table 2. Continued.

Immune system component	Technique	Description	Interpretation	Sample requirements	Materials needed	References
Humoral immunity	Radial immunodiffusion (RID)	Quantification of the diffusion of Ig (naturally circulating or post-challenge) through a gel containing anti-Ig antibody.	Larger diffusion distance: more Ig. Nonchallenged: many Ig: ability to maintain many Ig in preparation for fighting infection or currently fighting infection. Challenged: many Ig: ability to recognize foreign antigen and produce Ig to destroy the antigen.	Nonchallenged: single capture and handling: sampling done at time of capture. Nondestructive sample: blood (~50 μL). Challenged: multiple capture and handling: (1) antigen challenge, (2) post-challenge (5–10 days) sampling. Nondestructive sample: blood (~50 μL).	Antigen (if challenging), anti-subject Ig antibody for gel, purified subject Ig for standard curve, RID plates, microscope.	Mancini et al. 1965, Lü and Miller 1996, Spinu et al. 1999, Taylor et al. 2002.
Integrated immunity	Cytokine ELISA	Quantification of specific cytokines (e.g. interferons, interleukins, tumor necrosis factor) following antigen (e.g. LPS) challenge.	High levels of cytokines: ability to upregulate signaling between the various components of the immune system to mediate leukocyte differentiation, inflammation, and cytotoxic reactions *but* chronically elevated levels of cytokines indicate septic shock.	Single capture with multiple handling: antigen injection done at capture, and blood sampling done at selected interval (e.g. 90 min) postinjection. Nondestructive sample: blood (~200 μL).	Antigen, cytokine ELISA kit, 96-well plate reader.	Lee et al. 1992, Sacco et al. 1998, Baykal et al. 2000.

the field should step back and first try combining a variety of techniques to determine how the various components that make up the immune system interact. Because these types of experiments may require multiple sampling, large samples, or destructive sampling, preliminary experiments could be carried out in the laboratory. Once the basis for how the different components of the immune system function together is understood, field studies can be planned to examine how these interactions are modulated in response to changes in environmental conditions and physiological state. Moreover, greater knowledge of how the different components of the immune system work together could allow evolutionary ecologists and physiologists, who are interested in examining immunocompetence in a fitness context, to work with immunologists to develop more applicable tests of integrated immunity for use in the field.

Once the technical aspects of examining integrated immune function in the field are addressed, questions regarding the relationship between immune function modulation and fitness can be raised. However, because organisms modulate their immune responses to adaptively respond to their condition, physiological state, or environment (i.e. short-term declines in immune function may be adaptive at certain times, whereas enhanced immune function is required at other times), comparisons of the immune responses of individuals in different environmental or physiological conditions (e.g. nestlings vs. adults, nonbreeding vs. laying females, capital- vs. income-breeders, individuals in good vs. poor condition) can also be misleading. This may explain why results from studies that have examined the relationship between immune function and an index of fitness (e.g. return rate as an index of survival) are not consistent. In a meta-analysis using results from studies on immune function and survival in passerine birds, survival was correlated with stronger immune responses (Møller and Saino 2004). Similarly, in breeding female Common Eiders (*Somateria mollissima*), a capital breeder with precocial young, nonchallenged lymphocyte levels measured late in the breeding season were positively correlated with the probability of returning the next year (i.e. survival to the next breeding season; Hanssen et al. 2003). By contrast, when incubating female Common Eiders were challenged with multiple antigens, females that responded by activating the humoral component of the immune system by producing antibodies against sheep red blood cells and diphtheria, thus exhibiting a stronger immune response than nonresponders, were less likely to return to the breeding grounds in the next two years, and were assumed to have died (Hanssen et al. 2004).

Consequently, to properly understand how immune function and fitness are related, future studies need to examine integrated immunity (1) in a variety of organisms, (2) at various physiological stages, (3) throughout the lifetime of organisms, and (4) in a variety of environmental conditions. Furthermore, these studies must also measure aspects of fitness (e.g. mortality and lifetime reproductive success). While this task is complex, it is the future of evolutionary immunology.

Acknowledgments

I thank O. P. Love and T. D. Williams for stimulating discussions regarding the relationships between immunocompetence, life-history theory, and fitness. This manuscript benefited greatly from helpful comments by O. P. Love and two anonymous reviewers.

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Received 20 January 2005, accepted 23 July 2005
Associate Editor: A. M. Dufty, Jr.