Bottlenecks, budgets and immunity:
the costs and benefits of immune function
over the annual cycle of red knots
(*Calidris canutus*)

The research presented in this thesis was carried out at the Animal Ecology Group, Centre for Ecological and Evolutionary Studies at the University of Groningen, and the Marine Ecology Group at the Royal Netherlands Institute for Sea Research.

The printing of this thesis was partly funded by the University of Groningen and the Faculty of Mathematics and Natural Sciences.

Lay-out and figures: Dick Visser
Cover design: Deborah Buehler
Photos: Deborah Buehler
Printed by: Van Denderen BV, Groningen

ISBN: 978-90-367-3594-0
ISBN: 978-90-367-3595-7 (electronic version)

RIJKSUNIVERSITEIT GRONINGEN

Bottlenecks, budgets and immunity:
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Proefschrift

ter verkrijging van het doctoraat in de
Wiskunde en Natuurwetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. F. Zwarts,
in het openbaar te verdedigen op
vrijdag 7 november 2008
om 16:15 uur

door

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geboren op 19 januari 1977
te Toronto, Canada

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Delaware Bay

CHAPTER 1

General Introduction: migration, the immune system and the costs of immune function

Deborah M. Buehler

MIGRATION

This thesis was inspired by a fascination with migration. The sight of birds migrating into the sunset always leaves me awestruck. I suppose I am projecting my own curiosity and love of travel onto them - wishing that I too could cover thousands of kilometres on my own power and see the world from innumerable perspectives. But since the power of self sustained flight remains beyond my grasp, I need to fulfil my curiosity in a different fashion. And so, like many scientists before me, I ask and try to answer questions. How do the birds I see withstand days of non-stop flight? Do they get hungry, tired, and thirsty? How do they find their way? How do they prepare for the journey? How do they arrange the rest of their annual cycle around migration?

Researchers have been studying migration for years and many of the questions I listed above are areas of active study. However, a relatively new discipline in the study of migration addresses how migrants deal with the disease threats that they encounter during their travels and how they balance competing demands for resources during their busy annual cycle. This area of research is especially fascinating to me since my own love of travel has taught me that I am more susceptible to sickness when I arrive, disoriented and exhausted, in a completely new environment. How do the birds I study manage to stay healthy?

Before I can begin to tackle that question, I should define the type of migration that interests me. Migration is a complex biological phenomenon broadly defined as "the act of moving from one spatial unit to another" (Baker 1978). This research focuses on the seasonal migration of shorebirds which falls under the category of "calculated return migration" under Baker's (1978) hierarchical definition. Calculated return migration means the seasonal movement of individuals between different locations. In shorebirds this refers to yearly migrations between Arctic or boreal breeding areas and temperate, tropical or south temperate wintering areas.

This thesis weaves together four major strands: migration, immune function, red knots (as a model migrant) and the annual cycle. This general introduction continues with an overview of the immune system, the costs of immune function and the assays used in this research. Red knots as a study system and a discussion about bottlenecks in the annual cycle of migrants are presented in chapters 2 and 3.

THE IMMUNE SYSTEM

Our world contains a variety of infectious microbes and the living body provides a warm, moist and nutrient rich environment for these invaders, and is already home to flourishing populations of commensal microbes that must be kept in check. In birds and other vertebrates a complex network of overlapping and interlinked defence mechanisms, known as the immune system, has evolved to protect the body from microbial invasion.

Immune responses are complex and have been described in many ways. One of the most comprehensive descriptions proposes that immune responses fall along two

broad axes (Schmid-Hempel and Ebert 2003). The first axis refers to the degree of specificity of the immune response and its two extremes are *non-specific* and *specific*. The second axis refers to the temporal dynamics of the immune response, and its two extremes are *constitutive* and *induced*. Constitutive immune function is constantly maintained, providing a system of surveillance and general repair. An induced immune response is triggered only when a pathogen has established itself in the body. In general constitutive immune function is non-specific, whereas induced immune responses are specific to a particular pathogen. This association has lead to the two broad categories described in most immunology textbooks: innate and acquired immune function (Janeway et al. 2004). Innate immune function is immediate and general, and acquired immune function develops some time after initial infection, is specific to particular pathogens, and has memory. In reality, immune function can fall anywhere along the axes described by Schmid-Hempel and Ebert (2003). Therefore, in this introduction I try to represent both axes when describing the immune system (figure 1.1). Box 1.1 describes the major mediators of immune function referring to Janeway et al. (2004) unless otherwise indicated.

The path of a pathogen

A good way to understand the myriad interactions that occur during an immune response is to follow the path of a hypothetical pathogen (figure 1.1). The path of a pathogen begins outside the body where the invader must first overcome the body's *physical, chemical* and *behavioural barriers*. Many pathogens are removed via mechanisms, such as preening and grooming, or are denied access by the skin, the mucus and cilia of the respiratory tract. If they do gain entry, biochemical barriers such as acid in the gut and the rapid pH change between the stomach and the intestine may still prevent them from becoming established (Janeway et al. 2004).

Assuming the pathogen we are following manages to enter the body, it encounters surveillance cells of the immune system such as heterophils and macrophages (extracellular pathogens), and cytotoxic T-cells and natural killer cells (intracellular pathogens; arrows 1 in figure 1.1). These cells begin to engulf the invaders and release soluble mediators to attract more phagocytes and dendritic cells to the site of infection. For many pathogens the path ends here and these non-specific cells and soluble proteins can clear the infection within a few hours. However if the pathogen is persistent, macrophages release cytokines that induce the acute phase response (arrow 2 in figure 1.1). During the acute phase response the host feels lethargy, anorexia and fever, and the liver produces acute phase proteins and diverts amino acids away from normal processes (such as growth or reproduction). In addition, regular body cells increase protein turnover and MHC type I presentation to CD8 T-cells. At the same time dendritic cells, which have engulfed the pathogen, are migrating to the lymph nodes or spleen to present pathogen peptides on MHC type II receptors to CD4 T-cells for recognition (arrow 3 in Figure 1.1). Over the next few days those activated T-cells will proliferate and depending on the type of pathogen, will release cytokines for a cell-mediated response (intracellular pathogens), or an antibody based response (extracellular pathogens) via B-cells (arrow 4 in Figure 1.1). Cytokines and antibodies will feedback

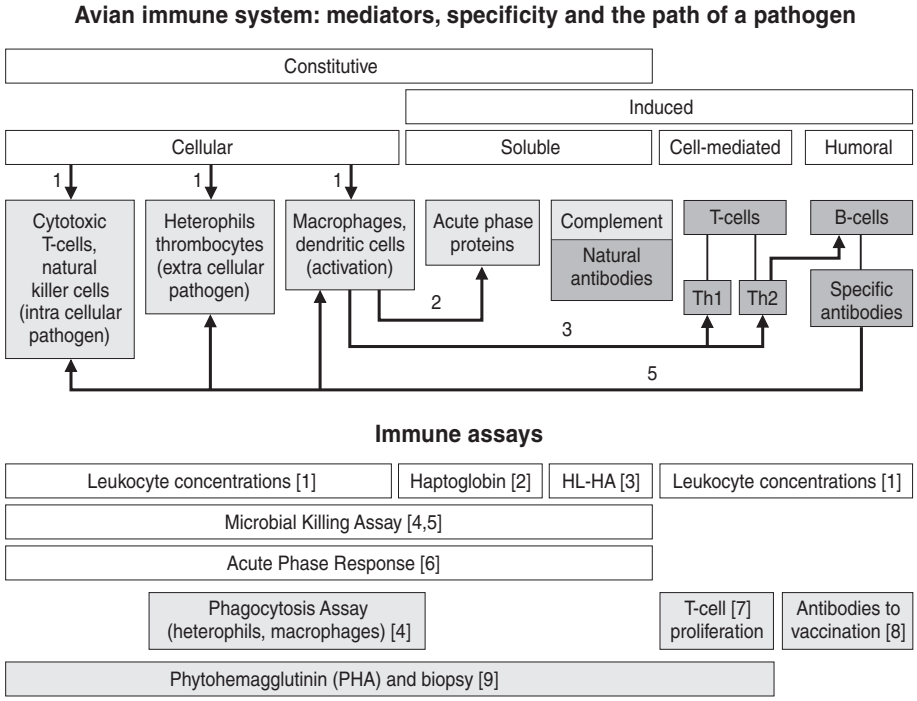


Figure 1.1. A simplified representation of the avian immune system. The cells and soluble mediators of immunity are shown below the categories and shading represents the specificity of each mediator. General mediators of immunity (innate immunity) are shaded light grey and specific mediators of immunity (acquired immunity) are shaded dark grey. Natural antibodies have relatively broad specificity and are represented with intermediate shading. Thick lines with arrows show the approximate path of a pathogen through the immune system during an immune response (see text). Above the mediators of immunity are a few basic categories. Constitutive and induced branches represent immunity that is constantly maintained and immunity that is triggered by a challenge, respectively. Cellular and soluble components refer to mediators that are cells or that are found in the body fluids. The terms cell-mediated and humoral immunity are by convention restricted to T-cell, B-cell and antibody mediated immunity. Immune assays are represented by boxes positioned below the aspects of immunity they quantify. Assays used in this thesis are shown first in white and other possible assays are shown below in grey. [1] Campbell 1995 [2] Matson 2006 [3] HL-HA = Hemolysis-hemagglutination, Matson et al. 2005 [4] Millet et al. 2007 [5] Tieleman et al. 2005 [6] Bonneaud et al. 2003 [7] Bentley et al. 1998 [8] Hasselquist et al. 1999 [9] Martin et al. 2006a

into the non-specific surveillance cells, greatly increasing the efficiency of phagocytosis by specifically marking the pathogen for destruction (arrow 5 in Figure 1.1). After the infection has been cleared, memory cells (both T and B types) will remain providing a swift and specific response in the event that the same pathogen is encountered again (Janeway et al. 2004). Thus, all branches of the immune system work in concert during an immune response, with constitutive innate immunity acting as the first line of defence and induced acquired immunity focusing and increasing the efficiency of innate immune mediators during the later stages of the response (Clark 2008).

THE COST OF IMMUNITY

The field of ecological immunology emerged in the in the mid-1990s when ecologists began to think of immune function in terms of costs and benefits, and began to use measures of immune function to test ecological hypotheses (Gustafsson et al. 1994; Sheldon and Verhulst 1996). Since then there has been a rapid increase in research examining how immune defence evolves and why immune function differs in different environments, individuals or species (reviewed in Lee 2006; Martin et al. 2006b). Research in the field of ecological immunology is all based on the idea that immunity comes with costs as well as benefits.

Having an immune system comes with the obvious benefit of enhanced disease resistance, but having an immune system also comes with costs. The most basic cost associated with having an immune system is an *evolutionary cost* (Schmid-Hempel 2003; Zuk and Stoehr 2002). The immune system evolves at the expense of another trait; for example, a functional change in a protein for use in immune defence, which renders it useless for other aspects of the host's biology. The evolutionary cost of immune function has been measured using organisms selected for differing degrees immune investment. For example, parasitoid-resistant fruit flies *Drosophila melanogast*er are less competitive than non-parasitoid-resistant flies (Kraaijeveld and Godfray 1997) and turkeys *Meleagris gallopavo* selected for higher body mass and egg production show reduced immune function (Nestor et al. 1996). Although the evolutionary costs of immunity are fascinating, this research is focused on ecological rather than evolutionary time (from the standpoint of a migratory shorebird), thus I do not discuss evolutionary costs further.

From an ecological standpoint, the cost of immunity can be divided into three. First, a *resource cost* paid in limited resources, such as energy or nutrients, important for both immune function and other aspects of host life (breeding, migration, reviewed in Schmid-Hempel 2003; Zuk and Stoehr 2002). Second, an i*mmunopathology cost* paid in collateral damage to the host. This arises when the immune system causes damage to the host as well as invaders (Råberg et al. 1998). For example, during heavy physical exercise muscle damage occurs and heat shock proteins are produced stimulating the immune system in the same way as damage caused by infection and triggering an immune response directed against the host (Weight et al. 1991; Winfield and Jarjour 1991). Finally, *opportunity cost* paid in lost opportunities because time must be allocated to immune system development or use. For example, the activation of sickness behavior causes temporary suspension of important life-history events such as breeding, molt or migration (Owen-Ashley and Wingfield 2007). These costs provide a conceptual framework and emphasize that fact that, due to the complexity of the immune system, there is no single cost of immunity.

Different aspects of the immune system have different costs during development, maintenance and use (Klasing 2004). Constitutive innate immunity has low development costs in terms of resources and time because the cells of the innate immune system do not require diversification or selection (in contrast to B and T-cells; Klasing 2004). The resource costs of maintaining constitutive innate immunity are moderate

because the majority of immune cells and proteins are "at rest" and replacement is slow and gradual (with the exception of heterophils). However, the immunopathology, resource and opportunity costs of using innate immunity, especially the acute phase response with its accompanying fever, anorexia and lethargy, can be very high (Klasing 2004). Induced acquired immunity on the other hand, has high development costs in terms of resources and time because functional antigen recognition sites are generated only rarely and high percentages of developing B and T lymphocytes must be discarded (Reynaud and Weill 1996). Once developed however, the maintenance and use costs of acquired immunity in terms of resources, collateral damage and time are quite low (Klasing 2004).

MEASURING IMMUNE FUNCTION

Why is it important to measure immune function and what can measures of immune function tell us? First, measures of immune function can help us to make inferences about pathogen pressure (i.e. in Chapters 5, 9, 10 and 11). Variation in immune parameters may reflect differences in the need (Lindström et al. 2004; Møller and Erritzøe 1998) or ability (Møller et al. 1998) to ward off infection. Second, because immune function is a costly but essential physiological process, measures of immune function can act as a proxy for investment in self maintenance in the context of life history trade-offs (i.e. in Chapters 3, 5, 6 and 7; Lee 2006; Lochmiller and Deerenberg 2000; Martin et al. 2008; Schmid-Hempel 2003; Sheldon and Verhulst 1996; Zuk and Stoehr 2002). Third, immune function is interesting from a mechanistic and functional perspective in its own right (i.e. Chapters 4 and 8).

How to measure immune function? Many strategies for measuring immune function exist and each has benefits and drawbacks (Salvante 2006). The assays chosen depend on the questions the researcher wants to address and practical constraints of sampling. For my research I focused on assays that measure constitutive immunity and the acute phase response. Constitutive immunity is effective at controlling multiple pathogen types and responds immediately to threats, making it an evolutionarily relevant first line of defence. This may be especially important for migrants encountering numerous novel environments throughout the annual cycle (Møller and Erritzøe 1998). Furthermore, mediators of constitutive immunity must be maintained even when not in use, generating costs that may be important for physiological trade-offs (Martin et al. 2008; Schmid-Hempel and Ebert 2003). From a practical standpoint, because a response is not induced and immunological memory is not stimulated, repeated measures of individuals throughout the annual cycle can be made. Finally, constitutive immunity can be measured from a single capture making it ideal for studies on free-living birds. Box 1.2 briefly describes the assays used in this thesis. Figure 1 shows, in a simplified manner, how these assays relate to the majors mediators of immune function. For reference, figure 1.1 also includes a few widely used or very promising assays not used in this thesis.

AIMS AND OVERVIEW OF THIS THESIS

This thesis aims to address a series of questions about immune function in a migrant bird. These questions can be thought of as *descriptive how questions, mechanistic how questions, functional why questions and historic why questions* (Piersma 1994), where "how questions" address the proximate causes of a biological phenomenon and "why questions" the ultimate and evolutionary causes (Mayr 1961; Orians 1962; Tinbergen 1963). Part I of this thesis introduces the study system and predictions, Part II focuses on assessing immunity and how it responds to different environmental conditions in a controlled environment, and Part III examines immune function in free-living birds.
More specifically, in chapter 2 we address the historical question "How did migration patterns in red knots evolve?" by providing a historical look at red knot flyways. In chapter 3 we provide a contemporary review of the red knot study system and examine nutritional, energetic, temporal and disease risk bottlenecks throughout the knot annual cycle. Chapter 3 addresses the functional question "Why is immune function seasonally variable and are bottlenecks part of the explanation?" In chapter 4 we address the mechanistic question "How does the stress of capture and handling affect immune response in knots?" In chapter 5 we provide a detailed description of constitutive immune function over the annual cycle and address the descriptive question "How does immune function vary over the annual cycle in a migrant shorebird?" We then delves deeper, examining the functional why question "Do birds use different immune strategies during different times of the year?" and the mechanistic question "How does temperature (energy expenditure) affect immune function in a migrant bird?" In chapters 6 and 7 we describe an experiment in which access to food was manipulated, addressing the mechanistic question "How do knots allocate resources to metabolic and immune function processes when access to food is limited?" In chapter 8 we address the functional question "Why is immune function seasonally variable and does melatonin play a role for migrant birds?" by looking at the relationship between immune function and melatonin. In chapter 9 we address the descriptive question "How do captive and free-living knots differ in immune strategy?" and the functional why question "Could differences in pathogen pressure in these two environments contribute to these differences?" In chapter 10 we address the descriptive question "How does immune function change during spring stopover in red knots and what can this tell us about pathogen pressure?" In chapter 11 we address the descriptive question "How do age and environmental factors affect immune function in free-living birds?" Finally in chapter 12, I provide a synthesis of the results, introduce a conceptual model for how animals arrive at optimal immune defence and suggest avenues for future research.

BOX 1.1. MEDIATORS OF IMMUNE FUNCTION

Cells of the immune system

Leukocytes (white blood cells) fall into two main categories: phagocytes and lymphocytes. The main function of *phagocytes* is to internalize and degrade foreign invaders. These cells use non-specific recognition systems that allow them to eliminate a variety of pathogens. Phagocytes belong to two main lines: the macrophages and the polymorphonuclear granulocytes. *Macrophages* are long lived cells that act as "professional" phagocytes. Their precursors, the *monocytes*, circulate in the blood and tissues providing surveillance. *Polymorphonuclear granulocytes* are phagocytes with lobed nuclei and include *heterophils* (neutrophils in mammals), *basophils* and *eosinophils*. *Heterophils* circulate in the blood and migrate into tissues during the early stages of inflammation. However, unlike macrophages, they are short lived cells whose main function is to phagocytose and destroy pathogens and then die. *Eosinophils* are less common in the blood than heterophils and appear to function mainly as cytotoxic cells with the ability to kill other cells as well as large extracellular parasites such as worms. *Basophils* circulate in very low concentrations and their main purpose is to mediate inflammation.

Lymphocytes are responsible for the specific recognition and memory of antigens during the later stages of an immune response. These cells occur as two major types: the B-cells and the T-cells. Antigen receptors on B and T-cells are specific to a single antigen, therefore the ability to recognize a wide variety of invaders rests on the development of extremely high levels of B and T-cell diversity. When a *B-cell* encounters its specific antigen it becomes activated, multiplies and differentiates into a plasma cell. This plasma cell produces huge quantities of soluble antibody which binds to the antigen marking it for destruction by phagocytes and greatly increases the efficiency and specificity of phagocytosis. After the infection has been cleared a few B-cells remain available as memory cells for that particular antigen and confer lasting immunity to it by generating a swift and specific acquired response if the pathogen is encountered again.

T-cells differ from B-cells because they recognize antigen only when it has been processed and is presented on the surface of an antigen presenting cell via major histocompatibility complex (MHC) molecules. When examining the interaction between T-cells and MHC molecules it is helpful to distinguish between extracellular and intracellular pathogens. *Extracellular pathogens* are found outside the body cells and are engulfed and processed by antigen presenting cells (dendritic cells, the macrophages and the B cells) in the skin, lymph nodes, spleen, thymus, and mucosal epithelia. These cells present antigen via MHC class II molecules to helper T-cells with CD4 receptors. *Intracellular pathogens* infect the body cells and antigens for these pathogens are found in the cytoplasm of infected cells and are displayed via MHC class I molecules (present on all cells not just immune cells) which are recognized by T-cells with CD8 receptors (which later mature into *cyto-*

toxic T-cells). The antigen presenting cells described above can process extracellular and intracellular pathogens for presentation to CD4 helper T-cells. Thus, CD4 helper T-cells are central to the immune response because they tailor the response to the type of pathogen. Type 1 helper T-cells (Th1) interact with cytotoxic T-cells, to instigate cell-mediated responses associated with local inflammation against interacellular pathogens. Type 2 helper T-cells (Th2) interact with B-cells and induce them to secrete antibody against extracellular pathogens. When the infection has been overcome, a small number of T memory cells persist in the body providing immunological memory.

Natural killer cells function much like cytotoxic T cells except that instead of killing cells that have started doing something they are not supposed to do (expressing foreign peptides on MHC class I molecules), they detect and kill cells that have stopped doing what they are supposed to do (expressing self antigens on MHC class I molecules). These two lymphocytes (cytotoxic T-cells and natural killer cells), in combination, are very effective in combating viral infections.

Soluble mediators of the immune system

Antibodies (Ab), also known as *immunoglobulins* (Ig), are soluble forms of B-cell antigen receptors and are highly specific. *Natural antibodies* (NAb) are a special type of immunoglobulin. They differ from induced specific antibodies in that they are present in the absence of exogenous antigenic stimulation (Ochsenbein et al. 1999). Furthermore, they are secreted by B-1 rather than B-2 cells (Baumgarth et al. 2005), they have broad specificity (are able to bind to more than one antigen), and they appear to confer little or no immunological memory (Janeway et al. 2004).

Cytokines are molecules involved in signalling between cells during an immune response and different cytokines are classified into categories. *Interferons* (IFNs) are important for limiting the spread of viral infections. *Interleukins* (ILs) are a large group of cytokines (IL-1 to IL-22) whose main function is to direct other cells to divide and differentiate (e.g. during clonal expansion of B-cells). *Colony-stimulating factors* (CSFs) are primarily involved in directing the division and differentiation of bone marrow stem cells and the precursors of leukocytes. *Chemokines* direct movement of cells around the body and attract immune cells to sites of infection during inflammation. *Tumour necrosis factors* (TNF-α and TNF-β) and *transforming growth factor-* β (TGF-β) are important in mediating inflammation, cytotoxic reactions and the acute phase response.

Complement is a group of about 20 proteins involved in inflammation. Complement can be activated directly by pathogens or indirectly by pathogen-bound antibody. There are three pathways of complement activation: the classical pathway, the mannose-binding lectin pathway and the alternative pathway. Whichever way it is activated, the complement system proceeds as a cascade reaction and generates protein molecules with three main effects: (1) opsonization (coating) of

microorganisms for uptake by phagocytes. (2) chemotaxis to attract other phagocytes to the site of infection. (3) lysis of the cell membranes of infected cells or gram-negative bacteria. In conjunction with natural antibodies, complement also provides an immediate defence against spreading infections such as viruses (Ochsenbein and Zinkernagel 2000).

Organs of the immune system

In addition to cells and soluble proteins, various organs are part of the immune system. *Primary lymphoid organs* such as the thymus, where T-cells develop, and the Bursa of Fabricius (birds) or bone marrow (mammals) where B-cells develop, regulate the production and differentiation of lymphocytes. *Secondary lymphoid organs*, the lymph nodes (ephemeral in birds) and the spleen, are "command centres" where antigen presenting cells interact with lymphocytes.

BOX 1.2. THE IMMUNE ASSAYS USED IN THIS THESIS

The *microbial-killing assay* is a functional measure of the capacity of blood to kill microorganisms *in vitro* (Matson et al. 2006b; Millet et al. 2007; Tieleman et al. 2005). This assay measures constitutive immunity integrated across circulating cellular and soluble blood components (figure 1.1). In the assay whole blood is mixed with a known concentration of microorganism, allowed to interact with the microorganism for a set amount of time, and then visualized by plating the solution on agar. I used three microbial strains whose genera are ubiquitous but are not highly pathogenic to minimize the problem of previous exposure in some individuals but not others. *Escherichia coli* a strain of gram negative bacteria that is commensal in the intestinal tract, but can cause infection in the respiratory tract in birds; *Candida albicans* a yeast-like fungus that causes candidiasis (thrush) in birds when ingested; and *Staphylococcus aureus* a strain of gram positive bacteria that normally inhabits the skin but causes inflammation if it enters a wound (United States Geological Survey 1999).

Leukocyte concentrations provide information on circulating immune cells which can be used as an indicator of health (Campbell 1995) and are also useful in multivariate analysis in terms of their relationship to functional measures of immunity such as microbial killing. As described in detail above, heterophils and eosinophils mediate non-specific immunity against novel pathogens and are important phagocytes; monocytes link innate and acquired defence; and lymphocytes mediate pathogen specific antibody and cell-mediated responses (Campbell 1995). I obtained leukocyte concentrations using a cell counting chamber in combination with standard blood smears (Campbell 1995).

The *hemolysis-hemagglutination assay* quantifies complement and natural antibody activity. As described above, the complement cascade and natural antibodies provide the first line of defence against spreading infections, including viruses (Ochsenbein and Zinkernagel 2000). In this assay serial dilutions of blood plasma are mixed with rabbit red blood cells. Hemolysis indicates the amount of haemoglobin released from lysed rabbit red blood cells as a result of complement action and hemagglutination reflects the action of natural antibodies.

Haptoglobin is an acute phase protein that binds iron (haem) to keep it from providing nutrients to pathogens and offers protection against harmful end products of the immune response (Delers et al. 1988). Elevated levels of haptoglobin indicate current infection, inflammation or trauma. I used a commercial kit which exploits the peroxidase activity of haptoglobin bound to haemoglobin to quantify haptoglobin levels.

The *acute phase response* is associated with changes in body temperature (hyperthermia in larger birds or hypothermia in small passerines), the secretion of acute phase proteins from the liver, and sickness behaviours including reduced food intake, body mass loss and reduced activity (Owen-Ashley and Wingfield 2007). It is considered one of the most costly types of immune function in terms of energy, immunopathology and opportunity costs (Klasing 2004). In this research I mimicked bacterial infection with lipopolysaccharide (LPS) from the cell wall of a strain of gram negative bacteria to induce an acute phase response (Bonneaud et al. 2003).

Delaware Bay

PART I

Study system and predictions

CHAPTER 2

Reconstructing palaeoflyways of the late Pleistocene and early Holocene Red Knot *Calidris canutus*

Deborah M. Buehler, Allan J. Baker and Theunis Piersma

ABSTRACT

Bird migration systems must have changed dramatically during the glacial–interglacial cycles of the Pleistocene and as novel habitats became available since the last glacial maximum. This study combines molecular dating of population divergence times with a review of polar-centred palaeovegetation and intertidal habitats world-wide to present a hypothesis for the evolution of Red Knot *Calidris canutus* flyways. Divergence dates from coalescent analysis of mitochondrial control region sequences indicate that *C. c. canutus* diverged from the most recent common ancestor (MRCA) of Red Knots about 20 000 (95% CI 60 000–4000) years ago. About 12 000 (95% CI 45 000–3500) years ago this MRCA diverged into two lineages, now represented by the North American breeding *C. c. roselaari, C. c. rufa* and *C. c. islandica* and the Siberian breeding *C. c. piersmai* and *C. c. rogersi*, respectively. Divergence times of these two Siberian breeding subspecies are about 6500 (95% CI 25 000–1000) years ago, and populations of the North American breeding subspecies are estimated to have diverged within about the last 1000 years. These divergence times suggest that all ancestral populations of knots emerged within the last glacial period of the Pleistocene via an eastward expansion into North America. This scenario implies that, contrary to contemporary opinions, *C. c. islandica* was not recently derived from *C. c. canutus* despite the fact that they are morphologically similar and that their contemporary migration routes overlap in the Wadden Sea. Instead, *C. c. islandica* is most closely related to the other North American breeding subspecies *C. c. roselaari* and *C. c. rufa*. Thus, *C. c. islandica* only recently pioneered its current migration route to Europe, following the amelioration of winter conditions in the Wadden Sea and the formation of staging habitat in Iceland. This implies that, in Red Knots at least, the Greenland/Iceland migratory route was established very recently from breeding grounds in the Americas with to wintering grounds in Europe and not *vice versa* as previously believed.

Ardea (2006) 94: 485-498

INTRODUCTION

During the glacial-interglacial cycling of the Pleistocene and as current habitats became available since the last glacial maximum, bird migration systems have changed dramatically (Alerstam 1990). Early paleogeographic studies on bird migration were interested in the effect of ice-free refugia on the present day distribution and morphological patterns of bird populations (Larson 1957, Ploeger 1968). However, the Quaternary ice ages have also left a genetic legacy (Hewitt 2000), and with the advent of molecular technology, to date population divergences, other studies have emphasized the importance of Pleistocene glaciations on population structure and hypothesized ways in which current population distributions might have arisen in shorebirds (Piersma 1994, Wenink et al. 1996, Kraaijeveld & Nieboer 2000) and passerines (Klicka & Zink 1997, Johnson & Cicero 2004, Zink et al. 2004, Lovette 2005). However, there have been no attempts to incorporate both molecular dating and an in-depth review of palaeovegetation and coastal geomorphology to examine the recent evolution of bird flyways in detail.

To examine the evolution of a flyway system for a particular species several criteria must be met. First, detailed knowledge of the specific habitat requirements of the species is needed. Second, these habitats should be identifiable in the fossil/geomorphic record to allow a reconstruction of past habitat distribution. Finally, data should exist over a sufficient scale to allow sensible reconstruction with reference to the global movements of the species (in the case of long distance migrants).

Red Knots *Calidris canutus* fulfil many of the requirements for an examination of flyway evolution, at least for the late Pleistocene and Holocene periods. Knots are divided into six subspecies on the basis of plumage colour, body size and migratory route: *C. c. canutus, C. c. piersmai, C. c. rogersi, C. c. roselaari, C. c. rufa* and *C. c. islandica* (Tomkovich 1992, 2001). Three of these subspecies are also genetically distinct on the basis of mitochondrial DNA (*C. c. canutus, C. c. piersmai, C. c. rogersi*) and a fourth lineage is comprised of *C. c. roselaari, C. c. rufa* and *C. c. islandica* (Buehler & Baker 2005). Overall, knots have very low genetic diversity both in the mitochondrial (control region) and nuclear (microsatellites and amplified fragment length polymorphisms AFLPs) genomes and although broad scale sequencing of nuclear genes may uncover structure among *C. c. roselaari, C. c. rufa* and *C. c. islandica* in the future, such structure has so far remained elusive (Buehler 2003).

Knots are selective feeders that use a specialized sensory mechanism to locate hard-shelled prey buried in soft sediments (Piersma et al. 1998). Thus in the non-breeding season, they are confined to intertidal coastal habitats with extensive stable soft sedimentary habitats (Piersma 1994, Piersma et al. 2005). In the breeding season they are cold tundra specialists and are thus confined to the High Arctic (Piersma & Davidson 1992). Both intertidal sediments and High Arctic tundra habitats are identifiable in the geological record and have been studied in many parts of the world. In the case of High Arctic tundra habitat, some studies have even generated comprehensive global summaries. Furthermore, arguments from first principals about sea level changes may allow us to generalize over gaps in the intertidal record.

Hypotheses regarding the timing of the population bottleneck in Red Knots have been proposed (Piersma 1994, Kraaijeveld & Nieboer 2000). Piersma (1994) posited that the bottleneck occurred during the Holocene deglaciation when boreal forest reached up to the ice sheets and intertidal areas may have been flooded by rising sea levels, caused by the melting of the ice sheets, at a rate too fast for the formation of stable intertidal sediments, which sustain the bivalve populations necessary for knots during the non breeding season. Kraaijeveld and Nieboer (2000), however, argued that breeding habitat for Red Knots was restricted during the climatic optimum in the early Holocene (7000 to 8000 years ago) much more so than during deglaciation, and thus the climatic optimum rather than deglaciation coincided with the population bottleneck in knots (Kraaijeveld & Nieboer 2000). To test these hypotheses this study integrates molecular estimates of population divergence time and a review of palaeovegetation for both cold tundra and intertidal areas, for the late Pleistocene and Holocene periods.

Habitat requirements for Red Knots

Red Knots are one of the most polar of all High Arctic breeders. They reproduce only on the northern most fringes of land, often within sight of the Arctic Ocean. In this habitat vegetation cover is sparse, usually between 4 and 25% (Olson et al., 1983), and conditions are harsh even in the summer (Piersma et al. 2003). On the tundra they eat mostly spiders and other arthropods which they obtain by surface pecking (Tulp et al. 1998). Outside the breeding season knots occur only on coastal sites with large expanses of intertidal substrate, and all current wintering habitats for knots are confined to marine intertidal areas with stable sediments and hard-shelled prey. Estuaries such as the Wadden Sea and the Wash in northwest Europe, and Bahia Lomas in Chile all contain extensive mudflats and an abundance of hard-shelled prey including *Macoma balthica, Mytulus edulis* and *Hydrobia ulvae* (van Gils et al. 2005a). Red Knots are extremely specialized feeders that use a unique bill-tip organ to locate hard objects in soft sediments (Piersma et al. 1998). Hard-shelled bivalves buried in the soft yet stable mud of intertidal flats are the ideal prey for knots and are ingested whole and crushed in a muscular gizzard (Piersma et al. 1999a, Battley & Piersma 2005). This specialized method of feeding, along with possible peculiarities in their immune system (Piersma 1997, 2003), may restrict Red Knots to very specific non-breeding habitats.

An exception to the habitat and diet restrictions described above occurs during northward migration at a stopover site in Delaware Bay, USA. There, Red Knots do not need to use their specialized feeding technique to locate buried bivalve prey because the beaches are covered with a nutritious and easy to find food source. In Delaware Bay *C. c. rufa* and *C. c roselaari* subspecies feed on Horseshoe crab *Limulus polyphemus* eggs. There are four extant species of horseshoe crab, of which *Tachylpeus tridentatus, Tachylpeus gigas* and *Carcinoscorpius rotundicauda* are all found in the coastal waters of southeast Asia including the Philippines and Indonesian (35°N to 5°S), while *Limulus polyphemus* inhabits the waters of the western Atlantic Ocean from Maine to the Yucatan (44.5°N to 21°N). Little is known about shorebird and horseshoe crab interactions in the Indo-Pacific. However, the dependence of shorebirds on horseshoe crab

eggs during spring migration in Delaware Bay has been well established (Tsipoura & Burger 1999, Schuster et al. 2003, Baker et al. 2004).

The temporal framework

Population structure and genetic diversity in Red Knots has been well studied using molecular techniques. Mitochondrial DNA, microsatellites and AFLP data have shown that knots have very little genetic diversity, and because this lack of diversity occurs across the genome, these data support the proposal that knots have undergone recent and severe population bottlenecks (Buehler 2003). The current hypothesis is that population bottlenecks occurred during the last glacial of the Pleistocene 115 000 to 10 000 years ago, as well as during the Holocene warming. Buehler and Baker (2005) proposed that *C. c. canutus* diverged from the most recent common ancestor (MRCA) of Red Knots about 20 000 (95% CI: 60 000–4 000) years ago. The MRCA of the lineage leading to *C. c. roselaari, C. c. rufa* and *C. c. islandica* subspecies was dated at about 12 000 (95% CI: 45 000–3500) years ago and the split between *C. c. piersmai* and *C. c. rogersi* at about 6500 (95% CI: 25 000–1000) years ago. These population divergences were estimated using a computer programme MDIV based on a coalescent model that jointly estimates the divergence time and migration rates among pairs of populations using DNA sequence data (Nielsen & Wakeley 2001). The method estimates several parameters using Markov Chain Monte Carlo simulations to generate posterior probability distributions. The 95% credibility intervals associated with the dates represent the interval that contains 95% of the posterior probability distribution.

Baker and Buehler (2005) used age of first reproduction (2 years) as an estimate for generation time in Red Knots. However, an improved calculation for generation time has recently become available. This new calculation takes into account the effect of adult survival on generation time and is calculated as follows: $g = \alpha + [\, s / (1–s) \,]$ where α is age of first reproduction and s is expected adult survival rate (Lande et al. 2003, Sæther et al. 2005). In a long-lived shorebird species such as the Red Knot an individual's contribution to the gene pool may be affected by its survival, thus we recalculated generation time for Red Knots as $g = 2 + [0.8 / (1 – 0.8)] = 6$ years, using an estimate of 0.8 for adult survival (Brochard et al. 2002). Because mutation rate per year is unaffected by generation time this new calculation does not change the population divergence time estimates given by Buehler and Baker (2005). However, a generation time of six rather than two years for reduces the estimates of effective population size threefold, strengthening the argument for a recent and severe population bottleneck in Red Knots.

In this paper we extend the hypothesis of Buehler and Baker (2005) by including a more detailed examination of Red Knot habitats during emergence of present day flyways and by considering how these habitats may have impacted seasonal migrations and long term range expansions. Finally, we reflect on whether Red Knot flyways might be representative of other High Arctic waders and how this relatively rapid evolution of flyways argues against strict genetic programming of migration in waders.

A SCENARIO FOR THE EVOLUTION OF RED KNOT FLYWAYS

Eastward expansion from Eurasia into North America

Figure 2.1 summarizes the current distribution of Red Knots and overlays on it a phenogram that summaries Red Knot population structure and the broad temporal framework presented above. Using these time periods as a guideline, we now present a scenario for the evolution of Red Knot flyways.

The Wisconsinan/Weichselian glacial from 115 000 to 10 000 years ago was characterized by many stadial and interstadial intervals of varying intensity and duration. Two cold and dry maxima occurred, one about 70 000 years ago and another 22 000 to 18 000 years ago. Between these two maxima conditions were more moderate and tundra habitat suitable for Red Knots was characteristic of northwest and central Europe (Bohncke 1993, Walker et al. 1993), northern Siberia (Serebryanny & Malyasova 1998,

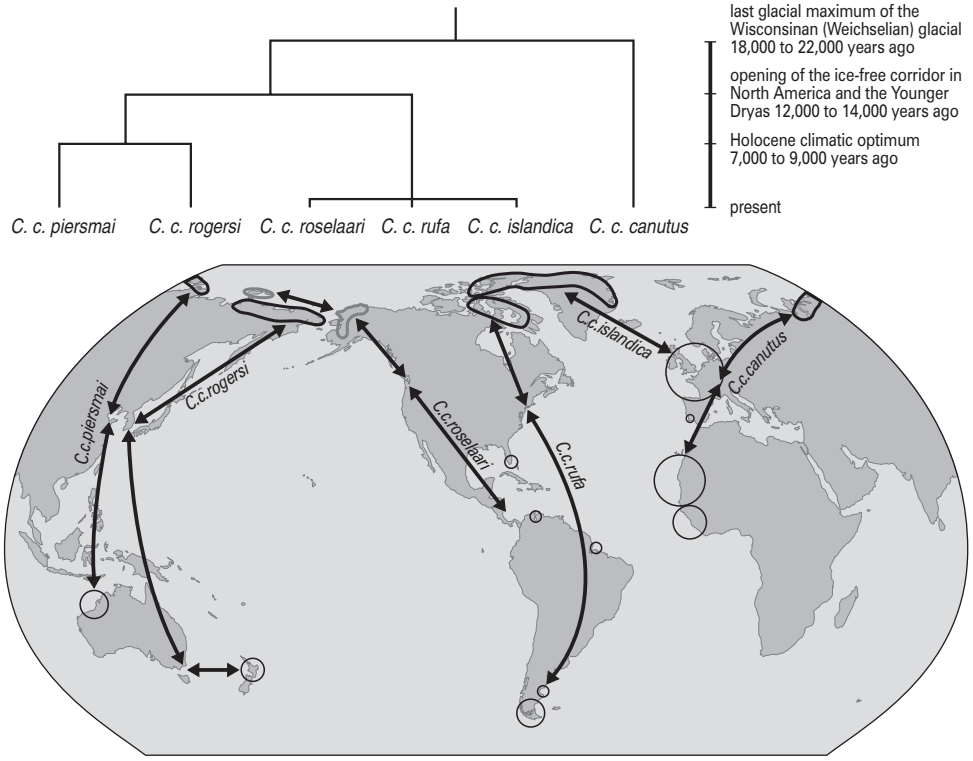


Figure 2.1. The global distribution of Red Knots (Piersma and Davidson 1992, T. Piersma pers. obs.). Shaded areas in the Arctic indicate breeding areas and circles indicate wintering areas. The size of the circle indicates the relative number of birds using the area. Migratory routes are colour coded to subspecies. Projected above the contemporary distribution of knots is a phenogram summarizing knot population structure and indicating major climatic events (with their approximate dates) that correspond roughly to divergence dates based on coalescent analysis of mitochondrial DNA. For population divergence date estimates and confidence intervals see text.

Hahne & Melles 1999), and small areas in northern Canada and Greenland (Möller et al. 1994, Lundqvist & Saarnisto 1995, Funder et al. 1998). Evidence also suggests that tundra existed south of the North American ice sheets, but this tundra may have been boreal in character and thus unsuitable for High Arctic waders such as Red Knots (Morgan et al. 1983). The area of arctic tundra during this time was greater than it is today and Red Knots were probably expanding their ranges (Kraaijeveld & Nieboer 2000). The last glacial maximum 22 000 to 18 000 years ago, however, was characterized by severe cold and aridity. During this period polar desert, characterized by < 4% vegetation cover and thus a harsher habitat than used by Red Knots today, separated the East Asian tundra along the Kolymskij Mountains (Adams & Faure 1997). This polar desert may have split a single ancestral population of knots into two groups, one breeding west of the polar desert somewhere in central Siberia and one breeding in Beringia (Fig. 2.2A).

Current continental shelf depths, estimates of sea level change, and the location of large rivers that deposit large quantities of sediment in to the interidal zone, can be used to make hypotheses about where suitable intertidal areas may have occurred during the last glacial maximum. Sea levels during the last glacial maximum have been estimated as 130 m lower than current sea levels (Pirazzoli 1996). As continental shelves range from 0 to 200 m below current sea level, intertidal areas may have existed at the edge of broad shelves. Furthermore, large rivers may have provided a constant source of intertidal sediment. We hypothesize that the Red Knot ancestral population breeding to the west of the polar desert may have staged in the Mediterranean Sea either at the inflow area from the Black Sea or at the mouth of the Nile River. Although the Mediterranean does not have large tidal ranges, contemporary knots are known to stage in areas with small tidal ranges provided there is sufficient food available, the Kneiss area in Tunisia being a good example (van der Have et al. 1997). This ancestral population probably wintered in areas along the west coast of Africa as *C. c. canutus* does today, but the exact location of these wintering grounds during the last glacial maximum is not known. Knots breeding in Beringia may have used the mouth of the Yangtze River and the Yellow Sea shores in China and the Koreas as a staging area and then continued south to wintering areas in Australia when the Yellow Sea shoreline systems froze over in winter.

Initial warming into our present interglacial began shortly after the last glacial maximum and gradually melted the ice in North America, thereby establishing an ice free corridor between the Laurentide and the Cordilleran ice sheets approximately 12 000 to 14 000 years ago (Pielou 1991, Adams & Faure 1997). During this initial warming period we hypothesize that the Beringian population of Red Knots expanded its range east into North America (Fig. 2.2B). Beringia has been used as a route into North America by a great number of species, including Humans *Homo sapiens* (Shutler 1983). Once in North America, these birds may have used the ice-free corridor between the Laurentide and Cordilleran ice sheets to pioneer a new and shorter migratory route to wintering grounds at the mouth of the Mississippi River on the Gulf of Mexico, and may also have migrated to the west of the Cordilleran ice sheet (Fig. 2.2B). Today a major North American flyway runs nearly parallel to the former ice free corridor follow-

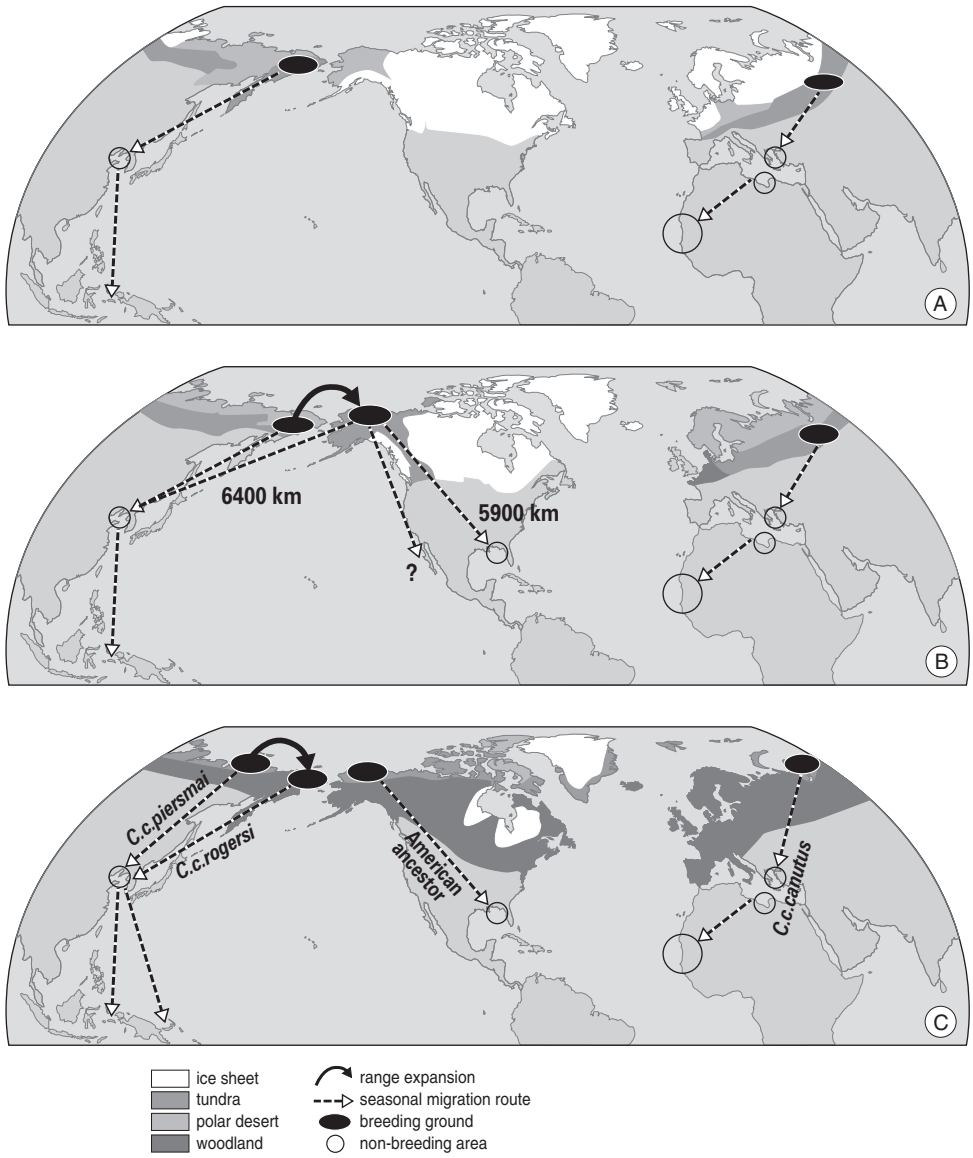


Figure 2.2. A graphical representation of our scenario for the evolution of Red Knot flyways. Palaeoreconstructions have been compiled from Adams and Faure (1997) and span from the last glacial maximum 18 000 to 20 000 years ago to present. In this figure polar desert refers to habitat characterized by < 4% vegetation cover, thus harsher than the 'polar desert' used by Red Knots today in which vegetation cover is usually between 4 and 25%. (A) Possible knot distribution during the last glacial maximum 18 000 to 20 000 years ago. (B) Possible expansion of knots into the North American Arctic. The great circle distances for the competing migratory routes are indicated. (C) Possible expansion of knots into the Chukotka Penninsula just before the Holocene climatic optimum, and the survival of knots in four isolated refugia during the optimum when suitable breeding tundra was greatly reduced (7000 to 9000 years ago).

ing the remnants of giant melt water lakes formed as the glaciers retreated. This hypothesis may explain why birds using this flyway do not follow a more straightforward migration route to breeding grounds in the central Canadian Arctic from the Gulf shores. Similar indirect migration routes have been attributed to Late Pleistocene range expansion, for example, in Swainson's Thrush *Catharus ustulatus* (Ruegg & Smith 2002). Finally, rapid cooling during the Younger Dryas (also about 12 000 years before present) may have played a role in isolating the Beringian and North American ancestral populations. During the Younger Dryas, polar desert crept south and may have geographically split the breeding populations (Fig. 2.2B).

Eurasia reached the climatic optimum of the current Holocene interglacial about 7000–9000 years ago (Adams & Faure 1997), during which woodland crept northward, reaching 300 to 400 km further north than its current boundary. Larches *Larix* spp. and birch *Betula* spp. trees were found in tundra in much of northern Siberia (Kremenetski et al. 1998) and shrub tundra covered northern Taymyr (Hahne & Melles 1999). In North America boreal forest reached right up to the remaining Laurentide ice sheet (Roberts 1989). Thus the Holocene optimum caused a large decrease in tundra breeding habitat and probably initiated local population bottlenecks in Red Knots. However, unlike Kraaijeveld and Nieboer (2000), we think it is unlikely that Red Knots were bottlenecked to a single refugial population in northern Canada and Greenland during the optimum. The subspecies currently inhabiting the High Canadian Arctic and Greenland has extremely low levels of nucleotide diversity making this area unlikely as the refuge for the ancestral population of Red Knots (Buehler & Baker 2005). Rather we propose that before the climatic optimum the Beringian breeding MRCA of *C. c. piersmai* and *C. c. rogersi* expanded its range into the Chukotka Peninsula. Later, during the climatic optimum, suitable tundra areas were isolated from one another by forest possibly causing the split between the *C. c. piersmai* and *C. c. rogersi* (Fig. 2.2C). We hypothesize that both populations probably followed migratory routes similar to what they do today using intertidal areas in the Yellow Sea and tropical Australia, (Battley et al. 2005) although the exact details of these routes will probably remain unknown.

A new migratory route into Europe

The most intriguing finding in this study is the possible establishment of the Greenland/Iceland migratory route from breeding grounds in North America to wintering areas in Western Europe. This idea contradicts conventional wisdom, which argues that this route most likely evolved via a northward range expansion into Iceland and then Greenland from staging and wintering areas in Western Europe. In this scenario, *C. c. canutus* and *C. c. islandica* are predicted to be sister subspecies (Piersma 1994). This idea has been supported in the past by morphological data which show *C. c. canutus* and *C. c. islandica* as similar in size and plumage colour (Tomkovich 1992), and very similar in their timing of annual cycle events including moult (Piersma & Davidson 1992). However, data presented in Buehler and Baker (2005) show *C. c. islandica* as genetically almost indistinguishable from *C. c. rufa* and *C. c. roselaari* birds. Furthermore, regardless of the exact dating, the North American breeders are deeply split from *C. c. canutus*. This distant relationship of *C. c. canutus* and *C. c. islandica* supports the

hypothesis of an expansion of knots across the North American Arctic from Beringia and the establishment of a new migratory route from the breeding grounds into Europe.

External morphological and plumage colouration similarities between *C. c. canutus* and *C. c. islandica* could have been acquired convergently by local adaptation on the breeding grounds, as both subspecies experience similar breeding conditions in the High Arctic of Northern Canada, Greenland and the Taymyr Peninsula. Similarities in winter plumage and the timing of moult could also have been acquired convergently as these subspecies partially share the nonbreeding grounds as well. The environmental conditions that these two subspecies experience differ from those experienced by *C. c. rufa* and *C. c. roselaari*, which occupy more dissimilar breeding ranges and wintering habitats. Indeed, this emphasizes that, when attempting to reconstruct population divergences, care should be taken to choose selectively neutral markers so that homologous and not convergent evolution can be examined. Genes under selection might be more likely to reveal genetic differences between the subspecies, but because of the possibility of convergence they would not represent historical population divergences. In this respect, we argue that the molecular markers used in Buehler and Baker (2005) may better represent the actual population structure of Red Knots than morphological characters.

Molecular markers give only an estimation of population divergence times, and for the mitochondrial DNA estimates presented in Buehler and Baker (2005) credibility intervals for the population divergence of *C. c. roselaari, C. c. rufa* and *C. c. islandica* include zero (95% CI: 0–16 500 years ago). Thus, we can only conclude that these populations diverged at some point between the last glacial maximum and the present. Contemporary Red Knots require cold tundra with sparse vegetation for breeding and extensive intertidal areas with soft sediments during the non-breeding season. Assuming that these current habitat requirements are representative of what was needed for survival in the past, we can now ask where these habitats occurred during the last glacial maximum and the subsequent Holocene warming.

Tundra vegetation has only recently colonized land left barren by receding ice sheets in the Canadian Arctic, allowing Red Knots to expand their breeding range east from northern Alaska. We propose that this eastward expansion of breeding ranges and the emergence of present day intertidal areas facilitated the evolution of the current flyways of *C. c. rufa* and *C. c. islandica*. How did *C. c. rufa* pioneer a migration route into the southern most reaches of South America? These birds were probably expanding their range eastwards in the central Canadian arctic as the ice sheets receded and tundra became available. As they migrated south they would have found suitable wintering habitats in the south eastern parts of North America already occupied by the earlier evolved *C. c. roselaari* population, possibly necessitating a flight across the Gulf of Mexico (Fig. 2.3A). In addition, the prevailing wind conditions may have played a role, with knots occasionally blown over the Atlantic Ocean during a season with particularly strong westerly winds. Once over the open water, individuals who had made it far enough south might have been aided by easterly equatorial trade winds facilitating migration to South America (Stoddard et al. 1983, Gauthreaux 1991, National Geographic Society 1992). In South America suitable staging areas may have existed at and

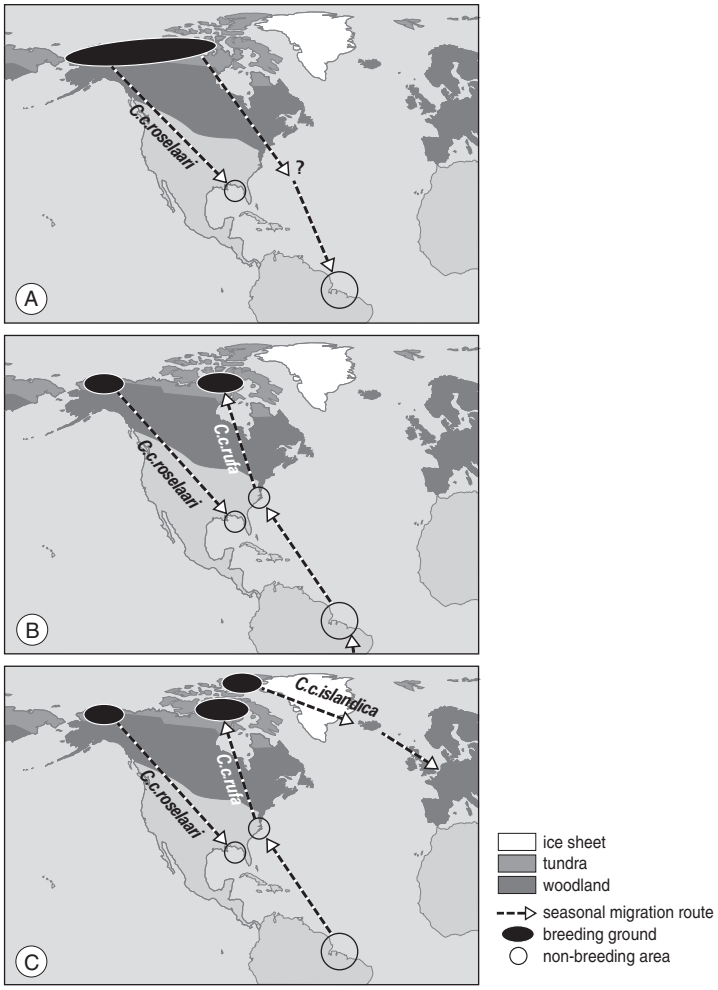


Figure 2.3. A graphical representation of the proposed range expansions and the establishment of new migratory routes in North America with subsequent expansion into Europe. (A) Possible expansion of knots eastward into the Canadian Arctic Archipelago followed by the establishment of a migratory route into South America. (B) The possible establishment of the contemporary *C. c. rufa* flyway for spring migration after the emergence of Horseshoe crab spawning grounds along the eastern seaboard of the United States. (C) Possible expansion of knots into the High Canadian Arctic and subsequent pioneering of a migratory route via Iceland into Europe.

around the mouth of the Amazon River, and by about 5000 years ago intertidal areas in Tierra del Fuego also became available for knots (Porter et al. 1984, Gordillo 1999).

The question then becomes how did the birds that wintered in South America return to their breeding grounds and where could they stage on spring migration? Birds of the *C. c. rufa* subspecies currently rely on spawning Horseshoe crabs in Delaware Bay to provide them with abundant food for their journey to the Arctic. The sandy

beaches along the north eastern seaboard of North America became suitable for Horseshoe crab spawning very recently and it is possible that huge numbers of crabs were not found in the area until a few thousand years ago (Schuster et al. 2003). This emergence of the North American seaboard as a staging area may have provided *C. c. rufa* pioneers with the stepping stone that they needed to reach breeding tundra in the Canadian Arctic (Fig. 2.3A).

As ice-free habitat emerged in the High Arctic islands of Canada and Northern Greenland, Red Knots currently considered *C. c. islandica* probably expanded their breeding range to the north and east (Fig. 2.3C). Genetic analysis of Greenland Eskimos also indicates relatively recent expansions into these areas (Saillard et al. 2000). But how might individuals of the *C. c. islandica* subspecies have pioneered a migratory route to wintering areas in Europe? The proximity of High Arctic breeding areas to the North Pole means that only a small eastwards shift in orientation (50°) would take birds breeding in Ellesmere Island and northern Greenland to Iceland rather than to the Atlantic coast of North America. The likely genetic template for migration is probably to follow a southward bearing, therefore such a shift in direction is feasible given severe orientation complications in regions close to the North Pole where nearly any bearing is south (Gudmundsson & Alerstam 1998). This new migratory route however, requires a hazardous crossing over the Greenland icecap, and Henningsson & Alerstam (2005a) point out that ecological barriers play a large role in shaping migratory routes. Nevertheless, the route from breeding grounds in Ellesmere Island and northern Greenland to staging areas in Iceland is nearly 1000 km shorter than a migration to the Bay of Fundy, and it does not require a full crossing of the Arctic Ocean. Furthermore, this link across the Greenland icecap is used today by other species of shorebirds including Ruddy Turnstones *Arenaria interpres*, Sanderlings *Calidris alba* and Ringed Plovers *Charadrius hiaticula*, indicating that migrant shorebirds are capable of crossing even severe ecological barriers (Henningsson & Alerstam 2005a). Thus, birds utilizing this new route were likely at a selective advantage either in terms of a shorter migration distance (Pienkowski & Evans 1985) or in terms of better staging and wintering areas in Iceland and Europe.

Favourable staging and wintering areas became available in the Wadden Sea, the British Isles and Iceland 4–5000 years ago (Vos & van Kesteren 2000, Simonarson & Leifsdottir 2002) and would thus have been available to *C. c. islandica* pioneers of a new migratory route (Fig. 2.3C). Current conditions in Iceland and northwest Europe continue to provide a suitable climate for staging and wintering birds at higher latitudes than the Atlantic coast of North America due to the moderating effect of the North Atlantic current.

CAN THIS HYPOTHESIS BE TESTED?

The palaeovegetation record is not perfect, and our hypotheses are based on population divergence times from a single genetic marker with broad 95% credibility intervals. Nevertheless, these divergence times correlate well with major events during the

Pleistocene, events which have had an impact on patterns of species distribution of many other species in the circumpolar Arctic (Henningsson & Alerstam 2005b). Clearly, the testing of this hypothesis of circumpolar expansion eastwards from Asia into North America and then into Europe is needed.

The first question to ask is whether such an expansion has occurred in other species. The circumpolar range expansion of the Herring Gull *Larus argentatus* complex has been the focus of much research as a classic example of the 'ring species model' (Mayr 1942). Recently, work on mitochondrial DNA variation among 21 gull taxa, has shown that the complex differentiated largely in allopatry following multiple segregation and long-distance-colonization events (Liebers et al. 2004). Although this finding argues against Mayr's 'ring species model', the results provide an example of an eastward expansion into North America much like that which we argue occurred in Red Knots. According to Liebers et al. (2004), two gull taxa (*L. glaucoides* and *L. smithsonianus*) colonized North America across the Bering Strait from an Aralo-Caspian refugium. Unlike in Red Knots, there is no subsequent development of a migratory route back to Europe. However, gulls are ecologically very different from knots and to the best of our knowledge there are no examples of this type of expansion in shorebirds. This is because very little genetic work has been done on shorebirds, at least at the intraspecific level, with the exception of Red Knots and Dunlins, which have very different demographic histories (Wenink et al. 1993, Wennerberg 2001, Buehler & Baker 2005).

An interesting way to test our hypothesis may be to expand upon the Red Knot versus Dunlin comparison presented by Buehler and Baker (2005) and examine population divergence times in shorebirds with circumpolar breeding ranges both in the extreme High Arctic such as the Red Knot and in the Lower Arctic such as the Dunlin. It is possible that the hypotheses of a breeding ground to wintering ground versus a wintering ground to breeding ground development of the Greenland/Iceland flyway need not be mutually exclusive. Red knots and Dunlins have different ecological requirements, with knots being more restricted to High Arctic breeding areas and marine wintering sites. It is possible that less constrained species such as Dunlins extended their breeding ranges towards Iceland/Greenland by jump dispersal and developed migration along the axis of this range expansion, as supported by the closer genetic relationship between birds using the Greenland/Iceland flyway to other European lineages than to North American lineages. In contrast, it is possible that High Arctic breeders, especially those with known low genetic variability such as Ruddy Turnstones and Sanderlings (Baker et al. 1994), may have expanded their ranges from recently bottlenecked ancestral stock under the same environmental conditions as Red Knots and may thus also have pioneered the Greenland/Iceland flyway from the breeding to the wintering grounds. If this hypothesis is correct for High Arctic breeders, we predict very recent population divergence times between lineages breeding in North America and older divergence dates from Eurasian breeding lineages of these species.

IMPLICATIONS FOR STRICT GENETIC PROGRAMMING OF MIGRATION

There is an element of speculation involved when examining what may have happened to birds pioneering new migratory routes, especially in the distant past. The idea of individuals establishing completely new migratory flyways is controversial. Sutherland (1998) has argued that migratory behaviour is either genetically or culturally determined and that the development of new migratory routes is more likely in species with culturally determined migration. However, the ideas discussed in this paper argue against an absolute genetic program for migratory routes in waders.

We discuss the recent engineering of six different migratory routes within the last glacial of the Pleistocene, at least two of which (those of *C. c. rufa* and *C. c. islandica*) probably occurred within the last few millennia as suitable breeding and wintering areas became available during deglaciation. Range expansions and concurrent development of new migratory routes in Arctic breeding species as ice receded after the last glacial period was not unique to Red Knots and must have occurred all over the northern hemisphere. This recent establishment of migratory routes must have occurred not only in species with culturally determined migration, but also in species like knots, in which juveniles perform their first migration unaccompanied by their parents, thus falling into the category of 'genetically determined migrants'. During periods of climatic upheaval such as the Pleistocene an absolute genetic program would result in birds flying to their deaths as breeding and wintering areas became ice covered or flooded, and we feel that this is unlikely in a whole suite of 'genetically determined migrants'. What may be more likely is a simple genetic template for migratory behaviour, such as a predisposition to follow a southward bearing, accompanied by learned behaviour that responds to the environment to perfect the new migratory route.

ACKNOWLEDGEMENTS

Alberto Castillo, Julia Schroeder and Yvonne Verkuil provided discussion and support in Groningen and Jim Wilson, Nigel Clark, Humphrey Sitters and Meinte Engelmoer provided helpful ideas at the 2004 IWSG meeting. At earlier stages of this project Christian Hjort was of great help. We thank Dick Visser and Sara Henningsson for help with the figures and maps and Bruno Bruderer for comments on the manuscript. This research was supported by Natural Sciences and Engineering Research Council of Canada (NSERC) Post Graduate Scholarships (PGSA-231177-2000 and PGSB-267701-2003) to DMB, a PIONIER grant to TP from the Netherlands Organisation for Scientific Research (NWO), and grants from NSERC (200-02) and the Royal Ontario Museum Foundation to AJB.

CHAPTER 3

Travelling on a budget: predictions and ecological evidence for bottlenecks in the annual cycle of long-distance migrants

Deborah M. Buehler and Theunis Piersma

ABSTRACT

Long-distance migration, and the study of the migrants who undertake these journeys, has fascinated generations of biologists. However, many aspects of the annual cycles of these migrants remain a mystery as do many of the driving forces behind the evolution and maintenance of the migrations themselves. In this article we discuss nutritional, energetic, temporal and disease risk bottlenecks in the annual cycle of long-distance migrants, taking a sandpiper, the red knot *Calidris canutus*, as a focal species. Red knots have six recognized subspecies each with different migratory routes, well known patterns of connectivity, and contrasting annual cycles. The diversity of red knot annual cycles allows us to discuss the existence and the effects of bottlenecks in a comparative framework. We examine evidence for bottlenecks focusing on the quality of breeding plumage and the timing of moult as indicators in the six subspecies. In terms of breeding plumage colouration, quality, and timing of prealternate body moult (from non-breeding into breeding plumage), the longest distance migrating of the knot subspecies, *C. c. rogersi* and *C. c. rufa* show the greatest impact of bottlenecking. The same is true in terms of prebasic body moult (from breeding into non-breeding plumage), which the in case of both *C. c. rogersi* and *C. c. rufa* overlaps with southward migration and may even commence in the breeding grounds. To close our discussion of bottlenecks in long-distance migrants, we make predictions about how migrants might be impacted via physiological "trade-offs" throughout the annual cycle, using investment in immune function as an example. We also predict how bottlenecks may affect the distribution of mortality throughout the annual cycle. We hope that this framework will be applicable to other species and types of migrants, thus expanding the comparative data base for future evaluation of seasonal selection pressures and the evolution of annual cycles in long-distance migrants. Furthermore, we hope that this synthesis of recent advancements in the knowledge of red knot annual cycles will prove useful in ongoing attempts to model annual cycles in migratory birds.

Philosophical Transactions of the Royal Society of London Series B 363: 247-266

INTRODUCTION: ANNUAL CYCLES AND LIFE HISTORY TRADE-OFFS IN AVIAN MIGRANTS

The Earth provides an ever fluctuating environment. Yearly, the earth revolves around the sun and its 23.5° tilt gives seasons. These seasons bring about fluctuations in resource availability and temperature that can be potent selective forces in the evolution of life (Alerstam 1990). Non-tropical animals display a variety of behavioural adaptations to cope with the challenge of seasonal survival. These adaptations influence the seasonal pattern of behaviour in the animal's life and influence the timing of major events throughout the year. In birds, these patterns of behaviour include breeding, moult, winter survival and migration, and are thought of as the bird's annual cycle.

This article focuses on annual cycles in migrant birds and considers migration as an adaptation for exploiting seasonal peaks of resource abundance while avoiding seasonal resource depression (Alerstam et al. 2003). Long-distance migration in particular allows migrants to exploit widely spaced resources during periods of high productivity. For example, shorebirds and gulls exploit an abundance of horseshoe crab *Limulus polyphemus* eggs on the beaches of Delaware Bay, USA, using a short window of opportunity during which their spring migration coincides with horseshoe crab spawning (Schuster et al. 2003). After Delaware Bay, they continue to Arctic tundra areas, where they are able to reproduce in remote and extreme environments only suitable for 2-3 months each year. During the short summer these habitats offer the advantages of long days, sufficient food resources and fewer pathogens and parasites than temperate breeding areas further south (Greiner et al. 1975; Mendes et al. 2005; Piersma 1997). However, to gain these benefits, migrants must perform demanding migrations often covering thousands of kilometres and requiring a complete change in physiology as they alternate between phases of the annual cycle (Piersma et al. 1999b; Piersma & Lindström 1997; Wingfield 2005).

Both migrants and residents must cope with different environments; however, the annual cycles of residents and migrants differ markedly. Residents must survive and find food in a changing seasonal environment requiring a high capacity for behavioural flexibility (Sol et al. 2005). Residents, however, do not have to carry out long and demanding travels. In contrast, migrants travel to a great diversity of habitats throughout their annual cycle. These travels reduce the amplitude of fluctuation in seasonal resource levels, but while travelling migrants must adjust to unfamiliar surroundings, balance conflicting demands between predator avoidance and fast fuel acquisition, cope with unfavourable weather, and determine the correct direction for the next leg of their journey (Piersma 1987; Piersma et al. 1990). Furthermore, they must satisfy nutritional demands not only for survival but also to fuel the energy cost of transport. Finally, all of this must be precisely timed to best exploit food resources along their migration route.

One way to look at differences in the annual cycles of residents and migrants and to examine annual cycles in general is through the framework of finite-state machine theory (Jacobs & Wingfield 2000; Wingfield 2007 this issue). This theory describes an organism's life cycle as a series of life history stages, such as breeding, moult, winter survival and migration. These stages are distinct, independent of one another and occur

in a set sequence that cannot be reversed. The finite nature of these life-history stages, and the states which occur within the stages, provides the analogy of the annual cycle as a finite-state machine (Jacobs & Wingfield 2000). Because migrants must pass through both northward and southward migration in addition to breeding, winter survival and moult, they have more life history stages than residents and this may mean less flexibility in timing throughout the annual cycle (Wingfield 2005).

This decrease in flexibility can be examined by looking at overlap between potentially costly life history stages such as migration or moult. Indeed, moult rarely overlaps with breeding or with the cruellest months of the winter (Dietz et al. 1992; Masman et al. 1988; Payne 1972). Furthermore, migration and moult rarely overlap (Payne 1972), and in some taxa such as waterfowl and grebes, moult completely constrains migration as it prevents the birds from flying (see Jehl 1990 for a review). In other taxa, however, flying remains possible but moult imparts an energetic cost, limiting overlap with migration. For example, in a split-brood experiment, blackcaps *Sylvia atricapilla* were kept in either a natural photoperiod or in an experimental photoperiod to advance and prolong moult (Pulido & Coppack 2004). In both groups the onset of migratory activity was significantly correlated with the termination but not with the onset of moult, and moult intensity at the onset of migration was low. If moult and migration do overlap, then active moult is suspended for the duration of active migration. This phenomenon is found in several shorebirds breeding at temperate and boreal latitudes in Eurasia and wintering in inland Africa, notably ruffs *Philomachus pugnax* (Koopman 1986) and black-tailed godwits *Limosa limosa limosa* (van Dijk 1980).

Another potential indicator of decreased flexibility in the annual cycle of migrants is decreased investment or a shift in immune function during potentially costly life history stages. This idea is based on the assumption that trade-offs exist between immune defence and other functions that share common resources and contribute to fitness (Sheldon & Verhulst 1996). Immunity can be divided into innate (nonspecific) and acquired (specific) responses, and further divided into constitutive (always present) and induced aspects. Due to the complexity of the immune system it is difficult to define a single ecological or evolutionary currency with which to measure the cost of immune function (Martin 2007 this issue). From a physiological standpoint, the costs of immunity can be subdivided into three components: development, maintenance and use, each of differing among different types of immunity (Klasing 2004). Inflammatory responses such as induced innate and cell-mediated acquired immunity are considered the most costly in terms of use because of the metabolic requirements of immune cells and due to indirect consequences such as tissue degradation or anorexia (Lochmiller & Deerenberg 2000; Klasing 2004). In contrast, mounting a specific antibody-mediated acquired response is thought to be less costly (Klasing 2004). Maintenance costs remain extremely difficult to measure, but it is thought that maintenance costs for constitutive immunity, both innate and acquired, are quite low (Klasing 2004; Lee 2006). Taking the complexities of the immune system into account, Lee (2006) provides a framework predicting a switch from reliance on inflammatory (cell-mediated acquired and constitutive innate) to specific (antibody-mediated acquired) immunity during high intensity effort or more demanding seasons.

Seasonal patterns in immune function have been well studied in temperate resident mammals (Nelson & Demas 1996) and research is accumulating in resident birds (Martin 2007 this issue). But how does immune function change throughout the year in migrants? To date, very little is known. Immune function has been studied in long-distance migrating ruffs and has shown that captive birds show decreased cell-mediated immune responses during the breeding versus the non-breeding season (Lozano & Lank 2003). However, to our knowledge, nothing is known about seasonal variability in immune function in long-distance migrants throughout the entire annual cycle, and it is beyond the scope of this paper to address this issue with empirical evidence. We do, however, make predictions about immune function in the context of trade-offs and bottlenecks in the annual cycle of red knots.

Closely tied to the concept of the annual cycle are annual routine models of optimal behaviour. Houston and McNamara (1999) introduced a modelling framework that takes into account the fact that optimal behaviour relies not only on isolated "decisions" but also on circumstances during the annual cycle as a whole (see also McNamara & Houston 2006 this issue). This approach has been used to model optimal migration timing and reproductive effort under varying circumstances (McNamara et al. 1998; McNamara et al. 2004). When first using this approach to model migration timing, McNamara et al. (1998) pointed out that the problem is that a lot of information about the organism and their annual cycles is required. They then suggested red knots *Calidris canutus* as a realistic species to model given that much information is known about these birds and their migrations. Well studied species are important in the development of realistic models because the details of their annual cycles are necessary as a framework from which to hang state variables used to examine interactions between factors affecting behaviour. Once developed, these models may be generalized and can be useful for species where such detailed knowledge is not available. Red knots remain an excellent species to model and nearly a decade later even more is known about their annual cycles. We hope that this review pushes forward theoretical as well as empirical studies on migrants and their annual cycles by providing a framework of bottlenecks via which selection pressures may act, and by synthesizing what is known about these bottlenecks in the annual cycle of a representative migrant.

The goal of this article is to examine ecological evidence for bottlenecks in the annual cycle of long-distance migrants and to make predictions using this framework. To establish the comparative setting, we first introduce red knots as a focal species and describe their subspecies and annual cycles. We then define possible nutritional, energetic, temporal and disease risk bottlenecks in their annual cycle and discuss possible evidence for these bottlenecks, looking in depth at the quality of breeding plumage and the timing of moult in the six subspecies as indicators. Finally, we make predictions about how these bottlenecks might impact long-distance migrants via physiological "trade-offs" such as investment in immune function, and how these bottlenecks may affect the distribution of mortality during the annual cycle. Throughout this paper we limit ourselves to the migration and annual cycle of adults. The selection pressures affecting juvenile birds may be very different from those affecting adults and are excluded from this discussion to limit the article to a manageable length.

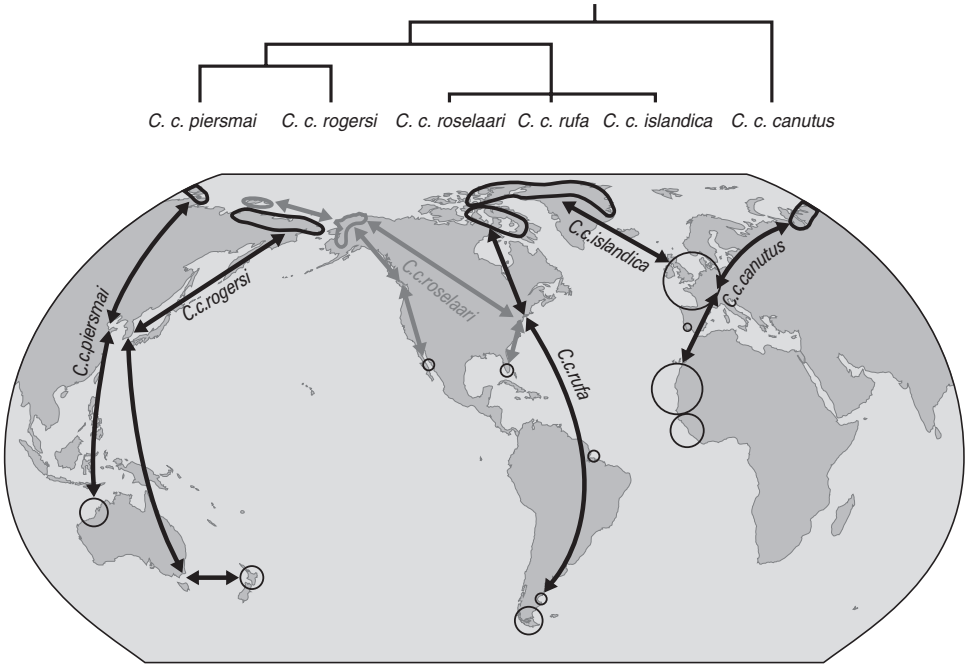
RED KNOT AS A MODEL SYSTEM

Red knots (hereafter referred to simply as knots) provide a beautiful study system for the investigation of bottlenecks throughout the annual cycle of a long-distance migrant. Knots are medium sized shorebirds with six subspecies and a web of migratory routes that spans the globe. Knot flyways cover a diversity of environments from the High Arctic, to the American and European North Temperate Zone, to the desert coasts of Africa and Australia, to the sub-Antarctic flats of Tierra del Fuego (Figure 3.1). It is this wide diversity of migratory routes, occurring within a single species, that make knots an ideal focal organism. Another benefit of knots as a focal species is that among the six subspecies, patterns of connectivity and population genetics are relatively well known (Figure 3.1).

As a species, knots share several common traits. All subspecies breed in harsh High Arctic habitats, all winter in coastal areas, and like most shorebird species, they lay a four egg clutch. On the breeding grounds incubation is shared, but females depart right after the eggs hatch leaving the males to care for the chicks until they fledge (Tomkovich & Soloviev 1996; Whitfield & Brade 1991). After fledging the males depart and the chicks undertake their first migration south independently. On the breeding grounds knots eat mostly spiders and arthropods obtained by surface pecking (Tulp et al. 1998) and on the wintering grounds they eat a variety of hard-shelled prey such as bivalves, gastropods and small crabs obtained by high frequency probing and the use of a specialized bill tip organ used to find hard objects in soft sediments (Piersma et al. 1993b; 1998). Prey are ingested whole and crushed by a muscular stomach (Piersma et al. 1999a; 1993b). Yet, beyond this brief description, the uniformity stops.

Knots are comprised of six distinct breeding populations, all of which are currently recognized as subspecies based on morphological characteristics and distinct migratory routes: *Calidris canutus canutus*, *C. c. piersmai, C. c. rogersi, C. c. roselaari, C. c. rufa* and *C. c. islandica*. Morphologically, knots have been subdivided by tarsus length, wing length and bill length as well as various fine points pertaining to plumage (Tomkovich 1992; 2001). Overall, *C. c. piersmai* is the smallest in size, followed by *C. c. rogersi*, then *C. c. islandica* and *C. c. canutus*, then *C. c. rufa* and finally *C. c. roselaari*, the largest subspecies. With respect to plumage characteristics, *C. c. canutus, C. c. islandica* and *C. c. piersmai* are the "darker" subspecies. *C. c. rogersi* have lighter bellies than both *C. c.* roselaari and *C. c. piersmai*, and *C. c. rufa* is the lightest in overall plumage (Figure 3.1).

Remarkably, in knots there is complete congruence between morphological typing and behavioural differences in migratory routes, leading to relatively well known patterns of connectivity. Evidence from long term ringing programs indicates that distinct flyways exist and correspond to separate breeding areas in the Arctic (Piersma & Davidson 1992). During the course of migration some subspecies share certain staging areas; for example, *C. c. rufa* and *C. c. roselaari* in Delaware Bay and the southeastern USA (Atkinson et al. 2005), and *C. c. islandica* and *C. c. canutus* in the Wadden Sea in Europe (Nebel et al. 2000). However, the subspecies can usually be distinguished using the timing of passage and their primary moult status. For example, during southward migration in South Carolina and Georgia, *C. c. roselaari* moult their primary feathers,



	C. c. piersmai	*C. c. rogersi*	*C. c. roselaari*	*C. c. rufa*	*C. c. islandica*	*C. c. canutus*
Body size (1=smallest) (1)	1.4	2.2	5.8	4.6	2.8	4.2
Plumage (6=darkest) (1)	4	2	3	1	5	6
Total migration distance (km) (2)	10400	15000	7500	15000	4650	9000
Max. single flight distance (km) (2)	6300	6500	5600	8000 (2)	2830	5100
Breeding latitude (2)	75–78°N	67–70°N	65–75°N	64–76°N	76–85°N	75–80°N
Population size	30000 (3)	100000 (3)	35000 (4)	30000 (5)	400000 (4)	300000 (4)

Figure 3.1. The global distribution of knots (updated from Piersma & Davidson 1992), highlighting morphological and behavioural congruence as well as genetic structuring in knot flyways. Migratory routes are colour coded to subspecies and dotted lines represent routes requiring further study. The South African wintering area present in Piersma & Davidson (1992) is not shown as knots no longer seem to winter there (L. Underhill pers. comm.). Shaded areas in the Arctic indicate breeding areas and circles indicate wintering areas. The size of the circle indicates the relative number of birds using the area. Projected above the contemporary distribution of knots is a phenogram summarizing knot population structure (Buehler & Baker 2005) and below a table outlining morphological, migration and population size details: (1) Bill, tarsus and wing length measurements were ranked and averaged for size score, and overall extent of redness and depth of colour were taken into account for rank plumage score (Tomkovich 1992, 2001); (2) Piersma et al. (2005); (3) P.F. Battley pers. obs.; (4) T. Piersma & B. Spaans, unpublished data; (5) Baker et al. (2004, 2005a).

whereas *C. c. rufa* do not because they still need to cross the Caribbean Sea to South America (B. A. Harrington pers. comm.). Wintering areas are geographically distant from one another and banding studies to date have shown that subspecies are not mixing on the wintering grounds (Piersma & Davidson 1992).

Close congruence has also been found between migratory routes and genetic differences between populations in knots. Genetic differentiation has been found between four groups: *C. c. canutus, C. c. piersmai, C. c. rogersi* and a North American breeding lineage comprised of *C. c. roselaari, C. c. rufa* and *C. c. islandica* (Figure 3.1; Buehler & Baker 2005; Buehler et al. 2006). The single discordance arises in the deep separation between *C. c. canutus* and *C. c. islandica* which are very close in terms of morphology and the timing of annual cycle events (Piersma & Davidson 1992). This incongruence may be explained by that fact that many genes code for size, plumage characteristics and annual cycling, and the fact that these genes are under strong selection. For example, the Arctic tundra areas used by *C. c. canutus* and *C. c. islandica* may be more similar than those used by *C. c. rufa*, possibly leading to selection for similar breeding plumage and size characteristics. Furthermore, as is discussed later, bottlenecks on the timing of moult are more similar in *C. c. canutus* and *C. c. islandica* than in the more closely related *C. c. islandica* and *C. c. rufa*. Because characteristics such as size and plumage are polygenetic (coded for by many genes) and are under selection they can evolve much faster than the single, and by definition selectively neutral genes, used in genetic typing.

The next few sections are dedicated to describing the six recognized subspecies of knot and details of their migrations; phenology and moult are summarized in Table 3.1 and Figure 3.2.

North temperate winterers

C. c. islandica. — *C. c. islandica* is one of the best studied subspecies. Its breeding grounds extend further north any of the other subspecies, spanning from 75°N to 85°N and covering northern Greenland and the Queen Elisabeth Islands west to Prince Patrick Island. Adults leave the breeding grounds between mid July and early August and fly non-stop (R. I. G. Morrison pers. comm.) to staging grounds in western Iceland where they refuel from late July to early August (Davidson & Wilson 1992). From Iceland the birds fly to the Wadden Sea where prebasic body moult (from breeding into non-breeding plumage) occurs in August and September and wing moult extends from August to mid October for adults. From October to December the birds disperse to wintering grounds, moving west from the Wadden Sea to Britain, and moving northwest within Britain (Davidson 2002), where they remain until March. In late March some of the birds return eastwards to the Wadden Sea, and some remain the UK estuaries where they undergo prealternate moult (from non-breeding into breeding plumage) and fuel for the journey north. From there they travel north to their final staging areas in Iceland and northern Norway (Davidson et al. 1986; Wilson & Strann 2005). In the last week of May there are synchronous departures to the breeding grounds where birds arrive in early June.

C. c. roselaari. —In contrast to *C. c. islandica*, the *C. c. roselaari* subspecies is the least studied of the six and as such details about its migration route and breeding areas are not well known. *C. c. roselaari* are thought to breed in northwest Alaska and Wrangel Island (67-73°N) probably from early June to mid July (Tomkovich 1992). From the breeding grounds they may migrate across the North American continent

perhaps using the large probably brackish lakes along this flyway as staging areas. It is also possible that *C. c. roselaari* birds fly non-stop over the continent and then intermingle with birds of the *C. c. rufa* subspecies on the Atlantic coast of North America. *C. c. roselaari* also uses the Pacific coast flyway and at least some individuals winter in California, USA and Baja California, Mexico (Tomkovich 1992, Page et al. 1997, Page et al. 1999). More investigation on this subspecies is needed to clarify the details of their migration routes. *C. c. roselaari* individuals that fly to Florida and the Gulf of Mexico, reach their wintering grounds in September. These birds probably spend September to April on the wintering grounds and begin prebasic body moult and wing moult as soon as they arrive. The duration of wing moult is not definitively known for this subspecies, but it is likely, given that wing moult does not render knots flightless at any stage, that wing moult continues into the end of November. Prealternate moult probably takes place from mid March to mid April and migration northwards can be traced around the west coast of the Gulf of Mexico by late April to early May and across the prairie provinces of Canada by late May (Morrison & Harrington 1992). In addition, stable isotope analysis has shown that some birds of the Florida wintering *C. c. roselaari* subspecies most likely join members of the *C. c. rufa* subspecies on the beaches of Delaware Bay in May where they take advantage of abundant horseshoe crab eggs (Atkinson et al. 2005).

Tropical winterers

C. c. canutus. —Birds belonging to the *C. c. canutus* subspecies breed in the coastal tundra of the Taymyr Peninsula (75°N to 80°N). From there they migrate to staging areas in the Wadden Sea where they pass from late July to early August (Nebel et al. 2000). The birds do not moult in the Wadden Sea, but continue on to wintering areas in the equatorial coasts of Mauritania and Guinea-Bissau, Africa where prebasic body moult occurs from late August until the end of October and wing moult takes place from late August to early December (Piersma et al. 1992, B. Spaans et al. unpublished data). Birds remain in the wintering areas from September through April, and prealternate moult occurs there from mid March through the end of April. Departure northwards begins in late April and the birds arrive in staging areas in the Wadden Sea in mid May for refuelling (Prokosch 1988). Staging areas in Portugal and France are used only briefly often as "emergency sites" when birds encounter headwinds and cannot make it directly to the Wadden Sea (Piersma 1987; Smit & Piersma 1989). *C. c. canutus* leave the Wadden Sea in early June passing southern Sweden and reaching the breeding grounds in mid June (Gudmundsson 1994).

C. c. piersmai. —*C. c. piersmai* is the most recently defined of the six subspecies (Tomkovich 2001) and breeds in the New Siberian Islands probably from early June to mid July. From there they most likely migrate to staging areas along the coast of eastern Asia but the details of this flight are not fully known. Their migrations then continue to wintering grounds mainly in tropical northwest Australia where they arrive by the end of August to early September (D.I. Rogers pers. comm.) and remain until the end of April. Wing moult takes place in the wintering areas from late August to early September and is completed between mid January and early February (D.I. Rogers,

pers. comm.). Prebasic body moult is faster beginning upon arrival in the wintering grounds in late August and early September and completed by October (Higgins & Davies 1996). Some birds arrive on the wintering areas already showing non-breeding body plumage indicating that some prebasic body moult might take place on staging areas (D.I. Rogers, pers. comm.). Prealternate moult takes place from late February until the end of March. *C. c. piersmai* differs from other subspecies in that there is a long time lag between the completion of prealternate moult and departure on northward migration (Battley et al. 2005). Furthermore, departure north from their wintering grounds takes place very late; for instance, on 5 May 2000 many birds were still on the wintering grounds (Battley et al. 2005). This late departure indicates that staging on the shores of the Yellow Sea on northward migration is very rapid, and only feasible if high quality prey are available (Battley et al. 2005). Knots in the Americas use horseshoe crab eggs as a high quality food source during northward migration and it may be possible that spawning horseshoe crabs in China and Southeast Asia (*Tachypleus tridentatus, T. gigas* and *Carcinoscorpius rotundicauda*) provide a food resource for *C. c. piersmai*. However, to date knots have not been observed feeding on horseshoe crab eggs in Asia and this possibility remains to be investigated further.

Trans-equatorial south temperate winterers

C. c. rogersi. —The *C. c. rogersi* subspecies breeds in the northern Chukotski Peninsula probably from early June to mid July. From there these knots most likely migrate to staging areas in the Sea of Okhotsk and the northern Yellow Sea in East Asia, but the details of this part of their migration require further study. Birds begin to arrive on the wintering grounds in southeast Australia and New Zealand in late September (Barter 1992). Prebasic body moult begins in unknown staging areas and ends on the wintering areas in southeast Australia and New Zealand from mid October to the end of November (Higgins & Davies 1996). The combination of moult and migration is unique to this subspecies and to *C. c. rufa* described below. The birds spend October to the end of March on the wintering grounds, and begin wing moult from mid October to the end of November. This wing moult is completed between the middle of January and early March (Higgins & Davies 1996; P.F. Battley pers .comm.). Prealternate moult begins from the middle of January to end of March (Battley 1997; Battley & Piersma 1997), but it may not be completed before departure and it is possible that this body moult is continued on staging areas during northward migration as seen in *C. c. rufa*. Departure northward takes place from mid March to the beginning of April (Battley 1997; P.F. Battley, pers. comm.) and the birds fly to staging areas in the northern Yellow Sea where they have been seen in early May, with New Zealand birds probably making an intermediate stopover en route.

C. c. rufa. —The *C. c. rufa* subspecies is one of the best studied groups and is known to breed in the central Canadian Arctic from Victoria Island southeast to South Hampton Island (65-75°N) from early June to mid July (Harrington 2001; Morrison & Harrington 1992). Prebasic body moult begins on the breeding grounds but is suspended after departure. This subspecies is most likely unique in beginning moult in the breeding grounds, although *C. c. rogersi* may also begin prebasic body moult on the

breeding areas. In addition, *C. c. rufa* is one of the only subspecies, along with *C. c. rogersi*, that suspends moult for migration.

Adults leave the breeding grounds towards mid July and peak in late July and early August at staging areas in James Bay and the Bay of Fundy. Adults also appear around the same time in the staging areas along the northeast coast of the United States seaboard and most have departed by late August to early September towards staging areas further south. From the second half of August to the first half of September *C. c. rufa* adults fly across the Atlantic to staging areas in Maranhão, northern Brazil and then on to staging areas in Rio Grande do Sul, southern Brazil. From there they depart to their wintering grounds in Tierra del Fuego where they arrive in late September through October already moulting into winter plumage (Baker et al. 2005a). Wing moult also takes place in the wintering grounds from arrival in late September and October until mid January (Baker et al. 2005a). *C. c. rufa* birds spend only three months in winter plumage, the shortest period among the subspecies, and they depart northward in February already moulting into summer plumage (Baker et al. 2005a). After departure from the wintering grounds, *C. c. rufa* birds migrate northward along the eastern Argentine coast with many individuals staging in Golfo San Matias (González et al. 1996) and often continuing onto Rio Grande do Sul, Brazil (Baker et al. 2001). From southern Brazil *C. c. rufa* are faced with a trans-Amazon crossing immediately followed by a trans-Atlantic crossing. The birds probably make a short stop in northern Brazil (Wilson et al. 1998, Augusto & Rodrigues 2000), but major refuelling in such a short period and on tropical mudflats is unlikely (Piersma et al. 2005). Furthermore it is possible that some birds make a spectacular 8000 km flight directly to the south eastern United States. Whichever route they choose, the birds make landfall in early May on the Atlantic coast of the United States and in staging areas in Delaware Bay they feed almost exclusively on the superabundant eggs of spawning horseshoe crabs (Tsipoura & Burger 1999). The use of horseshoe crab eggs as opposed to hard-shelled mollusc prey is unique to *C. c. rufa* and *C. c. roselaari* and has resulted in adaptations such as ingesting small stones to grind the leathery outer shell of the eggs (Piersma et al. 1993c). The birds depart Delaware Bay en masse around May 28 to 30 (Baker et al. 2001) and make a direct flight to the breeding grounds where they begin to arrive in the first week of June.

A portion of the birds using the *C. c. rufa* flyway stop migration in Maranhão, Brazil where they spend the winter (Baker et al. 2005b). This makes them, in fact, tropical winterers rather than trans-equatorial south temperate winterers. These birds were long thought to be *C. c. rufa* and are thus included in this section. However, stable isotope analysis is currently underway to examine the possibility that these birds might belong to the *C. c. roselaari* subspecies. Thus, most of the discussion about *C. c. rufa* in this paper focuses on the Tierra del Fuego wintering population.

Table 3.1. A summary of knot migrations, phenology and moult locations. (1) Piersma & Davidson 1992, (2) Higgins & Davies 1996, (3) Cramp & Simmons 1985.

Subspecies	Non-breeding mass peak (1)	Phenology Wing Moult (1,2,3)	Phenology Prebasic Moult (breeding to winter) (1,2,3)	Location Prebasic and Wing Moult (1)	Phenology Prealternate moult (winter to breeding) (1,2,3)	Location Pre-alternate Moult (1)
C. c. islandica	Yes	mid August to mid November	mid August to end September	Wadden Sea and United Kingdom Estuaries	late March to end April	Wadden Sea
C. c. roselaari	Yes - predicted	early September likely to end November	early September to end October	SE USA	mid March to mid April	SE USA
C. c. canutus	No	late August to early December	late August to end October	NW Africa	mid March to end April	NW Africa
C. c. piersmai	No	early September to early February	early September to end October	NW Australia	late February to end March	NW Australia
C. c. rogersi	No	late October to early March	possibly as early as August to end November	New Zealand (some prebasic body moult on southward migration and even breeding grounds)	early February to end March (possibly into northward migration)	New Zealand
C. c. rufa	No	mid October to mid January	begins mid July ends end November	Tierra del Fuego (some prebasic body moult on southward migration and breeding grounds)	begins mid February ends mid May	Tierra del Fuego (some prealternate body moult on northward migration)

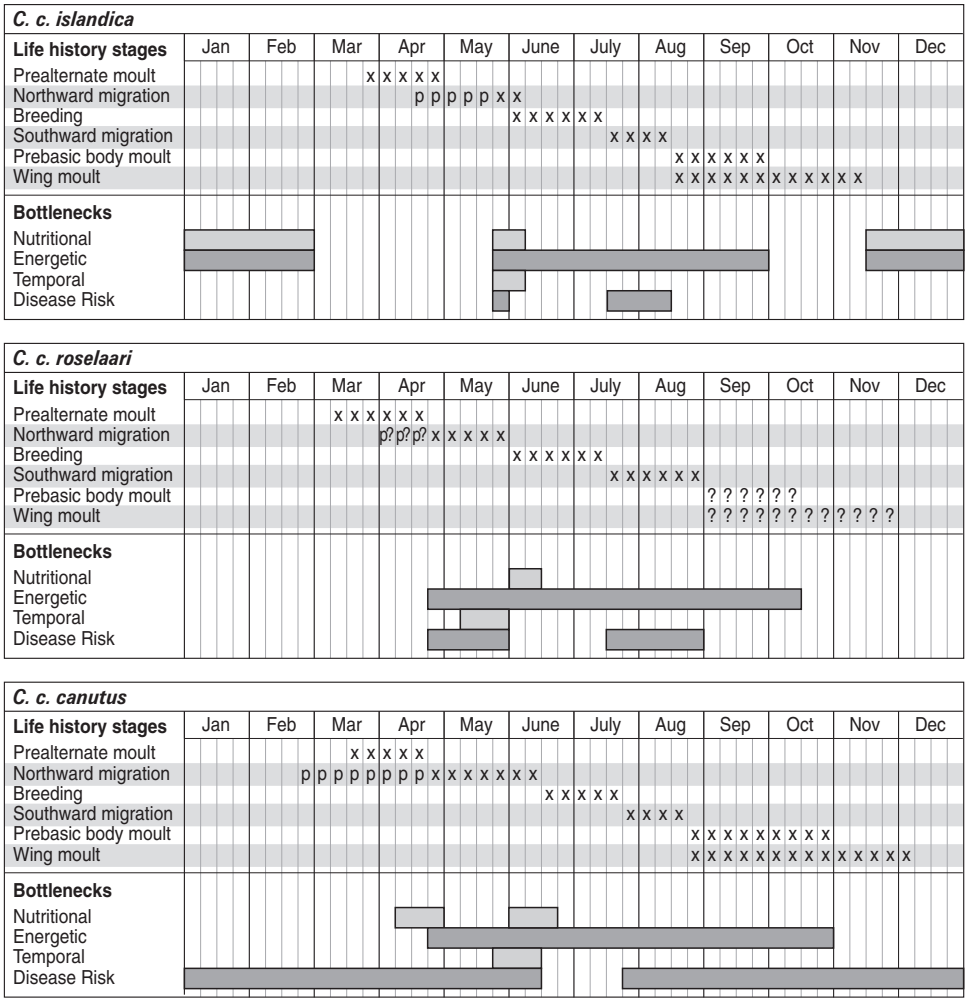
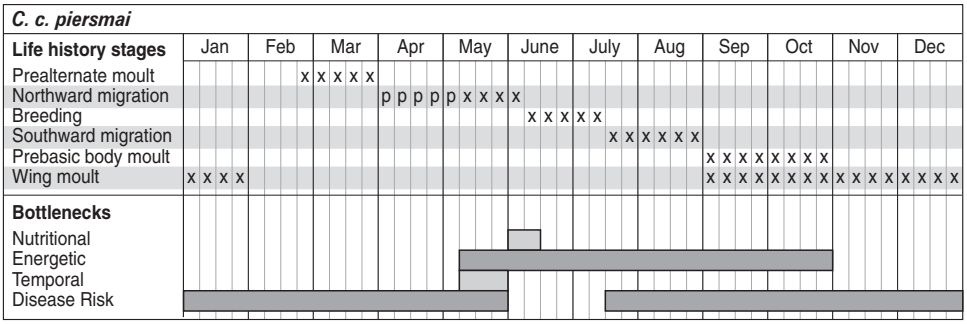
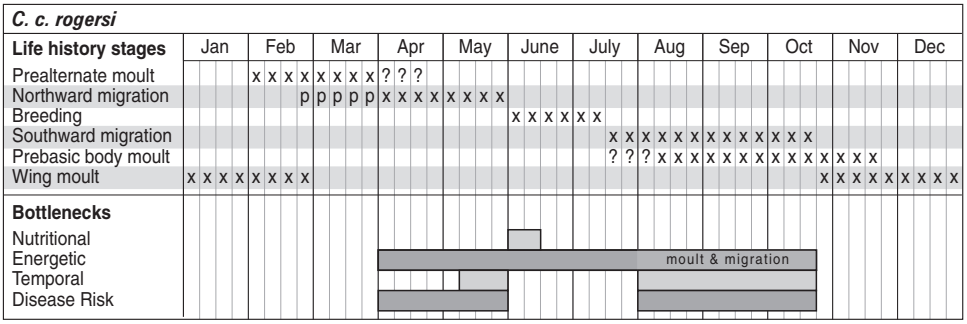
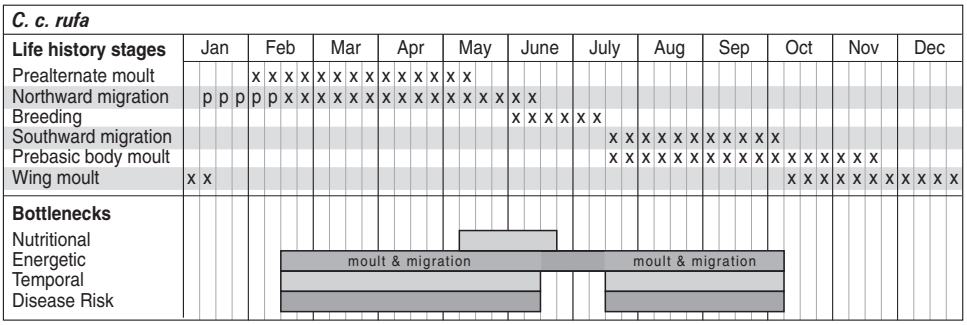


Figure 3.2. A graphical representation of the annual cycles and life history stages of the knot subspecies. For the life history stage "Northward Migration", "x" represents flight and staging, whereas "p" represents pre-migratory mass gain (extrapolated from Table 21.1 in Piersma et al. 2005). In all life history stages, "?" indicates parts of the annual cycle that are not known with certainty. Periods of bottleneck are shown below the life history stages. In this figure simultaneous prebasic and wing moult is highlighted as an energetic bottleneck, and periods where both moult and migration overlap are represented by different shading and are labelled in the *C. c. rogersi* and *C. c. rufa* subspecies. Periods of the annual cycle in which individuals experience three or more bottlenecks at a time are considered severely bottlenecked. For example, all subspecies are severely bottlenecked during the final stages of northward migration and arrival on the breeding grounds due to the overlap of energetic, temporal and disease risk bottlenecks.







x is flight and staging

p is pre-migratory mass gain for northward migration (extrapolated from Table 21.1 in Piersma *et al.* 2005)

? indicates parts of the annual cycle where life history stages are not completely known

BOTTLENECKS IN THE ANNUAL CYCLE OF KNOTS

We have now presented information on the annual cycles of long-distance migrants by example of the six subspecies of our focal migrant, the knot. We will now define possible bottlenecks in these annual cycles and make predictions regarding possible trade-offs as a result of these bottlenecks. For the purpose of this paper we focus on nutritional, energetic, temporal and disease-risk bottlenecks. Figure 3.2 summarizes these bottlenecks for each of the subspecies throughout their life history stages and Table 2 shows the predicted impact of these bottlenecks for long-distance migrant shorebirds, in general, throughout the annual cycle.

Nutritional Bottlenecks

A nutritional bottleneck is defined as a time when food resources are unpredictable, of low quality or found in low density. For knots nutritional bottlenecks in the wintering areas may be quantified by measuring the presence of a midwinter mass peak as an indication of food unpredictability (Piersma 1994). Furthermore, in view of the fact that knots ingest their shellfish prey whole, crushing the shells in their muscular gizzards and evacuating the crushed shell remains through the intestine (Piersma et al. 1993c, Battley & Piersma 2005), prey qualities at both wintering and stopover sites can be measured as the ratio of bivalve flesh and shell mass (van Gils et al. 2005b).

For long-distance migrants that breed in the High Arctic, and this includes all six subspecies of knots, the most severe nutritional bottleneck might be predicted upon early arrival on the Arctic breeding grounds (Figure 3.2). Although food is of high quality and abundant during reproduction, birds arrive in the Arctic before the peak in insect availability (I. Tulp & H. Schekkerman pers. comm.) and must endure starvation conditions after days of flight. Furthermore, the timing of snowmelt in the High Arctic is unpredictable and in some years the birds may arrive before the snow has gone. The existence of this bottleneck is evidenced by that fact that knots often carry a much greater fuel load on the last leg of migration than is necessary for the flight alone. This extra fuel is most likely an "insurance policy" against harsh conditions upon Arctic arrival (Morrison et al. 2005). After snowmelt, nutritional stress in the Arctic breeding grounds is expected to be lower, despite the extra energetic demands of reproduction, because food sources in the Arctic are abundant and long photoperiod offers more hours to feed (for example Schekkerman et al. 2003).

During both northward and southward migration, departures and arrivals are timed to exploit high quality and abundant food resources and, in general, prey quality at stop-over sites is higher than that at wintering sites (van Gils et al. 2005a). This is important since low quality prey necessitates an increase in gizzard size in knots because of the need to process more shell material. During migration, however, there is a trade-off between increased shell processing capacity and the costs of having to carry a heavy gizzard. Thus the combination of high prey quality and the knots' ability to adjust their gizzard size enable timely migrations (Battley & Piersma 2005; Battley et al. 2005) and due to generally abundant and high quality food, nutritional bottlenecks during migration should be low. The one exception to this rule may be a human-

related nutritional bottleneck during the final stopover in the *C. c. rufa* and *C. c. roselaari* subspecies flyways in Delaware Bay, USA (Figure 3.2). These subspecies feed on horseshoe crab eggs and recent over-harvesting of horseshoe crabs may be depleting food stocks for these birds with possible carry over effects throughout the annual cycle (Baker et al. 2004).

During the northern hemisphere winter knots use both temperate and tropical wintering areas. Nutritional bottlenecks in these areas are defined by both prey quality and prey predictability. For example, temperate wintering areas such as the Wadden Sea in northwest Europe, and coastal areas near Rio Grande, Tierra del Fuego, have high prey quality relative to tropical wintering areas such as Banc d'Arguin, Mauritania and Roebuck Bay, Northwest Australia (van Gils et al. 2005a). This higher prey quality in south temperate regions may offset the longer migrations and the higher thermoregulation costs required to migrate and survive there. Tropical wintering subspecies need not fly as far but may experience a moderate nutritional bottleneck as a result of predictable but low quality food resources. In the *C. c. canutus* subspecies for example, there is evidence that the birds may have trouble feeding at the rate maximizing levels necessary to gain fuel stores quickly before departure on northward migration (Figure 3.2; Piersma et al. 2005). Northern hemisphere winterers such as *C. c. islandica* appear to escape both trans-equatorial migrations and low prey quality found in tropical climes. However, thermoregulation is costly during the northern hemisphere winter (Wiersma and Piersma 1994) and cold weather can make food unpredictable if the mudflats freeze (Figure 3.2; Johnson 1985; Zwarts et al. 1996a). Evidence of this north temperate nutritional bottleneck in *C. c. islandica* is indicated by the presence of a mid-winter mass peak.

Energetic Bottlenecks

Energetic bottlenecks are defined as periods when Field Metabolic Rate, FMR approaches or even exceeds the maximum sustained metabolic rate of approximately five times Basal Metabolic Rate, BMR (Drent & Daan 1980; Hammond & Diamond 1997). Energetic bottlenecks can further be defined as periods of high energy turnover as quantified by fuelling rates on staging and wintering grounds (Piersma et al. 2005). For migratory shorebird species such as knots, energetic bottlenecks are expected during the flight phase of migration, when expenditure levels reach 7-8 times BMR (Kvist & Lindström 2001; Wiersma & Piersma 1994). In addition to the migratory period, knots are energetically bottlenecked during the reproductive period when energy is invested in breeding and where thermoregulation costs are high even during the Arctic summer (Figure 3.2; Piersma et al. 2003). Indeed, for tropical winterers such as *C. c. canutus* the highest thermoregulation costs of the year occur during breeding (Wiersma & Piersma 1994).

Only in the middle of the non-breeding season is energy expenditure relatively low because energy is not needed for either migration or reproduction. This may be why most knot subspecies postpone prebasic body moult and all subspecies postpone wing moult until arrival in the wintering areas. For, *C. c. islandica* knots wintering in temperate areas such as the Wadden Sea energy requirements for thermoregulation are high

even during winter, with expenditure levels of 4-5 times BMR (Figure 3.2; Wiersma & Piersma 1994). Perhaps this is why *C. c. islandica* performs prebasic body moult quite early in the season before the harsh winter weather begins.

In *C. c. rufa* and *C. c. rogersi*, moult and migration overlap and a strong energetic bottleneck is predicted during these periods of overlap (Figure 3.2). In fact, *C. c. rufa* individuals are particularly bottlenecked. They face relatively harsh conditions during both the Arctic summer and the austral summer in Tierra del Fuego, as well as an extremely long migration period, and extremely long periods of moult that stretch from the breeding areas through migration and into the wintering grounds.

Temporal Bottlenecks

Migrants must pass through more life history stages than resident birds within the annual cycle because they must perform northward and southward migration in addition to breeding, winter survival and moult. Furthermore, the migrations themselves require precise timing in order to fully exploit peaks in prey species abundance. Thus, timing is very important in the annual cycle of a migrant and time can be important in assessing the cost of migration (Hedenström & Alerstam 1997). A temporal bottleneck can be defined as a period in which migrants are "pressed for time" and can be measured using the synchrony of departure during migration and the amount of overlap between high energy demanding life history stages.

Temporal bottlenecks are most severe during the last leg of northward migration and during the breeding season (Figure 3.2). During this period knots of all subspecies are in a race against time because arrival on their Arctic breeding grounds must be precise in order to exploit the insect bloom for breeding, and to have time during the short Arctic summer to raise their young. This temporal bottleneck, based on the idea that the timing of migration becomes more constrained closer to the breeding grounds, is evidenced by increasingly synchronous departures as the breeding areas are approached in a number of waders including great knots *Calidris tenuirostris* and all subspecies of red knot (Battley et al. 2004).

During southward migration temporal bottlenecks may also come into play as the birds must arrive at staging areas in time to exploit food resources (Schneider & Harrington 1981; Zwarts et al. 1992) and must leave the Arctic and northern staging areas before the winter storms begin. In the Wadden Sea for example, crustacean density declines from July to September highlighting the importance of a timely arrival for *C. c. canutus* if they are to use this stopover before migration further south (van Gils et al. 2005c). However, the timing of northward migration may be more constrained than southward migration (O'Reilly & Wingfield 1995) and models have indicated that northward migration is more compressed (e.g. Figure 1 in McNamara et al. 1998). Finally, in the wintering grounds time pressure is low until fattening for northward migration commences and the race begins anew.

Migration distance can also increase temporal bottlenecks in the annual cycle; the longer the distance to be covered, the longer the migration life-history stage lasts. In the longest distance migrating *C. c. rogersi* and *C. c. rufa*, temporal bottlenecking is severe enough to necessitate an overlap between body moult and migration. In *C. c. rogersi*

this overlap occurs only during southward migration, but in *C. c. rufa* overlap occurs during both northward and southward migration. A further indication of temporal bottlenecking in *C. c. rogersi* and *C. c. rufa* is a complete lack of "down time" between the end of primary wing moult and preparation (via pre-migratory fuelling and prealternate moult) for migration and breeding (Figure 3.2).

Habitat and Behaviour Related Disease Risk Bottlenecks

In general, migrants encounter a wider diversity of environments, and possibly a wider diversity of pathogens and parasites throughout their annual cycle than do resident birds. This has lead to the prediction that migrant birds might have more robust immune defences than resident birds (Møller & Erritzøe 1998).

Among migrating species, it has been suggested that marathon migrants such as knots, may be have poorer immune resistance than shorter distance migrants (Piersma 1997; 2003). The hypothesis is based on the idea that in marathon migrants demanding migrations and a history of genetic bottlenecking on an evolutionary time scale may have led to poor immune resistance that further restricts them to low pathogen habitats with less disease risk (Piersma 1997; 2003). Furthermore, on an immediate time scale, the demanding migrations themselves may cause immunosuppression (Råberg et al. 1998). Chronic muscle damage has been shown to cause a state of mild inflammation followed by immunosuppression in overtraining athletes (Shephard & Shek 1998), and muscle damage (although slight) has been detected during migration in long-distance migrating western sandpipers *Calidris mauri* and bar-tailed godwits *Limosa lapponica* (Guglielmo et al. 2001).

Even within long-distance migrants restricted to low pathogen environments, like knots, some variability in pathogen pressure and immune function can be predicted throughout the annual cycle and between subspecies making use of different environments. In knots disease risk bottlenecks have two components: (1) habitat-related pathogen pressure pertaining to the variety and prevalence of pathogens along the flyway, (2) seasonal variation in flocking behaviour (aggregated or not aggregated). These two components are closely tied to individual seasonal investment in the immune system, which is discussed later in this article.

The highest levels of disease risk in knots are predicted during migration and during tropical wintering (Figure 3.2). During migration birds are aggregated into very dense and synchronously moving flocks and are passing through a variety of environments with novel pathogens. During tropical wintering pathogen prevalence is generally higher and pathogens are more varied, whereas pathogen pressure is expected to be lower in temperate wintering areas where conditions are colder and harsher. The lowest pathogen pressure is predicted in the Artic where conditions are generally cold and breeding birds are widely dispersed.

These predictions are based on two assumptions: (1) that pathogen pressure is higher in the tropics and (2) that disease risk is elevated when birds are densely aggregated during migration. Very little testing has been applied to these assumptions within long-distance migrants. However, in general, theory predicts that the spread, abundance and diversity of parasites, and thus disease risk, should be higher in hosts living

at high density or with frequent intraspecific contacts (Anderson & May 1992; Arneberg 2002; Roberts et al. 2002). Furthermore, research on blood parasites has long shown that bird species inhabiting high Arctic and marine habitats have lower parasites levels than species inhabiting lower latitude and aquatic environments (Bennet et al. 1992; Figuerola et al. 1996, Figuerola 1999; Greiner et al. 1975). This conclusion has recently been confirmed specifically within shorebird species. The prevalence of avian malaria was compared in shorebird species sampled in the Arctic, in temperate Europe, and in tropical West Africa and infected individuals were found mainly in tropical freshwater habitats (Mendes et al. 2005). In addition, a small population of knots that winter in tropical Brazil seem to suffer from high loads of feather lice and mites (Baker et al. 2005b).

ECOLOGICAL EVIDENCE

We need ecological evidence to evaluate the existence and impact of these proposed bottlenecks. A particularly promising way to do this is to look at various aspects of the expression of nuptial plumage and moult itself throughout the annual cycle (Hill 1995, von Shantz et al. 1999).

Breeding plumage: where and when to moult, and why fly dressed to impress?
Breeding plumage can be an important signal for sexual selection (Darwin 1871; see Baker & Parker 1979 for a review of theory). During breeding it is important for individuals to show their quality in order to ensure a high quality partner, and in many shorebird species, including knots, both partners are involved in mate selection (Piersma et al. 2001). Breeding plumage, its completeness and its colour, has been shown to be an honest quality signal. For example, in bar-tailed godwits the completeness of nuptial plumage during spring stopover in the Wadden Sea correlates positively with body mass (Piersma & Jukema 1993) and local survival (Drent et al. 2003), and negatively with the amount of intestinal cestode parasites (Piersma et al. 2001), although there may be an age component to these relationships (Battley 2007).

As their name suggests, breeding plumage in knots includes a rusty red colour acquired on the breast, belly, neck and face. Like many other sexually selected ornaments, this rusty colour is associated with pigments in the diet, namely melanin, and the darker the colour the better. We know that melanin occurs in two forms: eumelanins confer black and grey colours, and phaeomelanins are responsible for the chestnut and buff colours like the rusty red seen in knots. However, the physiological mechanisms that relate overall condition and melanin pigmentation are uncertain (McGraw 2005). For carotenoids, which stand as a model system to study honest signalling in pigments (von Schantz et al. 1999), one direct connection with condition is that individuals with more carotenoid pigmentation are better foragers and therefore more viable. Melanin pigments are large polymers synthesized endogenously from amino acids (Prota 1992) making the connection between the ingested building blocks of the pigment and colouration less direct. However, if not an indicator of foraging ability,

melanin may well serve as an indicator of oxidative stress and immune function (McGraw 2005). Melanins, like carotenoids, are potent antioxidants containing both oxidizing and reducing functional groups which give them the capacity to quench reactive oxygen and nitrogen free radicals via election donation or capture (Borovansky 1996). Melanin is also an immunostimulant via several mechanisms including phagocytosis, lysosomal enzyme activity, cytokine regulation and nitric oxide production in vertebrates (reviewed in Mackintosh 2001). Because they are synthesized internally, manipulation experiments to examine the direct anti-oxidative potential of melanin have been challenging (McGraw 2005). Nevertheless, increased pigmentation is correlated with improved health. For example, male house sparrows *Passer domesticus* that develop large patches of melanized throat feathers are in better body condition and show lower levels of parasitic infection than sparrows with smaller throat patches (Møller et al. 1996).

Both the immune system (especially the inflammatory response) and strenuous exercise such as migratory flight generate reactive metabolites and free radicals which contribute to oxidative stress (von Schantz et al. 1999). In migratory birds, pigmentation could then signal quality because pigments used by the immune response or for antioxidant purposes cannot be invested in breeding plumage. Thus, the "redness" of the plumage gives an indication of the condition of the individual at the time of moult. Unlike carotenoids present in wattles, combs and skin of birds, the investment of melanin into plumage is non-reversible. Once these nutrients are imbedded into the plumage they are no longer available, and are lost at the next moulting (Lozano 1994). Though in some species plumage colour is important for camouflage, in knots it is not life-saving body structure, thus if nutrients are directed away from investment in this "non-essential" ornament when energy is limited, then plumage colouration should be a particularity sensitive indicator of conditions faced by the individual at the time of moult (Hill 1995).

Thus, one would expect birds to moult into breeding plumage in areas with sufficient food, benign thermal conditions and the necessary amino acids for pigments – areas where they could best afford to invest energy into plumage colouration. Further, one would expect the birds to moult into breeding plumages as close to the breeding grounds as possible to avoid "wear and tear".

Is there ecological evidence supporting these predictions in knots and is there evidence of bottlenecking in certain subspecies? With respect to plumage characteristics, *C. c. canutus*, *C. c. islandica* and *C. c. piersmai* are the "darker" subspecies. *C. c. rogersi* have lighter bellies than both *C. c. roselaari* and *C. c. piersmai*, and *C. c. rufa* is the palest in overall plumage (Figure 3.1). Might pale plumage indicate a bottleneck? *C. c. rufa* moult into breeding plumage while simultaneously fuelling for northward migration, thus energy and nutrients must be shared between fat stores and deeper red plumage. Furthermore, *C. c. rufa* is one of the longest distance migrants of the six subspecies, together with *C. c. rogersi*, which similarly have pale breeding plumage. Finally, both of these subspecies fly many kilometres, from wintering grounds in the southern hemisphere, "dressed up" in their nuptial plumage. This may indicate a lack of sufficient food resources (nutritional bottleneck) or time (temporal bottleneck) to moult into

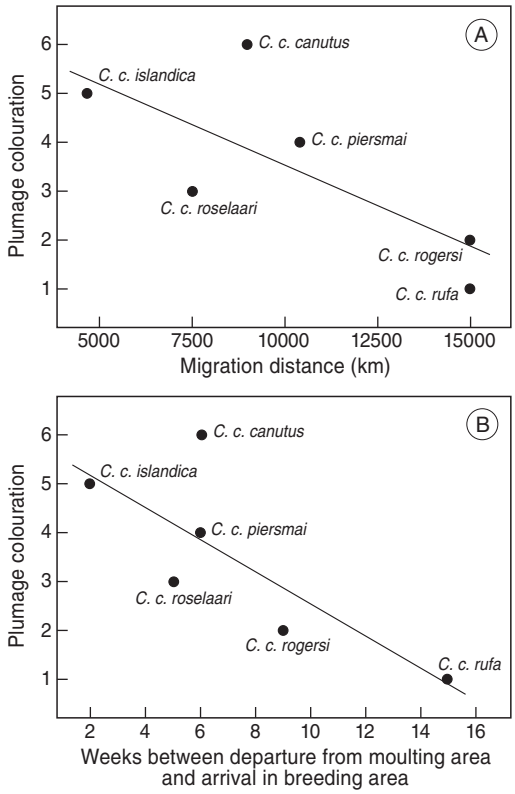


Figure 3.3. Trends between breeding plumage and aspects of migration. A) Breeding plumage colouration, where 6 signifies the darkest colouration (taking into account both overall extent of redness and depth of colour from Tomkovich (1992, 2001), and overall migration distance for a one way journey (Pearson correlation - 0.751, $P = 0.043$, one-tailed). B) Breeding plumage colouration (as in a) and the number of weeks between departure from the moulting area and arrival on the breeding area (Pearson correlation - 0.781, $P = 0.033$, one-tailed).

breeding plumage closer to the breeding grounds to avoid fading and "wear and tear" on their plumage. The "darker" subspecies (*C. c. canutus*, *C. c. islandica* and *C. c. piersmai*) also moult into breeding plumage on the wintering grounds, but they do so closer to the breeding grounds, both in terms of distance and in terms of time. Indeed, darker breeding plumage is associated with shorter overall migration distance, as well as with a shorter period between departure from moulting area and arrival in the breeding area (Figure 3.3).

Wing moult and prebasic body moult: what is the cost and where and when to moult?

Unlike prealternate moult which is thought to produce an honest signal for sexual selection, prebasic body moult and wing moult are necessary for survival in knots. The yearly renewal of body and flight feathers is essential for both thermoregulation and flight and this moulting period is a potentially costly portion of the annual cycle. A gen-

eral rise in energy metabolism during moult has been reported for a wide range of species (reviewed in King 1981 and Payne 1972) and factors that contribute to this increase in energy consumption include the synthesis of the feathers themselves, as well as the indirect cost of increased thermoregulation due to decreased insulation of the plumage during moult and increased exposure of blood engorged quills. Studies have been done on the cost of feather synthesis (e.g. Dietz et al. 1992; Lindström et al. 1993; Schieltz & Murphy 1997), and all indicate that feather production alone cannot entirely explain the increase in energy metabolism seen during moult. The Schieltz and Murphy (1997) study was particularly convincing as they experimentally plucked up to 36% of plumage in white-crowned sparrows *Zonotrichia leucophrys gambelii* and measured metabolic rate during regrowth. They found that under thermo-neutral conditions feather regrowth of even 36% of plumage did not increase oxygen consumption. Furthermore, a comparison between naturally moulting birds and birds that were plucked outside of the natural moulting period revealed that naturally moulting birds showed a 25-54% higher metabolic rate than plucked birds. This result strongly suggests that the energy cost of moult is caused by other metabolic changes (still undiscovered) associated with moult and not the process of feather growth itself. Whatever the cause, moult appears to be metabolically costly.

In addition to the physiological costs of moult, there are indirect costs associated with wing moult including an increased cost of flight due to a reduction of wing area and an increase in foraging effort and predation risk due to decreased manoeuvrability (review by Hedenström 2003). These indirect costs of wing moult have been uncoupled from the physiological costs of feather synthesis in an elegant experiment by Swaddle and Witter (1997). They simulated wing moult by using scissors to reduce primary length in non moulting birds and showed that this simulated moult resulted in reduced flight performance and body mass.

Given evidence for both direct and indirect costs of prebasic body moult and wing moult, one would predict that moult would be timed to avoid overlap with other costly life history stages. Furthermore, because prebasic body moult and wing moult differ we can make more specific predictions. For example, for temperate wintering subspecies, we would predict the timing of prebasic body moult to minimize overlap with short daylight and cold temperatures (Schieltz and Murphy 1997). In addition, given evidence for a reduction in flight performance, which would affect both predator avoidance and foraging, we would predict that wing moult would occur in an environment with sufficient food and low predation. Here again we can ask, is there ecological evidence supporting these predictions in knots, and is there evidence of bottlenecking in certain subspecies?

In terms of overlap between prebasic body moult and other costly life history stages, in general, knots delay body moult until arrival in the wintering areas. Only *C. c. rufa* is known to begin prebasic body moult on the breeding grounds. Moult is then suspended and is ultimately finished in the wintering areas 15,000 km away in Tierra del Fuego (Baker et al. 2005a). The reason why *C. c. rufa* commences prebasic body moult on the breeding grounds is unknown, but this phenomenon may be evidence of temporal bottlenecking throughout the flyway, especially a lack of adequate

time or possibly nutritional resources for complete moult in the wintering areas. Another possibility may be a carry-over effect from the need to moult into prealternate plumage very early in the season in this subspecies. By the end of breeding *C. c.rufa* birds will have carried their breeding plumage for nearly six months and a distance of 15,000 km! Perhaps a small "touch up" moult is necessary for migration, assuming that worn plumage offers poor thermal capacity or decreases flight performance. Birds of the *C. c. rogersi* subspecies also show overlap between prebasic body moult and migration (Figure 3.2) and moult on the breeding grounds is a possibility. As in *C. c. rufa*, the timing of moult and overlap with migration may indicate a temporal bottleneck throughout the flyway, possibly caused by the marathon distances covered by these two subspecies. Alternatively, moult and migration in *C. c. rufa* and *C. c. roselaari* might be a consequence of a shift in the temporal bottleneck relative to the other subspecies. *C. c. rufa* and *C. c. roselaari* breed in the warmest spring conditions of the six subspecies (Battley et al. 2005). It is possible that these subspecies might commence breeding slightly earlier, finish breeding slightly earlier and have the opportunity to start prebasic moult on the breeding areas, where the other subspecies do not.

In terms of the timing of prebasic body moult in relation to climatic conditions in the wintering areas, temperate winterers *C. c. roselaari* and *C. c. islandica* should moult as early as possible to avoid overlap with the cold dark winter months (see Summers et al. 2004). *C. c. islandica* birds do in fact perform prebasic body moult quite early, perhaps to minimize heat loss during body moult (although there is no evidence that thermogenic capacity of the feathers is reduced during moult under captive conditions, A. Gustowska & F. Vézina pers. comm.). The moult schedule for *C. c. roselaari* is not precisely known, but it seems likely that they too complete prebasic body moult quickly before the onset of the winter months. Trans-equatorial migrants such as *C. c. rogersi* and *C. c. rufa* would have the longest daylight hours for feeding and the highest temperatures during the height of the austral summer. Thus, they might be predicted to moult later in the non-breeding season than do tropical or north temperate winters. In fact, instead of postponing moult, *C. c. rogersi* and *C. c. rufa* begin moulting even before reaching the wintering areas, possibly because time constraints do not allow optimization in terms of when and where to moult. However, as discussed above, many factors may be involved in the moult and migration overlap displayed in these subspecies.

In terms of overlap between wing moult and other costly life history stages, all knot subspecies delay wing moult until arrival in the wintering areas, or very close to them in the case of *C. c. islandica* individuals, indicating that other times of the annual cycle may already be too nutritionally, energetically or temporally bottlenecked for overlap with costly wing moult.

The duration of wing moult may also be instructive in terms of ecological evidence for bottlenecking in knots. Stretching wing moult over as long a period as possible would have the advantage of diluting the physiological costs of feather synthesis as well as reducing the indirect costs on flight performance by reducing the size of the gap in the wing. Furthermore, there is evidence that a slower moult may also improve the quality of the feathers grown (Serra 2001). Even in the absence of statistically formal analyses, *C. c. rufa* and *C. c. islandica* both seem to have relatively short primary moult dura-

tions. *C. c. rufa* is temporally bottlenecked and spends only three to four months on the wintering grounds, undergoing wing moult from arrival until fattening for northward migration. *C. c. islandica* probably moult their primary feathers quickly to avoid elevated flight and foraging costs during the darker and colder months of winter when food is unpredictable and thermoregulatory costs are high (nutritional/energetic bottleneck).

FUTURE DIRECTIONS

In summary, as a species, knots appear to experience nutritional, energetic, temporal and disease risk bottlenecks throughout the annual cycle, and there appear to be critical periods in the course of this generally busy lifestyle. For example, in all subspecies timing seems to be paramount during departures from the final staging area before the breeding grounds, and all subspecies show synchronous departure during this period indicating little room for flexibility. Furthermore, the extent, type and timing of ecological bottlenecks vary between the subspecies, depending on the environments that they encounter throughout their annual cycle.

We have developed a framework to look at potential bottlenecks within the annual cycle of a long-distance migrant. We now use our framework to make predictions about investment in immunity in migrants, highlighting the idea that pathogen pressure and immune function are not constant throughout the annual cycle. We also discuss the occurrence of mortality in the annual cycle with reference to nutritional, energetic, temporal and disease risk bottlenecks.

Predictions for seasonal variation in immune function

We have discussed how disease risk may play an important role in the annual cycle of long-distance migrants. We would now like to make a few predictions about defence against disease, in relation to both the predicted risk of pathogens themselves, and the nutrition and energy requirements needed to maintain and use immune system defences. As discussed before, it is difficult to define a single currency with which to measure the cost of immune function. The discipline of ecological immunology is still in an exploratory phase (Klasing 2004) and we are only beginning to understand this enormously interactive branch of biology (Martin 2007 this issue), thus it is difficult to come up with simple predictions. Though it is clear that mounting an inflammatory immune response is costly (Lochmiller & Deerenberg 2000), knots and other migrants have to deal with several constraints acting together and it is not clear how they will respond to these constraints in the wild in terms of integrated trade-offs. Thus, until controlled laboratory experiments have been conducted, we will consider various different predictions to try to encompass the complexity of the immune system and the different conditions encountered during the annual cycle. It is important to keep in mind that this is a "cost- benefit" framework over the annual cycle in which individuals are aiming to maximize fitness. During high cost periods of the annual cycle such as breeding and migration, the cost of eliminating or preventing infection in terms of high cost inflammatory defences might outweigh the cost of living with the infection

(Viney et al. 2005). Thus, when we refer to reduced investment or a shift in the type of immune function we are not implying that this is non-adaptive, only that a trade-off is possible (Lee 2006).

Migration is a time of considerable energetic and temporal bottlenecking for knots and all subspecies endure several bottlenecks simultaneously during the last leg of northward migration and arrival on the breeding grounds (Figure 2). Furthermore, the risk of immunopathology is increased during strenuous activity such as prolonged flight (Råberg et al. 1998). As such, a trade-off between immune function, migration and preparation for reproduction can be hypothesized in terms of resource limitation and the risk of immunopathology. This hypothesis would predict relatively low immune function during the flight phase of migration during both northward and southward journeys and at arrival at stopover sites and the breeding grounds (Table 2). However, pathogen pressure differs between the flight and fuelling stages of migration with pathogen pressure relatively higher during fuelling because birds are landing in diverse habitats and foraging in dense aggregations. Examined from this angle, immune investment should be relatively high during the fuelling phase of migration. It is not yet known whether immune function is plastic between flight and fuelling; however, this apparent paradox may be resolved by down-regulation of costly inflammatory responses (such as the acute phase sickness response) and reliance on antibody-mediated acquired immunity during the both the flight and fuelling phases of migration (Table 3.2; Klasing 2004, Lee 2006). Indeed, a recent study shows no evidence for reduced antibody or phytohemagglutinin (PHA) swelling response in knots flown in a wind tunnel (Hasselquist et al. 2007). However, further controlled experiments measuring many immune parameters will be needed to test this hypothesis (reviewed in Martin 2007 this issue).

Breeding is also a period of energetic bottlenecking in knots. Furthermore, pathogen pressure during reproduction should be low because knots breed in the relatively low risk Arctic and are widely dispersed in the breeding areas (Piersma 1997). As such, the need for immune function in response to high pathogen pressure should be reduced. Thus, we predict low immune function during breeding for all subspecies (Table 3.2).

We predict immune investment to be highest during wintering when knots are no longer investing resources, energy or time in migration or reproduction (Table 3.2). During wintering, knots engage in only one potentially costly activity - moult. Studies on domestic fowl have demonstrated trade-offs between moult and immune activity (Kuenzel 2003). However, studies examining immune function and moult in wild birds are rare and often offer conflicting results (i.e. Silverin et al. 1999; Martin 2005) perhaps because they measured different types of immune function and may not be directly comparable. In general, during the wintering period we predict that knots should invest less in immune function during moult, though still more than during migration or reproduction.

A final consideration when making predictions about immune function is that it is probably a plastic response to the environment. As such predictions among subspecies can be made because different subspecies winter in different environments. Tropical winterers such as *C. c. canutus* and *C. c. piersmai* have low energetic demands, pre-

Table 3.2. Predicted impact of four types of bottleneck, as well as predicted immune function and mortality throughout the annual cycle.

	Nutritional Bottleneck	Energetic Bottleneck	Temporal Bottleneck	Disease Risk Bottleneck	Predicted Immune Function	Predicted Mortality
Northward Migration *Fuelling*	low	low	high	high risk	less inflammatory more antibody	low
Northward Migration *Fligh*	moderate	high	high	low risk	less inflammatory tmore antibody	high?
Northward Migration *Arrival*	high	high	high	low risk	low	high
Reproduction	low (high upon arrival)	high	high	low risk	low	high to moderate
Southward Migration *Fuelling*	low	low	moderate	moderate risk	less inflammatory more antibody	low
Southward Migration *Fligh*	moderate	high	high	low risk	less inflammatory tmore antibody	high?
Southward Migration *Arrival*	high	high	low	moderate risk	low	moderate
Wintering Temperate	moderate	moderate to high	low	low to moderate risk	high (moderate during moult)	low to moderate
Wintering Tropical	moderate	low	low	high risk	high (moderate during moult)	low

dictable food and relatively high pathogen pressure in winter and are predicted to invest the most in immune function relative to the other subspecies on the wintering grounds. *C. c. islandica* winter in more energetically demanding conditions with unpredictable food and lower pathogen pressure, thus are predicted to invest comparably less in immune function. Finally, *C. c. rogersi* and *C. c. rufa* have long migrations resulting in relatively short stays on wintering grounds coupled with simultaneous moult and fuelling, thus these subspecies are predicted to invest the least in immune function during winter in comparison to the other subspecies.

Predictions about the occurrence of mortality during the annual cycle of long-distance migrants

All the events within the annual cycle boil down to survival and reproduction. Due to the difficulty involved in measuring the reproductive success of knots in the High Arctic (Meltofte 2001; Piersma et al. 2006), we only briefly discuss indirect measures of reproductive success in relation to a larger discussion on mortality (the inverse of survival) over the annual cycle. To simplify this discussion we consider mortality from three broad sources: starvation, predation and disease (McNamara et al. 1998). These sources of mortality can be placed into our framework of bottlenecks without difficulty. Mortality from starvation is tied to both nutritional and energetic bottlenecks since a bird starves as a result of expending more energy than it takes in. Predation pressure on adult knots is closely linked with factors involved in our temporal bottleneck in which birds must time their migrations in order to maximize survival and reproduction, balancing the need for energy with increased predation risk. For example, predation may be higher during migratory fattening when a bird's chances of being captured by a predator are thought to increase as their fat stores increase due to wing loading and a lack of compensatory pectoral muscle increase (Lank & Ydenberg 2003, Dietz et al. 2007) and as their foraging intensity increases due to lack of vigilance (Dierschke 2003). Finally, death from disease is linked both with pathogen pressure, which can vary over the annual cycle, and to the amount of resources available to invest into immune defences.

Our framework considers four bottlenecks that overlap during different times in the year, and for different proportions of the year, for different subspecies. We define a period in which individuals experience three or more bottlenecks simultaneously as severely bottlenecked (Figure 3.2). Using this definition all subspecies are severely bottlenecked during the last leg of northward migration and arrival on the breeding grounds for a period of about a month when energetic, temporal, disease risk and sometimes nutritional bottlenecks occur together. In addition, *C. c. rogersi* and *C. c. rufa* are severely bottlenecked (energetic, temporal, disease risk) during migration. In *C. c. rogersi* this amounts to nearly 4 months and in *C. c. rufa* nearly 7 months of severe bottlenecking. Keeping this in mind, we predict high mortality in knots during the last leg of northward migration and arrival on the breeding grounds for all subspecies and during migration for *C. c. rogersi* and *C. c. rufa*. Furthermore, we might predict lower overall adult survival in *C. c. rogersi* and *C. c rufa* since they spend a greater proportion of the annual cycle severely bottlenecked.

Unlike resident birds and mammals that tend to suffer high mortality during severe winter weather (Nelson et al. 2002); migratory birds tend to have higher mortality during migration and reproduction, as predicted by our framework. For example, a study of black-throated blue warblers *Dendroica caerulescens* found that mortality during migration occurs at a rate at least 15 times higher than stationary periods (Sillett & Holmes 2002), with more than 85% of annual mortality occurring during migration. Beyond this study, however, few data exist on periods of high mortality in the annual cycle of migrating birds, even in a well-studied species such as the knot. Thus, at present we cannot test the hypothesis that mortality should be higher during the last leg of northward migration and arrival on the breeding grounds. Predictions can, however, be examined in more detail in *C. c. islandica* and *C. c. rufa* for which long-term demographic studies have been performed.

In *C. c. islandica* demographic studies go back as far as 1969 and birds show episodes of high mortality, closely tied to climate and starvation, during both wintering and arrival for reproduction (Boyd & Piersma 2001). For example, knot mortality was high during the very cold winter of 1962 to 1963. Within our framework, *C. c.* islandica is the only subspecies predicted to have high mortality during winter due to nutritional and energetic bottlenecks. Cold summers in 1972 and 1974 also had a great impact and resulted in high adult mortality and low juvenile recruitment. In addition, the summer of 1979 was one of the coldest on record in the Canadian breeding grounds of *C. c. islandica*, and between 1979 and 1980 there was a 29% drop in the numbers of *C. c. islandica* birds wintering in Britain and a high proportion of juveniles in the wintering flocks (Boyd 1992). This pattern indicates high adult mortality during northward migration and upon arrival, as predicted by our framework, but high fecundity in the few adults that survived to breed in the summer of 1979.

While demographic studies on *C. c. islandica* point to high mortality due to climate-related nutritional and energetic bottlenecks during reproduction and wintering, a study of *C. c. rufa* from 1997 to 2002 indicates a nutritional and energetic bottleneck tied closely with human interference during migration. Our framework predicts severe bottlenecking in *C. c. rufa* in over 50% of the year and all four bottlenecks overlap during May and early June. The *C. c. rufa* subspecies exploits horseshoe crab eggs on the beaches of Delaware Bay, USA, during northward migration. However, increased harvesting of horseshoe crabs may have decreased the abundance of food enough to exacerbate and prolong the already existent nutritional bottleneck in this subspecies to overwhelming levels. In fact, adult survival in *C. c. rufa* has dropped significantly from 84.6% from 1994 to 1997, to only 56.4% from 1998 to 2001 (Baker et al. 2004). Baker et al. (2004) also show that birds are failing to meet minimum fuelling requirements and the failure to fuel adequately may result in high adult mortality on the breeding grounds due to insufficient "emergency stores" upon arrival (cf. Morrison et al. 2005). High winter mortality due to disease could be predicted during migration in tropical stopover sites in Brazil for *C. c. rufa* or *C. c. roselaari* when stores are low and pathogen pressure is high. Indeed, the small population of birds that winter in Maranhão have high ectoparasite loads, indicating that pathogen pressure in the area is likely high (Baker et al. 2005b). Furthermore, a large die off of *C. c. rufa* birds, possibly as a result

of a viral infection combined with an infestation of Acanthocephalan worms, has been witnessed at the southern Brazil stopover of Lagoa do Peixe in April (Baker et al. 1999). High winter mortality due to disease could also be predicted during wintering for *C. c. canutus* and *C. c. piersmai*, but it is possible that low energetic costs and predictable food allow these subspecies to invest enough in immune defence to survive this risk.

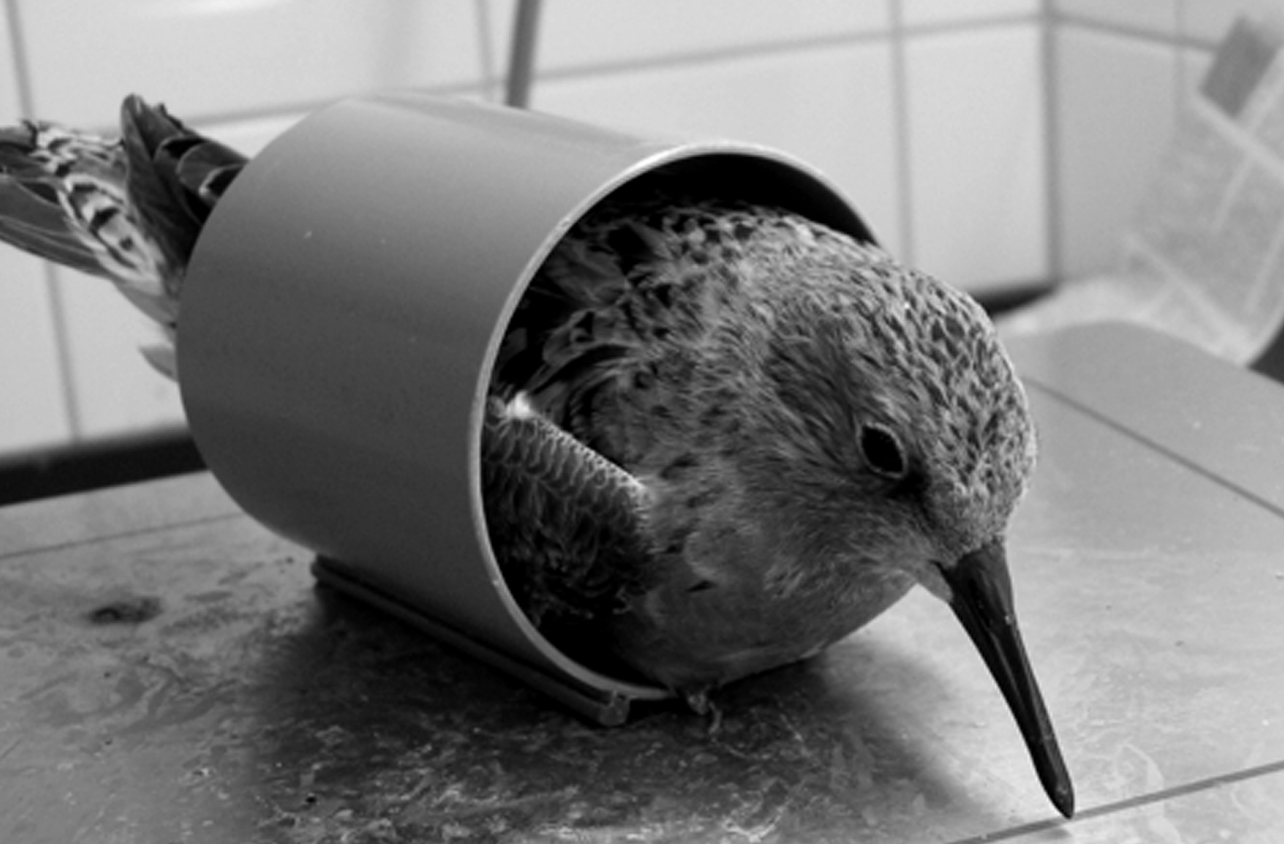
Large scale demographic studies examining mortality in knots throughout the annual cycle are now needed to integrate data from *C. c. islandica* during reproduction and wintering, and data from *C. c. rufa* during northward migration. Long-term survival studies are currently underway for five of the six subspecies (no program of focused studies on *C. c. roselaari* is taking place) and hopefully these studies can begin to address the questions about mortality within the annual cycle. Furthermore, technological advances in radio transmitting are close to providing transmitters small enough to track knots, and other migrants, throughout their annual cycle (A. Purgue, D.W. Winkler & K. Fristrup pers. comm.). This technology should allow us to discover when and where these birds die, and may also help us to discover whether mortality differs between habitats of differing quality or whether condition differences in one part of the annual cycle carry over into the next affecting survival and reproduction. This information can then be examined within the framework of ecological bottlenecks and other frameworks that theoretically model the annual cycle (e.g. Houston & McNamara 1999, McNamara & Houston 2006 this issue), helping to unravel the remaining mysteries of the annual cycle of long-distance migrants.

CONCLUDING REMARKS

As a heuristic tool for the examination of selection pressures during the annual cycle of long-distance migrant birds, we have reviewed nutritional, energetic, temporal and disease bottlenecks. We have applied this framework in a comparative way to the six subspecies knots, a globally distributed shorebird, and have come up with predictions on immune function and seasonal mortality patterns that we hope will prove testable in the near future. We hope this framework will be applicable to other species and types of migrants, thus expanding the comparative data base for future evaluation of seasonal selection pressures and the evolution of annual cycles in long-distance migrants. This description of the annual cycle of a representative migrant offers details not previously considered in models. We hope that these details can aid in the development of more realistic annual routine models for migrants and that these models can be generalized to examine factors that affect behaviour during the annual cycle, not only in well studied species, but also in species where such detailed knowledge is not available.

ACKNOWLEDGEMENTS

We would like to thank John McNamara and Alasdair Houston for inspiring this contribution and for helpful editorial comments. Danny Rogers, Luisa Mendes, Patricia González, Phil Battley and two anonymous reviewers gave valuable comments and feedback on this manuscript and Dick Visser finalized the figures. This work was supported by a Natural Science and Engineering Research Council of Canada (NSERC) Post Graduate Scholarship (PGSB-267701-2003) and a University of Groningen Ubbo Emmius Scholarship to DMB and operational grants by the Netherlands Organisation for Scientific Research (NWO), NIOZ and University of Groningen to TP.



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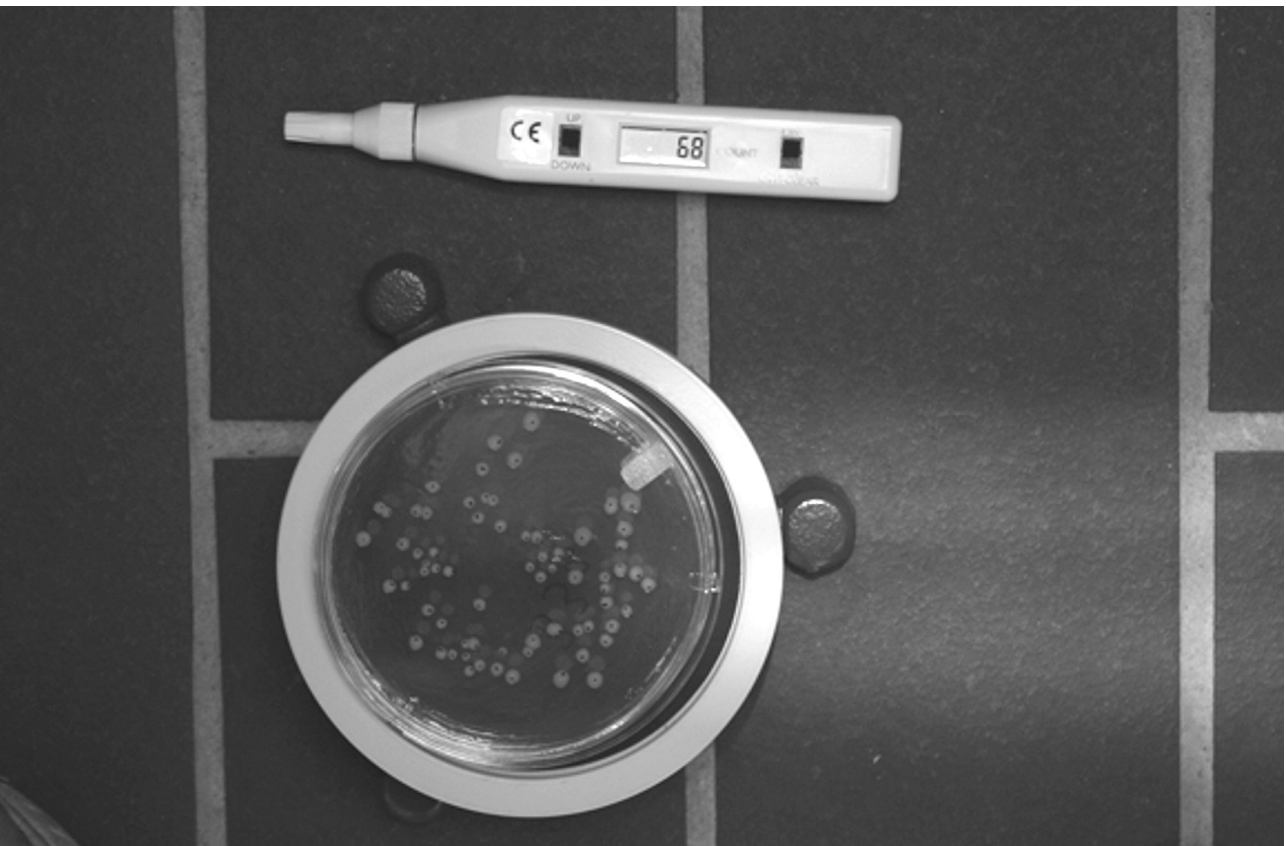

PART II

Assessing immunity and how it responds to different environmental conditions

CHAPTER 4

Constitutive immune function responds more slowly to handling stress than corticosterone in a shorebird

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ABSTRACT

Ecological immunologists are interested in how immune function changes during different seasons and under different environmental conditions. However, an obstacle to answering such questions is discerning the effects of biological factors of interest and investigation artifacts such as handling stress. Here we examined handling stress and its effects on constitutive (non-induced) immune function via two protocols on captive red knots (*Calidris canutus*). We investigated how constitutive immunity responds to handling stress, how quickly these changes take place, and the practical implications for researchers interested in sampling baseline immune levels. We found that *S. aureus* and *C. albicans* killing increased with handling stress while total leukocyte and lymphocyte concentrations decreased. However, although corticosterone increased significantly and rapidly in response to handling stress, none of the 10 measures of constitutive immunity that we tested differed significantly from baseline within 20 or 30 min of capture. Thus, researchers interested in baseline immune function should sample animals as soon as possible after capture, but studies in species not easily sampled in less than 3 min (such as red knots) could still yield useful results.

Physiological and Biochemical Zoology 81: 673-681

INTRODUCTION

Ecological immunologists are interested in how immune function changes during different seasons and under different environmental conditions (reviewed in Lee 2006). However, an obstacle to answering such questions is separating the effects of stress due to the biological factors of interest and investigation artifacts such as handling stress. Stress and immune function are linked via complex interactions between the neuroendocrine and immune axes (McEwen et al. 1997). This complexity means that the stress response suppresses some forms of immunity while enhancing others and that different stressors have differing effects (Apanius 1998). Therefore different stressors, such as acute and unpredictable stress (i.e. capture and handling or predator attack) and predictable life-cycle events that result in increased allostatic load (i.e. reproduction or migration, McEwen and Wingfield 2003), must be defined and considered separately. Furthermore, different facets of the immune system such as constitutive or induced, general or specific immunity must also be distinguished.

Field and experimental conditions often result in a certain amount of time between animal capture and blood sampling, thus determining the effect of acute handling stress on baseline immune function is particularly relevant. Like the well-known corticosterone response (for which a baseline sample must be obtained within 2-3 min after capture; Wingfield et al. 1995; Romero and Reed 2005), immunity may also change rapidly in response to acute stress. Few researchers have examined this question in wild birds and existing studies have focused on non-migratory species (Davis 2005; Matson et al. 2006b; Millet et al. 2007). Information on migratory species is lacking, and red knots (*Calidris canutus*) are an excellent model system for examining immune function in long-distance migrants (Buehler & Piersma 2008).

We examined the acute stress of capture and handling and its effects on constitutive (non-induced) immune function in captive red knots. We chose constitutive immunity because it can be measured from a single blood sample and can be tested repeatedly on the same individual. This repeated measures design and a captive approach allowed us to maximize our chances of detecting changes in immune function by examining within-individual changes and greatly decreased the sample sizes needed. We conducted two protocols, the first using staggered sampling to avoid resampling the same individual more than twice in a day, and the second using repeated sampling (more than twice a day) to compare different time points to a single baseline. The two experiments also differed in that protocol 1 was performed in spring when the birds were not in wing molt and protocol 2 was performed in fall when the birds were in the final stages of wing molt. We measured a total of 10 variables of constitutive immune function by quantifying microbial killing abilities (Millet et al. 2007), circulating cellular immunity (Campbell 1995), and complement and natural antibodies (Matson et al. 2005). In protocol 1 we also measured hematocrit and corticosterone, a stress hormone that increases rapidly in response to acute stress (Wingfield et al. 1995). We asked (1) How do different measures of constitutive immunity respond to the handling stress? (2) How quickly do these changes take place? (3) What are the practical implications of these changes when researchers are interested in baseline immune levels?

MATERIALS AND METHODS

Animals

A total of 19 adult red knots of the subspecies *C. c. islandica* were captured during fall migration or wintering in the Dutch Wadden Sea (53°31'N 6°23'E) and were brought into captivity at the Royal Netherlands Institute for Sea Research (NIOZ). At capture the birds were ringed, weighed, and aged as older than two years (Prater et al. 1977) and sexes were later determined using molecular techniques (Baker et al. 1999). The birds were housed in aviaries (1.5m by 4.5m by 2.3m) with a quarter of the aviary floor covered by an artificial sand flat flushed by salt water and a tray of fresh water for drinking and bathing. The birds had free access to food (Trouvit, trout food pellets, Vervins, France or mud snails *Hydrobia ulvae*). Bird handling was carried out under the auspices of the Animal Experiment Committee (DEC; protocol NIOZ.05.01).

Protocol 1: Staggered Sampling

Protocol 1 took place in April 2006 (n = 12, 8 females, 4 males, captured August 2004 to February 2005), just before pre-migratory fattening and when none of the birds were molting. Baseline was defined as within 3 min of entering the aviary (Piersma et al. 2000b) and the birds were left undisturbed at least the entire night and morning preceding sampling. We followed the standardized handling-stress protocol outlined in Wingfield et al. (1995) and examined multiple time points (baseline, 20 min, 60 min and 120 min) using a balanced repeated-measures design to neutralize the order in which the time point treatments were administered. Pairs of birds (one from each aviary holding six birds) were randomly assigned to one of six sequences (e.g. 20 min, 60 min, 120 min or 120 min, 20 min, 60 min). To avoid sampling the same individual several times in one day, protocol 1 was designed so that each bird was bled only twice (baseline and a given time point) on each sampling day. Six days were left between each sampling day for any given individual to minimize carry-over effects (which are likely to be small, see Piersma et al. 2000a). Therefore, the entire sampling protocol lasted 18 days with each bird experiencing all three time points.

Upon entering the aviary, we captured the bird in a net, sterilized the area around the brachial vein with 70% ethanol and collected about 300 μl of blood into pre-sterilized, heparinized capillary tubes (Fisher Emergo). After blood sampling we weighed the bird and then placed it into an individual keeping box (0.4m by 0.6m by 0.3m) at room temperature and without food or water until it was time to take the next blood sample. Keeping individuals in boxes and out of their normal environment mimicked prolonged periods of capture and handling. Immediately after each blood sampling session we made two blood smears and the remainder of the blood was transported in sterilized plastic boxes to the laboratory and processed within an hour.

Protocol 2: Repeated Sampling

Protocol 2 was carried out in October 2005 (n = 7, 4 females and 3 males, captured October 1995) when the birds were in the final stages of wing molt. We sampled one bird a day at four sampling times: baseline, 30 min, 90 min and 150 min. Blood samples

were obtained as above, but only about 200 μl of blood were taken since several small samples were needed to compare several time points to a single baseline.

Measuring immune function

We quantified microbial killing abilities (Tieleman et al. 2005, Millet et al. 2007), circulating cellular immunity (Campbell 1995), and complement and natural antibodies (Matson et al. 2005). These assays have been described extensively in the original methodological papers and we have included an extended immune methodology in Appendix 4.1. Briefly; the microbial-killing assay measures the capacity of blood to kill microorganisms *in vitro* and we used three strains: *E. coli* ATCC # 8739, *C. albicans* ATCC #10231, *S. aureus* ATCC # 6538: MicroBioLogics, St Cloud, MN. Higher "killing" equates with a greater capacity to limit infection by the particular strain of microorganism used in the assay (Millet et al. 2007). Circulating cellular immunity was measured using leukocyte concentrations which provide a description of circulating cellular immunity via differential concentrations of phagocytes and lymphocytes (Campbell 1995). Complement and natural antibodies were measured using a hemolysis-hemagglutination assay in which lysis and agglutination of rabbit red blood cells is quantified using serial dilution of plasma (Matson et al. 2005).

Hematocrit

We measured hematocrit by centrifuging 25 μl of blood in a capillary tube for 12 min at 12000 x g and reading the relative proportion of red blood cells to total volume.

Corticosterone

Plasma was obtained by centrifuging blood samples for 12 min at 12000 x g. The plasma was then stored at -80°C before shipment on dry ice to the Max Planck Institute for Ornithology, Andechs. Corticosterone concentrations were determined by direct radioimmunoassay (RIA) following Goymann et al. (2006) and the antibody was obtained from Esoterix Endocrinology, Calabasas Hills, California. Extraction efficiency (± SD), as calculated from trace amounts of tritiated hormone added to each sample, was 87.2 ± 4.4% (Perkin Elmer, NET 399) and the average limit of detection was 6.2 pg per tube (all samples were well above this limit). To minimize variance all samples were analyzed within one assay with an intra-assay coefficient of variation of 6.7%.

Statistics

Before performing statistical comparisons, all data were tested for normality using 1-sample Kolmogorov–Smirnov and histograms of each variable were examined visually. When data were not normally distributed we transformed them. Leukocyte concentrations were right skewed and these data were log (base 10) transformed. After transformation all variables conformed to normality both when testing the data themselves and when testing the residuals generated by parametric models. Eosinophil concentrations were very low (on average < 1% of the full differential) and were thus not included in statistical comparisons.

Protocol 1 had different baselines for each time point (between first stress and blood sample) and we first tested to see if the baselines themselves differed. Baselines

did not differ for corticosterone ($P = 0.3$) or any immune measure (all P's > 0.25; see Appendix 4.2 for individual baselines). Thus, we averaged the baselines for each bird and used a linear mixed model with time between first stress and blood sample (T_{stress}) as a fixed factor and bird as a random factor (Quinn & Keough 2002). This method allowed us to compare multiple time points, to control for the repeated measures nature of the study, to run various different covariates and to allow for missing values. However, to be rigorous we also performed tests between each individual baseline and its 20 min sampling point (Appendix 4.3). For these tests we also performed power analyses using the program G*Power to ensure that our power to detect significant changes between baseline and 20 min was moderate to high (Faul et al. 2007; Appendix 4.3).

When different time points were sampled on different days (protocol 1), we included the number of colonies on the inoculate control, the ratio of incubated over inoculate control, and the number of days since resuspending the micro-organisms as covariates for microbial killing (see Appendix 4.1 for details). Covariates were sequentially removed from the models when not statistically significant at the 0.05 level. We used SPSS v 14.0 for all statistical comparisons and report significance using both the conventional $\alpha = 0.05$ and a sequential Bonferroni correction (Rice 1989). All descriptions of the data in the text report mean ± SD. Graphs represent mean ± SE for clarity.

RESULTS

Body mass, hematocrit and corticosterone

We found no effect of T_{stress} on body mass (Table 4.1). Thus, we do not think that changes in immune function due to handling stress are confounded by concurrent effects on body mass. Hematocrit tended to decrease as T_{stress} increased (Table 4.1), but as the trend only neared significance after 120 min ($P = 0.06$), this was not likely the result of repeated bleedings. We found a strong, immediate and significant effect of T_{stress} on corticosterone (Table 4.1, Fig 4.1), indicating that our protocols successfully stimulated capture and handling stress.

Immune function

S. aureus killing increased in response to handling stress in both protocols, but in neither case was the response immediate (Figure 4.2A; Table 4.1). *C. albicans* killing also tended to increase (Figure 4.2B); however, this change was only significant in protocol 2 and then only by 150 min. In contrast, both total leukocyte and lymphocyte concentrations decreased with handling stress in protocol 1, but again the response was not immediate, occurring only after 20 min (Fig 4.2 C and D). In protocol 2 total leukocyte and lymphocyte concentrations also tended to decrease, but the trends were not significant after sequential Bonferroni correction (Table 4.1). Thrombocytes increased with handling stress, but only in protocol 2 and only by 150 min (Fig. 4.2E, Table 4.1). *E. coli* killing, monocyte concentrations, hemolysis and hemagglutination were not significantly affected by handling stress (Table 4.1).

Table 4.1. Summary of changes in body mass, corticosterone and immune function due to handling stress where sampling time (T_{stress}) is a fixed factor and individual bird (Bird) is a random factor. Statistical significance at a=0.05 is shown in italics and after sequential Bonferroni correction (Rice 1989) in bold.

Variable	T_{stress} Response	T_{stress}		Bird	
Protocol 1					
Body mass (g)	No change	$F_{3,33}$=0.39	P=0.764	$F_{11,33}$=14.41	**P<0.001**
Hematocrit	No change	$F_{3,33}$=2.36	P=0.089	$F_{11,33}$=3.99	**P=0.001**
Corticosterone (ng/ml)	Increases by 20 min	$F_{3,32}$=11.66	**P<0.001**	$F_{11,32}$=1.58	P=0.152
S. aureus (prop. killed)	Increases by 60 min	$F_{3,30}$=8.60	**P<0.001**	$F_{11,30}$=1.74	P=0.112
C. albicans (prop. killed)	No change	$F_{3,30}$=0.95	P=0.427	$F_{11,30}$=1.55	P=0.167
E. coli (prop. killed)	No change	$F_{3,31}$=2.14	P=0.116	$F_{11,31}$=9.84	**P<0.001**
Total leukocytes (per μl)	Decreases by 60 min	$F_{3,32}$=6.18	**P=0.002**	$F_{11,32}$=1.41	P=0.215
Heterophils (per μl)	No change	$F_{3,33}$=1.40	P=0.261	$F_{11,33}$=4.01	**P=0.001**
Lymphocytes (per μl)	Decreases by 60 min	$F_{3,32}$=6.24	**P=0.002**	$F_{11,32}$=1.06	P=0.421
Monocytes (per μl)	No change	$F_{3,29}$=1.87	P=0.157	$F_{11,29}$=1.46	P=0.200
Thrombocytes (per μl)	No change	$F_{3,33}$=1.22	P=0.319	$F_{11,33}$=2.06	P=0.054
Hemolysis (log_2)	No change	$F_{3,32}$=1.01	P=0.399	$F_{11,32}$=2.40	***P=0.026***
Hemagglutination (log_2)	No change	$F_{3,32}$=1.43	P=0.252	$F_{11,32}$=6.12	**P<0.001**
Protocol 2					
S. aureus (prop. killed)	Increases by 90 min	$F_{3,17}$=13.92	**P<0.001**	$F_{6,17}$=5.07	**P=0.002**
C. albicans (prop. killed)	Increases by 150 min	$F_{3,16}$=4.28	***P=0.021***	$F_{6,16}$=3.21	***P=0.029***
E. coli (prop. killed)	No change	$F_{3,17}$=1.03	P=0.403	$F_{6,17}$=13.05	**P<0.001**
Total leukocytes (per μl)	Decreases by 90 min	$F_{3,16}$=3.41	***P=0.043***	$F_{6,16}$=1.43	P=0.262
Heterophils (per μl)	Trent for increase by 90 min	$F_{3,16}$=3.13	P=0.055	$F_{6,16}$=2.76	***P=0.048***
Lymphocytes (per μl)	Decreases by 90 min	$F_{3,16}$=3.46	***P=0.041***	$F_{6,16}$=1.41	P=0.270
Monocytes (per μl)	No change	$F_{3,14}$=1.73	P=0.207	$F_{6,14}$=0.90	P=0.521
Thrombocytes (per μl)	Increases by 150 min	$F_{3,16}$=7.91	**P=0.002**	$F_{6,16}$=1.42	P=0.383

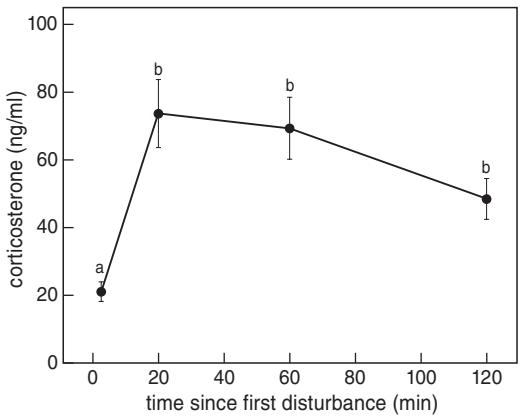

Figure 4.1. Corticosterone response to capture stress stimulated using the standard capture-stress protocol in protocol 1. Sampling times which differ significantly from one another (Tukey post-hoc tests) are indicated by different letters

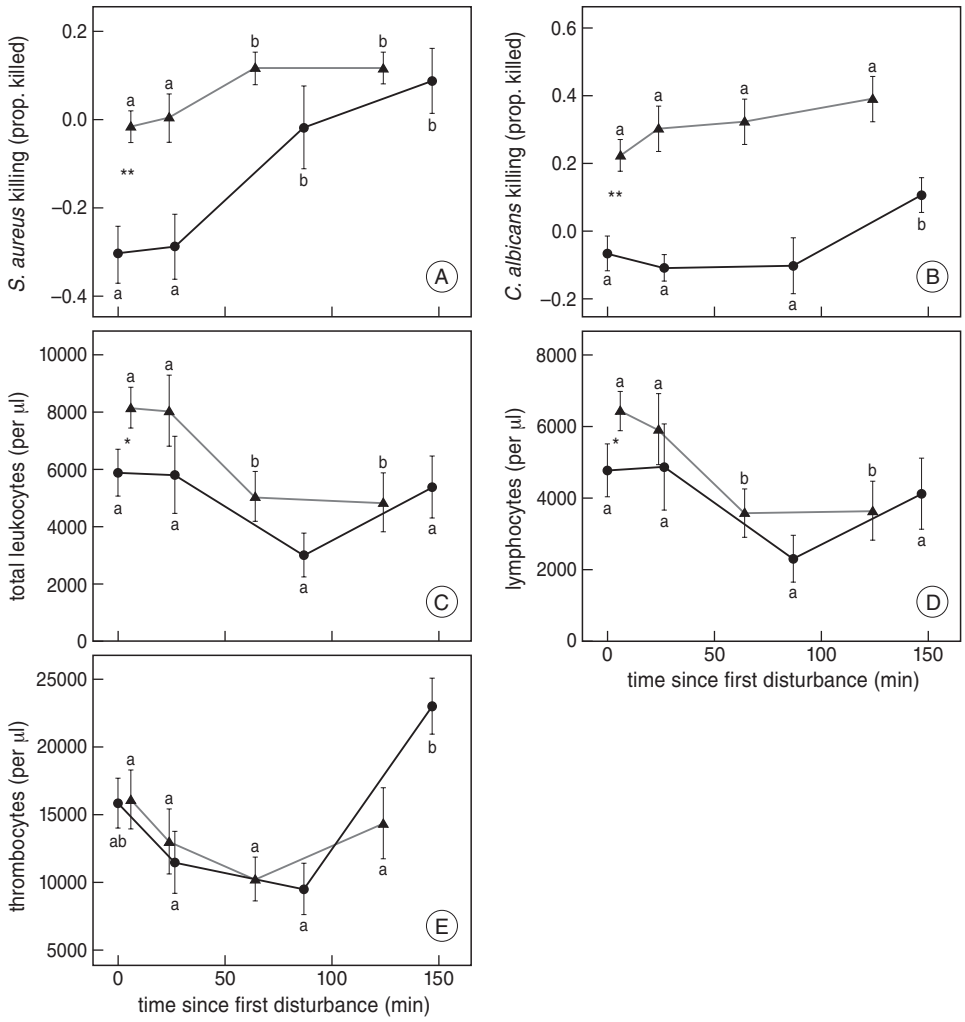

Figure 4.2. The response of different measures of constitutive immunity to capture stress. Triangles indicate Protocol 1 (n=12), circles indicate Protocol 2 (n=7) and error bars show ± 1 SE. For leukocyte concentrations statistics were performed on transformed values, but raw data are shown here for ease of interpretation. Sampling times which differ significantly from one another (Tukey post-hoc tests) are indicated by different letters and significant differences between the two protocols at baseline are indicated with (*) $P < 0.05$ and (**) $P < 0.01$. Complete statistics are shown in Table 4.1.

DISCUSSION

Constitutive immunity and handling stress

E. coli killing was not affected by handling stress, even after 2.5 hours. This is interesting as two other studies have found weak but significant changes in *E. coli* killing after 30 or 60 min of handling stress in certain species (Matson et al. 2006b; Millet et al. 2007). Matson et al. (2006b) found decreases in *E. coli* killing in three of five species of tropical passerines and suggest that the lack of effect in the other two species might be because these species lay very high or very low on an S-shaped dose response curve. However, the red knots in this study had an average baseline killing of 53.6 ± 23.5% falling in the middle of the dose response curve, thus an effect of stress should have been detectable. Millet et al. (2007) found a significant negative correlation between *E. coli* killing and corticosterone measured 30 min after capture. It is important to note, however, that both Matson et al. (2006b) and Millet et al. (2007) examined microbial killing of tropical birds which live in different environments and have different life histories than red knots. As data on microbial killing from more species becomes available, it may be possible to examine whether differing responses to handling stress reflect different life histories.

Both *S. aureus* and *C. albicans* killing increased after 60 or 90 min of handling stress. To our knowledge the effect of handling stress on microbial killing against these two strains has not been studied. *S. aureus* killing is generally carried out via phagocytosis (Matson et al. 2006b; Millet et al. 2007) and Millet et al. (2007) found a significant decrease in the ability of monocytes to phagocytose *S. aureus* after 30 and 60 min of handling stress. However, the phagocytosis assay described in Millet et al. (2007) examines phagocytosis by macrophages not heterophils (which are washed off prior to assaying). Heterophils are the most numerous phagocytes in birds (Janeway et al. 2004) and during acute stress, as lymphocytes are redeployed to the lymph system (Dhabhar et al. 1995), heterophils increase proportionally in the peripheral blood. This may explain the discrepancy between our results and those of Millet et al. (2007). If *S. aureus* and *C. albicans* killing are achieved mainly by heterophil phagocytosis, then killing may increase even though monocyte phagocytosis is decreased.

Total leukocyte and lymphocyte concentrations in red knots decreased after 60 min handling stress. This result is consistent with a similar experiment on house finches (*Carpodacus mexicanus*) showing that total leukocytes decreased over a 1-hour interval, both in birds bled at baseline and after 60 min, and in birds bled only after 60 min (Davis 2005). Our results are also consistent with the general dynamics of leukocyte redistribution during acute stress, in which heterophil traffic is increased and lymphocyte traffic is reduced in the blood stream (Dhabhar et al. 1995). This redistribution of cells enables mobilization of heterophils via the blood to form a first line of defense, and the redeployment of lymphocytes to the lymph system where they receive antigens released from sites of infection (Dhabhar et al. 1995).

The decrease in total leukocyte and lymphocyte concentrations that we measured may also have been caused by repeated bleedings. However, we feel that this is unlikely because concentrations did not decrease significantly between baseline and 20 min,

but only after 60 min. Furthermore, concentrations did not decrease more in protocol 2 than in protocol 1 despite the fact that the birds were bled four times in protocol 2 and only twice in protocol 1. Finally, in protocol 2, after 150 min (thus after four bleedings) leukocyte concentrations tended to increase (trend between 90 and 150 min in Fig 4.2 C and D) rather than decreasing further as would be predicted.

Constitutive immunity and corticosterone

We found a strong effect of handling stress on corticosterone (Fig 4.1) and as expected this effect occurred within 20 min of entering the aviaries. Similar baseline levels and increases in corticosterone due to handling stress have been found in both captive (Landys et al. 2004) and free-living red knots (Reneerkens et al. 2002). In contrast to the immediate response of corticosterone, immune measures appear to respond more slowly to handling stress. No measure of constitutive immune function was significantly different from baseline within 20 to 30 min of handling stress (Table 4.1 and Appendix 4.3).

A comparison of the protocols

The two protocols showed similar patterns in terms of the effect of handling stress on different measures of constitutive immunity (Table 4.1), indicating that both handling-stress protocols induced stress, and that in both April (without molt) and October (with some wing molt) red knots respond to handling stress in a similar manner. We expected that if the responses to the protocols differed at all, then protocol 2 would be more stressful because the birds were sampled repeatedly within a 2.5 hour period. The steeper increases in *S. aureus* killing and *C. albicans* killing indicate that this may be the case, at least for some measures (Fig. 4.2 A and B). However, because baselines differed for *S. aureus* and *C. albicans* killing, the intensity of the response may also be affected by how close the birds were to their maximum response at baseline. Thrombocytes are important for blood coagulation (Janeway et al. 2004), and also increased more steeply in protocol 2 than in protocol 1 (Fig. 4.2E), perhaps because we had inflicted four small wounds by 150 min.

The difference in baselines between the two protocols for *S. aureus* and *C. albicans* killing is interesting and is most likely due to seasonal changes in immune function over the annual cycle. We performed protocol 1 in April when the birds were not molting and protocol 2 in October during wing molt. Both *S. aureus* and *C. albicans* killing were lower in protocol 2, consistent with a study examining immune function over the annual cycle in knots, which found that *S. aureus* and *C. albicans* killing decreased during molt (Buehler et al. 2008a).

Practical implications for immune sampling

None of the 10 measures of constitutive immunity that we tested differed significantly from baseline within 20 or 30 min of handling stress, and this result was verified using two different protocols at two different times of the year and with different statistical methods. In contrast and as expected, corticosterone increased much more rapidly.

Our data are from captive birds. One may argue that captive birds are accustomed to handling stress and that free-living birds may be affected more quickly. However, we think this is unlikely since the captive birds in this experiment showed an immediate and significant increase in corticosterone indicating that they were indeed experiencing acute stress. Nevertheless, to test this definitively we would have had to run a capture and handling stress experiment on wild birds and such an experiment, with adequate sample sizes, was not achieved. Correlational data from free-living red knots, however, show no significant relationship between microbial killing or leukocyte concentrations (the measures most affected in captive birds) for capture and handling times 20 min or less (D. M. Buehler, unpublished data).

In conclusion, our results indicate that, in red knots, the indices of constitutive immunity that we measured do not change within capture and handling times less than 20 min. Thus, although birds should be sampled as soon as possible after capture, studies of baseline immune function in species not easily sampled in less than 3 min (such as red knots) should still yield useful results.

ACKNOWLEDGEMENTS

We thank Alberto Castillo and Steven Haan for lab assistance, Maarten Brugge for help with care of the captive knots and Judith van Bleijswijk for providing extra lab space. The manuscript was improved by two anonymous reviewers. Funding was provided by the Natural Sciences and Engineering Research Council of Canada (NSERC) PGSB-267701-2003, University of Groningen Ubbo Emmius Scholarship, and Schure-Beijerinck-Popping Fonds (DMB), by the Netherlands Organization for Scientific Research (NWO) and the University of Groningen (TP and BIT) and by the Royal Netherlands Institute for Sea Research (TP).

APPENDIX 4.1. SUPPLEMENTARY IMMUNE METHODS

Microbial killing capacity of whole blood

We followed the basic procedure outlined in (Millet et al. 2007) and performed the assay in a sterile working environment (a dead air box equipped with a UV Air Cleaner, Base Clear BV, KI-L046-M) using three microorganisms: *Escherichia coli*, a gram negative bacteria; *Candida albicans*, a yeast-like fungi; and *Staphylococcus aureus*, a gram positive bacteria. For each micro-organism we diluted the blood in CO2-independent media (#18045-054, Invitrogen) and added 20 μl of microorganism suspension reconstituted from lyophilized pellets (*E. coli* ATCC # 8739, *C. albicans* ATCC #10231, *S. aureus* ATCC # 6538: MicroBioLogics, St Cloud, MN) to a concentration of approximately 200 colonies per 75 μl of diluted blood–bacteria mixture. The mixture was incubated at 41°C (*E. coli*: 10 min., *C. albicans*: 60 min., *S. aureus*: 120 min.) and 75μl was spread onto agar plates in duplicate. The plates were stored upside down at 36°C, and the number of colonies per plate was counted the following day. For all strains we calculated the microbial killing capacity as one minus the number of colonies on blood plates relative to the number of colonies on inoculate control plates (200 μl of media mixed with 20 μl of microorganism without incubation).

Statistical considerations for microbial killing

Microbial killing was determined as the proportion of inoculated colonies killed after a strain-specific incubation time. This measure might be affected by factors which vary on a day-to-day basis such as the initial number of microbes (the inoculation concentration), the intrinsic microbial population trends during incubation (growth or death), and the microbial-suspension age (time since resuspension of the lyophilized pellets). In protocol 2, where different time points were measured on different days, we thus included covariates to control for inoculation concentration, microbial-suspension age and intrinsic microbial population trends during incubation in our statistical models.

These covariates were determined during a15-day pilot study conducted in July and August 2005. Each day we prepared three control suspensions of each microbe (200μl of media and 20μl of microbial-suspension) without blood. We plated the first immediately, without incubation, to determine day-to-day variation in the number of microbes inoculated. We incubated the second and third suspensions for two strain-specific incubation periods (10 and 20 min for *E. coli*, 30 and 60 min for *C. albicans*, and 60 and 120 min for *S. aureus*) to determine variation in intrinsic microbial population growth/death. We also examined the effects of microbial-suspension age by tracking changes related to the number of days since resuspension of the lyophilized pellet.

The number of *E. coli* colonies on the non-incubated control plates varied from 93 to 266 (mean ± SD = 176.1 ± 42.3; CV = 24%) over the 15-day period. Cultures tended to grow after 10 and 20 min of incubation; however growth was not significant (growth on 14 of 15 days, $F_{2,42} = 3.00$, $P = 0.06$). Microbial-suspension age contributed significantly to the number of plated colonies with a general increase for the first 2 days and then a decrease to 7 days ($F_{10,34} = 2.73$, $P = 0.01$).

The number of *C. albicans* colonies on the non-incubated control plates varied from 148 to 261 (mean ± SD = 190.6 ± 32.5; CV = 17%) over the 15-day period. Incubation time ($F_{3,56} = 4.03$, $P = 0.012$) significantly affected the number of colonies plated and on average there was a 13% increase in cell number after 30 min, a 6% increase after 60 min, and a 2.4% decrease after 120 min of incubation. Microbial-suspension age also significantly affected the number of colonies plated ($F_{10,49} = 3.24$, $P = 0.003$) with colony numbers decreasing for the first 3 days, increasing to 5 days and then decreasing again.

The number of *S. aureus* colonies on the non-incubated control plates varied from 132 to 253 (mean ± SD = 193.7 ± 38.7; CV = 20%) over the 15-day period and on average there was a 10% increase after 60 min and a 6% increase after 120 min ($F_{2,42} = 0.874$, $P = 0.425$). Microbial-suspension age contributed significantly ($F_{10,34} = 4.67$, $P < 0.001$) to the number of colonies on plates showing a general decrease in colonies as pellet age increased, with the steepest drop in the first 4 days.

Given the results of this pilot study we included the inoculation concentration and the number of days since pellet resuspension as covariates in our statistical analysis for all three strains. Furthermore, because *C. albicans* and *S. aureus* have 1 or 2 hour incubation times we controlled intrinsic microbial population trends during incubation using the ratio of control plate counts (i.e. in the absence of blood) before and after incubation (where a ratio greater than 1 signifies growth and less than one death). Because correlations between this ratio and the initial number of microbes added might result in co-linearity problems (i.e. the number of microbes added might contribute to whether growth or death occurs), we calculated tolerance for these covariates. In all cases tolerances were > 0.8 and thus well above the 0.1 collinearity cut-off (pg 128, Quinn & Keough 2002).

Leukocyte concentrations

We obtained differential leukocyte concentrations using blood smears. After staining, the smears were examined at 1000X magnification with oil immersion and the first 100 leukocytes were counted and classified as heterophils, eosinophils, lymphocytes or monocytes. The number of thrombocytes seen while counting the first 100 leukocytes was also recorded as an estimate of the relative number of thrombocytes per leukocyte. Basophils were extremely rare (< 0.5%) and were therefore not included in the counts. All counts were made in random order and blind to time point by DB for protocol 1 and NB for protocol 2. In combination with the blood smears, we obtained leukocyte concentrations using the indirect eosinophil Unopette method (Campbell 1995) following the manufacturers instructions (No. 5877; Becton Dickinson).

Hemolysis-hemagglutination assay

Plasma was obtained by centrifuging blood samples for 12 min at 12000 x g and storing the separated plasma at -80°C until the assay was performed. We performed the assay as described by (Matson et al. 2005). Hemolysis and hemagglutination were quantified by serial dilution. We placed 25 μl of plasma in the first and second rows of a 96-well plate and then from the second to the eleventh rows we performed ten 1:2

dilutions using Dulbecco's PBS (Mauck et al. 2005). We then added 25 μl of 1% of rabbit RBC suspension to each well, and incubated the plates at 37°C for 90·min. After incubation plates were tilted at a 45° angle and were scanned for agglutination after 20 min and lysis after 90 min. Sample order was randomized and all samples were assayed blindly with respect to origin. The resulting images from each sample were randomized with respect to day, plate, and row; and were scored blindly for lysis and agglutination by DMB.

APPENDIX 4.2

Appendix 4.2. Individual baselines for 20, 60 and 120 minute sampling points for protocol 2. Baselines did not differ for corticosterone (P = 0.3) or any immune measure (all *P*'s > 0.25). Thus, for statistical tests we averaged the baselines for each bird.

Variable	20 min		60 min		120 min	
	Mean	SD	Mean	SD	Mean	SD
Corticosterone (ng/ml)	25753.0	13589.7	24971.8	22817.4	17177.2	7320.2
S. aureus (prop. killed)	-0.03	0.22	-0.03	0.20	-0.01	0.15
C. albicans (prop. killed)	0.26	0.32	0.29	0.27	0.20	0.28
E. coli (prop. killed)	0.45	0.28	0.45	0.30	0.53	0.27
Total leukocytes (per μl)	12980.0	8992.8	9704.4	6325.5	10595.7	7947.4
Heterophils (per μl)	1010.5	848.2	1175.3	1100.0	666.6	233.3
Lymphocytes (per μl)	11012.3	8464.9	7829.1	5424.2	8821.3	7237.4
Monocytes (per μl)	816.4	986.0	669.7	562.9	844.6	714.2
Thrombocytes (per μl)	25314.6	20399.7	21677.6	20509.4	17786.1	10775.2
Hemolysis (log_2)	4.5	0.7	4.6	1.3	5.2	1.3
Hemagglutination (log_2)	2.0	1.2	2.0	0.9	1.9	0.9

APPENDIX 4.3

Appendix 4.3. Power analysis (using G*Power: Faul et al. 2007) and tests between individual baseline and 20 minute sampling points for protocol 2.

Variable	F	*P*	Effect Size	Power
S. aureus (prop. killed)	$F_{1,11} = 0.77$	$P = 0.814$	0.436	0.536
C. albicans (prop. killed)	$F_{1,11} = 1.40$	$P = 0.262$	0.362	0.400
E. coli (prop. killed)	$F_{1,11} = 1.16$	$P = 0.303$	0.537	0.713
Total leukocytes (per μl)	$F_{1,11} = 1.26$	$P = 0.286$	0.420	0.500
Heterophils (per μl)	$F_{1,11} < 0.01$	$P = 0.980$	0.490	0.632
Lymphocytes (per μl)	$F_{1,11} = 2.38$	$P = 0.151$	0.525	0.693
Monocytes (per μl)	$F_{1,10} = 1.48$	$P = 0.252$	0.317	0.320
Thrombocytes (per μl)	$F_{1,11} = 2.33$	$P = 0.155$	0.506	0.661
Hemolysis (log_2)	$F_{1,11} = 1.21$	$P = 0.295$	0.447	0.555
Hemagglutination (log_2)	$F_{1,10} = 4.81$	$P = 0.053$	0.419	0.503

CHAPTER 5

Seasonal redistribution of immune function in a migrant shorebird: annual cycle effects override adjustments to thermal regime

Deborah M. Buehler, Theunis Piersma, Kevin Matson and B. Irene Tieleman

ABSTRACT

Throughout the annual cycle, demands on competing physiological systems change, and animals must allocate resources to maximize fitness. Immune function is one such system and is important for survival. Yet, detailed empirical data tracking immune function over the entire annual cycle are lacking for most wild animals. We measured constitutive immune indices once a month for a year on captive red knots (*Calidris canutus*). We also examined temperature as an environmental contributor to immune variation by manipulating ambient temperature to vary energy expenditure. To identify relationships among immune indices we performed principal component analysis (PCA). We found significant repeatability in immune indices over the annual cycle and co-variation of immune indices within- and among-individuals. This co-variation suggests "immune strategies" as individual traits among-individuals and the use of different immune strategies during different annual cycle stages within-individuals. Over the annual cycle, both higher cost phagocyte-based immunity and lower cost lymphocyte-based immunity were high during mass change, but there was a clear shift towards lower cost lymphocyte-based immunity during peak molt. Experimental manipulation of temperature had little effect on annual variation in immune function. This suggests that other environmental factors, such as food availability and disease, should also be examined in the future.

The American Naturalist 2008: in press

INTRODUCTION

Throughout the annual cycle, demands on competing physiological systems change, and animals must allocate resources to maximize fitness (King 1974). Immune function represents one such physiological system and it contributes to host survival by limiting infection and by performing self maintenance duties such as clearing apoptotic cells. Given its importance, one might expect animals to maintain strong immune defenses throughout year. However, variability in immune defense is widespread (reviewed in Martin et al. 2008), and hypotheses about why this variation occurs include endogenous annual cycles, changes in disease threat and immuno-enhancement in anticipation of disease threat based on changes in environmental cues (Nelson et al. 2002). Variation may also occur because immune defense comes with costs as well as benefits. Building an immune system is developmentally costly and maintaining and using the system has energetic and immunopathology costs (Klasing 2004; Råberg et al. 1998; Schmid-Hempel and Ebert 2003). These costs have led to predictions about trade-offs between immune function and other activities (e.g. reproduction, migration, molt, thermoregulation; Lochmiller and Deerenberg 2000; Norris and Evans 2000; Sheldon and Verhulst 1996). However, evaluating these hypotheses is difficult without detailed empirical data (Hasselquist 2007).

Empirical data tracking immune variation over the annual cycle exist for humans and small mammals (Nelson et al. 2002), but are lacking for other animals. Avian studies, for example, have included comparisons between pairs of life cycle stages (e.g. breeding versus non-breeding or molt versus non-molt, see Martin et al. 2008 for a review), but have not detailed variation over the entire annual cycle. Furthermore, baseline data collected under controlled environmental, nutritional and pathogen pressure conditions are necessary to interpret data taken across seasons in the wild. In small mammals, such baseline data coupled with wild data show that complex physiology-environment interactions in the wild can mask underlying seasonal anticipatory patterns (Nelson et al. 2002).

In addition to the need for baseline data, controlled studies that manipulate environmental factors are needed to tease apart the relative importance of contributors to immune variation. Many environmental factors change over the annual cycle (i.e. temperature, pathogen pressure, photoperiod, food availability) and some, ambient temperature and photoperiod, have been linked to annual variation in immune function in mammals (Nelson et al. 2002). We focus on ambient temperature, which may also influence immune function in birds, especially species wintering in temperate regions where temperature greatly impacts energy expenditure (Wiersma and Piersma 1994). If immune function is traded-off when birds need to invest more on thermoregulation, then we predict that birds living in colder temperatures should show reduced immune function or a shift away from higher cost immunity (sensu Lee 2006, less phagocytosis/ inflammation).

Another environmental contributor to annual variation in immune function is pathogen pressure. Seasonal fluctuations in diseases have been detected in humans, mammals and birds (Nelson et al. 2002) and are likely to affect investment in immune

defense, but in the wild these patterns are extremely complex (Altizer et al. 2006). Even in captivity pathogen pressure is not easily manipulated; however, in some species it can be somewhat controlled by cleaning regimes (Buehler et al. 2008b). If pathogen pressure in captivity is relatively stable, then patterns of immune function observed in captivity may be interpreted in the context of anticipatory cycles, in species where annual patterns of pathogen pressure can be predicted.

Red knots (*Calidris canutus islandica*) are medium-sized (100-200 g) long distance migrant shorebirds with well studied annual cycles (Piersma 2007). In this subspecies, pathogen pressures are predicted to be highest during migration (Buehler and Piersma 2008) when the birds pass through a variety of environments where they might encounter novel pathogens (Møller and Erritzøe 1998). During breeding in the Arctic knots are widely dispersed decreasing the risk of disease transmission. Little is known about pathogen pressure during fall migration, molt and wintering; however it is likely lower than during spring migration (Buehler and Piersma 2008). Fall migration is less synchronous than spring migration and birds are aggregated in smaller flocks (Battley et al. 2004). Given appropriate photoperiod cues, captive red knots exhibit pre-migratory fattening, mass loss and molt cycles comparable to those of free-living birds (Jenni-Eiermann et al. 2002; Piersma et al. 1995). Furthermore, because captive knots do not migrate, anticipatory cycles corresponding to pathogen pressure, which might be masked by extreme physical activity in migrating birds, may also be detected.

The immune system can be divided along an innate (non-specific) and acquired (specific) axis and along a constitutive (non-induced) and induced axis (Schmid-Hempel and Ebert 2003). Immune defense may shift between or even within these axes to provide "optimal immune strategies" in different situations (Lee 2006; Martin et al. 2007b; Schmid-Hempel and Ebert 2003). We chose constitutive immunity to examine resource allocation over the annual cycle because it is effective at controlling multiple pathogen types and responds immediately to threats, making it an evolutionarily relevant first line of defense. Furthermore, mediators of constitutive immunity must be maintained even when not in use, generating costs that may be important in physiological trade-offs during the annual cycle (Martin et al. 2008; Schmid-Hempel and Ebert 2003). Finally, because a response is not induced and immunological memory is not stimulated, repeated measures of individuals throughout the annual cycle can be made. We quantified three categories of constitutive immune indices covering a range of protective functions. First, microbial killing ability which measures the functional capacity of blood to limit microbial infection (Millet et al. 2007; Tieleman et al. 2005). Second, leukocyte concentrations which provide information on circulating immune cells, and differential leukocyte counts which are useful in multivariate analysis in terms of their relationship to functional measures of immunity such as microbial killing. Heterophils and eosinophils mediate innate immunity against novel pathogens and are important phagocytes, monocytes link innate and acquired defence, and lymphocytes mediate pathogen specific antibody and cell-mediated responses of the acquired immune system (Campbell 1995). Third, levels of complement and natural antibodies (Matson et al. 2005) which provide a first line of defense against spreading infections via cell lysis, and link innate and acquired immunity (Ochsenbein and Zinkernagel 2000).

In this study we describe how immune function changes over the annual cycle of red knots and how ambient temperature influences this pattern. To do so, we sampled captive birds once a month over a complete annual cycle. We manipulated thermoregulatory costs using treatments of cold, warm and variable temperatures (the latter tracking outdoor conditions). We first establish the repeatability of immune indices, then examine how different indices co-vary, and finally analyze changes over the annual cycle and the effects of thermoregulatory costs.

MATERIALS AND METHODS

Birds, aviaries and experimental treatments

In August and September 2004 (n = 26) and February 2005 (n = 4) red knots, *C. c. islandica*, were captured in the Dutch Wadden Sea (53°31'N 6°23'E) and brought into captivity at the Royal Netherlands Institute for Sea Research (NIOZ). At capture the birds were ringed, weighed, and aged (all older than two years; Prater et al. 1977) and we later determined sexes using molecular techniques (Baker et al. 1999). We randomly assigned the birds (total n = 30, 21 females, 9 males) to one of three treatment groups in identical indoor aviaries (4.5m x 1.5m x 2.3m high). The birds had free access to food (mudsnails *Hydrobia ulvae*), freshwater for drinking, and before the start of measurements all birds had at least one month to acclimate to captivity. Photoperiod was set to track the seasonal changes in day length in the northern Netherlands for all cages. To verify that all study birds were healthy, we performed weekly assessments of body mass, feather condition and the skin of the feet, elbows (carpals) and keel for signs of local inflammation (e.g. bumblefoot).

The variable treatment birds (n = 12) were divided equally between two aviaries ventilated with outdoor air and maintained at outdoor temperature. During the experiment, variable treatment birds experienced a mean temperature of 15.4 ± 4.8°C (maximum of 25.5°C, 24 June 2005; minimum of 4.6°C, 6 March 2006). This treatment approximates temperatures experienced by free-living *C. c. islandica*, which spend ten months a year in the temperate climate of western Europe (Piersma 2007). Warm treatment birds (n = 12) were also divided between two aviaries and were maintained at 24.7 ± 1.8°C (i.e. within the zone of thermoneutrality; Piersma et al. 1995; Wiersma and Piersma 1994). Cold treatment birds (n = 6) were kept in a single aviary maintained at 4.9 ± 1.2°C. These temperature differences led to a 14% higher mass specific BMR in the cold than in the warm birds in February and March 2005 (Vézina et al. 2006). All groups were similar in terms of sex ratio and morphometrics (Vézina et al. 2006).

During the experiment, three birds died (all female). Two cold treatment birds that died in April and August 2005 were replaced sequentially in June (with a female captured in February 2005) and September (with a male captured in August 2005). Both were given at least a month to acclimate to captivity before entering the experiment. One warm treatment bird that died in February 2006 was not replaced because there was insufficient time to capture and acclimate a new bird before the end of the experiment.

Blood Sampling

Birds were sampled monthly with sampling order randomized by cage and individual. We always collected blood in the morning between 10:30 and 11:00 am (mean ± SD = 10:44 ± 5 min). Each day we sampled two birds within 20 min of entering an aviary (mean ± SD = 6.89 min ± 4.48). We thoroughly sterilized the area around the brachial vein with 70% ethanol and then collected about 600 _l of blood into pre-sterilized, heparinized capillary tubes. Immediately after sampling, we made blood smears and transported the remaining blood in sterile boxes to the laboratory for further processing within an hour of sampling.

Body mass and molt

Throughout the experiment, all birds were weighed and scored for molt and plumage color once a week. We scored wing molt by ranking the left primary feathers on a scale of 0 to 5 (0 for old feathers, 5 for fully grown new feathers, 1 to 4 for growing feathers depending on completeness; Ginn and Melville 1983). We scored body molt by examining the breast area and using the breast molt index (BMI; 0 = no growing feathers, 1 < 25% growing feathers, 2 = 25 to 50% growing feathers, 3 > 50% growing feathers; Piersma and Jukema 1993). Because we were interested in molt as a whole, we calculated total molt (TM) as the sum of BMI and the number of growing primaries (feathers in wing molt categories 1 through 4).

Measuring immune function

Microbial killing abilities

We followed the basic procedure of Millet et al. (2007) and Tieleman et al. (2005) and performed the assay in a sterile working environment (CleanAir CA/R3 Flow Cabinet, CleanAir Techniek B.V., Woerden, The Netherlands). To minimize the effects of different antigen-exposure histories, we used three ubiquitous microbial strains *Escherichia coli* (ATCC 8739), *Candida albicans* (ATCC 10231), and *Staphylococcus aureus* (ATCC 6538; see Appendix A from further details). First, we reconstituted lyophilized microbial pellets following the manufacturers instructions (MicroBioLogics, St Cloud, MN). Then, we diluted freshly-collected blood in CO_2-independent media (#18045-054, Invitrogen, The Netherlands). After subdividing the diluted blood samples for different incubation times (*E. coli* dilution 1:10, incubation 10 and 20 min; *C. albicans* dilution 1:10, incubation 30 and 60 min; *S. aureus* dilution 1:4, incubation 60 and 120 min) we added 20 μl of each microbial suspension to each subsample. These mixtures (totaling 220 μl) were incubated at 41°C and 75 μl was spread evenly onto agar plates in duplicate. Inverted plates were incubated at 36°C overnight; colonies were counted the following day.

For all strains we calculated the proportion of microorganisms killed as one minus the number of colonies on blood plates (described above) relative to the number of colonies on inoculate control plates (200 μl of media mixed with 20 μl of microorganism without incubation). These inoculate plates reflect the initial situation when the blood starts to act on the microorganism, thus best mimicking the situation of a bird responding to a pathogen.

Incubation times for *C. albicans* and *S. aureus* were long enough to allow microbial growth in some cases (i.e. negative killing – see Appendix 5.1). To take this into account, we prepared extra inoculate plates which were incubated the same amount of time as plates containing microorganism and blood. We included counts from these plates in our statistical models as "incubated controls" (see Statistics).

Circulating cellular immunity

At the end of each month blood smears were randomized and counted blind to treatment by a single observer (DMB). After staining (Giemsa Stain, Sigma-Aldrich, Germany), the smears were examined at 1000x magnification with oil immersion and the first 100 leukocytes were counted and classified as heterophils, eosinophils, lymphocytes or monocytes. We were unable to distinguish B and T cells on our blood smears, thus both are included in the lymphocyte count. Basophils were extremely rare (< 0.5%) and were therefore not included in the counts. The number of thrombocytes seen while counting the first 100 leukocytes was also recorded as an estimate of the relative number of thrombocytes per leukocyte. In combination with the blood smears, we obtained leukocyte concentrations using the indirect eosinophil Unopette method (Campbell 1995) following the manufacturers instructions (No. 5877; Becton Dickinson).

Complement and natural antibodies

We performed the assay as described by Matson et al. (2005). We pipetted 25 μl of plasma into the first and second rows of columns B to G in 96-well plates. Chicken serum (Gibco, ref: 16110-082) was used as a positive standard in columns A and H. Using Dulbecco's PBS (Mauck et al. 2005), we serially diluted the plasma and controls from row two to row 11 and left the 12th row as a negative control (PBS only). We then added 25 μl of 1% of rabbit red blood cell suspension to all wells and incubated the plates at 37°C for 90·min. After incubation, plates were tilted at a 45° angle and were scanned (Epson Perfection 4990 scanner) for agglutination after 20 minutes and lysis after 90 minutes. The scans were randomized with respect to sample origin, plate, and location within the plate and were scored blindly for lysis and agglutination by a single observer (DMB). Hemolysis, indicating complement and other lytic proteins, was identified by the absence of intact cells and the presence of free hemoglobin. Hemagglutination, indicating natural antibody activity, was identified by clumped red blood cells. Scores reflect the last plasma dilution in the dilution series (i.e. rows 2 to 11) exhibiting lysis or agglutination (see Matson et al. 2005 Fig 1, for an example).

Statistics

We used 1-sample Kolmogorov–Smirnov tests and visual examination of histograms to examine response variables and model residuals for normality. Although we calculated microbial killing as the proportion of colonies killed, the data were better described by normal than by binomial distributions (Appendix 5.1). Leukocyte data were right skewed and were log10 transformed. After transformation, all variables and the residuals of the models were normally distributed. Thus, we performed univariate analyses

using a linear mixed model with treatment and month as fixed factors and cage and bird as random factors. This approach allowed us to incorporate the nested and repeated measures design of our experiment (bird within cage within treatment, and individual birds sampled monthly) and to include all individuals, even those with missing values (Quinn and Keough 2002). In all comparisons, we ran the models including and excluding an effect of sex. Sex was never significant and never changed the outcome of the model, thus the statistics presented are from models excluding sex. We also included mass and time from cage entry to blood sampling as covariates in all models. For microbial killing capacities, we included the number of colonies inoculated, the ratio of incubated control and inoculation, and the microorganism suspension age (in days) as covariates. Covariates were sequentially removed from the models when not statistically significant at the 0.05 level. Effect sizes were calculated as generalized eta-squared (η_G^2; Olejnik and Algina 2003) and repeatability was calculated following Lessells and Boag (1987) using GLMs with fixed effects and covariates.

To gain insight into relationships between different measures of immune function we performed principle component analyses (PCA). We included only one time point for each killing measure (*E. coli* after 10 min, *C. albicans* after 60 min and *S. aureus* after 120 min) and excluded total leukocyte concentration since it is the sum of the differential concentrations. Eosinophil concentrations were also excluded due to a large number of 0 values. For the leukocyte concentrations we used the transformed data (though transformed and untransformed produce the same result). To account for the nested and repeated measures structure of our data, we performed the PCA on correlation matrixes with the data partitioned into within-individual (between-month) and among-individual levels following the procedure described in (Matson et al. 2006a). We used varimax rotation to maximize contrasts of variable loadings between factors (n.b. with this rotation, component scores calculated from the rotated loadings can be correlated; Joliffe 1995). We also tested the saliency criteria for these loadings (Cliff and Hamburger 1967), and saved scores for components with eigenvalues > 1 for further analysis. SPSS v 14.0 (2005) was used for univariate comparisons and we created the correlation matrices and ran the PCA using STATISTICA 7 (2004).

RESULTS

Patterns over the annual cycle

Body mass and molt

Body mass and molt data confirm previous findings that red knots in captivity retain fattening and molt schedules (Jenni-Eiermann et al. 2002). Birds fattened in early May, mass peaked in late May, and birds lost mass in June and re-stabilized mass in July (Fig 5.1A, Table 5.1). Molt data show prealternate molt (breast, back and head feathers) climaxed during April and May. Prebasic molt (all body, wing and tail feathers) peaked from July until September, but low levels of wing molt extended until January (Fig 5.1B, Table 5.1).

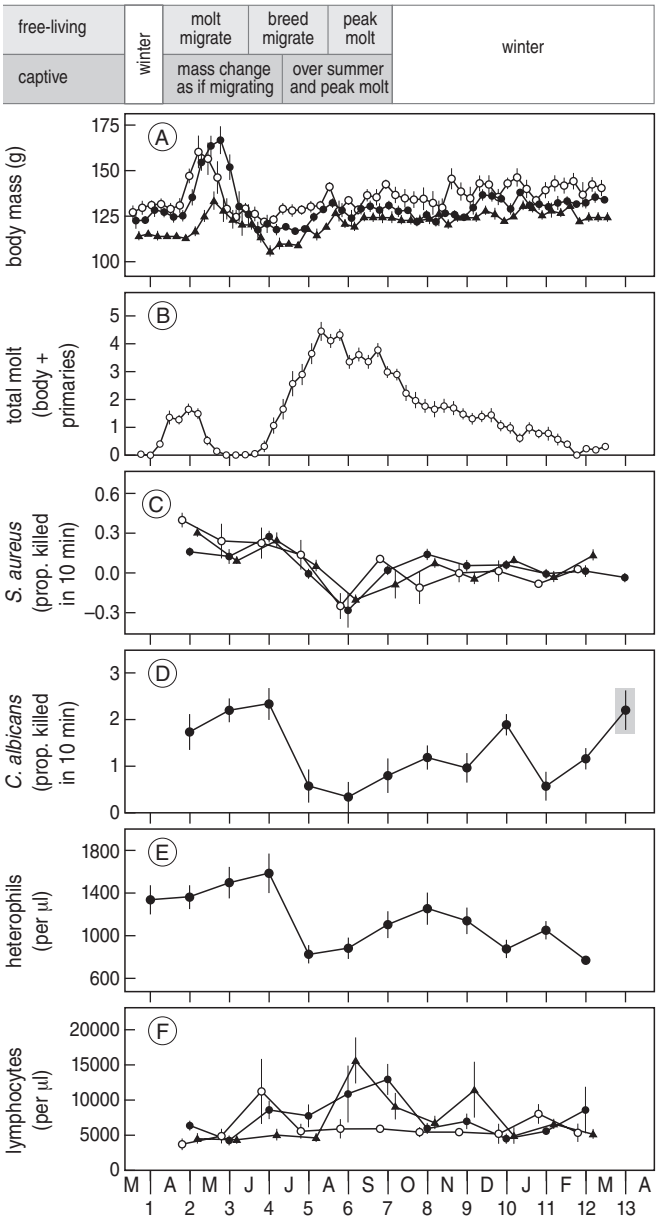

Figure 5.1. Trends in (A) body mass (B) molt and (C) to (J) constitutive immunity throughout the annual cycle. Where significant treatment by month interactions were detected, cold = open circles, variable = filled circles and warm = triangles. For all other variables the general trend is presented. For (E) to (H) statistics were performed on transformed values, but the raw data are shown for ease of interpretation. Symbols represent means and error bars show one SE. Due to repeated measures, error bars can only be used to infer statistical differences between treatments in (A), (C), (F) and (H), but not month to month differences. The annual cycle stages for free-living (Buehler & Piersma 2008) and captive birds (Jenni-Eiermann et al. 2002) are given above the graphs. Numbers under the months correspond to the labels in Fig 2C and boxed points in month 13 represent variable birds only.

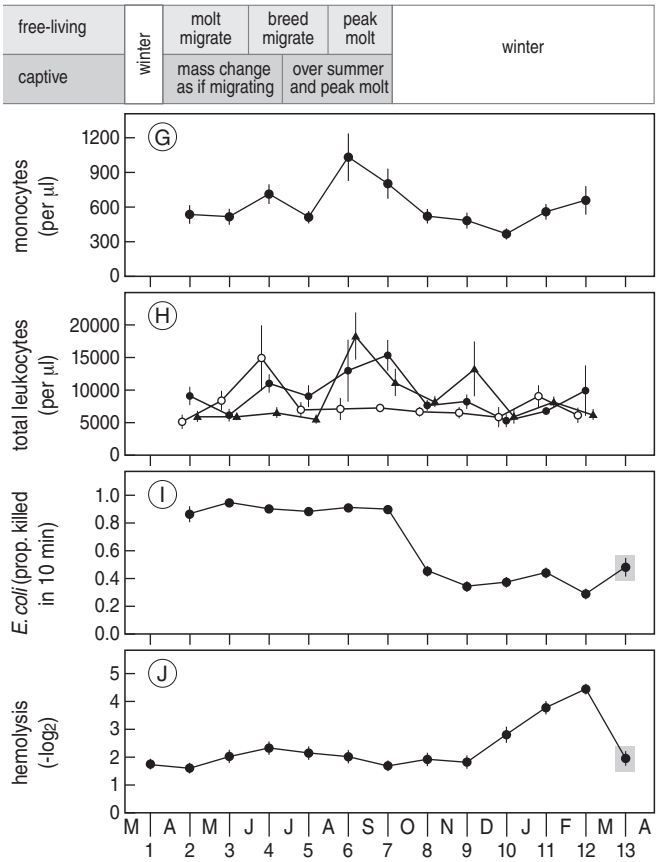

Table 5.1. Categorization of months based on monthly mass means, total molt means and Jenni-Eiermann et al. (2002).

	Month	Mean body mass ± SE	Mass description	Mean total molt ± SE	Total molt description	Category
1	March	123.1 ±1.8	Stable	0.10 ±0.17	Latent	Overwintering (B)
2	April	124.4 ±1.3	Mass gain	1.20 ±0.18	Body molt	Migration (E)
3	May	144.9 ±1.3	Peak mass	0.61 ±0.12	Latent	Migration (F)
4	June	121.2 ±1.0	Mass loss	0.35 ±0.10	Latent	Migration (G)
5	July	119.8 ±1.2	Lowest and rebound	2.74 ± 0.11	Wing and molt increasing	Over-summering (H)
6	August	127.4 ±1.1	Stable	4.01 ±0.10	Peak wing and body molt	Prebasic molt (A)
7	September	129.6 ±1.2	Stable	3.31 ±0.12	Wing and body molt decreasing	Prebasic molt (A)
8	October	127.6 ±1.2	Stable	1.97 ±0.11	Wing molt decreasing	Overwintering (B)
9	November	129.7 ±1.1	Stable	1.59 ±0.10	Wing molt decreasing	Overwintering (B)
10	December	132.7 ±1.4	Stable	1.22 ±0.13	Wing molt decreasing	Overwintering (B)
11	January	133.3 ±1.2	Stable	0.85 ±0.11	Latent	Overwintering (B)
12	February	132.9 ±1.0	Stable	0.38 ±0.10	Latent	Overwintering (B)

Table 5.2. Repeatability of immune function in red knots over the annual cycle (after Lessells & Boag 1987). Repeatability was calculated using models with fixed effects and covariates from individuals for which we had data for all 12 months (n = 27; 11 months for microbial killing). Because covariates differed for different immune indices, error degrees of freedom differ.

Response	Individual Repeatability				
	r	SE	df	F	*P*
Microbial Killing (proportion killed)					
S. aureus after 60 min	0.150	0.043	22,235	3.26	< 0.001
S. aureus after 120 min	0.216	0.053	22,235	4.72	< 0.001
C. albicans after 30 min	0.101	0.033	22,255	2.43	< 0.001
C. albicans after 60 min	0.097	0.032	22,258	2.68	< 0.001
E. coli after 10 min	0.400	0.070	22,258	9.10	< 0.001
E. coli after 20 min	0.469	0.072	22,258	11.64	< 0.001
White blood cell parameters (per μl)					
Total WBC	0.168	0.046	22,264	3.44	< 0.001
Heterophils	0.362	0.068	22,286	7.69	< 0.001
Eosinophils	0.061	0.024	22,286	1.79	0.020
Lymphocytes	0.196	0.050	22,264	3.94	< 0.001
Monocytes	0.135	0.040	22,286	2.85	< 0.001
Thrombocytes	0.049	0.021	22,286	1.61	0.043
Plasma parameters (log2)					
Hemolysis	0.291	0.063	22,286	5.92	< 0.001
Hemagglutination	0.107	0.034	22,286	2.44	< 0.001

Immune function

Repeatability and variability of individual immune measures

Immune function varied throughout the annual cycle at both the among- and within-individual levels. Among-individual differences explained 5 to 47% of total variation, depending on the index, and were significantly repeatable in all measures over the annual cycle (Table 5.2). At the within-individual level, all immune indices except hemagglutination varied significantly over months (Table 5.3).

Co-variation of immune variables among- and within-individuals

Among-individuals, PCA revealed three components with eigenvalues > 1 accounting for 70% of the total variability in the data (Table 5.4A). *S. aureus* killing, *C. albicans* killing, heterophil concentrations and hemolysis were positively correlated with PC1, *E. coli* killing, hemagglutination, and monocyte concentrations were positively correlated with PC2 (Fig 5.2A; monocytes also salient on PC3) and lymphocytes and thrombocytes were positively correlated with PC3. PC1 and PC2 scores were significantly correlated (Pearson r = 0.763, $P < 0.001$; after omitting the outlier, r = 0.655, $P < 0.001$; Fig 5.2B) as were PC2 and PC3 scores (r = 0.273, $P = 0.035$). PC1 and PC3 scores were not correlated (r = 0.173, $P = 0.186$).

Table 5.3. Immune function in red knots over the annual cycle. Effect sizes are generalized eta-squared (η_G^2; Olejnik & Algina 2003). Significance at 0.05 is in italic and at 0.01 in bold. Degrees of freedom vary among indices because in some birds we did not obtain the full set of immune indices from every bird in every month. Statistics for interactions are in the text.

Response	Among-individuals												Within -individuals			
	Treatment - fixed				Cage(Treatment) - random				Bird(Cage(Treat)) - random				Month - fixed			
	df	F	*P*	η_G^2	df	F	*P*	η_G^2	df	F	*P*	η_G^2	df	F	*P*	η_G^2
Microbial Killing (proportion killed)																
S.aureus 60 min	2,2	0.71	0.58	< 0.01	2,25	0.44	0.65	< 0.01	26,278	3.31	< **0.01**	**0.18**	10,278	11.6	< **0.01**	**0.24**
S.aureus 120 min	2,2	0.89	0.53	0.01	2,26	0.49	0.62	0.01	26,258	4.14	< **0.01**	**0.18**	10,258	18.2	< **0.01**	**0.31**
C.albicans 30 min	2,2	6.50	0.14	0.02	2,25	0.24	0.78	< 0.01	26,278	2.82	< **0.01**	**0.19**	10,278	2.88	< **0.01**	**0.07**
C.albicans 60 min	2,2	2.28	0.29	<0.01	2,25	0.13	0.88	< 0.01	26,278	2.66	< **0.01**	**0.18**	10,278	3.87	< **0.01**	**0.10**
E.coli 10 min	2,2	0.49	0.72	< 0.01	2,26	0.13	0.88	< 0.01	26,279	9.31	< **0.01**	**0.13**	10,279	130.1	< **0.01**	**0.71**
E.coli 20 min	2,2	0.32	0.76	< 0.01	2,26	0.33	0.72	< 0.01	26,281	11.80	< **0.01**	**0.22**	10,281	77.8	< **0.01**	**0.57**
White blood cell parameters (per μl)																
Total leukocytes	2,2	0.19	0.84	< 0.01	2,25	2.07	0.15	0.03	26,285	3.03	< **0.01**	**0.17**	11,285	4.38	< **0.01**	**0.10**
Heterophils	2,2	0.73	0.93	< 0.01	2,26	2.94	0.7	0.07	26,307	6.55	< **0.01**	**0.28**	11,307	8.27	< **0.01**	**0.15**
Eosinophils	2,2	0.82	0.55	0.01	2,25	1.15	0.33	0.01	26,219	1.60	*0.04*	*0.12*	11,219	6.39	< **0.01**	**0.21**
Lymphocytes	2,2	0.40	0.72	< 0.01	2,25	1.07	0.36	0.02	26,285	3.50	< **0.01**	**0.19**	11,285	4.64	< **0.01**	**0.11**
Monocytes	2,2	0.08	0.92	< 0.01	2,25	4.79	***0.02***	***0.06***	26,305	2.35	< **0.01**	**0.15**	11,305	2.65	< **0.01**	**0.07**
Thrombocytes	2,2	0.70	0.59	0.02	2,24	4.87	***0.02***	***0.04***	26,307	1.47	0.07	0.09	11,307	4.53	< **0.01**	**0.12**
Plasma parameters (-log2)																
Hemolysis	2,2	7.50	0.14	0.07	2,25	0.35	0.71	0.01	26,310	5.57	< **0.01**	**0.20**	11,310	25.6	< **0.01**	**0.38**
Hemagglutination	2,2	4.40	0.13	0.01	2,25	0.08	0.92	0.01	26,311	2.05	**0.01**	**0.17**	11,311	1.27	0.24	0.04

Table 5.4. Principal component loadings after varimax rotation (A) among-individuals and (B) within-individuals. Bold faced loadings are the highest loading for a measure across the PCs and underlined loadings meet the saliency criteria for that PC.

	Response	PC1	PC2	PC3
(A)	*S.aureus* killing (proportion killed)	**0.870**	0.067	-0.178
	C.albicans killing (proportion killed)	**0.907**	0.152	0.102
	E.coli killing (proportion killed)	0.149	**0.872**	-0.104
	Heterophils (per μl)	**0.835**	0.242	0.001
	Lymphocytes (per μl)	0.178	-0.311	**0.808**
	Monocytes (per μl)	0.186	**0.610**	0.523
	Thrombocytes (per μl)	-0.243	0.239	**0.734**
	Hemolysis (-log2)	**0.542**	0.337	0.099
	Hemagglutination (-log2)	0.415	**0.654**	0.022
	Totals			
	Variance (%) per component	32.1	21.3	17.0
	Cumulative variance (%)	32.1	53.4	70.4

	Response	PC1	PC2	PC3
(B)	*S.aureus* killing (proportion killed)	-0.429	**0.780**	0.113
	C.albicans killing (proportion killed)	-0.351	**0.810**	-0.018
	E.coli killing (proportion killed)	0.290	0.178	**0.776**
	Heterophils (per μl)	0.040	**0.730**	0.458
	Lymphocytes (per μl)	**0.836**	-0.401	0.192
	Monocytes (per μl)	**0.852**	0.019	0.252
	Thrombocytes (per μl)	**0.788**	-0.088	-0.051
	Hemolysis (-log2)	0.496	**0.714**	-0.133
	Hemagglutination (-log2)	0.044	0.033	**-0.952**
	Totals			
	Variance (%) per component	29.8	27.9	20.6
	Cumulative variance (%)	29.8	57.7	78.3

Within-individuals (among-months) PCA also revealed three components with eigenvalues > 1 accounting for 78% of the total variability in the data (Table 5.4B). Lymphocytes, monocytes and thrombocytes were positively correlated with PC1; *S. aureus* killing, *C. albicans* killing, heterophil concentrations and hemolysis were positively correlated with PC2; *E. coli* killing was positively correlated and hemagglutination was negatively correlated with PC3.

Changes in immune function over the annual cycle

Changes in immune indices corresponded to physiological changes that knots experience over a captive annual cycle (Fig 5.1). *S. aureus* killing, *C. albicans* killing and heterophil concentrations peaked during mass change and dropped during peak molt (Fig 5.1C, D and E). Conversely, lymphocyte and monocyte concentrations showed a

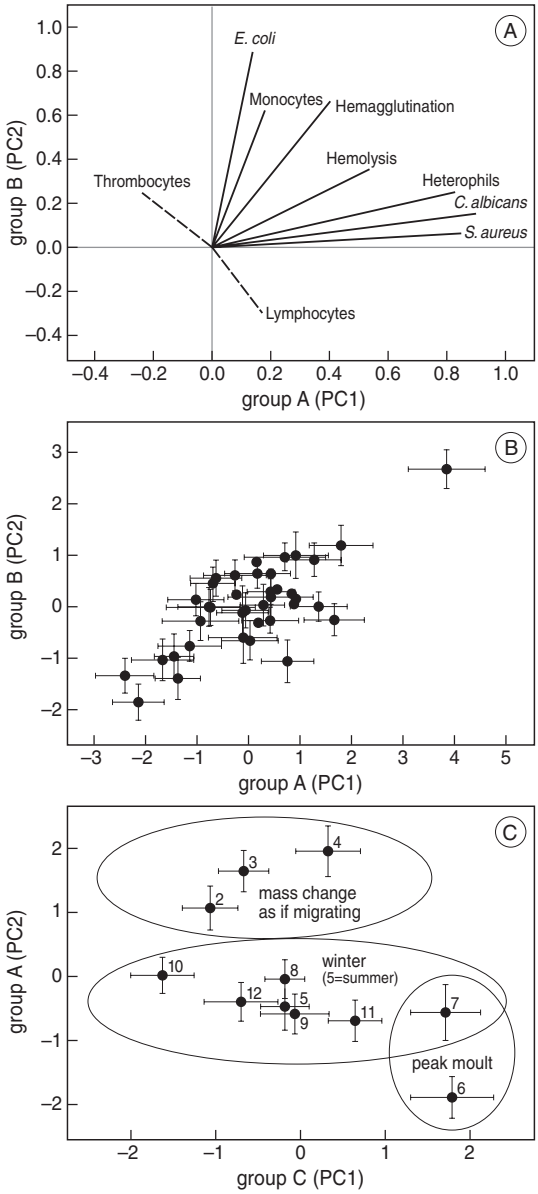

Figure 5.2. Relationships among immune indices based on principal components analysis among-individuals. Axes labels correspond to the alphabetical groups in Table 5. In (a) vectors are the PC1 and PC2 loadings of each measure (dashed vectors are best explained by PC3). Vector length indicates the strength of the relationship and the angle between two vectors gives the degree of correlation (adjacent = highly correlated, orthogonal (90°) = uncorrelated, and opposite (180°) = negatively correlated). In (b) the relationship between PC1 and PC2 scores among-individuals is shown and points (mean ± SE) represent individual birds. In (c) relationships between PC1 and PC2 scores within-individuals indicate redistribution of immune strategies over the annual cycle. Points represent months (mean ± SE) and numbers correspond to the months in Fig 1. Annual cycle stages are based on Jenni-Eiermann et al. (2002) and Table 5.1.

small increase during mass change, but peaked during peak molt (Fig 5.1F and G). Total leukocyte concentrations (the sum of all other leukocyte types) closely matched the pattern in lymphocytes because lymphocytes made up on average 74% of the total leukocyte count (Fig 5.1H).

E. coli killing and hemolysis also changed over the annual cycle; however, these changes did not occur during mass change or molt (Fig 5.1I and J). *E. coli* killing dropped between September and October and then rebounded modestly by April 2006 (boxed point in Fig 5.1I). In contrast, hemolysis increased from December through February and then trended back towards original levels by April 2006 (boxed point in Fig 5.1J).

We plotted monthly mean scores from PC1 and PC2 to further examine annual patterns among immune measures (Fig 5.2C). These scores cluster into three physiologically-relevant periods: (1) mass change (gain, loss and re-stabilization associated with migration) (2) peak wing and prebasic molt, and (3) winter (see Jenni-Eiermann et al. 2002 and Table 5.1 for categorization). During mass change PC2 scores are high, whereas during peak molt PC2 scores are low and PC1 scores are high. In month 5, an "over-summering" transition month between mass change and peak molt for captive red knots, PC2 scores are intermediate.

Effects of temperature treatments

Body mass and molt

Body mass was significantly affected by temperature treatment. Cold birds were consistently heavier than warm birds; variable birds were intermediate (outside the fattening period; Tukey post-hoc Cold > Variable > Warm, $P < 0.001$). A significant week by treatment interaction ($F_{104,1379} = 3.26$, $P < 0.001$) resulted from variable birds gaining more pre-migratory mass than cold birds or warm birds (Fig 5.1A, see Vézina et al. 2007). Molt was not affected by treatment (Molt: $X^2 = 2.33$, df = 2, $P = 0.31$).

Immune function

Annual cycle effects (reflected by month in Table 5.3) dominated over the effect of our experimental manipulations of thermoregulatory costs (treatment in Table 5.3). Significant month by treatment interactions were found only for *S. aureus* killing after 120 min ($F_{20,258} = 1.83$, $P = 0.02$, $\eta_p^2 = 0.09$), lymphocyte concentrations ($F_{20,285} = 1.86$, $P = 0.01$, $\eta_p^2 = 0.10$) and total leukocyte concentrations ($F_{20,285} = 1.98$, $P < 0.01$, $\eta_p^2 = 0.13$). With *S. aureus* killing, the interaction, which was driven by significantly lower killing ability in the variable treatment in April ($F_{2,3} = 30.97$, $P = 0.008$), is clearly less important than the overall temporal trends (Fig 5.1C). For lymphocyte and total leukocyte concentrations, cold birds peaked in June and July during lowest mass; warm and variable birds peaked in August and September during peak molt (Fig 5.1 F and H); however, Tukey post-hoc tests revealed no significant treatment effect in any month. No other immune measures showed significant treatment effects (Table 5.3) or significant treatment by month interactions (all p's > 0.10).

DISCUSSION

This study explored immune function over the annual cycle of red knots and examined how ambient temperature influenced this pattern. Our results established the repeatability of immune indices at the level of individual birds and showed that immune indices co-varied similarly among- and within-birds over time, suggesting functional "immune strategies". We discuss these strategies in terms of their possible costs and protective benefits at the among- and within-individual levels. We then discuss how these strategies shift during different stages of the annual cycle in terms of physiological trade-offs and predicted pathogen pressure in the wild. Finally, we discuss the relative lack of temperature effect on these patterns.

Relationships among immune indices

We examined relationships among nine immune indices with a range of protective benefits and hypothesized energetic and immunopathology costs (Table 5.5). Principal component analyses identified three axes (PCs) comprised of the same indices; however, the ordering of the PCs differed within- and among-individuals. For clarity we define these axes as group A, B and C (Table 5) and refer to them as such throughout the discussion.

Group A comprised *S. aureus* killing, *C. albicans* killing, heterophils and hemolysis (Table 5.4A, B). *S. aureus* and *C. albicans* killing are primarily the result of phagocytosis (Millet et al. 2007) and heterophils are the most numerous phagocytic cells in birds (Janeway et al. 2004). Heterophil phagocytosis produces harmful reactive oxygen and nitrogen species (Splettstoesser and Schuff-Werner 2002) and energy requirements may be heightened by their high turnover rates (Janeway et al. 2004). Complement, which mediates lysis, is linked to inflammation (high immunopathology costs) and must be tightly regulated to prevent host tissue damage (Janeway et al. 2004). Thus, we associate group A with more costly immunity in terms of collateral damage to the host (immunopathology cost) and energy requirements when a host encounters a pathogen and the constitutive immunity we measured is put to use (Table 5.5, Klasing 2004, Buehler et al. 2008b). Functionally, these indices are associated with protection from extracellular bacteria and yeast infections. Thus, this strategy (Group A) may be favored under conditions where diversity of these pathogens is high and encounters with new strains are frequent (Schmid-Hempel and Ebert 2003).

Group B comprised *E. coli* killing and natural antibodies (Table 5.4A, B). *E. coli* killing relies on soluble components in blood plasma (Matson et al. 2006b; Millet et al. 2007) rather than more costly phagocytosis (Splettstoesser and Schuff-Werner 2002). Natural antibodies link innate and acquired immune systems (Ochsenbein and Zinkernagel 2000). These molecules exhibit weak affinities and low specificities, but are important for initiating acquired antibody responses, which are highly specific and unlikely to be self-reactive. Thus, relative to group A, we interpreted group B as moderate in terms of immune cost (Table 5.5). Functionally, *E. coli* killing represents the ability to control or prevent infection by some gram-negative bacteria, and natural antibodies are a constitutive component of the acquired immune system providing a first line of defense against pathogen attack (Ochsenbein et al. 1999). Interestingly, hemol-

Table 5.5. Summary and clarification of the nine independent indices measured and hypothesized costs and classifications for the groupings identified by principal components analysis. [1] Millet et al. (2007) [2] Janeway et al. (2004) [3] Splettstoesser & Schuff-Werner (2002) and [4] Matson et al. (2005).

Group	Specific Index	Description and Function	Strategy	Hypothesized Cost	Among-bird	Within-bird
A	*S. aureus* killing *C. albicans* killing Heterophils Hemolysis	Bacteria defense - mostly phagocytosis [1] Yeast defense - mostly phagocytosis [1] Short-lived phagocytes associated with inflammation [2, 3] Complement mediated lysis during inflammation [4]	Constitutive innate immunity (phagocytosis and inflammation)	High - high energy costs due to short lived cells and high immunopathology costs due to inflammation	PC1	PC2
B	*E. coli* killing Hemagglutination	Bacteria defense - little phagocytosis [1] Natural antibody mediated clumping of invaders [4]	Constitutiv innate immunity (soluble factors)	Moderate - lower energy and immunopathology costs for killing by soluble factors rather than phagocytosis	PC2	PC3
C	Lymphocytes Monocytes Thrombocytes	Basis of specific induced immunity [2] Long-lived phagocytes and antigen presenting cells for specific immunity [2] Important for blood clotting [2]	Constitutive *potential* for induced acquired immunity	Low - low energy costs due to longer lived cells and low immunopathology costs due to less host damage	PC3	PC1

ysis did not correlate with group B. Complement and other lytic proteins are also soluble and have correlated with natural antibodies in other analyses (Matson et al. 2006a, Buehler et al. 2008b). The inclusion of monocytes in group B at the among-individual level is also puzzling; however, because monocytes meet the saliency criteria for group C among-individuals and clearly fall into group C within-individuals (Table 5.4B) we discuss them with group C below.

Group C comprised lymphocytes, monocytes and thrombocytes (Table 5.4A, B). Lymphocytes form the basis of cell-mediated (T-cell) and antibody-mediated (B-cell) acquired immunity (Janeway et al. 2004). Antibody-mediated immunity in particular is considered low cost due to its high efficiency and high specificity, which lowers energy and immunopathology costs (Klasing 2004). Monocytes mature into long-lived macrophages and antigen-presenting-cells important for initiating acquired immune responses, and thrombocytes are important for blood coagulation (Janeway et al. 2004). Thus, relative to groups A and B, we interpreted group C as lower cost because of its association with acquired immunity (Table 5.5). Functionally, lymphocyte and monocyte based immunity might be favored in conditions where energy demands are high or where pathogen pressure is associated with reoccurring infections rather than with novel pathogens (Schmid-Hempel and Ebert 2003).

Immune function as an individual trait

Our immune indices co-varied similarly among- and within-birds over time suggesting functional "immune strategies" at multiple levels of organization. This idea is supported by a study describing species-specific immune strategies in mammals (Martin et al. 2007b) and by a study describing similar co-variation among constitutive immune indices in captive and free-living red knots (Buehler et al. 2008b). We found that the proportion of variability explained by each strategy differed among- and within-individuals (Table 5.4A and B). Higher cost indices (Table 5.5) explained more variability among-birds suggesting that individuals are best distinguished by more costly immunity. Furthermore, the positive correlation between group A and B indices among-birds (Fig 5.2B), indicates that individuals maintain either high or low levels of more costly immune strategies. It is also notable that *E. coli* killing and natural antibody levels are positively correlated among-individuals (Table 5.4A), but negatively correlated within-individuals (Table 5.4B). This indicates, like the relationship between group A and B strategies, that individuals maintain either high or low soluble constitutive defenses, but that across the year there is an antagonistic relationship between *E. coli* killing and natural antibody levels.

Redistribution of immune function in different annual cycle stages

Over the annual cycle, limited resources must be allocated to immune defense, predicting an "optimal" portfolio of defenses (Schmid-Hempel and Ebert 2003). Our results suggest that different strategies may be used during different periods of the year (Fig 5.2C).

The period of mass change in captive birds is associated with fattening and spring migration in the wild. Because fattening and migration are energy demanding activities,

trade-offs with immune function may be predicted when resources are limited. However, our results indicate that group A indices, which are associated with higher energy and immunopathology costs, where high during the period of mass change. This may be due to the fact that our birds had *ad libitum* access to food and were not experiencing resource limitation. Studies manipulating food availability are underway to examine this possibility (D. M. Buehler et al. submitted, chapter 7 of this thesis) and studies on free-living birds will be necessary to test whether trade-offs occur under resource limited conditions in the wild. From a disease perspective, fattening and spring migration are considered high risk (Buehler and Piersma 2008). During migration birds pass through a variety of environments where they might encounter novel pathogens (Møller and Erritzøe 1998) and during stopovers they feed in dense flocks facilitating disease transmission (Altizer et al. 2006). Thus, during this period, the protective benefits of group A indices might outweigh their costs.

The period of body mass stabilization in captive birds is associated with over-summering or breeding in the wild. During this period we found an abrupt drop in group A indices (month 5 in Figs 5.1C, D, E and 5.2B), which may represent a transition period between mass change and peak molt in captive birds. From a disease perspective, in the wild, once the knots establish a breeding territory they are widely dispersed. Thus, transmission risk and the change of encountering novel pathogens may be decreased (Buehler and Piersma 2008).

Peak molt occurs at approximately the same time in captive and free-living knots (Fig 5.1). During molt, feathers must be synthesized by keratinocytes (Haake & Sawyer 1986) and experiments have demonstrated that hydrogen peroxides, such as those generated during respiratory burst and phagocyte ingestion (Splettstoesser and Schuff-Werner 2002), inhibit keratinocyte proliferation (O'Toole et al. 1996). Furthermore, growing feathers are a potential cause of dermal inflammation (Silverin et al. 1999), which can be costly in terms of immunopathology (Råberg et al. 1998). Thus, immune strategies that counter infection with less phagocytosis should be favored during molt. Our results support this idea as we found a clear shift from group A to group C indices during peak molt (Fig 5.2C) indicating a decrease in phagocytosis-based defenses and a greater role for lymphocytes and monocytes.

Looking at the annual cycle as a whole our data suggest two main strategies. Group A indices represent defenses that are necessary during periods of high pathogen pressure, but that are costly enough to warrant down-regulation when pathogen pressures are lower and the costs of these defenses outweigh their benefits. Conversely, Group C indices represent relatively low cost immunological functions that may be necessary year-round. To fully test the ideas presented here, studies on free-living birds and induced immune responses will also be required.

No trade-off between constitutive immunity and thermoregulatory costs

Contrary to predictions that ambient temperature might affect immune function in knots, this study suggests temperature in itself is relatively unimportant to overall annual variation. Higher thermoregulatory costs alone do not seem to necessitate trade-offs between constitutive immune function and thermoregulation. Nevertheless,

cold birds did respond physiologically to low temperatures. They were heavier than warm birds (Fig 5.1) and exhibited 14% higher mass specific BMR than warm birds in February and March 2005 (Vézina et al. 2006). Exposure to cold for a year also led to larger spleen size in cold birds (100% heavier than warm birds, fresh mass, F. Vézina unpublished data 2006). The spleen is an important organ for the presentation and recognition of antigens in induced acquired immunity (Janeway et al. 2004, but see Smith and Hunt 2004), indicating that thermoregulatory costs may impact aspects of immunity that were not measured.

Another explanation for the limited effects of temperature treatments on measured immune indices may be that the *ad libitum* feeding regime allowed cold-treatment birds to compensate for increased energy expenditure via higher food intake (Vézina et al. 2006). Energy expenditure is only one component of the energy budget, and future experiments that simultaneously manipulate energy intake are needed to examine the effect of limited resources on immune function. Furthermore, future experiments manipulating other environmental factors that vary over the annual cycle (e.g. photoperiod and pathogen pressure) will help resolve the proximate causes of variation in immune function in wild birds.

ACKNOWLEDGEMENTS

We thank François Vézina, Magali Petit, Kirsten Jalvingh, Ania Gustowska and Maarten Brugge for help with captive knots, Chris Pool of the Animal Experiment Committee of the Royal Netherlands Academy for Arts and Sciences for overseeing experimental procedures (protocol NIOZ.05.01), and Anne Dekinga, Bernard Spaans, Maarten Brugge and the crew of the research vessel Navicula for catching birds and fishing Hydrobia. We thank Alberto Castillo, Daliborka Barjaktarov, Steven Haan, Mieke Lange and Eeke Marks for lab assistance and Judith van Bleijswijk for lab space. We thank Dick Visser for excellent help with finalizing the figures. This article was greatly improved by comments from François Vézina, Arne Hegemann, Barbara Helm and several anonymous reviewers. Funding was provided by the Natural Science and Engineering Research Council of Canada (NSERC PGSB-267701-2003), the University of Groningen and Schure-Beijerinck-Popping Fonds to DMB, the Netherlands Organization for Scientific Research and the University of Groningen to TP and BIT, and the Royal Netherlands Institute of Sea Research to TP.

APPENDIX 5.1. SUPPLEMENTARY METHODS FOR MICROBIAL KILLING ASSAY

Choice of micro-organisms

By quantifying the capacity of whole blood to kill micro-organisms *in vitro*, the microbial killing assay measures innate immunity integrated across circulating cellular and plasma components (Millet et al. 2007). In order to minimize the effects of different antigen-exposure histories, we used three common microbial strains, none of which are highly pathogenic (*E. coli* ATCC 8739, *C. albicans* ATCC 10231, *S. aureus* ATCC 6538).

Escherichia coli, gram negative bacteria, are often commensal in the intestinal tract, but can cause infection in the respiratory tract in birds. *Candida albicans* are yeast-like fungi that can cause candidiasis in birds when ingested. *Staphylococcus aureus*, gram positive bacteria, are normal inhabitants of skin but can result in inflammation and infection if they enter a wound (United States Geological Survey 1999).

The *E. coli* strain we used is primarily killed by plasma components of the blood (Matson et al. 2006b; Millet et al. 2007), whereas cellular components of blood are needed for the effective killing of *C. albicans* and *S. aureus* (Millet et al. 2007). Nonetheless, we always used whole blood in order to ensure all assays equally integrated cellular and soluble immune components.

Optimal blood dilutions and incubation times for red knots

We performed pilot studies (in February 2005 for *E. coli* and *S. aureus* and March 2005 for *C. albicans*) to determine the optimal dilutions and incubation times for each strain. We found that on average a 1:10 blood:media dilution killed 62.3% of *E. coli* colonies after 10 min, 76.3% after 20 min and 94.0% after 30 min (n = 3). For *C. albicans* we found killing took longer; on average a 1:10 blood:media dilution killed 23 % after 30 min, 50% after 60 min, 62% after 120min, and 71% after 180 min (n = 2). Killing of *S. aureus* was the lowest, even after increasing the blood concentration. On average a 1:4 blood:media dilution killed only 15.7% of *S. aureus* after 60 min and 32% after 120 min. After 180 min of incubation, killing was back down to 31%, n = 3 indicating that increased incubation did not increase killing. From this pilot study we chose the blood dilutions and incubation times listed in the main text, which gave killing proportions nearest to the centre of the S-shaped dose response curve described in Matson et al. (2006b).

Variation in inoculation concentrations

We quantified the variation in the number of microorganisms added to diluted blood at the start of each assay. For each strain and from the same stock suspension, we produced eight replicate control suspensions (20μl microbial stock in 200μl media) and plated each control suspension in duplicate. Following standard procedure, we calculated the average of each pair of control plates. We found that the coefficient-of-variation among our 8 replicates was 5.8% for *E. coli* (average colonies ± SD: 515.7 ± 30.1, n = 8; for this trial we were targeting 500 colonies), 9.2% for *C. albicans* (167.0 ± 15.4, n = 8) and 9.2% for *S. aureus* (178.5 ± 16.4, n = 8).

Normal versus binomial treatment of microbial killing data

Because microbial killing capacity is calculated as a proportion, the data might better fit a binomial, rather than a normal, distribution. However, for *C. albicans* and *S. aureus* killing, the data were in fact better fit by a normal distribution because in many cases (*C. albicans*: 56% at 30min and 19% at 60min; *S. aureus*: 48% at 60min and 33% at 120min) the bacteria grew in the presence of blood producing "negative killing", or in other words more bacterial colonies with blood than without blood. Biologically, this indicates that the microorganisms actually benefited from the presence of the blood, possibly by using nutritive blood components as food.

This "negative killing" violates the assumption of a binomial distribution bounded by 0 and 1. For *E .coli*, only 3 out of 353 observations showed "negative killing" and when these three observations were set to 0 or excluded, the *E. coli* killing data were well approximated by either a binomial or a normal distribution. Thus, we analysed the *E. coli* killing data using both a GLM in SPSS 14 (2005) and a generalized linear model with a binomial distribution and log link function in MLWIN (Rasbash et al. 2004). The output of the two models was identical and because we examined all other response variables with the GLM we present only GLM output.

CHAPTER 6

Limited access to food and physiological trade-offs in a long distance migrant shorebird part I: energy metabolic, behavior and body mass regulation

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ABSTRACT

Previous experiments showed downregulation of basal metabolic rate (BMR) in birds facing energetic challenges. We alternatively exposed two groups of red knots (*Calidris canutus*) to either 6h or 22h of food availability for periods of 22 days. Six hours access to food led to a 6-10% loss of body mass over the first 8 days, with nearly all DEE supported by body nutrient stores during the first 2 days. Birds responded by increasing feeding behaviour and food intake but the response was slow. There were no gains of mass before day 15, suggesting a digestive bottleneck and a period of physiological adjustment. Food restricted birds exhibited a decrease in pectoral muscle thickness and BMR in association with a loss of body mass. Although a decrease in BMR saves energy, the savings represented only 2-7% of the daily energy spent in excess of that acquired during the deficit period. Knots did not downregulate mass-independent BMR. Based on recent independent findings and the pattern of mass gain when switched from 6h to 22h access to food, we suggests that these birds routinely maintain nutrient stores as a buffer against periods of energy shortages, precluding the need for downregulation of mass-independent BMR.

Submitted

INTRODUCTION

Animals facing energy challenges have to develop strategies to maximize survival, and some of these strategies may involve significant physiological transformations (Piersma and Drent 2003; Secor and Diamond 1998). Because pushing the upper limit of daily energy expenditure (DEE) may bear important fitness consequences (Drent and Daan 1980), ecological constraints can lead to differential allocation of resource and energy. Examples of differential allocation in animals are common and visible at multiple levels of integration. Masman et al. (1986) and Weathers and Sullivan (1993) suggested that energy reallocation may happen between demanding seasonal activities such as reproduction and wintering. Savings may also be achieved through behavioral adjustments, for example by decreasing locomotor activity to cope with the costs of molt (Robin et al. 1989), pregnancy and lactation (Butte et al. 2004; Poppitt et al. 1993; Speakman et al. 2001) or egg production (Ettinger and King 1980; Vézina et al. 2006). Within-individual energy reallocation can also happen passively with the heat produced as a byproduct of digestion (Bech and Praesteng 2004; Chappell et al. 1997; MacArthur and Campbell 1994; Rashotte et al. 1999) or locomotion (Bruinzeel and Piersma 1998) compensating part of thermoregulatory costs. Energy reallocation has also been recognized at the level of internal physiological systems (Wikelski and Ricklefs 2001) where, for example, changes in metabolic intensity (i.e. energy consumption per unit mass) of certain tissues may be opposite to changes in total mass of the organ from which they are part (Vézina and Williams 2005) or to changes in metabolic intensity and mass of other tissues (Selman and Evans 2005).

Recent studies of animals experimentally forced to increase work for food rewards have shown them to reallocate energy through down-regulation of night-time resting whole metabolic rate (Bautista et al. 1998; Deerenberg et al. 1998; Nudds and Bryant 2001; Vaanholt et al. 2007; Wiersma et al. 2005; Wiersma and Verhulst 2005) and/or mass-specific basal metabolic rate (BMR, Bautista et al. 1998; Deerenberg et al. 1998). We define BMR here as the energy consumption of a resting, post-absorptive animal measured at thermoneutrality during the inactive phase of the day. Wiersma et al. (2005) manipulated foraging costs in European starlings (*Sturnus vulgaris*) by forcing the birds to fly various distances for food reward. Individuals forced to work hard for their food exhibited a 43% increase in DEE accompanied by a 20% loss of body mass, including a significant reduction of pectoral muscle size. The authors demonstrated that these birds, experiencing an energy shortage, adjusted their energy budget to the experimental environment partly by downregulating nighttime resting metabolic rate and by attaining metabolic stability earlier in the night. They examined 6 hypotheses capable of explaining why birds were not increasing foraging efforts even more to maintain stable body mass. Their conclusion was that the physiological costs of increased work may take the form of a decreased capacity for other vital functions such as somatic self-repair and immune function. Other studies confirmed that both self-maintenance (Wiersma et al. 2004; Wiersma and Verhulst 2005) can indeed be compromised by intense foraging efforts or elevated DEE, thus reflecting another form of, perhaps more extreme, within-individual physiological trade-offs.

Whereas these experiments manipulated food availability through energy expenditure by increasing workload per food reward, in free-living conditions there may also be cases where food is temporarily unavailable for extended periods of time, independently of actual foraging effort. An obvious example is daytime foragers having to fast overnight (e.g. Lehikoinen 1987). Temporary food unavailability may also go beyond natural daily cycles. For example, ground foraging bird species wintering in northern latitudes may face temporary food unavailability during and after heavy snow falls (e.g. Doherty and Grubb 2002; Doherty and Grubb 2003). These ecological conditions can force animals to face negative energy balance for extended periods of time. In such cases, within-individual energy reallocation is a likely means to adjust DEE to restricted food, and downregulation of nighttime metabolic intensity may be part in this process (Graf et al. 1989; Ketterson and King 1977; Laurila et al. 2005; Shapiro and Weathers 1981).

Shorebirds are interesting in this context because species specialized on intertidal prey face time limitations in food availability on a daily basis (van Gils et al. 2005b; van Gils et al. 2006). Tides make food completely unavailable twice a day and, when facing bad weather, the birds may even endure several days of fasting (Zwarts et al. 1996b). The red knot (*Calidris canutus* L.), an intertidal molluscivore during the non-breeding season, is one of the well-known shorebird species routinely coping with such ecological constraints (Piersma 2002; Piersma 2007). Knots have been studied extensively in the context of their foraging ecology and long-distance migration (Piersma 2002; Piersma 2007) and demonstrate an extraordinary capacity to adjust phenotypic traits, including mass-independent metabolic rate, in response to demanding ecological conditions (Piersma et al. 2004). Thus, in the context of limited time access to food resource, red knots are an excellent model to study within individual resource and energy allocation strategies.

We subjected groups of red knots to two experimental regimes of food availability, 22h and 6h of food in excess, following an experimental design similar to Wiersma et al. (2005) where groups of birds experienced the two treatments in reverse order. Six hours of food availability roughly mimics food restriction of one natural tide cycle and is thought to represent a significant energy challenge for knots as pilot experiments showed that short term exposure to this treatment led to significant loss of body mass (M. Petit. and F. Vézina unpublished data).

This is the first part of a two-section experiment where we were specifically interested in within-individual energy allocation and trade-offs. To monitor these adjustments we studied, in a first step, individual variation in body mass, pectoral muscle thickness as an indicator of lean body mass, BMR and behavioral changes. We were particularly interested to find out whether red knots downregulate mass-independent BMR when facing food shortage. In a second step, we monitored the effects of changes in length of food availability on several parameters of constitutive and induced immunity (see companion paper by Buehler et al. submitted, chapter 7 of this thesis).

MATERIALS AND METHODS

Experimental animals

Twenty-four adult red knots (subspecies *C. c. islandica*) were used for this experiment (13 females, 11 males; PCR sexing, Baker et al. 1999). The birds were captured in the Wadden Sea in September 2006 and brought into captivity at the NIOZ experimental shorebird facility. Knots were maintained in indoor aviaries (4.5m x 1.5m x 2.3m, length x width x height) and experienced natural photoperiod as well as stable ambient temperatures of 12.7 ± 0.5˚C during the experiment. The cages were equipped with an artificial mudflat flooded with running sea water to allow the birds to probe the sediments. The floor of the cage was also flooded with running salt water to avoid health problems caused by dry feet. Red knots kept under these experimental conditions maintain their seasonal cycles of molt and fattening which remain in synchrony with those in free-living individuals (Piersma et al. 1995; Piersma et al. 2000a; Piersma 2002). The birds were fed in excess, with no time limitation during the period preceding the experiment, with a protein-rich trout food diet (*ad libitum* access; 45% protein, 8% fat, 12% fibers, 3% cellulose, 11% water) and had *ad libitum* access to fresh water. During the experiment, food was still provided in excess but for limited time periods as described below. The birds were maintained in four separate cages containing 6 individuals and were routinely checked (once a week) for health condition, molt score and weight. All birds were comparable in terms of structural body size (i.e. no difference between groups in principal component 1 reflecting variations in length of bill, total head, tarsus, and tarsus plus toe, ANOVA, $P = 0.9$, Rising and Somers 1989; Freeman and Jackson 1990; Senar and Pascual 1997). The experiment was carried out from mid January to the end of March 2007. Knots show stable body mass and plumage phenotypes during this period (Piersma et al. 2000a). The experiment was carried out under an Animal Experiment Committee permit (DEC; NIOZ.07.01).

Time restriction in food availability

We randomly divided the birds in two experimental groups composed each of 12 birds held in separate cages. We worked with two time limitations on food access; food was available either for 6 or 22 hours, therein called 6h and 22h treatments respectively. We removed food from the cages between 11:00h and 13:00h each day, providing a constant time cue for food reappearance. Birds exposed to the 22h treatments therefore had access to food from 13:00h to11:00h the following day. Birds exposed to the 6h treatment had their food taken away again at 17:00h and brought back at 9:00h the following morning.

Experimental sequence

Our respirometry setup allowed the measurement of two birds per day. Therefore, we stacked the measurements over time beginning the experiment one cage per day and performed all measurements per cage in relation to the cage-specific starting day. Consequently, birds from different cages experienced exactly the same time sequence of manipulation. During the 18 days before applying the time limitation treatments, we

Table 6.1. Schedule of measured variables within time block.

Day into time block	Variable measured in each cage
0	Block 1: Start of the food treatments Block 2: Inversion of the food treatments
2 and 3*	Behavioral observations, body mass on day 2
4	BMR on two birds
6 and 7	Food intake
8	Body mass and BMR on two birds
12	BMR on two birds
15–16*	Behavioral observations, body mass on day 15
18–19	Food intake
21	Body mass

* Behavioral observations in the morning, body mass in the afternoon.

measured all parameters to obtain baseline levels of our variables and to confirm that all birds were comparable in pre-experimental conditions. Values recorded during this measurement series will therefore be referred to as baseline levels. During baseline, food was available 24h hours per day.

The experiment was divided in two time bocks lasting 22 days and referred to as block 1 and block 2. We repeated the exact same measurement sequence in each time block with the only difference being that treatments were switched between experimental groups 7 days after the end of block 1, thus marking the beginning of block 2. Within each time block, we measured the different parameters according to the schedule described in Table 6.1 (see companion paper by Buehler et al. submitted, chapter 7 of this thesis, for a graphical representation of the experiment).

Basal metabolic rate

We measured BMR using the same equipment and technique as described by Piersma et al. (2004), Vézina et al. (2006) and Vézina et al. (2007). Briefly, on the day of BMR measurement, two birds were taken out of their cage at 11:00h and maintained in a plastic holding box (32 cm x 40 cm x 69 cm H x W x L) in a separate room. At 15:30h, fasted birds were weighed to the nearest 0.1g before being placed in a metabolic chamber for overnight BMR measurements, which began at 16:00h. During measurements, the birds were maintained in the dark at 25°C, a temperature within the zone of thermoneutrality (Piersma et al. 1995; Wiersma and Piersma 1994), and received a flow of dry air at 50 L/h. Measurements lasted until 09:00h the following morning. Birds were then weighed a second time and released in their cage. Reported body mass for BMR was calculated as an average of first and second mass measured. VO_2 and VCO_2 were calculated taking into account the presence of CO_2 in reference air as described in Piersma et al. (2004). We used the lowest 10 minutes of VO_2 measured as BMR with a sampling interval of 30 seconds. Average RQ over all the trials was 0.70 ± 0.004. Therefore, energy consumption was estimated using a constant equivalent for fat of

19.8 kJ/L O_2 and then converted to Watts (Gessaman and Nagy 1988; Piersma et al. 1995; Piersma et al. 1996; Piersma et al. 2004; Weber and Piersma 1996). Calculations were performed with Warthog Systems LABANALYST X (Riverside, CA, USA). O_2 and CO_2 analyzers were calibrated on a daily basis using span gases and testing the system by calculating VO_2 and VCO_2 from burning a known mass of pure alcohol in the chamber revealed that the system was accurate to 4% (F. Vézina unpublished data).

Measurement of muscle thickness

We measured the thickness of the pectoral muscles (pectoralis and supracoracoideus together) using an ultrasound scanner (model AQUILA, Pie Medical Benelux, Maastricht, The Netherlands) fitted with an 8 MHz linear probe and using ultrasonic gel to make contact with the animal skin. Measurements were made according to Dietz et al. (1999) and Lindström et al. (2000). All measures were performed blindly, with the observer also being unaware of the experimental treatment of specific birds. Pectoral muscle sizes are presented as muscle thickness (cm) measured from the skin to the sternum. Measurement trials with this apparatus and observer (AD) revealed high repeatability of the measurements (calculated according to Lessells and Boag 1987, $r = 0.97$). Dissection data showed that pectoral muscle thickness measured by this technique is also correlated with total lean dry body mass in this species ($r^2 = 0.40$, $n = 18$, $P < 0.01$, F. Vézina unpublished data).

Food intake

Because all individuals of a cage were feeding on the same food tray, we could only measure food intake for six individuals at a time. Food intake was calculated as the amount of food given minus the amount of food left the next day and then converted to units of dry matter. We measured dry matter content by taking three 30 g sub-samples of food every day and drying it to constant mass in an oven at 60°C. We measured food intake in series of two consecutive days, considering these as duplicate measurements for a given time point. This data was recorded twice per time block (Table 6.1) and we report food intake on a per day and per bird basis. Trout chow contains 8.25% water, 10.82% ash, has a digestibility of 0.509 and a caloric density of 22.63 kJ/g ash-free dry mass (J. Samuels, A. Dekinga, T. Piersma, unpublished data). We used these values to convert food intake to energy expenditure equivalents (see Discussion).

Behavioral observations

At specific time points during an experimental block (Table 6.1), we recorded four individual behaviors by scanning observations; feeding, resting, self care and locomotor activity. We define "feeding" as a bird either eating or drinking, "resting" as a bird standing immobile, with a leg up or with its beak tucked under the wing, "self care" as preening and bathing activities and "locomotor activity" as a bird walking or flying. Preliminary observation confirmed the findings of Reneerkens et al. (2002) that red knots under 6h of food availability exhibit very low levels of aggressive interactions (occasional). We therefore also included the few occurrences of aggressive behaviors in the category "locomotor activity". We conducted behavioral observations once a day for

an hour beginning at 09:00h, two days in a row and twice per experimental block (Table 6.1). During an observation period, each bird was scanned every two min. through a one-way window and had its specific behavior recorded according to our definitions by a single observer MP.

Statistical analysis

Data were analyzed by general linear mixed models, using repeated measures ANOVA for body mass, food intake and behaviors. We used the same approach for muscle thickness and BMR and added body mass as covariate to generate mass independent least square means (i.e. repeated measures ANCOVA). Because we inverted the food access treatments between groups at the experiment mid-point, we could not simply test for treatment effects by adding a variable "time treatment" in our models. Instead, we considered the effect of the time sequence for specific variables (i.e. each block has 4 body mass measures, 2 behavior measures, etc.) and its interaction with experimental group (group A experiencing 6h and then 22h of food availability and group B experiencing the reverse sequence). We then used post-hoc Tukey analysis to compare least square means within interaction and detect treatment effects (see figures). In all cases, we considered the random effect of social group unit (i.e. variable "cage") nested in "experimental group". Except for food intake, which was measured per cage, we also considered individual variation by including the random variable "bird ID" nested in "cage" and "experimental group". Food intake and behavioral data were always measured over two consecutive days. Potential differences between these replicates were considered and controlled for by including in our models the variables "sample number" nested in the "time sequence". For clarity we discuss these data as if both replicates were collected on the first of the two days. Normality of residuals was confirmed by visual inspection. All data are reported as mean ± SE.

RESULTS

Body mass

We found very clear patterns of body mass variation in relation to changes in time access to the food (Figure 6.1, Table 6.2). Body mass at baseline was significantly different between groups with birds of group A being on average 4% heavier than individuals forming group B. This difference was obviously not related to the treatment to come and body mass did not differ between groups at group formation (one-way ANOVA $F_{1,22}$ = 0.7, P = 0.4, data not shown). During the first experimental block, birds of group A, exposed to the 6h treatment, showed a rapid decline in body mass with a 6.9% and 10.4% loss in body mass relative to baseline level by day 2 and day 8 respectively. The birds then went into a recovery phase where, by day 21, body mass was at the same level as at the second day of the food restriction treatment (day 2 = 131.3 ± 1.1 g, day 21 = 132.2 ± 1.1 g), but still 6.2% lower than baseline level (Figure 6.1). Birds of group B showed no significant changes in body mass in the first experimental block, when exposed to the 22h treatment (Figure 6.1).

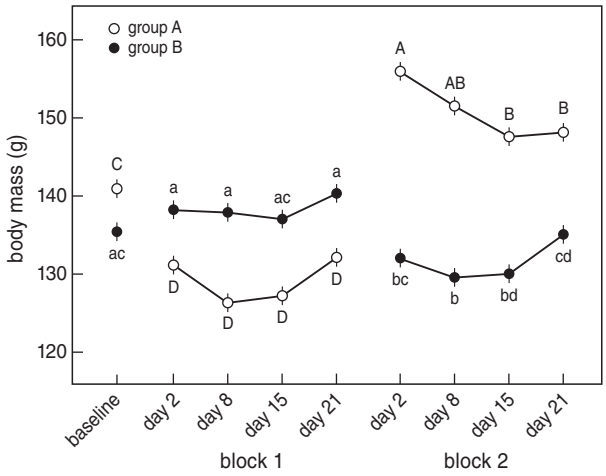

Figure 6.1. Body mass of red knots forming groups A and B measured during baseline and at four time points during experimental blocks 1 and 2. Letters indicate significant differences as tested by a post-hoc Tukey analysis. Although all comparisons were tested (see text), only differences within experimental groups are presented for clarity. Capital letters = post-hoc analysis within group A, lower case, post-hoc analysis within group B.

During the second experimental block however, birds of group B now exposed to 6h of food access showed a pattern of body mass loss and recovery very similar to the one exhibited by individuals of group A during the first experimental block (Figure 1). Indeed, comparing the two groups when exposed to the 6h treatment, post-hoc Tukey analysis revealed no significant differences between least square mean body masses at day 2 and 21 or day 8 and 15 across groups (analysis not shown in Figure 1 to avoid confusion). Therefore, birds of the two experimental groups showed comparable average body masses when under the food limitation treatment. However, because group B had a lower average body mass at the beginning of the experiment, this translated in a smaller body mass loss relative to baseline when compared to group A (-2.5% and -4.2% by day 2 and 8 of block 2 respectively). Compared to average body mass during block 1, mass loss in group B was -4.6 % at day 2 and -6.4% at day 8. By the end of the 6h treatment, body mass in birds of group B was back to baseline level but still 2.4% lower than average levels during block 1 (Figure 6.1).

Birds of group A, when switched from 6h to 22h of food availability, showed an impressive increase in body mass. Two days after inverting the treatments, least square mean body mass was 10.7% higher than baseline levels. This is a 23.5% and 18.1% increase in body mass relative to the lowest and last measure of the 6h treatment. Body mass in this group then gradually decreased and stabilized by day 15, but remained 4.9% higher than baseline body mass for this group (Figure 6.1).

Table 6.2. Mixed GLM analysis testing for effects of experimental conditions on body mass, muscle thickness, BMR and time at which BMR was found in the night

Independent variables	Body mass			Muscle thickness			BMR			Time of BMR		
	df	F	*P*	df	F	*P*	df	F	*P*	df	F	*P*
Cage (Group)	2, 20	0.6	0.6	2, 20.2	1.9	0.2	2, 20.7	0.5	0.6	2, 9.9	4.4	< 0.05
Bird id (Cage(Group))	20, 198	140.9	< 0.0001	18, 38	1.6	0.1	20, 43	9.7	< 0.0001	20, 43	0.7	0.8
Group	1, 2	1.3	0.4	1, 1.6	6.6	0.2	1, 2.2	0.007	0.9	1, 2.3	0.6	0.5
Time sequence	9, 198	25.9	< 0.0001	2, 38	3.5	< 0.05	2, 43	0.4	0.7	2, 43	0.8	0.5
Group x Time sequence	9, 198	78.5	< **0.0001**	2, 38	0.6	**0.5**	2, 43	0.3	**0.8**	2, 43	0.09	**0.9**
Body mass	-	-	-	1, 38	3	0.09	1, 43	21.9	< 0.0001	-	-	-

P values in bold are referred to in the text

Table 6.3. Mixed GLM analysis testing for effects of experimental conditions on food intake and various behavioral variables

Independent variables	Food intake			Feeding			Locomotor activity			Resting			Self care		
	df	F	*P*	df	F	*P*	df	F	*P*	df	F	*P*	df	F	*P*
Cage (Group)	2, 23	0.2	0.8	2, 20.2	1.1	0.4	2, 20.9	4.1	< 0.05	2, 20.7	1.5	0.2	2, 21.3	1.5	0.3
Bird id (Cage(Group))	-	-	-	22, 201	0.8	0.7	22, 201	1.3	0.2	22, 201	1.1	0.4	22, 201	2	< 0.01
Group	1, 2	0.8	0.5	1, 3.1	4.3	0.1	1, 1.9	0.3	0.6	1, 2.1	0.5	0.6	1, 1.4	5.6	0.2
Sample number (Time sequence)	5, 23	0.8	0.6	5, 201	0.4	0.8	5, 201	8.4	< 0.0001	5, 201	2.8	< 0.05	5, 201	1.5	0.2
Time sequence	4,23	7.3	< 0.001	4, 201	8.1	< 0.0001	4, 201	2.6	< 0.05	4, 201	20.2	< 0.0001	4, 201	1.2	0.3
Group x Time sequence	4,23	21.6	< **0.0001**	4, 201	39.1	< **0.0001**	4, 201	38.1	< **0.0001**	4, 201	17.9	< **0.0001**	4, 201	5.1	< **0.001**

P values in bold are referred to in the text

Food intake and feeding activity

Food intake did not differ between experimental groups during baseline, but changed in response to the food access treatments (Figure 6.2A, Table 6.3). Birds of group A, exposed to the 6h treatment in the first experimental block, exhibited a 24.9% reduction in food intake relative to baseline levels by day 6. Twelve days later, at day 18, the birds had adjusted to the food availability schedule and had increased food intake to an average level 12.2% above baseline (Figure 6.2A). Although this latter difference is not statistically significant, it nevertheless represents a significant 49.3% increase in food intake between day 6 and day 18. Interestingly, birds forming group B and exposed to the 22h hour treatment during the first block, exhibited a gradual but non-significant decline in food intake with the amount of food consumed being 3.7% and 18.2% lower than baseline levels by day 6 and 18 respectively. By the end of the experimental block, food consumption was statistically comparable to the daily amount consumed by birds on the 6h schedule at day 6 (Figure 6.2A).

When birds of group B were switched to the 6h treatment in the second experimental block, food intake decreased a further 13.9% by day 6 but this change was not significant (Figure 6.2A). As for group A, these birds then responded to the restriction in food access with a significant 44.7% increase in food consumption bringing them back a level not significantly distinguishable from baseline level by day 18. Switching the birds of group A to 22h hours of food availability during the second experimental block led to the exact same pattern of food consumption as the individuals of group B during the first experimental block. At day 6, food intake was statistically comparable to baseline level and declined, although not significantly, by 13.7% from day 6 to 18, making it statistically undistinguishable from food intake of birds in the early stages of the 6h food restriction.

Individual feeding activity somewhat mirrored the patterns found for food intake (Figure 6.2B, Table 6.3). During baseline, the two experimental groups were spending comparable amount of time in feeding activities. During block 1, however, birds of group A exposed to the 6h treatment increased the time spent feeding by a factor 5.4 by day 2. This was obviously not enough, given that food intake was still low by day 6 (Figure 2A). By the time we recorded their behavior again at day 15, individual feeding activity had increased 10 fold relative to baseline levels. Birds of group B, exposed to the 22h treatment during the first experimental block showed no significant changes in feeding activities relative to baseline. When switched to the 6h treatment, however, feeding activity increased by 6.5 and 9.2 fold by days 2 and 15 respectively compared to their activity level during block 1 (2.1 and 3.0 fold increase relative to baseline). In the meantime birds of group A, now exposed to the 22h treatment, had decreased their feeding activity down to levels statistically indistinguishable from their baseline conditions.

Locomotor activity, resting and self care

Locomotor activity exhibited by individual birds varied according to changes in food access (Figure 6.3A, Table 6.3). Groups A and B differed with regard to the time spent in locomotion during baseline. As supported by a significant cage effect on this variable

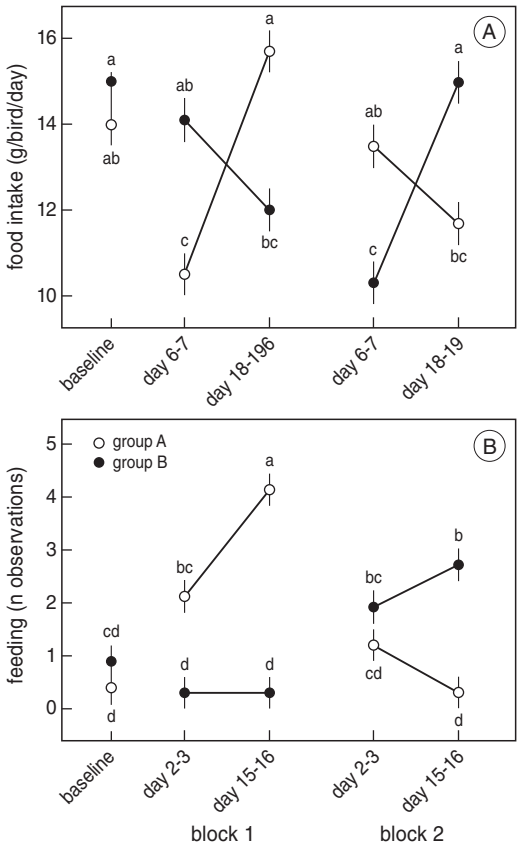

Figure 6.2. Variation in food intake (A) and feeding behavior (B) in red knots forming group A and group B measured during baseline and at two time points during experimental blocks 1 and 2. Letters indicate significant differences as tested by a post-hoc Tukey analysis. All comparisons are presented.

(Table 6.3), our observations indicated that this difference was mainly due to one social sub-group of 6 birds kept in one cage. This social sub-group, forming one half of group B, showed very high levels of locomotor activity during baseline. Indeed, restricting the analysis to the baseline period, one way ANOVA revealed a significant cage effect on locomotor activity ($F_{3,44} = 29.5$ $P < 0.0001$) with post-hoc Tukey analysis confirming that birds from all cages except one had comparable low levels of locomotor activity (not significantly different from group A at baseline, Figure 6.3A). The fourth sub-group, however, were 12.4 times more active than the average of the three other sub-groups at baseline. Nevertheless, the pattern of movement recorded during the experiment appeared independent from this difference between social sub-group, as removing the "active" cage data from the analysis did not affect the observed pattern (data not shown). We therefore kept all birds in the analysis but, given that 3 out of 4 cages showed comparable baseline levels of activity, we considered baseline level of group A as our comparative reference.

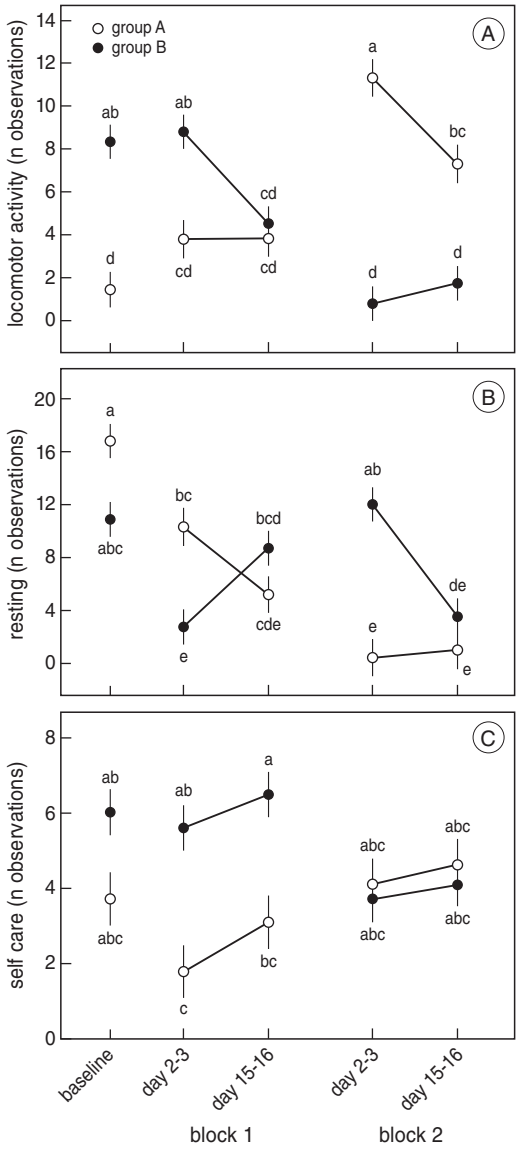

Figure 6.3. Variation in locomotor activity (A), resting (B) and self care (C) behaviors in red knots forming group A and group B measured during baseline and at two time points during experimental blocks 1 and 2. Letters indicate significant differences as tested by a post-hoc Tukey analysis. All comparisons are presented.

During block 1, birds of group A that were exposed to the 6h treatment exhibited no significant changes in locomotor activity relative to baseline levels (Figure 6.3A). Group B showed a significant 5.9 fold increase in locomotor activity by day 2 but, by day 15, activity was back to levels undistinguishable from baseline. These patterns

were reversed during block 2. Birds from group B, now exposed to 6h of food availability showed low levels of locomotor activity that did not differ significantly from baseline levels while birds from group A, having access to food 22h per day, exhibited a 7.5 fold increase in locomotor activity relative to baseline (2.9 fold relative to day 15 of block 1). By day 15, this latter group had decreased locomotor activity to levels statistically comparable to that recorded during the 6h treatment but nevertheless 4.9 times higher than baseline.

Resting behavior did not differ significantly between groups during baseline, but was affected by the food access treatments during the experiment (Table 6.3, Figure 6.3B). Interestingly, the two groups did not respond to the 6h and 22h treatments in the same way. During block 1, birds of group A that were exposed to 6h of food access showed a general decline in time spent resting with a 38.7% and 68.5% decrease relative to baseline by day 2 and 15 respectively. During block 2, these birds had access to food 22h a day but yet spent even less time resting. Indeed, resting behavior did not change within block 2 and was on average 96.2% lower than baseline level. Group B, exposed to 22h of access to food during the first experimental block exhibited an initial 75.2% decrease in time spent resting but then were back to baseline levels by day 15. During block 2, however, these birds showed the same response as the birds of group A when exposed to the 6h treatment. An initial level of resting comparable to baseline levels (day 2) and then a 67.9% decrease relative to pre-experimental conditions (day 15).

The time spent in self-care behavior did not differ between experimental groups during baseline (Figure 6.3C). Although birds showed a response to the food availability treatments (Table 6.3), within group post-hoc Tukey analysis showed that none of the changes were significantly different from their specific baseline starting point (Figure 6.3C). There was a clear tendency for a decrease in self-care behavior in both groups when food access was limited to 6h.

Pectoral muscles and BMR

Both whole pectoral muscle thickness and whole BMR varied within group according to the change in food availability (group x time sequence interaction: muscle $F_{2,39} = 17.8$ $P < 0.0001$, BMR $F_{2,44} = 17.5$ $P < 0.0001$). However, changes in these variables were linked to variation in body mass. Indeed, when including body mass as a covariate in the models, although its effect was at the margin of significance for muscle thickness ($P = 0.09$), the interaction term group x time sequence revealed to be non significant (Table 6.2, interaction term group x body mass and time sequence x body mass were not significant and are not included in Table 6.2). This indicates that the recorded variation in lean mass, as measured by pectoral muscle thickness, and the variation in BMR simply followed within-individual changes in total body mass and that mass independent values were not significantly affected by treatments. Therefore, birds exposed to 6h of food availability did not downregulate mass-independent BMR. We also tested whether food access treatments would result in birds reaching basal levels of metabolic rate at different times in the night. A non-significant interaction term "group x time sequence" showed that this was not the case ($P = 0.9$, Table 6.2). Therefore, under the 6h treatment, birds did not reach BMR earlier in the night.

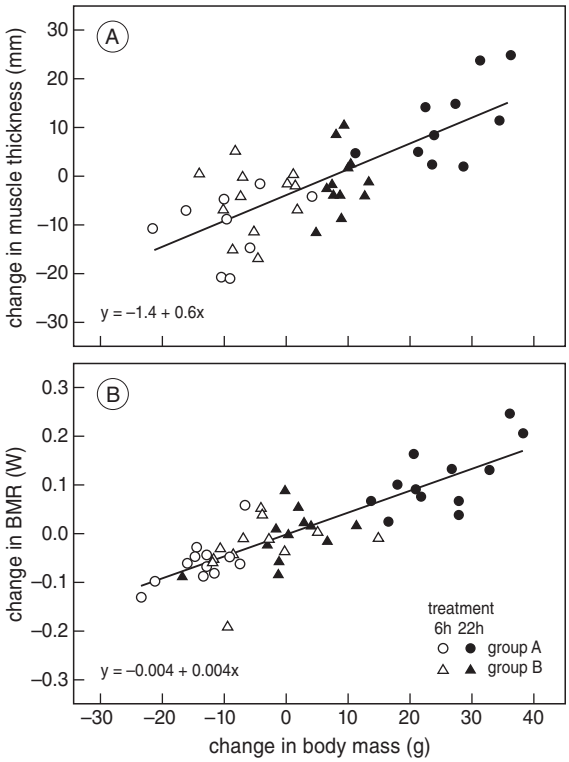

Figure 6.4. Relationships between the change in body mass and both the change in muscle thickness (A) and the change in BMR (B). Changes are calculated as the differences between values measured during baseline and block 1 as well as block 1 and block 2. All individuals are represented twice in the figure (see text for details). Circles = group A, squares = group B, open symbols = 6h treatment, closed symbols = 22h treatment.

We calculated the actual individual changes in body mass, pectoral muscle thickness, and BMR as the difference between baseline and block 1 and the difference between block 1 and block 2, therefore providing two time periods per individuals. Repeated measure ANCOVA, considering the effects of individual birds and social subgroups, showed a significant relationship between the change in body mass and both the change in pectoral muscle thickness ($F_{1,19} = 25.5$ $P < 0.0001$, Figure 6.4A) and BMR ($F_{1,22} = 40.3$ $P < 0.0001$, Figure 6.4B). There were no significant interaction terms "time period x body mass". Therefore, independent of the experimental sequence (22h to 6h or 6h to 22h), a given change in body mass resulted in the same variation in pectoral muscle thickness and BMR.

DISCUSSION

Energy challenge

Knots exposed to six hours of food availability underwent a clear decline in body mass over the first 8 days of treatment, direct evidence of negative energy balance. By two days into this treatment, time spent feeding had increased relative to pre-treatment conditions but energy intake at this point was still not enough to balance the energy budget and body mass kept declining for a further 6 days. At day 6 food intake was still 25-30% lower than at baseline. Only two weeks after we initiated the 6h treatment did the knots show a stable body mass, suggesting that mass stability had been attained between day 8 and 15 (Figure 6.1). By day 18, food intake and feeding activity had increased 169% from levels at day 2, allowing the birds to achieve a positive energy budget and gain body mass.

Loss of body mass reflects a negative energy balance because the animal has to metabolize endogenous nutrient stores to fuel energy requirements. We do not have DEE estimations for block 1 and block 2, but converting daily mass loss into energy units allows for calculating the daily energy spent in excess of that acquired during the day. As shown by the relationship between changes in pectoral muscle thickness and change in body mass (Figure 6.4A), mass variations in knots are not simply reflecting variations in fat content. Indeed, both lean and fat body components are changing with gain and loss of body mass in migratory shorebirds (Lindström and Piersma 1993). Using dissection data from indoor captive knots ranging in mass 95-150 g (F. Vézina unpublished data), we estimated the lean and fat content of our birds at baseline and at each weighing day into the 6h treatment by regression analysis (predicting lean mass by second degree polynomial regression, lean dry: $r^2 = 0.92$, n = 18, $P < 0.0001$, lean wet: $r^2 = 0.76$, n = 18, $P < 0.0001$). Using energy equivalents of 39.4 kJ/g for fat and 17.8 kJ/g for dry protein (Schmidt-Nielsen 1990), we then estimated the energy contained in the lean and fat components of mass loss and gain over the whole 6h treatment period.

Figure 6.5 presents the average energy spent in excess above daily energy assimilation early into the 6h treatment, and the average energy accumulated in body components later in the experimental session, when birds were recovering body mass. It becomes clear then that a major imbalance in the energy budget occurred during the first eight days into the 6h treatment. Assuming a stable daily energy budget during baseline conditions, food intake data suggests an average baseline DEE of 1.66 W for group A and 1.72 W for group and B. The average excess energy spent during the first two days of the 6h treatment was 1.78 W and 1.22 W for group A and B respectively, thus representing 107% of average baseline DEE for group A and 70% of average baseline DEE for group B. Although these values are only rough estimates, they indicate that nearly all daily energy expenditures during the early phase of the 6h treatment were fuelled by body nutrient stores.

The initial body mass loss in the 6h treatment could reflect a learning period necessary for the birds to assimilate the permanence of the new feeding conditions and change their feeding behavior. However, this hypothesis seems counter adaptive given the significant loss of body stores and the recorded increase in feeding activity by the

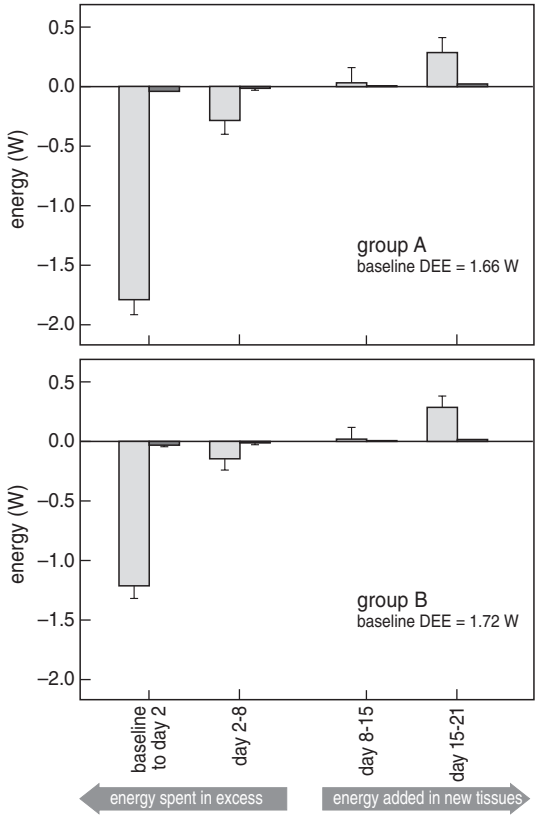

Figure 6.5. Estimated average energy spent in excess of that acquired or accumulated in new tissue on a daily basis during the 6h treatment for group A (light grey bars top) and group B (light grey bars bottom). Also shown by the dark grey bars is the estimated average change in BMR resulting from individual variation in body mass throughout the experimental time period. Excess energy expenditure and accumulation is calculated from lean and fat component of mass loss or gain estimated at each time point using equations from dissection data (F. Vézina and T. Piersma unpublished data). Individual variation in BMR was calculated from linear equations specific to baseline, block 1 and block 2. Baseline DEE was calculated from energy content of the food consumed and assumes a balanced energy budget during that period. See text for further details

second day into the treatment. Why then did the birds not balance their energy budget earlier? Part of the answer may come from an experiment performed by Bautista et al. (1998). They exposed starlings (*Sturnus vulgaris*) to experimental treatments differing in the amount of work to perform for food reward and found that birds having to work "hard" (flying about 5 times more distance per reward than individuals exposed to an "easy" treatment) extracted more energy from their food when exposed to this work regime. This paper thus provided a clear evidence of physiological adjustments of digestive functions under constrained energy budget. Similarly, knots are known for their digestive flexibility. Experiments have shown reversible changes in the size of certain digestive tract components (e.g. gizzard) when birds were switched between a soft

trout food diet, as used in the present experiment, and a natural winter diet of hard shelled blue mussels (*Metilus edulis*; Dekinga et al. 2001; Piersma et al. 2004). Van Gils et al. (2003), van Gils et al. (2005a) and van Gils et al. (2005b) further showed that, depending on shellfish quality, knots in the wild may suffer a digestive bottleneck due to the amount of shell to be processed per unit digestible content thus requiring flexible adjustments of digestive organs for the animals to meet their daily energy needs (van Gils 2003; van Gils 2005b). Previous studies showed that the reversible adjustment in knots' gizzards takes about 5-10 days (Dekinga et al. 2001). In starlings, stability in nutritional variables was attained on average after 17 days into the treatment (stability in the data was recorded the last 6 days of a 23 days experimental period, Bautista et al. 1998). In the present study, birds reached a stable body mass between day 8 and day 15 when exposed to the 6h treatment. Our finding could therefore reflect either or both an increase in digestive efficiency and adjustments in size of digestive organs such as the gizzard (both not measured) to accommodate more food to be processed per unit time.

A recent study by Reneerkens et al. (2002) also reported an initial loss of body mass in captive red knots feeding on trout chow for 6 hours per day. Although changes in mass were not the main focus of their study, the authors reported that birds had stabilized and regained body mass already within a week. This time difference between the experiments suggests flexibility in the adaptive process suggested above. Reneerkens et al. (2002) study was carried out on outdoor captive birds during wintertime. Therefore, their birds experienced a colder environment and higher thermostatic costs than individuals in our experiment, possibly increasing the energy deficit and inducing a faster response. Nevertheless, the pattern of body mass loss found by the two studies shows that, even with food in excess, the 6h treatment represented a significant energy challenge for red knots as would be expected from a transient lack of food resources in natural conditions.

Do knots downregulate mass independent BMR as an energy saving strategy?
Recent evidences suggests that animals submitted to experimentally increased daily energy demands for food rewards may compensate part of the extra energy expenditure through downregulation of whole or mass-specific basal or resting metabolic rate (Bautista et al. 1998; Deerenberg et al. 1998; Nudds and Bryant 2001; Tiebout 1991; Vaanholt et al. 2007; Wiersma et al. 2005; Wiersma and Verhulst 2005). Of course, a decrease in whole BMR may simply reflect a declining body mass, common to more than half of the studies to date (See Table 1 in Wiersma and Verhulst 2005) and also found in the present experiment. Such a reduction of BMR most likely reflects an overall loss of lean tissue, including metabolically active internal organs (e.g. Vaanholt et al. 2007). In contrast, a decrease in mass-independent BMR would reflect a downregulation of tissue metabolic intensity. In our study, individual changes in body mass were positively associated with changes in BMR, but we found no indications that birds under energetic stress decreased metabolic intensity. Mass-independent BMR was not related to treatment and birds having access to food six hours per day did not reach BMR earlier in the night.

Interestingly, studies that have highlighted a decrease in mass-independent metabolic rates in response to elevated work load almost all reported downregulated metabolism when measured at temperatures below thermoneutrality (Deerenberg et al. 1998; Nudds and Bryant 2001; Tiebout 1991; Vaanholt et al. 2007; Wiersma and Verhulst 2005, but see Bautista et al. 1998). In cases where metabolic rate of post absorptive animals were measured at thermoneutrality (i.e. BMR), correcting for body mass resulted in no significant effects of increased work load in two cases (Wiersma and Verhults 2005; Wiersma et al. 2005) and supported downregulation of metabolic intensity in one case (Bautista et al. 1998). Part of this discrepancy could be due to differences in statistical body mass correction techniques (mass-specific vs ANCOVA approach, Packard and Boardman 1988; Packard and Boardman 1999), but overall these results suggests that a decrease in metabolic intensity seems more frequent when the animals are measured under cold ambient temperatures. Perhaps, controlled hypothermia plays a significant role in this finding (e.g. Rashotte and Henderson 1988).

Despite our observations, recent evidence suggested that knots can also downregulate metabolic intensity under thermoneutral conditions. Piersma et al. (2004) shifted diet from trout chow to blue mussels and measured energy expenditure during the adaptive change in digestive organs. Birds expressed the typical increase in gizzard size together with an increase in overall lean and total body mass but showed a decline in whole BMR. Taken together, their and our results suggest that, although leading to an energetic deficit, our time restriction on food access may not have been perceived as a an energetic offence requiring downregulation of metabolic intensity (or downregulation of constitutive immune function, see also companion paper by Buehler et al. submitted, chapter 7 of this thesis).

Considering the findings discussed above, one could ask if the decrease in whole BMR resulting from the loss of body mass saves enough energy to compensate the excess requirements during food shortage, thus alleviating the need for a downregulation of metabolic intensity. To answer this question we estimated how much energy individual birds would save in terms of BMR reduction resulting from their individual changes in body mass during the 6h treatment. To do so, we calculated individual BMR from individual body mass at each weighing time (using the linear equation specific to baseline, block 1 and block 2) and calculated the differences from one period to another for each bird. As shown in Figure 6.5, the average energy economy due to the decrease in BMR is minimal relative to the average energy spent in excess for the two time periods where the birds exhibited negative energy balance (2.2% and 7.1% of excess energy expenditure from baseline to day 2 and day 2 to day 8 respectively for group A, 2.5% and 7.1% for the same time periods for group B). Therefore, although loss of body mass led to energy savings by reducing whole BMR, this economy was not enough to compensate the negative energy balance exhibited by the birds early in the treatment. Comparing measured BMR values for baseline, block 1 and block 2 revealed that average whole BMR decreased by 0.06 watts (-6.8%) between baseline and block 1 in group A and by 0.03 watts (-3.5%) between block 1 and block 2 in group B (calculating from baseline gives the same difference for this group). Assuming all compo-

nents of DEE are additive, this energy economy would only decrease estimated baseline DEE by 3.6% and 1.7% for group A and B respectively. Clearly, the recorded change in whole BMR did not contribute much in compensating the energy shortage in the early phase of the 6h treatment. If knots used an energy saving strategy to reduce the extent of the energy shortage, it was most likely part of the non-BMR component of DEE. This is somewhat supported by the decrease in non-feeding behavior found here and reported in other studies (see Table 1 in Wiersma and Verhulst 2005).

Storing a nutrient buffer after a crisis

One of the very interesting findings in this experiment was the impressive "overshoot" in body mass of birds forming group A during block 2 (Figure 1). These individuals had experienced time restriction in food availability resulting in energy imbalance and, now having access to food 22h per day, their body mass increased to a point 11% heavier on average than pre-experimental baseline conditions (18% above the end of block 1 body mass). This happened in 2 days, most likely helped by an improved digestive capacity. Body mass then declined throughout block 2 until it stabilized at a lower level that was still 5% above baseline. Therefore, not only birds regained their pre-experimental body mass, but they also accumulated and maintained additional body stores.

Pectoral muscles mass in knots tracks changes in total body mass (Lindström et al. 2000). In fact, Dietz et al. (2007) showed that variations in pectoral muscles of free-living knots are tightly coupled with body mass variation in such way that individuals below a certain average mass threshold (148 g) maintain an optimal pectoral muscle mass in order to keep constant flight capacity. Above this mass threshold however, the relationship between pectoral muscle mass and body mass has a shallower slope such that birds become relatively heavier per unit mass of flight muscles, therefore logically experiencing an increased relative flight cost. At 160 g average body mass, knots show signs of decreased maneuverability likely to impair their escape capacity (Dietz et al. 2007). Remarkably, in the present experiment, the increase in body mass in group A following the 6h treatment reached a maximal average mass of 156.1 ± 1.1 g, near but not above the maneuverability break point and then decreased to stabilize at average body mass 147.9 ± 1.1 g, the highest possible body mass before paying extra flight costs. Why then not let body mass decline to pre-experimental levels?

We suggest that knots depend on their evaluation of environmental "stability" and constantly maintain a certain amount of body stores to support energy needs in periods of high demand. Given that knots weighing less than 148 g on average show tightly adjusted pectoral muscles mass and constant flight capacities (Dietz et al. 2007), these birds have the option of carrying a certain amount of body stores that can be used as a buffer against periods of energy shortage. In the present study, birds from the two experimental groups consumed 8-14 g (8-10%) of their initial body stores before reaching a stable or positive energy budget. Although these birds clearly went trough an initial period of energy deficit, they did not decrease metabolic intensity as an energy saving mechanism. It is thus possible that the mass loss recorded under the 6h treatment was in fact reflecting the energy required by the birds to adjust their digestive phenotype to increase energy intake within a shorter daily time period.

This nutrient buffer would likely be adjusted to the nature of the immediate environment such as food availability, quality and predictability. In the present case, shortly after a period of deficit, our birds increased their body mass near to the point where maneuverability problems become apparent. Although being that heavy provides a large "nutritional buffer" it is not cost free in terms of movement. Therefore, as time provided reinsurance of condition stability (i.e. no change in food availability during block 2), body mass declined over the next 15 days and stabilized below the flight cost threshold. Prolongation of the experimental period would probably have resulted in the birds eventually reaching baseline level of body mass. Understanding the fine-tuning of this response necessitate further study.

That birds maintain energy stores to face periods of energy shortage is of course not new, and is very well known in the wintering passerine literature (e.g. Lehikoinen 1987). However, in small species it is understood that winter variations in body mass are mostly reflecting accumulation and use of fat stores (Blem 1976; Blem 1990; King 1972; Lehikoinen 1987; Merom et al. 2005). In the present case, birds used both fat and lean component of body nutrient stores and data on captive and free-living shorebirds, including knots, is consistent with the bodily buffer hypothesis. Knots experimentally acclimated to cold winter-like conditions (4°C) maintain a body mass about 16 g (13%) heavier than birds maintained in thermoneutral conditions (25°C, Vézina et al. 2006), a difference comparable to winter and summer body mass of knots captured in the UK (Figure 33 in Piersma 1994). Similarly, dunlins (*Calidris alpina*) wintering in northern, colder, estuaries of the UK are heavier both in terms of total and lean body mass than individuals spending their winters in estuaries south of the country (Davidson 1986a; Davidson 1986b). Captive dunlins also exhibit short-term adaptive variations in body mass in response to experimental fluctuations in ambient temperature, wind and rain (Kelly et al. 2002). Furthermore, recent evidence suggests that the phenomenon in red knots would also take place during long distance spring migration to the high Arctic breeding grounds. Morrison et al. (2007) showed that the heaviest knots on departure from their Iceland stopover sites, those that have accumulated the largest stores, are more likely to survive stormy weather on arrival in the Arctic. Thus, the amount of stores accumulated during the fuelling period would not only have the purpose of supporting flight and maintenance costs during the migratory journey but also of supporting the immediate needs on arrival. This later finding is consistent with data of Morrison et al. (2005) showing that part of body stores in knots are probably used to support internal organ regrowth the first two weeks after arrival on the High Arctic breeding grounds.

ACKNOWLEDGEMENTS

We are grateful to members of the shorebird and benthos group of the Marine Ecology and Evolution department at the NIOZ for useful comments on the data presented in this paper. We also thank Bernard Spaans for catching the birds, Anne Dekinga and Maarten Brugge for help taking care of the captive knots, and J. Reneerkens constructive discussions about effects of food limitation in knots. This research was supported by an NIOZ operating grant to T.P. and a post-doctoral VENI grant from the Netherlands Organization for Scientific Research (NWO) to F.V.

CHAPTER 7

Limited access to food and physiological trade-offs in a long-distance migrant shorebird part II: constitutive immune function and the acute phase response

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ABSTRACT

In response to unbalanced energy budgets, animals must allocate resources among competing physiological systems to maximize fitness. Constraints can be imposed on energy availability or energy expenditure, and adjustments can be made via changes in metabolism or trade-offs with competing demands such as body mass maintenance and immune function. This study investigates changes in constitutive immune function and the acute phase response in shorebirds (red knots) faced with limited time access to food. We separated birds into two experimental groups receiving either 6h or 22h food access and measured constitutive immune function. After 3 weeks we induced an acute phase response and, after a week of recovery, switched the groups to opposite food treatments and measured constitutive immune function again. We found little effect of food treatment on constitutive immune function, suggesting that even under resource limitation a baseline level of immune function is maintained. However, birds enduring limited access to food suppressed aspects of the acute phase response (decreased feeding and mass loss) to maintain energy intake, and down-regulated thermoregulatory adjustments to food treatment to maintain body temperature during simulated infection. Thus, under resource limited conditions, birds save energy on the most costly aspects of immune defense.

Submitted

INTRODUCTION

In situations of energetic constraint, where energy availability is limited or energy expenditure is increased, animals must balance demands on competing physiological systems by allocating resources to maximize fitness (King 1974; Willmer et al. 2000). In response to changing energy balance, animals can reallocate resources via metabolic adjustments such as down-regulation of night-time metabolic rate (e.g. Bautista et al. 1998; Deerenberg et al. 1998; Wiersma et al. 2005), or via trade-offs with other vital functions such as somatic self repair (Wiersma and Verhulst 2005) or immune function (e.g. Sheldon and Verhulst 1996; Verhulst et al. 2005).

The immune system is important for survival, but maintaining and using that system carries energetic and immunopathological costs (Klasing 2004). Because of this combination of importance and cost, trade-offs between immune function and other costly activities (locomotion, reproduction, thermoregulation etc.) have been predicted during times of energy constraint (Norris and Evans 2000; Sheldon and Verhulst 1996). Immune function can be thought of in terms of maintenance (constitutive immunity) and use (induced immunity), and can be further divided into innate (non-specific) and acquired (specific) branches (Schmid-Hempel and Ebert 2003). Aspects of induced-innate immunity such as inflammation and fever during the acute phase response are considered particularly costly (Klasing 2004). Previous studies on the effect of energy constraint on immune function have focused on induced acquired immunity. For example, in chickens enduring 40% of *ad libitum* food restriction lymphocyte proliferation was suppressed (Hangalapura et al. 2005), in yellow-legged gulls, *Larus cachinnans* enduring 33% of *ad libitum* food restriction wing web swelling to phytohaemagglutinin (PHA) was decreased (Alonso-Alvarez and Tella 2001), and in deer mice *Peromyscus maniculatus* enduring 70% of *ad libitum* food restriction secondary antibody production was decreased (Martin et al. 2007c). Data are now needed for the effects of energy constraint on both constitutive and induced immunity to gain insight into the energetic challenges posed by immune maintenance and use.

Previous studies examining adjustments to energy constraint manipulated energy balance by restricting food consumption to some percentage of *ad libitum* or by increasing foraging effort (e.g. Bautista et al. 1998; Wiersma et al. 2005). However, in the wild food may become temporarily unavailable, independent of overall quantity or foraging effort. For example, in red knots *Calidris canutus*, a shorebird species specializing on intertidal prey, birds face daily time limitations on food availability due to tides and wind conditions (van Gils et al., 2006). Furthermore, during migration they encounter feast and famine conditions, and the *C. c. islandica* subspecies, which winters in the northern hemisphere, may face days of fasting if estuaries freeze (Buehler and Piersma 2008; Piersma 2007). In a previous experiment on red knots, Buehler et al. (2008a) manipulated energy expenditure to study immune function trade-offs. They found little effect of increased energy expenditure on constitutive immune function and suggested that *ad libitum* food access may have allowed birds to maintain baseline levels of immunity even when living at winter-like ambient temperatures (Vézina et al. 2006; Buehler et al. 2008a). Here we limit time access to food and examine the other side of the energy budget equation - energy availability.

Because single assays of immune function are difficult to interpret and different immune indices are often uncorrelated (Adamo 2004; Matson et al. 2006a), we examine multiple indices of constitutive and induced immunity and perform principle component analyses (PCA) to examine relationships between the indices. We quantify constitutive immunity by measuring microbial killing ability, leukocyte concentrations, levels of complement and natural antibodies, and baseline haptoglobin. To examine induced immunity we mimic bacterial infection with lipopolysaccharide (LPS) to induce an acute phase response. We focus on the acute phase response as an index of induced immune function and examine it in the context of limited food access. However, in the context of constitutive immune function, LPS injection can also be seen as a treatment, thus we perform our constitutive immune assays before and after LPS injection to examine the effect of LPS on constitutive immune indices from a mechanistic standpoint.

This study is the second of a two-section experiment examining physiological trade-offs in red knots faced with limited time access to food. In the first section, metabolic and behavioral adjustments are examined (Vézina et al. submitted) and here we examine the effects of limited time access to food on constitutive immune function (lower cost immune maintenance) and on the acute phase response (higher cost immune use). We separate birds into two experimental groups receiving either 6h or 22h of *ad libitum* food access and measure constitutive immune function. After 3 weeks of treatment we inject LPS to induce an acute phase response and, after a week of recovery, we switch the groups to opposite food treatments and measure constitutive immune function again. If immune maintenance is affected by food availability then we predict a decrease in constitutive immune function in the 6h food treatment that will be reversed when the birds return to 22h food access. If immune use is affected by food availability then we predict a decrease in acute phase response symptoms in birds in the 6h food treatment during LPS injection.

MATERIALS AND METHODS

Animals

Red knots of the subspecies *C. c. islandica* were captured with mist-nets in the Dutch Wadden Sea (53°31'N 6°23'E) in September 2006 and were brought into captivity at the Royal Netherlands Institute for Sea Research (NIOZ). At capture the birds were ringed, weighed, and aged as older than two years (Prater et al. 1977). Sexes were later determined using molecular sexing (13 females, 11 males; Baker et al. 1999). The birds were housed in indoor aviaries (1.5m x 4.5m x 2.3m) with a quarter of the aviary floor covered by an artificial sand flat flushed by salt water and a tray of fresh water for drinking and bathing.

The experiment took place from mid January until the end of March 2007, during a period of stable body mass and when the birds were not molting (Jenni-Eiermann et al. 2002; Buehler et al. 2008a). Throughout the experiment, photoperiod was similar for all birds and followed the natural cycle of the northern Netherlands. Ambient temper-

ature was held constant at 12.7°C ± 0.5°C (room temperature, ca. 8°C under the lower critical temperature; Wiersma and Piersma 1994). Bird handling and all experimental protocols were carried out under the auspices of the Animal Experiment Committee of the Royal Netherlands Academy of Sciences (KNAW DEC; protocol NIOZ.07.01).

Experimental design

Birds (n = 24) were divided into experimental and control groups (n = 12) balanced for sex and with two replicates (n = 6) to control for social group effects. All birds were comparable in terms of structural body size (i.e. no difference between groups in principal component 1 reflecting variations in length of bill, total head, tarsus and tarsus plus toe ANOVA $P = 0.9$; Vézina et al. submitted). Although all birds were healthy at baseline (day 0), over the course of the experiment a total of five birds showed signs of illness or infection. Four birds developed inflammation of the wings or feet and one bird showed an elevated white blood cell count. These birds were left in their cages to keep group sizes consistent; however, their immune measures were not included in the dataset (leaving n = 19, 9 females, 10 males).

We manipulated access to food following (Reneerkens et al. 2007) using a predictable feeding regime (i.e. food was available at the same times everyday). Birds had access to food (Trouvit trout food pellets, Vervins, France) either 6h a day (9:00 to 11:00 and 13:00 to 17:00) or 22h a day (22h versus 24h of food did not result in significant changes in body mass (Vézina et al. submitted) or constitutive immunity (D. M. Buehler, unpublished data). To minimize differences in digestive state (fed or fasted) during blood sampling, food was removed from all cages at 11:00 (1h before blood sampling) and was left out until 13:00 to allow time for cage cleaning.

The experiment was divided into sections (baseline, block 1, LPS and recovery, and block 2; Figure 7.1). During block 1, group A experienced the 6h food treatment and group B the 22h food treatment. The treatments remained the same during the induction of the acute phase response and a week of recovery (LPS and recovery). We then switched the groups following a design similar to (Wiersma et al. 2005) such that group A returned to 22h food access and group B experienced the 6h food treatment (block 2). Blood samples were taken 6 times over the experiment: at baseline, day 2, day 15 (2 weeks after treatment began), day 22 (after LPS injection), day 29 (2 days after food treatment switch) and day 42 (2 weeks after switch).

Blood Sampling

All blood samples were taken at 12:00, within 12 min of entering an aviary (mean ± SD = 5.4 ± 2.9) and were spaced at least one week apart to minimize carry-over effects. A previous experiment on captive red knots has shown that samples taken within 30 min of entering an aviary reflect baseline values for microbial killing, leukocyte concentrations, and levels of complement and natural antibodies (Buehler et al. 2008c). Similarly, correlative data on haptoglobin concentrations show that samples taken within 25 minutes of entering an aviary reflect baseline values (D. M. Buehler, unpublished data). At sampling we thoroughly sterilized the area around the brachial vein with 70% ethanol and then collected about 400μl of blood into pre-sterilized, heparinized capillary

tubes (capillary tubes were individually packaged and sterilized under UV light). Immediately after sampling, we made blood smears and transported the remaining blood in sterile boxes to the laboratory for processing within an hour of sampling.

Hematocrit

We measured hematocrit by centrifuging 25μl of blood in a capillary tube for 12 min at 12000 x g and reading the relative proportion of red blood cells to total volume.

Measuring immune function

Constitutive Immunity

Microbial killing abilities

The microbial killing assay measures the functional capacity to limit microbial infection (Millet et al. 2007; Tieleman et al. 2005). We followed the basic procedure outlined in Millet et al. (2007) and performed the assay in a sterile working environment (a dead air box equipped with a UV Air Cleaner, Base Clear BV, KI-L046-M). To gain a broad understanding of microbial killing we use three microorganisms: *Escherichia coli*, a gram negative strain of bacteria killed mainly by soluble blood components (Merchant et al. 2003; Millet et al. 2007); *Candida albicans*, a yeast-like fungus; and *Staphylococcus aureus*, a gram positive strain of bacteria killed mainly by cellular blood components (Millet et al. 2007). For each micro-organism we diluted the blood in CO_2-independent media (#18045-054, Invitrogen) to a volume of 200 μl and added 20 μl of microorganism suspension reconstituted from lyophilized pellets (*E. coli* ATCC # 8739, *C. albicans* ATCC #10231,*S. aureus* ATCC # 6538: MicroBioLogics, St Cloud, MN). The dilution was optimized to a concentration of approximately 200 colony forming units per 75 μl of diluted blood–bacteria mixture. We incubated the mixture at 41°C (*E. coli*: 10 min, *C. albicans*: 60 min, *S. aureus*: 120 min) and spread 75μl onto agar plates in duplicate. We then stored plates upside down at 36°C and counted the number of colonies per plate the following day. We quantified the number of microorganisms in the inoculation with control plates (200 μl of media and 20 μl of bacteria-suspension only, plated immediately without incubation) and calculated microbial killing capacity as one minus the experimental plate colony count divided by the control plate colony count.

Circulating cellular immunity

Leukocyte concentrations provide information on circulating immune cells and can be used as an indicator of health (Campbell 1995). Blood smears were stained (Giemsa Stain, Sigma-Aldrich, Germany), randomized and counted blind to treatment by a single researcher (FEV) at 1000X magnification with oil immersion. The first 100 leukocytes seen were classified as heterophils, eosinophils, lymphocytes or monocytes following Campbell (1995). Basophils were extremely rare (< 0.5%) and were therefore not included in the counts. While counting the first 100 leukocytes, thrombocytes were also recorded as an estimate of the relative number of thrombocytes per leukocyte. In combination with the blood smears, we obtained leukocyte concentrations using the indirect eosinophil Unopette method (Campbell 1995) following the manufacturers instructions (No. 5877; Becton Dickinson).

Complement and natural antibodies

Complement and natural antibodies provide a first line of defense against spreading infections via cell lysis, and link innate and acquired immunity (Ochsenbein and Zinkernagel 2000). We performed the assay as described by Matson et al. (2005). We pipetted 25 μl of plasma into the first and second rows of 96-well plates. Using Dulbecco's PBS (Mauck et al. 2005), we serially diluted the plasma from row two to row 11 and left the 12th row as a negative control (PBS only). We then added 25 μl of 1% of rabbit red blood cell suspension to all wells and incubated the plates at 37°C for 90·min. After incubation, plates were tilted at a 45° angle and were scanned (Epson Perfection 4990 scanner) for agglutination after 20 minutes and lysis after 90 minutes. The scans were randomized with respect to sample origin, plate, and location within the plate and were scored blindly for lysis and agglutination by a single observer (DMB) using the criteria outlined in Matson et al. (2005).

Haptoglobin

Haptoglobin is an acute phase protein that binds iron (haem) to keep it from providing nutrients to pathogens and offers protection against harmful end products of the immune response (Delers et al. 1988). Haptoglobin was quantified in mg/ml following the 'manual method' instructions provided with a commercially available assay kit (#TP801; Tri-Delta Diagnostics, Inc., Morris Plains, NJ). We pipetted 7.5 μl of plasma into rows 2 to 11 and 7.5 μl of prepared calibrators (0-2mg/ml) into rows 1 and 12 of 96-well plates. We then added 100 μl of diluted haemoglobin (Reagent 1), followed by 140 μl of chromogen/substrate solution (Reagent 2) to each microwell using a multi-channel pipette. We incubated the plates for 5 minutes at room temperature and then read absorbance at 630 nm using a microplate reader (Molecular Devices Spectra Max 340). To facilitate calculation of haptoglobin concentration in the plasma, we generated a calibration curve by plotting absorbance (630 nm) versus haptoglobin concentration (mg/ml) in the calibrators.

Acute Phase Response

The acute phase response is associated with changes in body temperature (hyperthermia in larger birds or hypothermia in small passerines), the secretion of acute phase proteins from the liver, and sickness behaviors including reduced food intake, body mass loss and reduced activity (Owen-Ashley and Wingfield 2007). We induced an acute phase response after the birds had been in their treatments for 3 weeks (Figure 7.1). We subdivided the 6h and 22h treatments into injected and uninjected groups, balanced for sex (each cage had 3 injected and 3 uninjected birds). Injected birds received 500 μl of 0.25mg/ml LPS in saline (Sigma L 7261, source strain *Salmonella typhimurium* ATCC 7823) intra-peritoneally for a dosage of 1mg/kg. This dosage was the same as those used to elicit responses in Japanese quail, *Coturnix coturnix japonica* (Koutsos and Klasing 2001) and pigeons, *Columba livia* (K. Matson, pers. comm. 2006). Uninjected birds received the same handling procedure as injected birds, but the skin on their abdomen was not broken (Koutsos and Klasing 2001).

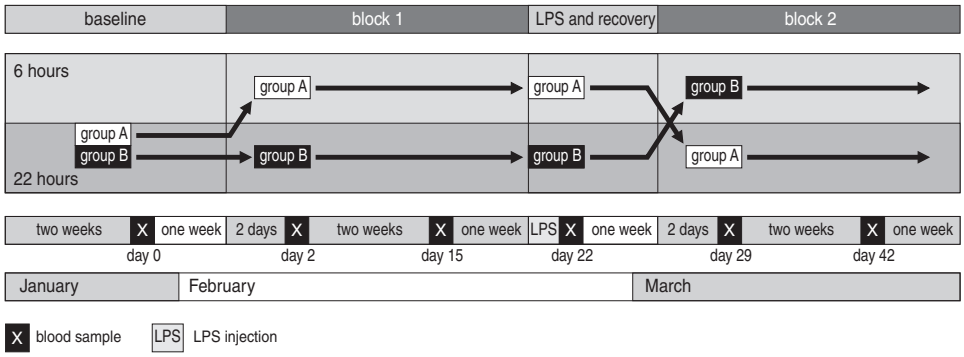

Figure 7.1. The experimental set-up. We separated birds into two experimental groups receiving either 6h or 22h of food *ad libitum* and measured immune function 2 and 15 days after the treatment began (block 1). After 3 weeks we induced the acute phase response with lipopolysaccharide (LPS) and measured immune function (day 22). We then switched the food treatments and measured immune function 2 days and 2 weeks after the switch (days 29 and 42; block 2) after the switch. Group A (n = 8) experienced 6h then 22h food and group B (n = 11) 22h then 6h food.

We measured body mass and body temperature (cloacal insertion of a high accuracy thermocouple, Omega 450 ATT, calibrated against a certified mercury thermometer) at injection and 5h and 17h after injection. We also took blood samples to examine changes in constitutive immunity one week before (day 15) and 17 hours after injection (day 22). The timing of immune sampling followed that used in Matson et al. (2005) and Millet et al. (2007). In order to keep blood-sampling times consistent with the rest of the experiment, LPS injections took place at 19:00 in the evening. To monitor sickness behavior, we took 30min videos of each cage at 9:30 the day of injection and again at 9:30 the day after injection. The timing of these observations (10h before and 14h after injection) is similar to that used by Bonneaud et al. (2003). Video cameras were placed outside the cages and were focused through a one-way mirror so that they did not interfere with the birds' activity. Before the experiment began, we individually marked each bird with color rings to allow individual observations, and all observations were made by a single researcher (FEV). Behaviors were scored as feeding (eating and drinking), activity (flying and walking), and self-care (preening and bathing). Time budgets were obtained by scoring the number of seconds the birds performed the behaviors during the observation period, and any time not feeding, active or self-caring was classified as rest.

Statistics

Before performing statistical comparisons, we used 1-sample Kolmogorov–Smirnov tests and visual examination of histograms to test for normality. Leukocyte and haptoglobin data were log10 transformed (due to 32% zero values eosinophils were excluded from further analysis), hemolysis was squared, and hemagglutination was square-root transformed. After transformation, all variables and the residuals of the models were normally distributed.

To examine treatment effects over the course of the experiment, while taking into account the fact that we inverted the food access treatment at the midpoint of the experiment, we performed a general linear model with group and day as main effects and social group (cage) and bird as random effects. The group variable took into account the sequence of the treatments (i.e. group A at 6h in block 1 and 22h in block 2, and group B at 22h in block 1 and 6h in block 2, see Figure 7.1) and in all cases we considered the effect of social group as cage nested in group. We first looked for significant group by day interactions, which indicated that the groups responded differently under the two food treatments. We then used Tukey post-hoc tests to determine the significance of treatment effects and to examine the timing and reversibility of within-individual effects. For all analyses, we included LPS (injected or uninjected) as a covariate to take into account any carry-over effects from the LPS injections in block 2.

To examine whether food treatment affected the LPS-induced acute phase response, we tested the treatment by LPS interaction in a general linear model with food treatment and LPS (injected and uninjected) as main effects and cage as a random effect. We used the same model to examine the effect of LPS injection on constitutive immunity, temperature, body mass and behavior by testing for an LPS effect after the LPS treatment.

To gain insight into relationships between measures of constitutive immunity, we performed principle component analyses (PCA). Total leukocyte concentrations were excluded (since they are the sum of the differential concentrations) and we used transformed data for this analysis (both transformed and untransformed produce the same result). To take into account the repeated measures structure of the data, we used an among-bird correlation matrix following the procedure described in Matson et al. (2006a). We used varimax rotation to maximize the contrasts of the variable loadings, tested the saliency criteria for these loadings (Cliff and Hamburger 1967), and saved scores for components with eigenvalues > 1 for further analysis (Kaiser 1960). We used SPSS v 14.0 (2005) or JMP v 5 (2002) for all statistical comparisons, but created the correlation matrix for the PCA using STATISTICA 7 (2004).

RESULTS

Effects of food treatment

Body mass and hematocrit

Our food treatments clearly affected body mass (group by day interaction $F_{4,67} = 95.3$, $P < 0.001$; see Figure 1 and Table 2 in Vézina et al. submitted for a week-by-week analysis). During block 1, birds in group A showed a rapid decline in body mass after exposure to the 6h treatment, whereas birds in group B showed no significant change (Figure 6.1 in Vézina et al. submitted). Furthermore, during block 2, birds in group B, now experiencing the 6h food treatment, showed a pattern of body mass loss very similar to birds in group A during block 1 (Figure 1 in Vézina et al. submitted). This pattern indicates that our 6h treatment produced a negative energy balance (Vézina et al. submitted) and changes in hematocrit further demonstrate this point (group by day interaction $F_{4,67} = 5.71$, $P = 0.001$). During block 1, hematocrit in birds in group A

(6h treatment) showed a significant decrease by day 15, whereas hematocrit in birds in group B (22h treatment) did not differ from baseline (Figure 7.2A). During block 2, birds in group B (6h treatment) showed a significant decrease in hematocrit by day 29, whereas hematocrit in birds in group A (22h treatment) returned to baseline levels (Figure 7.2A).

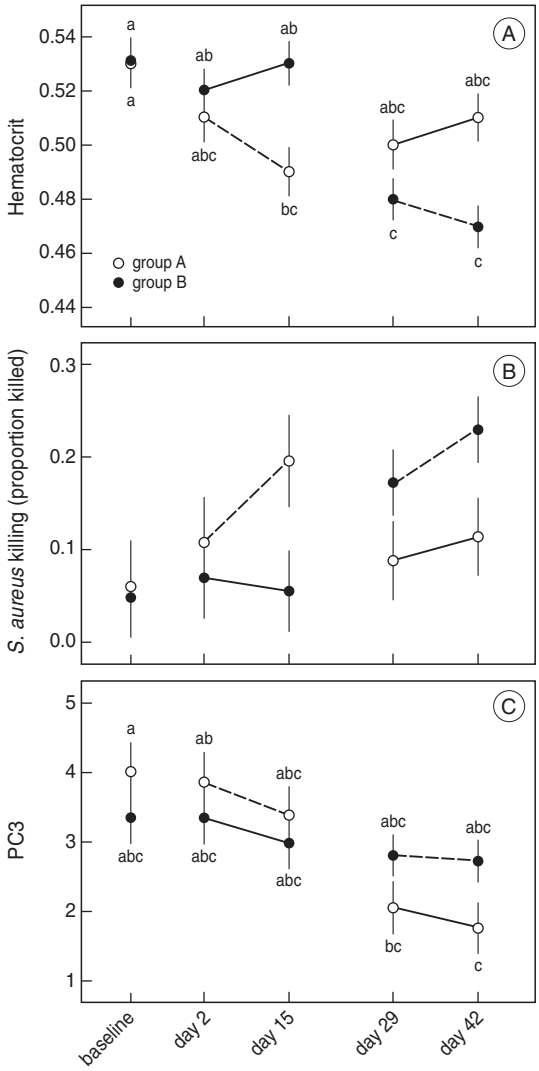

Figure 7.2. Trends over the whole experiment in variables with statistically significant group by day interactions: (A) hematocrit (group*day: $F_{4,67} = 5.71$, $P = 0.001$), (B) *S. aureus* killing (group*day: $F_{4,67} = 3.42$, $P = 0.01$) and (C) PC3 (group*day: $F_{4,66} = 2.66$, $P = 0.04$). Symbols represent least squared means and bars ± one SE. Different letters indicate significant Tukey post-hoc tests for between group and within-bird differences. Where no letters appear no significant post-hoc differences were found. Group A (n = 8, open circles) and group B (n = 11, closed circles). Dashed lines indicate 6h food access and solid lines 22h food access.

Table 7.1. Principal component loadings after varimax rotation. Bold faced loadings are the highest loading for a measure across the PCs and underlined loadings meet the saliency criteria for that PC.

Response	PC1	PC2	PC3	PC4
S. aureus (prop. killed)	**0.922**	0.108	-0.033	-0.237
C. albicans (prop. killed)	**0.802**	-0.086	0.111	0.016
E. coli (prop. killed)	0.471	**0.793**	-0.188	-0.099
Heterophils (per μl)	**0.702**	0.088	-0.057	0.238
Lymphocytes (per μl)	-0.096	0.124	**0.862**	0.286
Monocytes (per μl)	0.115	-0.163	**0.904**	-0.127
Thrombocytes (per μl)	0.248	0.317	0.130	**0.650**
Hemolysis (log2)	0.007	**0.864**	-0.159	0.219
Hemagglutination (log2)	-0.151	**0.799**	0.234	-0.170
Haptoglobin (mg/ml)	-0.140	-0.221	0.011	**0.776**
Totals				
Variance (%) per component	23.3	22.3	17.1	13.2
Cumulative variance (%)	23.3	45.6	62.7	75.9

Constitutive immunity

Principal component analysis identified four PCs with eigenvalues > 1 that cumulatively accounted for 75.9% of the total variation in constitutive immunity (Table 7.1). Loadings on these PCs showed that *S. aureus* killing, *C. albicans* killing, and heterophils correlated with PC1 (23.3% of total variation), *E. coli* killing, hemolysis and hemagglutination correlated with PC2 (22.3% of total variation), lymphocytes and monocytes correlated with PC3 (17.1% of total variation), and thrombocytes and haptoglobin correlated with PC4 (13.2% of total variation).

During the baseline period, groups A and B, and social groups (cages) did not differ significantly with respect to microbial killing, complement, natural antibodies or hematocrit (all *P*'s > 0.17) confirming homogeneity of group composition. However, upon analyzing the blood smears (after the experiment was completed) we found that group A had significantly higher lymphocytes concentrations ($F_{1,2} = 16.7$, $P = 0.04$) and marginally higher total leukocyte concentrations ($F_{1,2} = 13.2$, $P = 0.06$) than group B. Group A also had higher PC3 scores than group B at baseline ($F_{1,2} = 14.9$, $P = 0.02$). Thus, for these measures we examined the effect of limited time access to food using both the difference before and after treatment (to control for differences at baseline) and absolute differences between the groups during the 6h and 22h treatments. Both analyses gave the same results.

Only one index of constitutive immunity, *S. aureus* killing, was affected by limited time access to food (group by day interaction $F_{4,67} = 3.42$, $P = 0.013$). In block 1, *S. aureus* killing increased in group A (6h treatment), but remained constant in group B (22h treatment; Figure 7.2B). During block 2 this pattern reversed, *S. aureus* killing increased in group B (6h treatment) and returned to baseline in group A (22h treatment; Figure 7.2B). However, Tukey post-hoc tests showed that between treatment and within-bird differences did not reach statistical significance.

Considering all indices of constitutive immunity together using PCA scores, only PC3 (lymphocytes and monocytes) showed a statistically significant group by day interaction ($F_{4,66} = 2.66$, $P = 0.04$). However, Tukey post-hoc analysis indicated that this interaction was caused by a significant drop in PC3 from baseline to day 42 in group A only (Figure 7.2C); a different pattern from the reversible food treatment effect seen in hematocrit and *S. aureus* killing.

Acute Phase Response – sickness behavior and body temperature

Injected and uninjected birds did not differ significantly on any temperature or behavioral indices (all *P*'s > 0.30) before LPS injection (day 15) confirming homogeneity of injection group composition.

LPS and food treatment had interactive effects on feeding behavior, body mass and body temperature (Figure 7.3A to C). For feeding behavior, injected birds in the 22h treatment showed a trend for decreased feeding relative to uninjected birds, whereas injected birds in the 6h treatment showed a trend for increased feeding relative to uninjected birds (Figure 3A; LPS*Treat: $F_{1,13} = 8.01$, $P = 0.01$). Furthermore injected birds in the 6h treatment fed more than both injected and uninjected birds in the 22h treatment. For body mass, injected birds in the 22h treatment showed a trend for mass loss compared with uninjected birds whereas injected and uninjected birds both lost mass in the 6h treatment (Figure 7.3B; LPS*Treat: $F_{1,13} = 4.85$, $P = 0.04$). For body temperature, no significant difference was found between injected and uninjected birds in the 22h group, but in the 6h group injected birds increased or held body temperature steady, whereas uninjected birds decreased temperature (Figure 7.3C; 6h after injection LPS*Treat: $F_{1,13} = 14.38$, $P = 0.002$ and 17h after injection LPS*Treat: $F_{1,13} = 6.07$, $P = 0.03$).

LPS injection, but not treatment, affected activity and resting behaviors during the acute phase response. Injected birds in both food treatments decreased activity (LPS: $F_{1,14} = 7.72$, $P = 0.01$; Treatment: $F_{1,2} = 0.13$, $P = 0.75$, Figure 7.3D) and showed a trend for increased rest (LPS: $F_{1,14} = 4.23$, $P = 0.06$; Treatment: $F_{1,2} = 0.004$, $P = 0.95$).

Birds with symptoms of infection

Over the course of the experiment a total of five birds showed signs of illness or infection. Of the five, four displayed inflammation of the foot or wing and became ill during 6h food access (three in Group A and one in Group B) and one had an elevated leukocyte count and became ill upon returning to 22h food after three weeks on 6h food. Of the three birds in Group A that became ill during 6h food, two recovered when back on 22h food. Analysis of samples from the foot and wing lesions indicated bumblefoot and avian pox infections (T. Kuiken, pers. comm.) Bumblefoot is caused by *S. aureus* bacteria (although not the strain used in our assay) and avian pox is caused by several strains of avipoxvirus (USGS 1999).

LPS effects on constitutive immunity

Injected and uninjected birds did not differ significantly on any constitutive immune indices (all *P*'s > 0.160) before LPS injection (day 15). However, by chance, injected

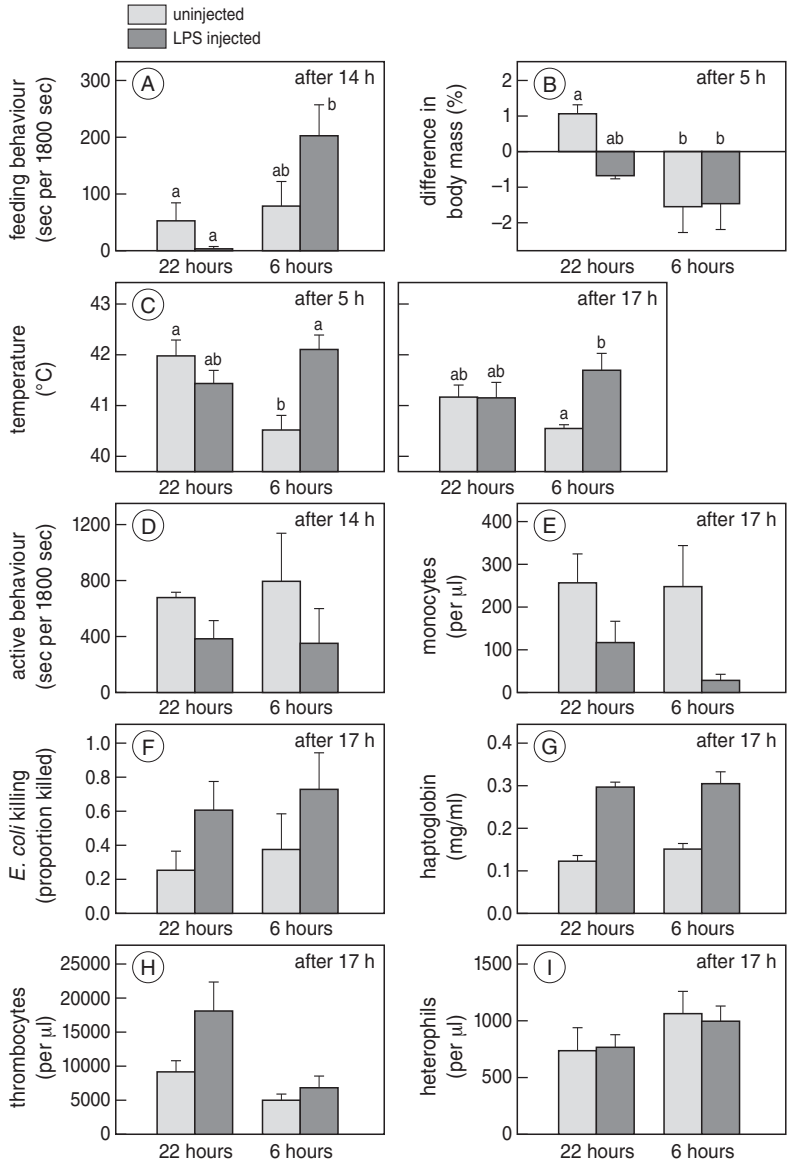

Figure 7.3. The effect of limited time access to food on the lipopolysaccharide (LPS) induced acute phase response: (A) feeding (B) body mass (C) temperature (D) activity (E) monocytes (F) *E. coli* killing (G) haptoglobin (H) thrombocytes (I) heterophils. Light grey bars = uninjected (22 hours, n = 5, 6 hours, n = 4); dark grey bars = LPS injected (22 hours, n = 6, 6 hours, n = 4). Bars are least squared means and error bars ± one SE. In (A) to (C) where the food treatment*LPS interaction was significant, different letters indicate significant Tukey post-hoc tests and interaction statistics are given in the text. For (D) to (I) LPS and treatment main effects are presented in the text.

birds in the 6 hour group had higher hematocrit than uninjected birds, thus we analyzed the change in hematocrit before and after injection. We found that LPS injection resulted in a 10% drop in hematocrit in injected birds, but no change in uninjected birds (LPS: $F_{1,14} = 13.80$, $P = 0.002$).

LPS injection affected several measures of constitutive immunity; inducing a decrease in monocytes (LPS: $F_{1,14} = 8.78$, $P = 0.01$; Treatment: $F_{1,2} = 0.14$, $P = 0.74$, Figure 7.3E), but increases in *E. coli* killing (LPS: $F_{1,14} = 5.788$, $P = 0.03$; Treatment: $F_{1,2} = 1.04$, $P = 0.41$) and haptoglobin concentration (LPS: F1,14 = 105.1 $P < 0.001$; Treatment: $F_{1,2} = 0.88$, $P = 0.44$; Figure 7.3F to G). LPS also induced an increase in thrombocytes (LPS: $F_{1,14} = 7.88$, $P = 0.01$) and the data indicate a stronger effect in the 22h treatment, although neither the interaction nor the treatment effect are statistically significant (treatment*LPS: $F_{2,13} = 0.54$, $P = 0.82$; Treatment: $F_{1,2} = 7.30$, $P = 0.11$; Figure 7.3H). Food treatment, but not LPS, affected heterophils, which were higher in the 6h treatment (both injected and uninjected) than in the 22 hour group after LPS injection (Figure 7.3I, Treatment $F_{1,2} = 15.53$, $P = 0.04$; LPS: $F_{1,14} = 0.10$, $P = 0.75$). Analysis using the PC scores showed trends for LPS induced increases in PC1 (LPS: $F_{1,14} = 4.50$, $P = 0.05$; Treatment: $F_{1,2} = 0.02$, $P = 0.89$) and PC4 (LPS: $F_{1,2} = 3.98$, $P = 0.06$; Treatment: $F_{1,2} = 0.004$, $P = 0.95$) and decreases in PC3 (LPS: $F_{1,2} = 4.46$, $P = 0.05$; Treatment: $F_{1,2} = 0.23$, $P = 0.68$). No treatment*LPS interactions were significant for either individual immune indices ($P > 0.8$) or for PC scores ($P > 0.75$).

DISCUSSION

This study investigated constitutive immune function and the acute phase response in red knots faced with limited time access to food. Our 6h food treatment led to a clear energy deficit, producing reversible adjustments in body mass, feeding behavior (Vézina et al. submitted), and hematocrit (an indicator of nutritional status; Campbell 1995). Nevertheless, we found little affect of food treatment on constitutive immune function; although aspects of the more costly acute phase response were suppressed. Here we discuss these results and anecdotal evidence suggesting the importance of disease itself as an indicator of trade-offs. We also discuss the effect of LPS injection on constitutive immunity from a mechanistic standpoint.

Little effect of limited time access to food on constitutive immune function

Of the indices of constitutive immune function measured only *S. aureus* killing was affected by limited access to food, and the effect was not statistically significant at the between group or within bird levels (post-hoc tests n.s.). Even when all indices of constitutive immunity were considered together, none of the PCs were significantly affected by food treatment. PC3 decreased over the course of the experiment in group A regardless of food treatment, whereas PC3 scores remained constant in group B, indicating that the effect was not driven by our by food treatments. These results suggest that constitutive immunity, which is constantly maintained and ready for immediate

action against pathogen threats, is not traded-off during periods of limited food availability. Maintaining basic immune function in the face of limited access to food makes sense since the red knot lifestyle includes frequent periods of limited food availability (Buehler and Piersma 2008; Piersma 2007; van Gils et al. 2006). Rather than trading-off constitutive immunity during periods of negative energy balance, birds seem to adjust their behavior and pay for deficits using energy stores (Vézina et al. submitted). A similar situation is seen in the maintenance of mass independent basal metabolic rate during limited food access (Vézina et al. submitted).

On the other side of the energy budget equation, Buehler et al. (2008a) manipulated energy expenditure by making birds live at winter-like ambient temperatures. They also found little effect on constitutive immune function. Thus, it appears that although acclimation to situations of increased energy expenditure and limited food access requires adjustments in body mass and feeding behavior (Vézina et al. 2006; Vézina et al. submitted); a basic level of constitutive immunity remains robust within the natural range of temperatures and food availabilities experienced by red knots in the wild. Contrary to the prediction that constitutive immunity would either decrease or not change in birds experiencing limited access to food, *S. aureus* killing increased during the 6h treatment. This result may be a mechanistic response to the stress of limited food availability. Our 6h treatment led to a clear energy imbalance (Vézina et al. submitted) and this energetic deficit and associated adjustments in feeding behavior likely caused physiological and social stress. During periods of acute stress there is a redistribution of lymphocytes from the blood to the lymph system (detectable from 30 min after stress and lasting up to a few weeks if stress is continued; Dhabhar and McEwen, 1997) leaving a higher proportion of circulating phagocytes. Since cellular components in whole blood are thought to be important in *S. aureus* killing (Millet et al. 2007), this relative increase in phagocytes may be connected to increases in phagocytosis based killing.

The acute phase response and energy balance under limited time access to food

Significant LPS by food treatment interactions were detected in several aspects of the acute phase response, indicating that birds in the 6h and 22h treatments responded differently to the LPS challenge. Feeding behavior and patterns of mass loss suggest that anorexia (decreased feeding behavior; Owen-Ashley and Wingfield, 2007), usually seen as part of the acute phase response (Klasing 2004; Owen-Ashley and Wingfield, 2007), was not present in food-restricted birds exposed to LPS. While injected birds in the 22h treatment tended to decrease feeding and lose mass relative to uninjected birds, injected birds in the 6h treatment tended to increase feeding and lost approximately the same amount of mass as uninjected birds. Because body mass was taken when birds in the 6h treatment did not have access to food, it is not surprising that both injected and uninjected birds lost mass. The lack of anorexia in LPS-injected 6h-birds is consistent with the energy limitation hypothesis (Owen-Ashley and Wingfield, 2007) stating that the acute phase response may be suppressed if reductions in energy reserves caused by sickness behavior result in body mass values low enough to threaten survival. Our birds lost mass at a rapid rate during the first few days of limited food access and this

weight loss was slowed via behavioral adjustments including vastly increased feeding during periods when food was available (Vézina et al. submitted). Thus, during the acute phase response, birds in the 6h treatment may not have been able to afford to suppress feeding activity.

Our body temperature data are intriguing since we expected either fever (hyperthermia) in both food treatments (i.e. LPS effect alone), or the suppression of fever in injected birds in the 6h group to save energy (i.e. if an interactive effect was present). Instead, our data indicate no significant response in the 22h treatment and decreased temperature in uninjected birds in the 6h treatment (Figure 7.3C). In many bird species hypothermia is used as a strategy to conserve energy during periods of restricted food availability (Hainsworth et al. 1972; MacMillen and Trost 1967; Rashotte and Henderson 1988). We measured body temperature at times when birds in the 6h treatment did not have access to food. Thus, the lower body temperature seen in the uninjected birds is likely reflecting an energy conserving strategy in response to limited food access. However, in the injected birds this energy conserving strategy may have been over ridden by the acute phase response.

The difference in body temperature between injected and uninjected birds in the 6h treatment was 1.5ºC and 1ºC after 5h and 17h, respectively (Figure 7.3C). According to the equation $MR = c(Tb - Ta)$, where MR is metabolic rate, c is thermal conductance (0.045W/ºC for red knots; Wiersma and Piersma 1994), Tb is body temperature and Ta is ambient temperature (12.7°C ± 0.5°C in the cages), and assuming that birds in the 6h treatment remain hypothermic all day, uninjected birds saved about 0.07W on thermoregulation. This suggests a 4.2% saving on overall daily energy expenditure (DEE; average baseline DEE is 1.66W for group A; Vézina et al. submitted). This saving is relatively small, but the fact that it is traded-off during the acute phase response suggests that, in contrast to constitutive immunity, the acute phase response represents an energetic challenge for red knots.

Susceptibility to disease

Although we did not specifically test for it, anecdotal evidence suggests that birds facing limited access to food are more susceptible to disease. Four of the five birds that showed signs of inflammation or illness did so while enduring limited food availability (three in Group A and one in Group B), and of the three birds in Group A, two recovered upon returning to 22h food. These data bring to light the important point that poor resource conditions can exacerbate relatively mild disease (Chandra and Chandra 1986). Thus, although animals exhibiting disease symptoms should be excluded from analysis of constitutive immunity (because they represent an immune response rather than immune maintenance), these individuals remain important for biologically relevant thinking about variation in immune function. In the future experiments and monitoring of wild birds should include both measures of immune function and documentation of disease. Furthermore, where ethically possible, studies examining disease susceptibility or manipulating pathogen pressure will help our understanding of biologically viable levels of immune defense.

Table 7.2. A summary of hypothesized costs and immune strategies: [1] inferred using Klasing (2004), [2] Janeway et al. (2004), [3] Matson et al. (2006b), Millet et al. (2007), [4] Matson et al. (2005), [5] T. Kuiken, pers. comm. Groupings within constitutive immunity from this study and Buehler et al. (2008a and b).

Specific Index	Description and Function	Use Cost [1]	Strategy
Lymphocytes	Form the basis of specific immunity [2]	Low	Constitutive, leukocytes associated with specific (acquired) immune defense
Monocytes	Long-lived phagocytes and presenting cells for specific immunity [2]		
Thrombocytes	Important for blood clotting [2]		
E. coli killing	Bacteria defense - mostly without phagocytosis [3]	Moderate	Constitutive, soluble factors associated with non-specific and immediate defense
Hemagglutination	Natural antibody mediated clumping of invaders [4]		
Hemolysis	Complement mediated lysis and opsinization [4]		
S. aureus killing	Bacteria defense - mostly by phagocytosis [1]	High	Constitutive, associated phagocytosis with non-specific and immediate defense
C. albicans killing	Yeast defense - mostly by phagocytosis [3]		
Heterophils	Short-lived phagocytes [2]		
Temperature	Kills invaders with heat [2]	High	Induced, inflammation and systemic non-specific defense
Haptoglobin (induced)	Acute phase protein [2]		
Decreased feeding	Starves invader while saving energy [2]		

The effect of LPS injection on constitutive immune indices

The injection of LPS increased *E. coli* killing and tended to decrease *C. albicans* killing (though not statistically significant $P = 0.32$, data not shown), similar to responses reported in chickens (Millet et al. 2007). In addition, monocytes decreased, possibly as they migrated out of the blood to the tissues (Dhabhar and McEwen 1997), and thrombocytes increased, especially in the 22h group, perhaps in anticipation of blood clotting at the site of the injection wound (Janeway et al. 2004). Finally, concentrations of the acute phase protein haptoglobin were nearly doubled in injected birds (Figure 7.3G). Haptoglobin concentration increases during an acute phase response because the liver increases secretion of acute phase proteins in response to cytokines (Eckersall 1995). Thus, this strong increase in haptoglobin indicates the successful induction of an acute phase response.

Optimal immune adjustments in energetically constrained situations

The pathogen thwarting benefits of immune defenses must be balanced against their energetic and immunopathological costs (Norris and Evans 2000; Råberg et al. 1998; Schmid-Hempel and Ebert 2003; Sheldon and Verhulst 1996) especially in energetically constrained situations. To illustrate the costs and benefits of immune indices meas-

ured in this study, we summarize constitutive immune groupings identified by PCA (this study, Buehler et al. 2008a and b) with the acute phase response (Table 7.2). We exclude thrombocytes and baseline haptoglobin because these indices were not measured in all studies. Table 7.2 shows that constitutive immune indices cluster into three different cost and benefit strategies: a low cost strategy associated with lymphocytes and monocytes; a moderate cost strategy comprised of soluble factors (*E. coli* killing, hemolysis and hemagglutination); and a high cost strategy comprised of phagocytosis-based defense (*S. aureus* killing, *C. albicans* killing and heterophils). However, despite these groupings, this study shows that a baseline level of constitutive immune function is constantly maintained, while aspects of the acute phase response appear to be down-regulated during resource limitation. This suggests that all constitutive immune strategies are lower cost than the acute phase response (fever, anorexia and induced haptoglobin) and implies that in energetically constrained situations, birds optimize by saving energy on the most costly aspects of immune defense.

ACKNOWLEDGEMENTS

We thank Alberto Castillo for help in the lab, Bernard Spaans for catching the birds, Anne Dekinga and Maarten Brugge for help taking care of the captive knots, and J. Reneerkens for much advice about food limitation. Funding was provided by the Natural Sciences and Engineering Research Council of Canada (NSERC), University of Groningen Ubbo Emmius Scholarship, and Schure-Beijerinck-Popping Fonds to DMB, by the Netherlands Organization for Scientific Research and the University of Groningen to TP and BIT, and by the Royal Netherlands Institute for Sea Research to TP

CHAPTER 8

No evidence for melatonin-linked immunoenhancement over the annual cycle of an avian species

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ABSTRACT

The winter immunoenhancement hypothesis associates long winter nights and increased exposure to melatonin with enhanced immune function during winter when resource availability is low and chances to become sick are high. In addition to providing a seasonal signal, increased exposure to melatonin could therefore be adaptive for species experiencing tough conditions during the winter. This idea has found some support in studies of resident mammals, but has not been tested in birds. In birds, the link between day length and melatonin over the annual cycle is weaker, and contributions of melatonin to seasonal timing are unclear. Furthermore, many species, especially migrants, do not experience the toughest conditions of their annual cycle in winter. In this study we tested whether the winter immunoenhancement hypothesis holds in an avian species, the red knot *Calidris canutus*. We found that duration and amplitude of daily melatonin rhythms varied significantly over the annual cycle, but that this variation was not linked to day length and did not correlate with annual variation in immune function. Our findings do not support the winter immunoenhancement hypothesis in knots and further question whether the link between short days and increased exposure to melatonin can be generalized in birds. Furthermore, the data question whether the idea that immune function should be bolstered in winter can be generalized to systems where winter is not the toughest time of the year. Unlike resident mammals, red knots do not experience high disease risk and internal competition for resources during winter, but rather during spring migration and arrival on the breeding grounds. To assess whether melatonin functions as the proximate driver of annual variation in immune function, comparative studies examining mammalian and avian species with a range of ecologies will be instructive.

Submitted

INTRODUCTION

In many species, disease risk, resource availability and immune defense vary over the annual cycle (reviewed in Altizer et al., 2006 and Martin et al., 2008). If variability in disease risk is predictable, and assuming that immune defense is costly to maintain and use (Råberg et al., 1998; Schmid-Hempel and Ebert, 2003; Klasing, 2004), it is potentially adaptive for immune responses to become adjusted to seasonal fluctuations in disease risk. One hypothesis derived from this reasoning is the winter immunoenhancement hypothesis (Nelson and Demas, 1996; Nelson et al., 2002, also known as the melatonin-induced immunoenhancement hypothesis; Hasselquist, 2007). Melatonin is an indole amine secreted mainly by the pineal gland primarily at night. In mammals the duration of melatonin secretion is proportional to night length, and animals thus experience the longest exposure to melatonin during short days. The duration of elevated melatonin in mammals functions as a seasonal signal for the timing of annual cycles (Prendergast et al., 2002). Because melatonin can enhance the immune system (reviewed in Nelson et al., 2002), it has been proposed that immune function is enhanced in winter. This could be adaptive for animals experiencing harsh winter conditions, as melatonin-linked immunoenhancement might counteract the effects of thermal stress and low food resource availability which would otherwise suppress immunity (Nelson and Demas, 1996).

The winter immunoenhancement hypothesis predicts that disease risk and immune function should peak when days are short and melatonin levels are highest (Nelson et al., 2002). This hypothesis is based mainly on data from mammals that have tightly linked day length and melatonin rhythms, and that reside in the temperate zone year round and thus experience harsh conditions during the winter (Nelson et al., 2002). The mammal data indicate that day length affects many aspects of the immune system, although some measures increase (Yellon et al., 1999; Bilbo et al., 2002a; Mann et al., 2000; Nelson et al., 2002), while others are attenuated (Yellon et al., 1999; Bilbo et al., 2002b) under short days. Thus, the mammal data provide partial support for winter immunoenhancement (Nelson et al., 2002). The present study tests whether the hypothesis can be extended to bird species.

Testing the winter immunoenhancement hypothesis requires an annual profile of melatonin to assess whether melatonin exposure peaks when days are short, and an annual profile of immune function to verify that immune indices increase under short days. To date such data on melatonin and immune function in birds are scarce (reviewed in Hasselquist, 2007). Changes in melatonin profiles have been compared for selected seasons under temperate light conditions, but have not been tracked over the entire annual cycle (Brandstätter et al., 2001; Hau et al., 2002; Fusani and Gwinner, 2005). Studies involving detailed sampling of melatonin over the entire annual cycle have focused on extreme photoperiodic situations, e.g., polar light (Miché et al., 1991; Reierth et al., 1999) and constant equatorial day lengths (Gwinner et al., 1993). In contrast to predictions of the winter immunoenhancement hypothesis, these studies showed that peaks and troughs in plasma melatonin concentration did not necessarily coincide with summer and winter solstices (Miché et al., 1991; Gwinner et al., 1993; Reierth et al., 1999; Brandstätter et al., 2001; Hau et al., 2002; Fusani and Gwinner, 2005).

From an immune standpoint, avian studies tracking immune function over the annual cycle show no general pattern of either immunoenhancement or attenuation during short days. When cell-mediated immune function was measured, two of seven studies showed increases during short days (Bentley et al., 1998; Martin et al., 2004), whereas the others showed opposite trends or no change at all (reviewed in Hasselquist, 2007). When humoral immune function was measured, one of three studies on showed decreases during short days (Hasselquist et al., 1999), and again the other studies showed opposite trends or no change at all (reviewed in Hasselquist, 2007). Furthermore, experimental studies linking melatonin and immune function via melatonin implants or photoperiodic manipulation have also produced incongruent results in terms of melatonin-linked immunoenhancement (Giannessi et al., 1992; Bentley et al., 1998; Moore and Siopes, 2000; Majewski et al., 2005). This inconclusive situation is further complicated because studies examining seasonal fluctuations in immune function in birds did not provide measures of melatonin, so that no combined information for a single species is available. Thus in birds, evidence for generalizable annual cycles of melatonin or immune function and day length, and evidence that melatonin covaries with immune function throughout the year, is still lacking.

We address this deficit in the present study of an avian species, the red knot. We test the winter immunoenhancement hypothesis by using detailed melatonin and immune function datasets collected over entire annual cycles. Red knots are long-distance migrant shorebirds with distinct annual phenotype cycles that are maintained even in captivity (Cadée et al., 1996; Piersma, 2007; Buehler et al., 2008a). We examined whether melatonin and several measures of constitutive immunity were related by correlating melatonin data with monthly measurements of immune function presented in Buehler et al. (2008a). If the winter immunoenhancement hypothesis holds in red knots, we predict peak exposure to melatonin during the shortest days of the year and positive correlations between melatonin exposure and indices of constitutive immune function.

MATERIALS AND METHODS

Birds

Red knots of the subspecies *C. c. islandica*, were captured in the Dutch Wadden Sea (53°31'N 6°23'E) and brought into captivity at the Royal Netherlands Institute for Sea Research (NIOZ). All birds were kept under local photoperiodic and temperature conditions. In the wild, *C. c. islandica* knots spend nearly ten of twelve months of the year in western Europe (Buehler and Piersma, 2008). They depart for breeding grounds in Greenland and the Canadian Arctic only from late May and return in early August. Thus, they experienced natural conditions for most of the year, but days were shorter than on their Arctic breeding grounds during mid summer. At capture the birds were ringed, weighed, and aged (all older than two years; Prater et al., 1977) and sexes were later determined using molecular techniques (Baker et al., 1999). The birds used to study seasonal rhythms of melatonin (n = 6) were captured on 6 November 1994 (all

males) and on 27 October 1995 (two females and a male) and kept in outdoor aviaries (4.5m x 1.5m x 2.3m high) with *ad libitum* access to protein-rich trout pellets (Piersma et al., 2000a). The birds used to study seasonal rhythms of immune function (n = 12) were part of the control group (variable treatment) in a larger study examining the effect of ambient temperature on immune function over the annual cycle (Buehler et al., 2008a). The birds were captured on 22 August and 16 September 2004 (six females and four males) or on 8 February 2005 (one female and one male), and were housed, in flocks of six, in indoor aviaries identical to the outdoor aviaries described above. They experienced outdoor temperatures and had *ad libitum* access to a natural diet of shellfish (mudsnails *Hydrobia ulvae*).

All birds were weighed and scored for molt once a week. Because we were interested in molt as a whole, we calculated total molt as the sum of a breast molt index (Piersma and Jukema, 1993) and the number of growing primaries (feathers in wing molt categories 1 through 4; Ginn and Melville, 1983). To verify that all study birds were healthy, we also performed weekly assessments of feather condition and the skin of the feet, elbows (carpals) and keel for signs of local inflammation (e.g. bumblefoot).

Sampling

The birds used to study seasonal rhythms of melatonin were sampled bimonthly from early March 1997 to late February 1998. Sampling took place over a 24 hour session, starting and ending at midday with seven bleedings at four hour intervals (12:00, 16:00, 20:00, 00:00, 04:00, 08:00 and 12:00). This fixed clock time schedule simplified blood sampling and data analysis, but reflected different circadian times as the year progressed. Assessment of the health of birds indicated that the bleeding schedule had no harmful effects (Piersma et al., 2000a). We always collected blood within a quarter of an hour (usually within a few minutes) after entry into the aviary by puncturing the brachial wing vein and collecting 150 to 250 μl of blood into heparinized capillary tubes. We centrifuged the blood within two hours of sampling at 6,900*g for 15 min and stored the plasma at -80°C until processing.

The birds used to study seasonal rhythms of immune function were sampled monthly from March 2005 to March 2006. We always collected blood in the morning between 10:30 and 11:00 am (mean ± SD = 10:44 ± 5 min) and always within 20 min of entering an aviary. We thoroughly sterilized the area around the brachial vein with 70% ethanol and then collected about 600 μl of blood into pre-sterilized, heparinized capillary tubes. Immediately after sampling, we made blood smears and transported the remaining blood in sterile boxes to the laboratory for further processing. Whole blood was used for the microbial killing assay within an hour of sampling and the remaining blood was centrifuged at 6,900*g for 12 min and the plasma stored at -80°C (Buehler et al., 2008a).

Quantifying melatonin

We quantified melatonin by radioimmuneassay (Van't Hof and Gwinner, 1996). We extracted plasma overnight in chloroform with 1 M NaOH, the next morning the extractions were pulse centrifuged and the chloroform layer was aspirated off. Again

chloroform was added, then after at least 2 hours sitting with the chloroform the top layer was aspirated off. The extraction was then dried under nitrogen and redissolved overnight in 0.1 M Tricine buffer. We then performed another extraction with Petroleum ether (pertoleum benzine) to remove fat. We added Petroleum ether to the extractions on dry ice and then aspirated off the upper Petroleum ether phase. To determine extraction efficiency, we added 2000 cpm of 3H-melatonin to each extraction tube. After extraction, melatonin was measured by radioimmunoassay using sheep anti-melatonin antiserum (G/S/704-8483, Stockgrand Ltd., Guildford, UK) and 3H-labelled melatonin with a specific activity of 3148.7 (GBq/mM from NEN, Du Pont). For each individual bird over the 24-h blood sampling session we examined the duration of melatonin secretion (melatonin duration) and daily melatonin peak to determine whether these two indices of melatonin are correlated in knots and to test whether they correlate with immune function. Melatonin duration was calculated as the number of hours between samplings with detectable melatonin (above the assay detection limit of 17 pg/ml). For example, if we saw melatonin greater than 17 pg/ml from 20:00 to 6:00 then melatonin duration was at least 10 hours.

Quantifying immune function

We measured immune function by quantifying microbial killing abilities against Escherichia coli, Candida albicans and Staphylococcus aureus (Tieleman et al., 2005; Millet et al., 2007), leukocyte concentrations (heterophils, lymphocytes and monocytes, Campbell, 1995), and complement and natural antibody levels (Matson et al., 2005). These indices represent constitutive immunity, which is always present and thus able to respond immediately to threats from multiple pathogen types. Constitutive immunity is an animal's first line of defense and thus an evolutionarily relevant branch of immunity for testing the winter immunoenhancement hypothesis. Furthermore, because a response is not induced and immunological memory is not stimulated, repeated measures of individuals throughout the annual cycle can be made. The assays have been described extensively in the original methodological papers and in Buehler et al. (2008a) for the immune data presented here. Briefly, the microbial killing assay measured the capacity of whole blood to kill microorganisms *in vitro*. Higher "killing" equates with a greater capacity to limit infection by the particular strain of microorganism used in the assay (Millet et al., 2007). Leukocyte concentrations obtained using the indirect eosinophil Unopette method (Campbell 1995; No. 5877; Becton Dickinson) provided a description of circulating cellular immunity (Campbell, 1995). Complement and natural antibodies measured using a hemolysis-hemagglutination assay (Matson et al., 2005) assessed the link between innate and acquired immunity and the first line of defense against spreading infections, including viruses (Ochsenbein and Zinkernagel, 2000).

Statistical Analysis

We used Kolmogorov–Smirnov tests and visual examination of histograms to examine response variables and model residuals for normality. Peak melatonin and melatonin duration were right skewed and were log 10 transformed to achieve normality.

To examine melatonin variation over the annual cycle we performed univariate analyses using a linear mixed model with week as a fixed factor and bird as a random factor. We ran the models including and excluding an effect of sex. Sex was never significant and never changed the outcome of the model, thus the statistics presented are from models excluding sex. Univariate and principal component analyses of variation in immune function over the annual cycle are presented in Buehler et al. (2008a). To show general patterns in immune function over the annual cycle we calculated principal component scores for the birds in this study using the within-bird component loadings presented in Buehler et al. (2008a). Measures of microbial killing were not available for March and early April thus principal component scores for that month were calculated using heterophil data only.

Although different birds and different years were used to examine melatonin and immune function, annual patterns of body mass and total molt showed nearly identical patterns (Fig. 8.1). Thus, for every individual we averaged the bi-weekly melatonin measurements which most closely matched the calendar dates on which we took the monthly measurements of immune function. Then, for both melatonin and immune function, we averaged individual values to create monthly means. We then ran Pearson correlations on these monthly means to test for relationships between melatonin and immune function over the annual cycle. Melatonin sampling was not performed during August and early September, thus the monthly mean matched to immune sampling for the period (late August/early September) is an interpolation (Fig. 8.2 B and C).

RESULTS

Melatonin cycles of knots had very low amplitudes, and melatonin duration and daily peaks showed similar patterns (were positively correlated) over the annual cycle, but did not correlate with day length (Fig. 8.2 A to C). Both measures were lowest in late June and early July when days were long and increased as day lengths shortened. However, contrary to predictions, values dropped in December when day lengths were shortest and increased sharply in January and early February as day lengths slowly began to increase again. This seasonal variation was significant for both melatonin duration (Week: $F_{19,91} = 7.00$, $P < 0.001$; Bird: $F_{5,91} = 3.90$, $P = 0.003$) and melatonin peak (Week: $F_{19,91} = 6.34$, $P < 0.001$; Bird: $F_{5,91} = 2.98$, $P = 0.015$).

Seasonal patterns in immune function are described in detail in Buehler et al. (2008a), all immune indices except natural antibody agglutination showed significant annual fluctuation. Here we present plots of principal component scores for the birds considered in this study calculated using the within-bird component loadings presented in Buehler et al. (2008a). In general, highs and lows for principal components of immune function did not correspond to changes in day length or variation in melatonin (Fig. 8.2A and D to F). Indices associated with the potential for acquired immunity (lymphocytes and monocytes, within-bird PC1, Group C in Buehler et al., 2008a) peaked during peak molt (Fig. 8.2D). Indices associated with phagocytosis and inflammation (phagocytosis based microbial killing, heterophils and lysis, within-bird PC2,

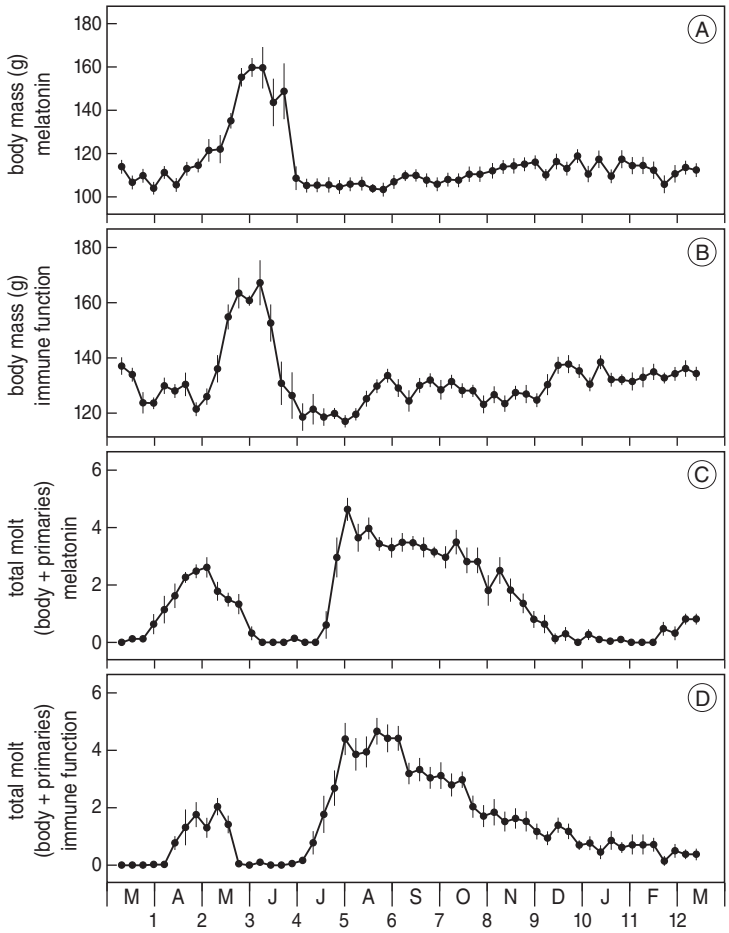

Figure 8.1. Patterns in body mass and molt over the annual cycle of red knots. Months of the year are shown along the x-axis and numbers match the monthly means presented in Figures 8.2 and 8.3. Body mass from birds used to examine melatonin (A), body mass from birds used to examine immune function (B), molt from birds used to examine melatonin (C), molt from birds used to examine immune function (D).

Group A in Buehler et al., 2008a) were highest from May to early July when free-living birds are migrating and arriving on breeding grounds, and were lowest in late August and early September during peak molt (Fig. 2E). Finally, indices associated with soluble factors of constitutive immunity (*E. coli* killing and agglutination, within-bird PC3, Group B in Buehler et al., 2008a) fell from September to March (Fig. 8.2F).

We found no relationship between either melatonin duration (Fig. 8.3) or daily melatonin peak and immune function, and none of the Pearson correlations were statistically significant (Table 8.1).

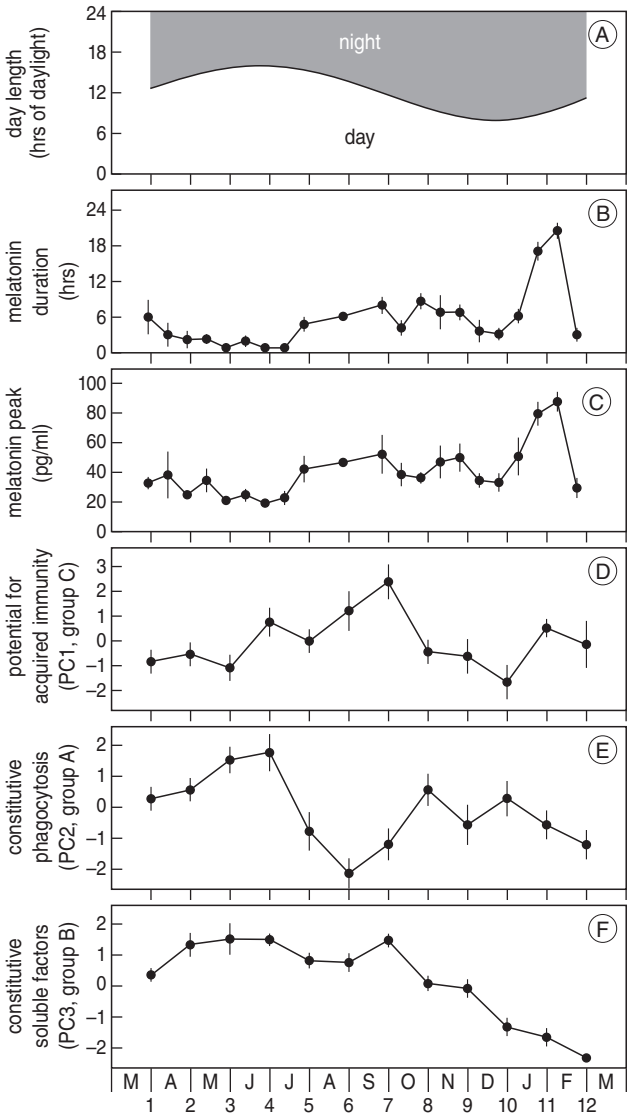

Figure 8.2. Patterns of melatonin and immune function over the annual cycle of red knots. Day length at 50°N, compiled from the information in Figure 5 in (Piersma and Davidson 1992) (A) melatonin duration (B) daily melatonin peak (C). For (B) and (C) statistics were performed on log 10 transformed values, but the raw data are shown for ease of interpretation. Symbols show means and error bars show one SE. Months of the year are shown along the x-axis and numbers match the monthly means presented in Figure 8.3. Melatonin sampling was not performed during August and early September, thus in (B) and (C) the point in late August (without error bars) is an interpolation. Measures of microbial killing were not available for late March and early April thus principal component scores for month 1 in (E) were calculated using heterophil data only.

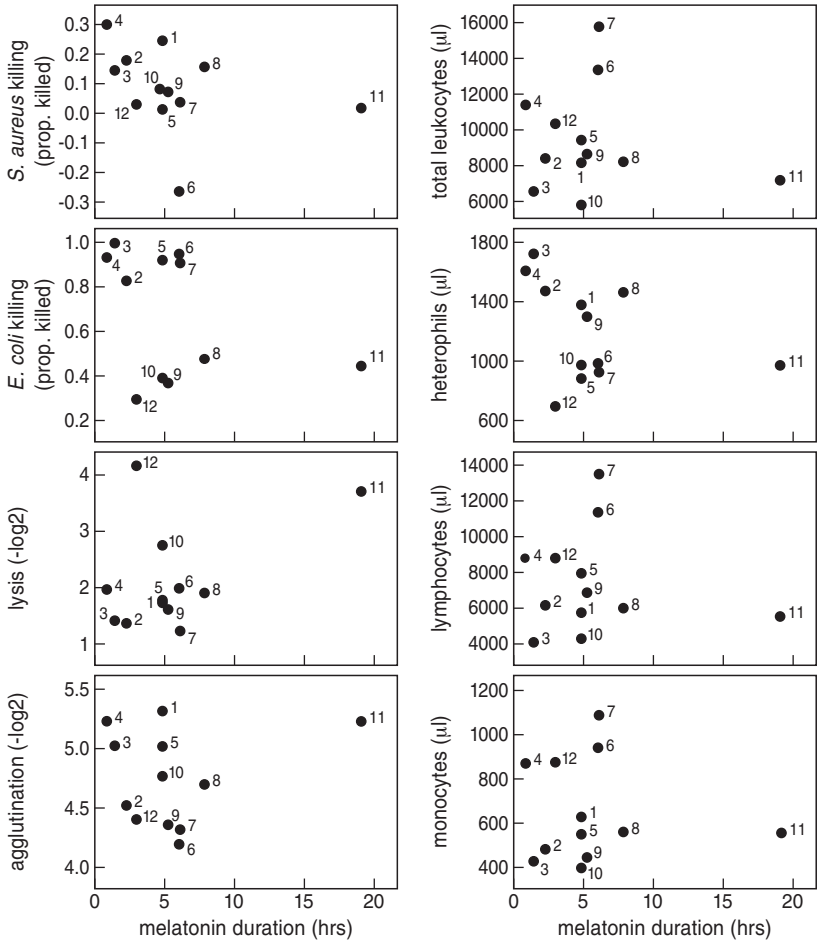

Figure 8.3. Relationships between melatonin duration and indices of immune function. Dots represent monthly means and numbers reflect the months of the year given in Figures 8.1 and 8.2. Pearson correlations are given in Table 8.1.

Table 8.1. Summary of Pearson correlations between indices of constitutive immune function and melatonin in red knots.

	Melatonin duration (hrs)		Melatonin peak (pg/ml)	
	Correlation	*P*	Correlation	*P*
S.aureus (prop. killed)	-0.36	0.31	-0.46	0.15
E.coli killing (prop. killed)	-0.37	0.26	-0.36	0.31
Lysis (-log2)	0.44	0.15	0.39	0.21
Agglutination (-log2)	0.17	0.60	0.06	0.85
Total leukocytes (per µl)	-0.13	0.68	-0.06	0.84
Heterophils (per µl)	-0.36	0.26	-0.48	0.12
Lymphocytes (per µl)	-0.08	0.81	0.01	0.98
Monocytes (per µl)	-0.08	0.82	-0.60	0.85

DISCUSSION

In this study we examined whether predictions made by the winter immunoenhancement hypothesis hold in an avian species, the red knot. Our data address two predictions: first, exposure to melatonin peaks during shorts days, and second immune function also peaks during short days and correlates with melatonin exposure. We found significant variation in melatonin and immune function over the annual cycle, but this variation did not fluctuate in conjunction with day length (Fig. 8.2). Furthermore, we did not find any significant relationship between melatonin and immune function (Fig. 8.3). These results do not provide evidence for winter immunoenhancement in red knots.

The winter immunoenhancement hypothesis is based on the premise that the short days of winter increase the duration of melatonin secretion which in turn could bolster immune function (Nelson, 2004). However, unlike in mammals, the role of melatonin in the annual cycle of birds is unclear (Dawson et al., 2001; Kumar, 2001). Experimental application of melatonin generally does not affect seasonal timing, although *in vivo* and *in vitro* studies showed that melatonin secretion of the avian pineal encodes day length information in house sparrows (*Passer domesticus*; Brandstätter et al., 2001). Our findings add to the growing evidence that close links between day length and melatonin secretion cannot be generalized in birds (e.g. Miché et al., 1991; Gwinner et al., 1993; Reierth et al., 1999; Brandstätter et al., 2001; Fusani and Gwinner, 2005). In knots melatonin duration and peak were correlated and highest in January and February when day lengths were increasing (Fig. 8.2 A to C). Similarly, in Svalbard ptarmigans *Lagopus mutus hyperboreus*, and house sparrows *Passer domesticus* melatonin levels peaked in spring (January to April; Reierth et al., 1999 and March/April, Brandstätter et al., 2001). Thus, seasonal patterns of melatonin secretion may be influenced by non-photic factors, for instance diel activity patterns at different times of year (Reierth et al., 1999; Fusani and Gwinner, 2005). Red knots are tidal foragers, thus like polar ptarmigan, they are active during periods of darkness (van Gils and Piersma, 1999; van Gils et al., 2006) and nocturnal activity patterns change over the year (T. Piersma, unpublished data). The knots in this study had low daily melatonin peaks ranging from 17 to 116 pg/ml, comparable to nocturnal and polar birds (range 16 to 110 pg/ml; Miché et al., 1991; Taniguchi et al., 1993; Van't Hof and Gwinner, 1998; Reierth et al., 1999; Hau et al., 2002 wild sample in Alaska), but lower than diurnal birds (range 200 to 900 pg/ml; Miché et al., 1991; Taniguchi et al., 1993; Van't Hof and Gwinner, 1998; Reierth et al., 1999; Hau et al., 2002 captive sample in Seattle). The mismatch between melatonin and day length, and low levels of melatonin in species active at night, suggest that avian melatonin rhythms may be fitted to lifestyle with a flexible link to day length cues. This flexibility may be related to the complexity of the avian circadian system which consists of several interacting oscillators (Gwinner and Brandstätter, 2001; Kumar, 2001).

A second premise of the winter immunoenhancement hypothesis is that immune function should be bolstered during winter when days are short, because winter is the most stressful period of the annual cycle (Nelson, 2004). This premise does not hold

for migrants or other animals that do not have a predictably tougher time in winter compared with the rest of the year. For example, in red knots the toughest periods of the year are predicted during spring migration and arrival on the breeding grounds (Buehler and Piersma, 2008). Thus if knots do bolster immune function in anticipation of higher disease risk and lower resource availability, this enhancement would be predicted during spring and early summer and when we found low levels of melatonin (Fig. 8.2 A to C). Certain immune indices, especially those associated with phagocytosis, are elevated during this period (Fig. 8.2E; Buehler et al., 2008a). Furthermore, in the birds used to study melatonin, a courser measure of leukocyte levels (buffy coat) also showed a slight rise during this period (after an initial drop while acclimating to captivity; Piersma et al., 2000a). However, since we found no relationships (positive or negative) between melatonin and immune function (Fig. 8.3, Table 8.1) we suggest that melatonin is an unlikely mechanism for this immunoenhancement.

Assessment of immunoenhancement due to seasonal increases in melatonin exposure is complicated by the fact that immune function is not a monolithic entity (Nelson et al., 2002; Adamo, 2004; Matson et al., 2006). The immune system can divided into four quadrants by two axes: the first concerning degree of specificity from non-specific to specific and the second concerning temporal dynamics from constitutive to induced (Schmid-Hempel and Ebert, 2003). In mammals there appears to be a trend towards increased aspects of induced acquired immunity (lymphocyte proliferation and the cytolytic killer cell capacity; (Yellon et al., 1999; Bilbo et al., 2002a; Mann et al., 2000; Nelson et al., 2002) with exposure to short days. In contrast, aspects of constitutive innate immunity (phagocytosis and oxidative burst) and aspects of the induced innate sickness response decrease with exposure to short days (Yellon et al., 1999; Bilbo et al., 2002b). In birds the data are ambiguous, even within different branches of immune function. This study and Buehler et al. (2008a) show that in knots measures of constitutive immunity show fluctuations that do not coincide with changes in day length. Studies measuring aspects of induced specific immunity are also incongruent. Two of seven studies on cell-mediated immunity showed increases during short days (Bentley et al., 1998; Martin et al., 2004), whereas one of three studies on humoral immunity showed decreases (Hasselquist et al., 1999) the other studies showed opposite trends or no change (Hasselquist, 2007). Finally, a study examining induced innate sickness response indicated that sickness duration was decreased during short days, but only in males (Owen-Ashley et al., 2006).

In summary, our study provides no support for winter immunoenhancement in an avian species. In knots, short days were not linked to increased exposure to melatonin or enhanced immune function. Furthermore, in knots winter does not coincide with the toughest times of the year. More research will be needed to provide alternative proximate mechanisms for annual variation in immune function. Comparative studies examining species with a range of ecologies including migratory and tropical birds and mammals will be instructive.

ACKNOWLEDGEMENTS

We thank François Vézina for logistical and intellectual support, Maarten Brugge, Ania Gustowska, Kirsten Jalvingh, Magali Petit, Ciska Raaymakers and Timo Verbeek for help with captive knots, and Maarten Brugge, Anne Dekinga, Piet Duiven, Bernard Spaans and the crew of the research vessel *Navicula* for catching birds. We thank Alberto Castillo, Daliborka Barjaktarov, Steven Haan, Mieke Lange and Eeke Marks for lab assistance and Judith van Bleijswijk for lab space. Finally we thank Barbara Helm for constructive comments on earlier drafts of this article. This research was carried out with permission from the Animal Experiment Committee of the Royal Netherlands Academy for Arts and Sciences. Funding was provided by the Natural Sciences and Engineering Research Council of Canada (NSERC), the University of Groningen and Schure-Beijerinck-Popping Fonds to DMB, the Max Planck Gesellschaft to EG, the Royal Netherlands Institute for Sea Research and a Netherlands Organization for Scientific Research (NWO) PIONIER grant to TP, and NWO and the University of Groningen to TP and BIT. Discussions with Eberhard Gwinner inspired the collection of the melatonin data used in this study. He died in September 2004 and we miss him.

Mauritania

PART III

Immune function in free-living birds

CHAPTER 9

Captive and free-living red knots exhibit differences in non-induced immunity suggesting different immune strategies in different environments

Deborah M. Buehler, Theunis Piersma and B. Irene Tieleman

ABSTRACT

Experiments on captive animals, in which conditions can be controlled, are useful for examining complex biological phenomena such as immune function. Such experiments have increased our understanding of immune responses in the context of trade-offs and pathogen risk. However, few studies have examined how captivity itself affects immune function. We used microbial killing, leukocyte concentrations and complement-natural antibody assays to examine non-induced (constitutive) immunity in captive and free-living red knots *Calidris canutus*. Univariate and multivariate analyses indicated that captive and free-living birds differed in their immune strategies. Captive birds showed reduced *S. aureus* killing, *C. albicans* killing, heterophils, and eosinophils. In a principal component analysis, the affected variables fell onto a single axis, that reflected phagocytosis and inflammation based immunity. We discuss possible reasons for this result in an immune cost and protective benefit framework.

Journal of Avian Biology 39: 560-566

INTRODUCTION

Experiments on captive animals, in which conditions can be carefully controlled, are useful for examining complex biological phenomena such as immune function. For example, experiments on wild birds recently taken into captivity have been important for understanding ecological questions such as how immune function differs in birds with differing reproductive (e.g. Casto et al. 2001), or migration (e.g. Mendes et al. 2006) strategies. Immune defences may play an important role in mediating life-history trade-offs, and measures of immune function have been used to address ecological and evolutionary questions about adaptations of animals to different environmental conditions (reviewed in Lee 2006). However, in the wild, numerous environmental factors act simultaneously, making it difficult to identify which factors influence immune variables, and necessitating experiments on captive animals. Yet, captivity itself presents animals with a different environment and little is known about whether or how this affects immune function.

Captive conditions can differ from wild conditions in many ways and here we focus on two that could affect immune function: energy balance and pathogen risk. In terms of energy balance, resource availability is often high in captivity because free access to food is provided, whereas energy expenditure is often low because housing conditions restrict activity. In contrast, in the wild, food availability can be unpredictable and food must be actively obtained. These differences may be important for immune function because maintaining and using an immune system carries energetic costs (Klasing 2004). Because of these costs, trade-offs between immunity and other costly physiological processes or activities (i.e. growth, reproduction, migration,) have been predicted under conditions of resource limitation (Sheldon and Verhulst 1996, Piersma 1997, Lochmiller and Deerenberg 2000, Norris and Evans 2000). Captive birds, without resource limitations, could be released from trade-offs that are inevitable in the wild. Thus, one might predict that immune function in captive birds might reflect more costly strategies than in wild birds.

Conditions in captivity and the wild may also differ in pathogen risk. Pathogen risk is expected to influence immune function since immune investment is a balance between costs and benefits, and investment is wasted in an environment without pathogens to defend against (Schmid-Hempel and Ebert 2003). Indeed, certain measures of immune function have been shown to correlate with pathogen risk (e.g. Lindström et al. 2004, Martin et al. 2007a). Unfortunately, pathogen risk is difficult to quantify, and studies comparing captive and free-living animals have focused on the transfer of specific diseases from domesticated captives to free-living animals (i.e. Deem et al. 2005), or on common diseases of captivity (Fujita and Kageyama 2007), rather than on overall pathogen risk. Nevertheless, housing of captive animals is cleaned regularly, presumably to decrease exposure to pathogens or at least to limit pathogen diversity (USGS 1999). Thus, one might predict that immune function may be down-regulated or focused on more specific immune defences in captivity because fewer pathogens would be encountered and fewer pathogens would be novel.

The immune system can be divided into innate (non-specific) and acquired (specific) arms and further divided into constitutive (non-induced) and induced branches

(Schmid-Hempel and Ebert, 2003). Constitutive levels of innate immunity can be measured from a single capture because an immune response is not induced prior to measurement. Such assays are ideal for studies on free-living animals and the assays we chose are all non-induced in this methodological sense. We used three techniques: microbial killing (Tieleman et al. 2005, Millet et al. 2007), leukocyte concentrations (Campbell 1995) and complement-natural antibody assays (Matson et al. 2005). Microbial killing measures the capacity to limit microbial infection and is a functional measure of immunity, thus is expected to be flexible in different conditions (Millet et al. 2007). Leukocyte concentrations provide information on circulating immune cells which can be used as an indicator of health (Campbell 1995). Differential leukocyte counts are also useful in multivariate analysis in terms of their relationship to functional measures of immunity such as microbial killing. Heterophils and eosinophils mediate innate immunity against novel pathogens and are important phagocytes, monocytes link innate and acquired defence, and lymphocytes mediate pathogen specific antibody and cell-mediated responses of the acquired immune system (Campbell 1995). The complement cascade and natural antibodies link innate and acquired immunity and provide the first line of defense against spreading infections, including viruses (Ochsenbein and Zinkernagel 2000).

Given differences between captive and wild conditions, the question of whether or how captivity affects immune function is important to put experiments conducted on captive animals into better ecological perspective and to provide insight into the relationship between environmental context and immunity. Our study investigates whether differences in immune function exist between captive and free-living red knots *Calidris canutus* (Linnaeus 1758) by comparing birds recently taken into captivity (< 1 year in captivity) with free-living birds. We consider two predictions: first, more costly immune strategies in captive birds because they have regular access to food and are potentially released from trade-offs; or second, down-regulated immune function in captive birds because they are exposed to fewer or a smaller variety of pathogens.

MATERIALS AND METHODS

Birds and sampling

Red knots are medium-sized shorebirds (100 to 200 g) that acclimate well to captive conditions (Piersma and Ramenofsky 1998), and retain natural mass change and moult cycles in captivity (Jenni-Eiermann et al. 2002, Reneerkens et al. 2007). This indicates that any differences in immune function between captive and free-living knots should represent acclimation to captive conditions rather than cessation of normal physiological rhythms in captivity.

We studied a total of 27 red knots of the subspecies *C. c. islandica* (as determined using the criteria in Nebel et al. 2000), captured in the Dutch Wadden Sea (53ºN, 5ºE). Birds were ringed and weighed at capture and sexes were later determined using molecular techniques (Baker et al. 1999). Birds also were aged on the basis of plumage characteristics (Prater et al. 1977), and to minimize age effects, we used only adults

(older than two years) for both free-living and captive samples. Furthermore, because some measures of non-induced immunity can be affected by current infection, we assessed body mass, condition of the feathers, leukocyte counts, and the skin of the feet, carpal and keel bones for signs of inflammation (bumblefoot) to verify that birds (captive and free) were healthy.

Captive birds

Birds in the captive sample (n = 12) were captured in August and September 2004 (6 females, 3 males) or February 2005 (1 female, 1 male) and kept at the Royal Netherlands Institute for Sea Research (NIOZ). These birds were part of a larger study examining the effect of ambient temperature on immune function over the annual cycle (Buehler et al. 2008a). For this study we only consider data from birds in the variable temperature treatment, which was set up as a control group (ambient temperature mirroring outdoor conditions). The birds were housed in aviaries 1.5 m by 4.5 m by 2.3 m with smooth walls (no mesh) and a smooth floor that was constantly flushed with filtered salt water. A quarter of the aviary floor was covered by an artificial sand flat and also flushed by salt water, and a tray of fresh water was provided for drinking and bathing. The birds had free access to mud snails *Hydrobia ulvae* which can make up a large proportion of their natural diet (van Gils et al. 2003). We cleaned organic waste and flushed the aviary floors with fresh water daily. At weekly intervals, we removed the birds for weighing and moult score while the aviaries were disinfected using bleach.

Free-living birds

We sampled free-living red knots (n = 15) in early September 2005 (7 females, 2 males) and late July 2006 (4 females, 2 males). The birds were captured in mistnets opened and monitored at night on the rising and falling tides.

Blood Sampling

Blood sampling of both captive and free-living birds occurred in the period corresponding to southward migration (July, August and September) to minimize seasonal differences between groups. Captive birds were sampled in July and August 2005 and samples were taken within 13 minutes of entering the aviary (mean ± SD = 5.5 ± 4.1). Free-living birds were sampled in September 2005 and July 2006, immediately upon removal from mistnets. We checked the nets every five to 15 minutes meaning that the absolute longest a bird could hang in a net was 15 minutes, thus all samples were taken within 20 min of the bird hitting the net (mean ± SD = 14.2 ± 4.8). Time-series experiments show no change in any of our immune indices within 20 min of capture (Buehler et al. 2008c) thus we assume that both of our samples represent baseline immune function. However, as a further precaution we also included the time between capture and blood sampling in our statistical analyses.

For both captive and free-living samples, we collected 300 to 600 μl blood into presterilized heparinized capillary tubes (Fisher Emergo) after sterilizing the area around the brachial vein with 70% ethanol. Immediately after sampling we made two blood

smears and the remainder of the blood was transported in sterilized plastic boxes to the laboratory and processed within an hour of sampling.

Because blood samples in captive birds were taken during the day and free-living birds were caught at night, we ran a repeated measures study (analysed using GLM to account for covariates) in which we sampled nine birds (5 females, 4 males) at 10:30 and 02:30, times which represented our captive (10:38 ± 5 min) and free-living (02:00 ± 85 min) sampling times. To avoid resampling the birds on the same day, we followed a rotating design in which each bird was sampled once a day and then allowed a day of recovery. Immune function did not differ with time of sampling (Appendix 1), which is not surprising since red knots can be active at night in captivity (T. Piersma, unpublished data) and are known to feed at night in the wild (e.g. Sitters et al. 2001, van Gils et al. 2005c, Leyrer et al. 2006, Rogers et al. 2006).

Measuring immune function

Microbial killing capacity of whole blood

The microbial-killing assay is a functional measure of the capacity of blood to kill microorganisms *in vitro* and measures immunity integrated across circulating cell and plasma protein components. We use three microorganisms: *Escherichia coli*, a strain of gram negative bacteria; *Candida albicans*, a strain of yeast-like fungi; and *Staphylococcus aureus*, a strain of gram positive bacteria. The use of several microorganisms gives a broad understanding of microbial killing because their killing relies on different mechanisms. *S. aureus* and *C. albicans* are killed mainly by cells via phagocytosis (Millet et al. 2007), whereas *E. coli* killing relies mainly on soluble blood components (Merchant et al. 2003, Millet et al. 2007).

We followed the basic procedure outlined in Millet et al. (2007) and performed the assay in a sterile working environment (a dead air box equipped with a UV Air Cleaner, Base Clear BV, KI-L046-M). For both captive and free-living birds samples were transported to the lab in pre-sterilized capillary tubes sealed with pre-sterilized clay in pre-sterilized Tupperware containers, and blood was processed within one hour of collection. Briefly, for each micro-organism we diluted the blood in CO_2-independent media (#18045-054, Invitrogen) and added 20 μl of microorganism suspension reconstituted from lyophilized pellets (*E. coli* ATCC # 8739, *C. albicans* ATCC #10231, *S. aureus* ATCC # 6538: MicroBioLogics, St Cloud, MN) to a concentration of approximately 200 colonies per 75 μl of diluted blood–bacteria mixture. The mixture was incubated at 41° C (*E. coli*: 10 min., *C. albicans*: 60 min., *S. aureus*: 120 min.) and 75μl was spread onto agar plates in duplicate. The plates were stored upside down at 36° C, and the number of colonies per plate was counted the following day.

For all strains we calculated the microbial killing capacity as one minus the number of colonies on blood plates relative to the number of colonies on inoculate control plates (200 μl of media mixed with 20 μl of microorganism without incubation). We used the inoculate for our calculations because it reflects the initial situation at the time that the blood starts to act, mimicking the biology of a bird responding to a pathogen.

Leukocyte concentrations

Leukocyte concentrations provide a description of circulating cellular immunity. Blood smears were randomized and counted blind to treatment using the criteria in Campbell (1995) by a single observer (DMB). After staining (Giemsa Stain, Sigma-Aldrich, Germany) the smears were examined at 1000x magnification with oil immersion and the first 100 leukocytes were counted and classified as heterophils, eosinophils, lymphocytes or monocytes. Basophils were extremely rare (< 0.5%) and were therefore not included in the counts. In combination with the blood smears, we obtained leukocyte concentrations using the indirect eosinophil Unopette method (Campbell 1995) following the manufacturers instructions (No. 5877, Becton Dickinson).

Hemolysis-hemagglutination assay

Complement and other lytic enzymes lyse extracellular pathogens and work with natural antibodies to facilitate initial pathogen recognition and initiate acquired immune responses (Ochsenbein and Zinkernagel 2000). We performed the assay as described by Matson et al. (2005). Hemolysis reflects complement action from the amount of haemoglobin released from lysed rabbit red blood cell and the hemagglutination reflects the interaction between natural antibodies and the antigens on rabbit red blood cells. Hemolysis and hemagglutination were quantified by serial dilution. We placed 25 μl of plasma in the first and second rows of a 96-well plate and then from the second to the eleventh rows we performed ten 1:2 dilutions using Dulbecco's PBS (Mauck et al. 2005). We then added 25 μl of 1% of rabbit red blood cell suspension to each well, and incubated the plates at 37°C for 90·min. After incubation plates were tilted at a 45° angle and then digitally scanned (Epson Perfection 4990 scanner) for agglutination after 20 min and lysis after 90 min. The scans were randomized with respect to sample origin, plate, and location within the plate and were scored blindly by a single researcher (DMB) for lysis and agglutination using the criteria outlined in Matson et al. (2005).

Statistics

All data and residuals of parametric models were tested for normality using 1-sample Kolmogorov–Smirnov tests and histograms were examined visually. *E. coli* killing data were left-skewed and were squared to achieve normality. Leukocyte concentrations were right-skewed and were logarithmically (base 10) transformed. After transformation all variables conformed to normality.

Because our free-living birds were caught in September 2005 and July 2006 we examined each immune measure over three groups (captive, free-living 2005 and free-living 2006) coded as a fixed factor "captivity" in a general linear model. Where "captivity" was significant we used Tukey tests to determine the significance of group differences. We included sex in our models as a fixed factor (models including and excluding sex produced the same result), and body mass and time between capture and blood sampling as covariates. For microbial killing we also included the number of colonies on the inoculate control as a covariate. Covariates were sequentially removed from the models when not statistically significant.

To gain insight into the complex relationships among measures of immunity (Matson et al. 2006b) we performed a principle component analysis. We excluded total leukocyte concentrations from the analysis since they are the sum of the differential concentrations and thus caused problems with co-linearity. We used varimax rotation to maximize the contrasts of the variable loadings between factors, tested the saliency criteria for these loadings (Cliff and Hamburger 1967), and saved scores for components with eigenvalues > 1 for further analysis (Kaiser 1960).

We used SPSS 14.0 for all statistical comparisons. We report mean ± SD in the text and mean ± SE in the graphs.

RESULTS

Captive birds showed lower *S. aureus* killing ($F_{2,23} = 14.92$, $P < 0.001$), *C. albicans* killing ($F_{2,23} = 19.95$, $P < 0.001$), heterophil concentrations ($F_{2,24} = 7.59$, $P = 0.003$) and eosinophil concentrations ($F_{2,29} = 6.68$, $P = 0.006$) compared with free-living birds (Fig. 9.1). We found no statistically significant difference for total leukocyte count ($F_{2,24} = 1.72$, $P = 0.20$), lymphocytes ($F_{2,24} = 1.34$, $P = 0.28$) or monocytes ($F_{2,24} = 0.30$, $P = 0.74$; Fig. 9.1). For *E. coli* killing ($F_{2,23} = 6.14$, $P = 0.007$), hemolysis ($F_{2,24} = 22.47$, $P < 0.001$) and hemagglutination ($F_{2,24} = 6.32$, $P = 0.006$) differences between the free-living groups were larger than differences among captive and free-living groups (Fig. 9.1). Free-living individuals caught in 2005 killed more *E. coli* than those caught in 2006, but showed less lysis and agglutination.

The principal component analysis identified three PCs with eigenvalues > 1 that cumulatively accounted for 77% of the total variation. The patterns of loadings on these PCs, revealed that *S. aureus* killing, *C. albicans* killing, heterophil count and eosinophil count correlated with PC1 (29.6% of total variation) and lymphocytes and monocytes correlated with PC2 (24.9% of total variation; Table 9.1, Fig. 9.2A). Paralleling the univariate analysis, captive and free-living birds differed significantly on PC1 ($F_{2,24} = 16.68$, $P < 0.001$) with captive birds significantly lower (captive versus Sep05, $P < 0.001$, captive vs. Jul06, $P = 0.008$; Fig. 9.2B), but did not differ on PC2 ($F_{2,24} = 0.303$, $P = 0.74$; Fig 2b). *E. coli* killing, hemolysis and hemagglutination correlated with PC3 (22.0% of total variation; Table 9.1).

DISCUSSION

We found that captivity reduced certain measures of immune function, but not others, suggesting that birds may use different immune strategies, representing "optimum" immunity, in different environments (Schmid-Hempel and Ebert 2003). Here we discuss our results, by first considering the results of the principal component analysis, then by examining the costs and benefits of the immune measures reduced by captivity, and finally by proposing a hypothesis about why captive and free-living birds may use different strategies.

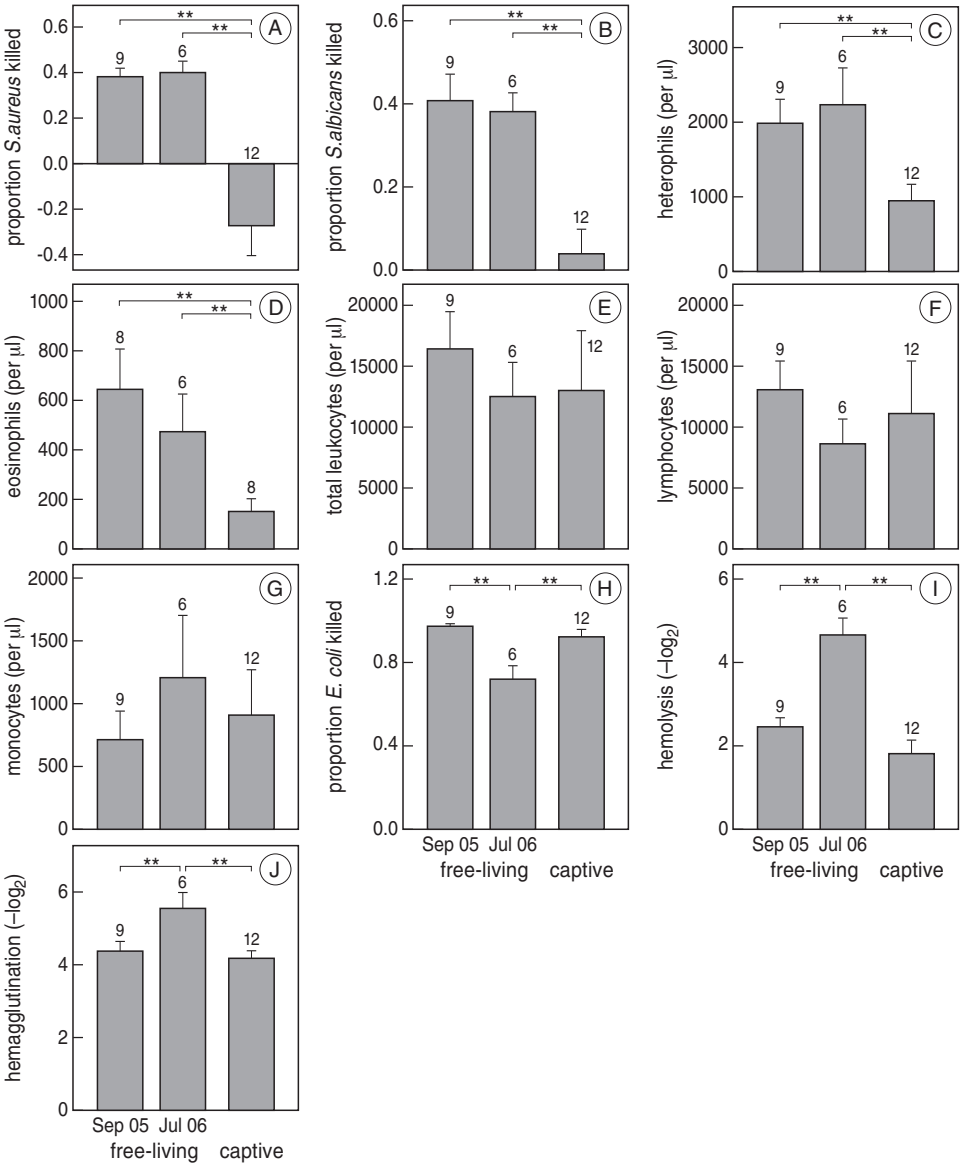

Figure 9.1. Immune function in captive versus free-living red knots. For *E. coli* killing and leukocyte concentrations statistics were performed on transformed values, but raw data are shown here for ease of interpretation. Bars indicate mean ± SE and sample sizes are indicated above the bars (lower for eosinophil count because zero values are not included). Significance from post-hoc Tukey tests is presented here as $P < 0.05$ (*) and $P < 0.01$ (**).

Table 9.1. Principal component loadings after varimax rotation. Bold faced loadings are the highest loading for a measure across the PCs and underlined loadings meet the saliency criteria for that PC.

Response	PC1	PC2	PC3
Microbial killing (proportion killed)			
S. aureus killing	**0.578**	0.491	0.373
C. albicans killing	**0.884**	-0.027	0.195
E. coli killing	0.174	-0.190	**-0.849**
Leukocyte parameters (per μl)			
Heterophils	**0.605**	0.561	0.361
Eosinophils	**0.809**	0.277	-0.067
Lymphocytes	0.208	**0.901**	-0.034
Monocytes	-0.009	**0.863**	0.075
Plasma parameters (log2)			
Hemolysis	0.492	0.047	**0.707**
Hemagglutination	0.462	-0.126	**0.664**
Totals			
Variance (%) per component	29.6	24.9	22.0
Cumulative variance (%)	29.6	54.5	76.6

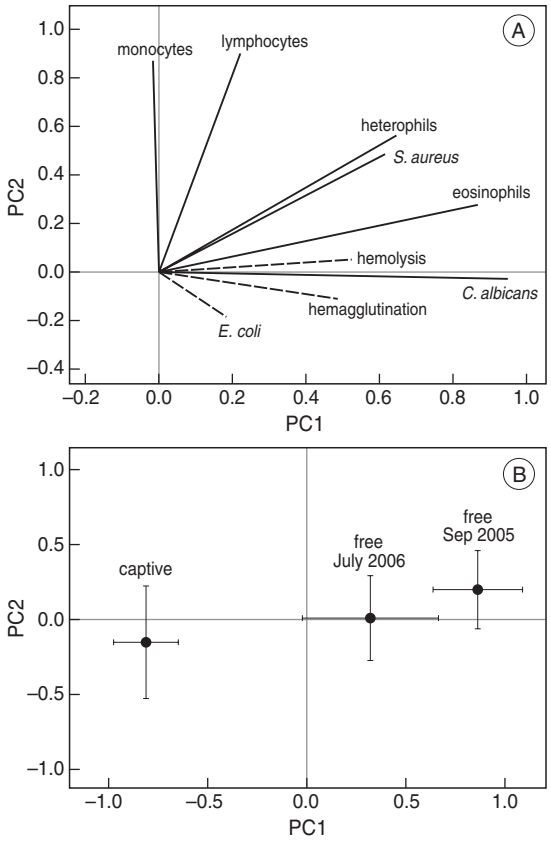

Figure 9.2. (A) Relationships between immune measures. The axes represent the first two components of a principal component analysis. Vectors are the loadings of each immune measure and the length of the vector indicates the strength of the relationship (the R2 value) or how much of the variation in an immune measure is explained by the two axes. Dashed vectors are measures best explained by PC3 (see Table 9.1 for component loadings). The angle between two vectors gives the degree of correlation between them. Adjacent vectors are highly correlated with each other, orthogonal (90°) vectors are uncorrelated, and vectors pointing in opposite directions (180°) are negatively correlated. (b) The groupings of principal component scores (captive birds, free-living birds caught in September 2005 and free-living birds caught in July 2006). For each group the mean ± SE of PC1 is plotted against the mean ± SE of PC2. Captive and free-living birds are distinguished on PC1 but not on PC2.

The groupings found in the principal component analysis closely match those found at both the individual level and within birds over the annual cycle in Buehler et al. (2008a). Our principal component analysis indicated that *S. aureus* killing, *C. albicans* killing, heterophil concentrations and eosinophil concentrations correlated with PC1 and were lower in captive than in free-living knots. Lymphocyte and monocyte concentrations correlated with PC2 and did not differ between captive and free-living knots. *E. coli* killing, hemolysis and hemagglutination correlated with PC3 and did not differ between captive and free-living birds, but separated free-living birds sampled in 2005 from those sampled in 2006, perhaps indicating environmental differences (e.g. food, temperature, rainfall) between years. *E. coli* killing differs from *S. aureus* and *C. albicans* killing because like hemolysis and hemagglutination, it relies mainly on soluble blood components rather than phagocytosis (Merchant et al. 2003, Millet et al. 2007).

The measures of immune function affected by captivity, *S. aureus* and *C. albicans*killing, heterophils and eosinophils, are associated with immediate and non-specific immunity. *S. aureus* and *C. albicans* killing are carried out mainly through phagocytosis (Millet et al. 2007) and heterophils are the most numerous phagocytic cells in birds (although thrombocytes and monocytes are also phagocytes, Campbell 1995). In terms of cost, heterophil phagocytosis may have high immunopathology costs due to the large amount of reactive oxygen and nitrogen species that they produce during particle ingestion (Splettstoesser and Schuff-Werner 2002). Energy costs may also be high due to the high turnover rates of heterophils (Janeway 2004). In terms of benefits, these measures are associated with protection from bacteria and yeast, pathogens with high replication rates and substantial damage potential if not stopped quickly. These measures of immune function were hypothesized by Buehler et al. (2008a) to represent an immune strategy necessary during periods of high pathogen pressure, but costly enough to warrant down-regulation when pathogen pressures are lower.

We considered two predictions regarding immune function in captive and free living red knots. First, more costly immune strategies in captive birds because they have regular access to food and a lower workload, potentially releasing them from trade-offs, and second down-regulated immune function in captive birds because they are exposed to fewer or a smaller variety of pathogens. In light of the immune cost and protective benefit discussion above, our results do not support the idea that captives are released from trade-offs, because immune function in wild birds reflected more costly strategies than in captive birds. However, our results do support the proposal that immune function may be down-regulated in captivity. In the wild, where encounters with bacteria, yeasts and other novel pathogens are high, the protective benefits of immediate, non-specific immune function might outweigh the costs, and immune function represented by PC1 might be favoured (Schmid-Hempel and Ebert 2003). This may be especially true during migration when large aggregations of birds make disease transmission easier (Altizer et al. 2006). However, in captivity where, at least with captive knots, cleaning regimes are likely to keep all but a few common diseases of captivity at bay, the costs of immediate, non-specific immune function might outweigh the benefits (Schmid-Hempel and Ebert 2003).

We put forth this idea as a hypothesis, but we acknowledge that this study does not fully test it since we did not measure pathogen pressure. Preliminary data examining pathogen pressure over a range of microbes in red knot habitats show about 135% more microbial colonies per gram mud in the Wadden Sea (n = 5) than in our cages (n = 4), and higher coliform density in the Wadden Sea (27.3 ± 30.4, n = 4) than in our cages (1.5 ± 1.5 colonies per Petrifilm^{3M}, n = 4; D. M. Buehler unpublished data). These data tentatively support the idea of higher pathogen pressure or pathogen diversity in the wild, but more data are needed.

Few studies have examined immune function in relation to pathogen pressure in captive and free-living animals. The studies we could find focused on nuisance diseases caused by captive conditions, which we argue would favour protection by induced and specific, rather than immediate and non-specific, immune function. For example, in chimpanzees *Pan troglodytes* the bacteria *Clostridium perfringens* was more prevalent in captive than in wild animals likely due to the high calorie and low fiber captive diet (Fujita and Kageyama 2007). In blue-fronted parrots *Amazona aestiva* antibody titers to *Salmonella pullorum* were higher in captive birds (Deem et al. 2005). Again this is not surprising as pet parrots in Bolivia are often in close contact with poultry that carry *Salmonella* and their higher antibody titers indicate that the disease is being fought mainly by specific immune function.

An alternative explanation for our results is the more conventional idea that captive birds are stressed and have depressed immune systems, However, we feel that this alternative is unlikely for red knots because they acclimate well to captive conditions (Piersma and Ramenofsky, 1998) and retain natural cycling of corticosterone in captivity (Piersma et al. 2000b).

We examined immune function using indices that could be taken from a single capture, thus we could not measure induced responses. Therefore, we encourage further research examining induced measures (e.g. specific antibody titres, acute phase responses) to explore differences in immune function between captive and free-living animals. Furthermore, we suggest further study of immune function using animals kept under different captive conditions, or the manipulation of pathogen pressure in captivity to build upon the ideas discussed here.

ACKNOWLEDGEMENTS

We thank Daliborka Barjaktarov, Nina Bhola, Alberto Castillo and Steven Haan for help in the lab, Judith van Bleijswijk for providing lab space, Daliborka Barjaktarov, François Vézina, Magali Petit, Anne Dekinga, Bernard Spaans and the crew of the H. M. Navicula for support in the field, and Maarten Brugge for help with the captive knots. Arne Hegemann, Nick Horrocks, Kevin Matson, Luisa Mendes and several anonymous reviewers gave valuable comments on this article. Funding came from the Natural Sciences and Engineering Research Council of Canada (NSERC), the University of Groningen and the Schure-Beijerinck-Popping Fonds to DMB, the Netherlands Organization for Scientific Research (NWO) and the University of Groningen to TP and BIT, and the Royal Netherlands Institute for Sea Research (NIOZ) to TP.

Appendix 9.1. Summary of immune function measured at 10:30 and 02:30. Statistics were performed on transformed values for leukocyte measures but raw means are shown here for ease of interpretation. Eosinophils were very rare and are thus excluded from the analysis.

	Mean ± SD		Statistics	
Variable	10:30	2:30	$F_{1,8}$	*P*
Microbial killing *S. aureus* (proportion killed)	0.17 ± 0.34	0.07 ± 0.14	0.67	0.44
Microbial killing *C. albicans* (proportion killed)	0.15 ± 0.13	0.03 ± 0.14	3.93	0.08
Microbial killing *E. coli* (proportion killed)	0.51 ± 0.39	0.58 ± 0.33	0.44	0.53
Total leukocytes (per μl)	10745.4 ± 9856.3	13291.7 ± 8710.1	0.44	0.53
Heterophils (per μl)	988.1 ± 600.9	1089.5 ± 569.3	0.25	0.63
Lymphocytes (per μl)	9243.3 ± 9690.4	11374.5 ± 7966.8	0.41	0.54
Monocytes (per μl)	463.7 ± 242.7	763.4 ± 657.8	0.89	0.37
Thrombocytes (per μl)	22853.3 ± 22279.0	25563.9 ± 23990.8	0.36	0.56
Hemolysis (-log2)	3.10 ± 1.16	3.22 ± 0.56	0.33	0.58
Hemagglutination (-log2)	5.40 ± 0.82	5.33 ± 0.35	0.03	0.86
H:L ratio	0.17 ± 0.13	0.14 ± 0.09	0.16	0.70
Hematocrit (proportion RBC)	0.46 ± 0.05	0.45 ± 0.04	0.14	0.72
Young RBCs (proportion in 5000 RBCs)	0.04 ± 0.02	0.04 ± 0.01	0.94	0.36

CHAPTER 10

Increasing immune indices in Red Knots suggest high pathogen pressure during stopover in Delaware Bay

Deborah M. Buehler, B. Irene Tieleman and Theunis Piersma

ABSTRACT

Each year thousands of shorebirds use Delaware Bay, USA, as a final stopover before migration to breeding areas. The bay provides them with an abundant food source, horseshoe crab (*Limulus polyphemus*) eggs, which they use to gain mass very rapidly. However, because the birds feed in dense mixed-species flocks, which may facilitate pathogen transmission, pathogen pressure in Delaware Bay may be high. We therefore predicted increased immune indices in birds over the course of stopover. To test this, we measured constitutive immune function in Red Knots (*Calidris canutus*) during stopover in Delaware Bay. We found lower total leukocytes, complement mediated lysis and haptoglobin in new arrivals (<133 grams) than in fuelling birds (≥ 133 grams). This result supports the idea of high pathogen pressure in Delaware Bay and suggests that fuelling birds have an increased rate of infection or are up-regulating of immune indices in response to pathogen pressure.

Submitted

INTRODUCTION

Each year millions of shorebirds migrate through a small number of highly productive staging areas before the last leg of their journey to breeding areas (van Gils et al. 2005a). These sites provide important food resources, but may also have high pathogen pressures. Delaware Bay, USA is one such stopover, where birds rapidly gain mass by feeding on an abundance of horseshoe crab (*Limulus polyphemus*) eggs (Castro and Myers 1993, Haramis et al. 2007). However, from a pathogen pressure perspective, Delaware Bay may be high risk for shorebirds. In the bay, birds feed in dense mixed-species flocks of Red Knots (*Calidris canutus*), Sanderlings (*Caldiris alba*), Semipalmated Sandpipers (*Calidris pusilla*), Ruddy Turnstones (*Arenaria interpres*) and Laughing Gulls (*Larus atricilla*; Botton et al. 1994). On bay beaches, flocks are so dense that it is common for birds to be feeding on substrate covered with the feces of other birds (D. M. Buehler pers. obs.). Thus, the spread of diseases, especially those with fecal-oral transmission, may be high (Altizer et al. 2006). As an example, avian influenza has been detected in both shorebirds and gulls in Delaware Bay (Krauss et al. 2007).

To capitalize on their well described stopover ecology, we focused on Red Knots in this research. Knots from different wintering populations use the Delaware Bay area, and stable isotope analysis indicates that shorter distance migrants eat mussels on the Atlantic coast; whereas longer distance migrants feed on crab eggs within the bay (Atkinson et al. 2006). We captured birds on bay beaches, thus our samples likely contain knots from stopover sites in South America. There, in contrast to dense mixed-species flocks in Delaware Bay, knots feed in single-species flocks dispersed over large areas of restinga (González et al. 1996). Red Knots arrive in Delaware Bay exhausted after migrations of over 8000 km (Piersma et al. 2005), and must achieve weights of at least 180 grams by late May or early June in order to reach the Arctic on time and with sufficient stores to breed successfully (Baker et al. 2004, Morrison et al. 2005). During migration, knots first use fat stores and then cross a "breakpoint" and begin protein catabolism (van der Meer and Piersma 1994). At stopover sites, this process is reversed and birds first recover protein before depositing fat. Atkinson et al. (2007) model the relationship between the total mass of an individual and whether it is depositing protein or fat. They find that newly arrived birds under 133 grams are recovering protein and gain very little fat (15%), whereas birds over 133 grams gain approximately 84% fat. In this way, body mass indicates a Red Knot's progression during stopover.

Given the high disease potential in Delaware Bay we predict increasing immune function over the course of the stopover, such that newly arrived birds, which are still recovering protein, should have lower immune indices than birds that have been in the bay longer and are depositing fat. Increased immune indices in wild birds, with unknown health status, can mean that birds are fighting a current infection or that they have up-regulated immune function to avoid infection in an environment with high pathogen pressure. We measure constitutive (non-induced) immune function because it represents the birds' first line of defense and is likely the most important defense during short stopovers where there is not enough time to mount an acquired response (Schmid-Hempel and Ebert 2003). Furthermore, it does not require keeping birds in

captivity or recapturing birds during this very sensitive time in their migration. Specifically we measured complement and natural antibody levels (Matson et al. 2005), haptoglobin concentrations (Matson 2006) and leukocyte concentrations (Campbell 1995).

MATERIALS AND METHODS

Birds

As part of an ongoing monitoring program in Delaware Bay, birds were captured using cannon nets between 16 and 28 May 2007. At capture biometrics were taken and birds were banded, weighed and aged as adults based on plumage characteristics (Prater et al. 1977). Sexes were later determined using molecular techniques (Baker et al. 1999). A total of 108 birds were caught (63% male, 37% female); however, one bird escaped before body mass was taken and for two others we were unable to collect a sufficient volume of blood (n = 105).

Stopover progression

Our data do not tell us exactly how long a given bird has been in Delaware Bay. However, the dynamics of fueling can be used as an indicator of a bird's stopover progression. Newly arrived individuals weighing less than 133 grams are recovering protein, whereas individuals weighing more than 133 grams are gaining mostly fat (Atkinson et al. 2007). This difference in the physiology of fuelling gives an indication of how long a bird has been in the bay. Thus, we used body mass to categorize birds into a protein recovery group (< 133 grams) and a fuel storage group (≥ 133 grams).

Blood sampling

We collected 200 to 400 μl of blood into heparinized capillary tubes (Fisher Emergo) after sterilizing the area around the brachial vein with 70% ethanol. To obtain baseline values for leukocyte concentrations, blood samples used for this assay were always taken within 25 min (mean ± SD = 14.8 ± 5.5 min.) of cannon net firing (first stress for the birds). Time-series experiments show no change in leukocyte counts within 30 min of capture (Buehler et al. 2008c). Immediately after sampling we made two blood smears and the remainder of the blood was stored on ice and transported to the laboratory. Blood samples not used for leukocyte analysis were taken within two hours of capture (73.0 min ± 51.8 min.). Complement and natural antibody titers are insensitive to capture and handling times up to two hours (Buehler et al. 2008c). Because the sensitivity of haptoglobin has not been tested, we included time between capture and sampling as a covariate in all statistics with haptoglobin as the response variable. Plasma was obtained by centrifuging blood samples for 10 min at 12000 x g. The plasma was stored at -20°C in Delaware, transported frozen and stored in The Netherlands at -80°C until processing.

Immune Assays

Leukocyte concentrations

Leukocyte concentrations provide information on circulating immune cells and current infection (Campbell 1995). After staining (Giemsa Stain, Sigma-Aldrich, Germany) blood smears were examined at 1000X magnification under oil immersion and the first 100 leukocytes were counted and classified as heterophils, eosinophils, lymphocytes or monocytes. Basophils were extremely rare (< 0.5%) and were not included in the counts. Eosinophils were included in the counts, but because they had a high proportion of zero values were excluded from further analysis. While counting the first 100 leukocytes, thrombocytes were also recorded as an estimate of the relative number of thrombocytes per leukocyte. Blood smears were randomized and counted blind to stopover progression by a single observer (DMB) using the criteria in Campbell (1995) Total leukocyte concentrations were obtained in combination with the blood smears using the indirect eosinophil Unopette method (Campbell 1995) following the manufacturers instructions (No. 5877; Becton Dickinson). Sample sizes for total leukocyte concentrations are smaller than for other assays due to the need to sample birds within 25 min of capture (n = 38).

Hemolysis-hemagglutination assay

The complement cascade and natural antibodies provide a first line of defense against spreading infections via cell lysis, and link innate and acquired immunity (Ochsenbein and Zinkernagel 2000). The amount of haemoglobin released from lysed rabbit red blood cells (hemolysis) indicates complement action and the agglutination of rabbit red blood cells indicates natural antibody activity. Following the procedure outlined in Matson et al. (2005), we placed 25 μl of plasma in the first and second rows of a 96-well plate and then from the second to the eleventh rows we performed ten 1:2 dilutions using Dulbecco's PBS (Mauck et al. 2005). We then added 25 μl of 1% of rabbit red blood cell suspension to each well, and incubated the plates at 37°C for 90 min. After incubation we tilted the plates 45° and then digitally scanned (Epson Perfection 4990 scanner) them for agglutination after 20 min and lysis after 90 min. The scans were randomized and were scored blindly by a single observer (DMB) for lysis and agglutination using the criteria outlined in Matson et al. (2005).

Haptoglobin assay

Haptoglobin is an acute phase protein that binds iron (haem) to keep it from providing nutrients to pathogens (Delers et al. 1988). Haptoglobin was quantified from blood plasma following the 'manual method' instructions provided with a commercially available assay kit (#TP801; Tri-Delta Diagnostics, Inc., Morris Plains, NJ). Sample sizes for haptoglobin are smaller than for complement and natural antibodies because we did not have enough plasma to conduct the assay for five birds (n = 100).

Statistical analyses

We used general linear models or non-parametric tests to examine the effect of stopover progression (protein recovery or fuel storage) on immune function. We

included sex in our models as a co-factor (sex was never statistically significant and models including and excluding sex produced the same result) and time between capture and blood sampling as a covariate for haptoglobin concentrations. Covariates were removed from models where $P > 0.05$. Haptoglobin concentrations were logarithmically (base 10) transformed to achieve normality of the data and model residuals. Agglutination and lysis were not normally distributed and transformation did not improve the situation so we show the results of both parametric and nonparametric models. Leukocyte data and model residuals were normally distributed; however, we show the results of both parametric and nonparametric models as count data are often skewed. We used SPSS 14.0 for statistical tests and report mean ± SD in the text.

RESULTS

We found lower total leukocytes, complement mediated lysis and haptoglobin in birds recovering protein than in fuelling birds (Fig. 10.1A to C, Table 10.1). Natural antibody mediated agglutination did not differ between protein recovery and fuel storage (Fig. 10.1D, Table 10.1). Like total leukocytes, heterophils, lymphocytes, monocytes and thrombocytes all showed the same pattern of lower concentrations during protein recovery, but these trends did not reach statistical significance (Fig. 10.2, Table 10.1).

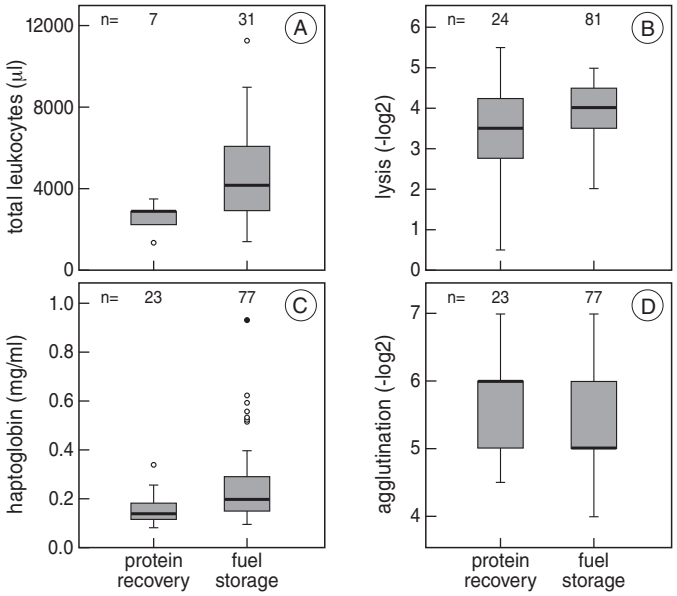

Figure 10.1. Total leukocyte concentrations (A), lysis (B) and haptoglobin (C) were lower during protein recovery than during fuel storage. Agglutination (D) did not differ during the two phases of stopover progression. Box plots show the median (thick line), interquartile range (boxes), range (whiskers), outliers (open dots) and extremes (black dots). See Table 10.1 for statistics.

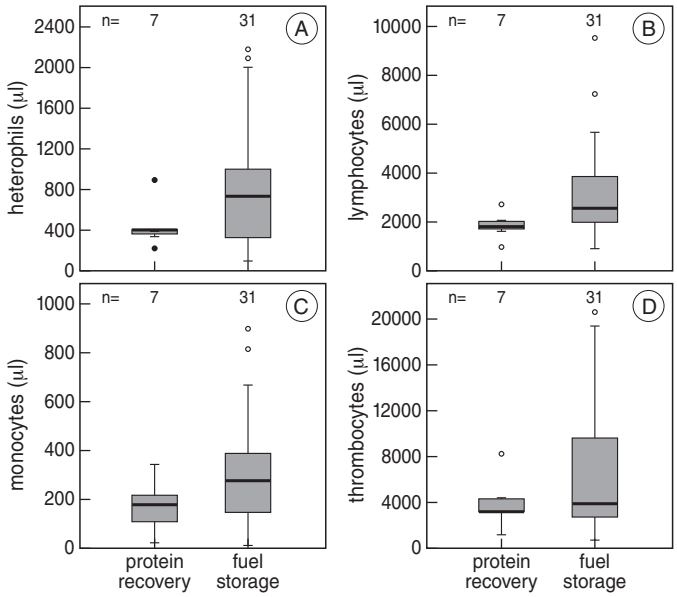

Figure 10.2. Heterophils (A), lymphocytes (B), monocytes (C) and thrombocytes (D) also show a trend for lower concentrations during protein recovery than during fuel storage. Box plots show the median (thick line), interquartile range (boxes), range (whiskers), outliers (open dots) and extremes (black dots). See Table 10.1 for statistics.

DISCUSSION

Constitutive immune function increased from protein recovery to fuel storage in Red Knots during stopover in Delaware Bay. This general increase in leukocytes, haptoglobin and complement suggests an increased rate of actual infection or an up-regulation of immune indices to protect against infection in an environment with high pathogen pressure. It may also suggest that birds arrive with depleted constitutive immunity, which they need to rebuild while fueling. Although we do not have the data to tease these possibilities apart, it is clear that birds that have progressed further in their fueling, and have likely been exposed to pathogen pressure in Delaware Bay longer, have higher constitutive immunity. High levels of constitutive immune function during the period of spring migration have also been found in captive Red Knots that do not actually migrate and have *ad libitum* access to food (Buehler et al. 2008a). Our data suggest that this increase in captivity might indicate that captive birds bolster immune function in anticipation of high pathogen pressure in the wild.

The lack of difference in natural antibody titers between protein recovery and fuel storage is not surprising. Natural antibodies are unique among the immune indices measured in this study in that they are not plastic over the annual cycle (Buehler et al. 2008a). Furthermore, natural antibody levels do not seem to be affected by current

Table 10.1. Statistical tests for differences in immune indices between protein recovery and fuel storage. Statistics were performed on log 10 transformed haptoglobin values. For all other variables the results of both parametric and non-parametric analyses are shown. Significance at the $P < 0.05$ level is shown in bold and trends where $0.1 > P > 0.05$ are shown in italics.

Response	Parametric GLM			Non-parametric tests			
	df	*F*	*P*	*U*	*W*	*z*	*P*
Total leukocytes (per μl)	1,37	5.24	**0.03**	41.5	69.5	-2.52	**0.01**
Heterophils (per μl)	1,37	2.70	0.11	68.0	96.0	-1.53	0.13
Lymphocytes (per μl)	1,37	3.94	*0.06*	45.0	73.0	-2.39	**0.02**
Monocytes (per μl)	1,37	2.37	0.13	69.0	97.0	-1.49	0.14
Thrombocytes (per μl)	1,37	1.73	0.20	85.0	113.0	-0.88	0.39
Lysis (-log2)	1,104	4.95	**0.03**	756.0	1056.5	-1.67	*0.09*
Agglutination (-log2)	1,104	1.73	0.20	827.0	4148.0	-1.22	0.22
Haptoglobin (mg/ml)	1,99	6.20	**0.01**	transformed and covariate			

infection (Matson et al. 2005). Therefore they are not likely to increase as a result of actual infection or in response to pathogen pressure.

An intriguing detail of our data is a marked increase in the variability of leukocyte counts and haptoglobin between birds under 133 grams and those above 133 grams. This variability might indicate differences in individual quality in birds using Delaware Bay. High quality birds may be able to up-regulate immune function to higher levels or, conversely, low quality birds may have higher scores because they are sicker. Either way, this increased variability during fueling suggests individual differences in the ability of birds to resist infection. These quality differences may be linked to arrival time and the need for late arriving individuals to "catch up" (Baker et al. 2004, Atkinson et al. 2007). Late arriving birds may trade-off immune function for fuel storage since there are negative fitness consequences associated with leaving Delaware Bay below 180 grams (Baker et al. 2004). This trade-off is likely to be even more extreme in years with insufficient crab eggs such as 2000, 2003 and 2005 (Atkinson et al. 2007) when birds are crowded into even denser flocks because fewer of the bay's beaches contain eggs (N. Clark, pers. comm.). Larger datasets with data from early and late arriving birds will allow closer examination of this variability.

ACKNOWLEDGEMENTS

We thank Alberto Castillo for invaluable help in the field and lab; the British Trust for Ornithology and the Delaware National Estuaries Program (especially Kevin Kalasz and Karen Bennet) for logistical support; Oliver Haddrath for the molecular sexing and Dick Visser for finalizing the figures. Funding was provided by the Natural Science and Engineering Research Council of Canada (NSERC) PGSB-267701-2003, a University of Groningen Ubbo Emmius Scholarship, a Netherlands Organization for Scientific Research (NWO) travel grant, and Schure-Beijerinck-Popping Fonds to DMB, NWO and the University of Groningen to TP and BIT, and the Royal Netherlands Institute for Sea Research to TP.

CHAPTER 11

Age and environment affect constitutive immune function in red knots (*Calidris canutus*)

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ABSTRACT

We studied age and environmental effects on constitutive immune function (natural antibody and complement levels, haptoglobin and leukocyte concentrations) in red knots (*Calidris canutus*). We compared *C. c. islandica* and *C. c. canutus* in the Wadden Sea and found no difference in immune function. However, *C. c. canutus* on their wintering grounds in Banc d'Arguin had higher natural antibody and lower complement levels than *C. c. canutus* in the Wadden Sea. This suggests that immune function is determined more by the surrounding environment than by subspecies. We also compared age classes in the Wadden Sea and found that first year birds had significantly lower natural antibody levels than adults, but that second year birds no longer differed from adults. Finally, we examined the interaction of age and environment in Banc d'Arguin. We found that first year birds (but not adults) in a low quality habitat had higher leukocyte concentrations than first year birds or adults in a high quality habitat. Variation in immune function between different sites likely reflects tradeoffs between available resources and defence needs in different environments, as well as differences in individuals differentially distributed within sites. Future studies, which examine these factors on wild birds, will be important for our understanding of how animals function in their natural environment.

Submitted

INTRODUCTION

Animals must survive in environments which differ in resources, resource demands and possibly pathogen risk. These environments change over time, either because seasons change within an environment, or because animals migrate between environments. Furthermore, individuals may be differentially distributed among environments (i.e. on the basis of quality or age), and may differ in prior experience (i.e. recent migration) and future needs (i.e. continued migration or moult). All of these factors may affect immune function or current infection status. Thus, spatial and temporal variation in immune function likely reflect trade-offs between available resources and defence needs in different environments, differences in individuals within sites, and interactions between these factors.

Red knots provide a good model system to examine spatial and temporal variation in immune function (Buehler and Piersma 2008), and in this study we focus on the *C. c. islandica* and *C. c. canutus* subspecies. *C. c. islandica* breed in northern Greenland and northeast Arctic Canada and winter in western Europe, whereas *C. c. canutus* breed on the Taymyr Peninsula in central Siberia and winter in West Africa (Piersma 2007). The *C. c. islandica* and *C. c. canutus* flyways overlap in the Wadden Sea during southward migration.

Overlap in the use of the Wadden Sea provides a unique opportunity to compare different subspecies in a common environment, allowing us to examine the contributions of subspecies and environment to immune function. *C. c. islandica* and *C. c. canutus* experience the same conditions in the Wadden Sea, and have bred in similar Arctic areas and migrated similar distances from the breeding grounds before arrival (Piersma et al. 2005). After this period of overlap, however, *C. c. canutus* continue migration to wintering areas in Banc d'Arguin, while *C. c. islandica* remain in western Europe. These two environments differ in many respects including food quantity and quality (Piersma et al. 1993a; Zwarts et al. 1990) and climate factors such as temperature and salinity (Wolff and Smit 1990) which affect thermoregulatory costs (Wiersma and Piersma 1994) and may affect pathogen pressure. Thus, if the immediate environment is a dominating factor in the determination of immune function, we do not predict differences in immune indices between subspecies in the Wadden Sea; however, we do predict differences within *C. c. canutus* between the Wadden Sea and Banc d'Arguin.

The situation in the Wadden Sea also provides an opportunity to compare different age classes in a common environment. Knots of different ages have had different past experiences, and first year birds may still be developing their immune system. First year birds arrive in the Wadden Sea at about two months of age, having just completed their first migration (Piersma and Davidson 1992). Second year birds are one year old and have not migrated, but rather over-summer in the Wadden Sea (T. Piersma pers. obs.). Adults are at least two years old and have just reproduced and migrated. If recent migration (or reproduction) has an effect on immune function, then we predict differences between second year birds and adults; and if immune system development is not yet complete at two months of age, then we predict differences between first year birds and other age classes.

The Banc d'Arguin also provides an opportunity to look at age and environment interactions and their relationship with immune function. Within Banc d'Arguin, knots of the *C. c. canutus* subspecies segregate into two roosting and feeding areas: Ebelk Aiznay and Baie d'Aouatif (see map in Fig. 11.3). Despite the close proximity of these sites, knots show high fidelity to these areas (Leyrer et al. 2006a). The way that birds segregate between the sites, as well as survival data, indicate differences in habitat quality. Adults, which arrive in Banc d'Arguin earlier than first year birds, have first choice of habitat and make up a larger proportion of the birds at the Ebelk Aiznay site and a smaller proportion of the population at Baie d'Aouatif (Leyrer et al. 2006b). Furthermore, from 2003 to 2008, annual adult survival in Ebelk Aiznay averaged 0.88 ± 0.01 percent (mean ± SE), which translates to an average lifespan of 7.9 years, whereas annual survival in Baie d'Aouatif was 0.77 ± 0.05 percent and lifespan was only 3.9 years (B. Spaans et al., unpublished data).

The immune system can be divided along an innate (non-specific) and an acquired (specific) axis, and along a constitutive (non-induced) and induced axis (Schmid-Hempel and Ebert 2003). In this study we focus on constitutive immune function because it represents a first line of defense that is important for migrants encountering novel environments and for young birds who have not yet built up acquired induced responses. Furthermore, levels of constitutive immune function can be measured from a single capture making it ideal for studies on free-living birds. Specifically we measured complement and natural antibody levels (Matson et al. 2005), haptoglobin concentrations (Matson 2006) and leukocyte concentrations (Campbell 1995). The complement cascade and natural antibodies provide a first line of defense against spreading infections via cell lysis, and link innate and acquired immunity (Ochsenbein and Zinkernagel 2000). Haptoglobin is an acute phase protein that binds iron (haem) to keep it from providing nutrients to pathogens (Delers et al. 1988). Leukocyte concentrations provide information on circulating immune cells and can be used as an indicator of health (Campbell 1995).

In this study we examine age and environmental effects on constitutive immune indices in two subspecies of red knot. Specifically we ask: How do constitutive immune indices differ between subspecies in the same environment and within subspecies in different environments? Do first year, second year and adult *C. c. islandica* differ in immune function in the Wadden Sea? Does habitat quality affect immune function in *C. c. canutus* in Banc d'Arguin and does this effect differ between first year birds and adults?

MATERIALS AND METHODS

Capture methods and samples

All birds in this study were captured at night using mist nets and were ringed, weighed and aged on the basis of plumage characteristics (Prater et al. 1977) at capture. Sexes were determined using molecular techniques (Baker et al. 1999). In the Dutch Wadden Sea (53°15'N, 5°15'E), a total of 202 birds (31% male and 69% female) were caught during fall migration (July to September) between July 2004 and July 2006. In Banc

d'Arguin, Mauritania (19°54'N, 16°17'W) a total of 80 birds (41% male and 59% female) were caught between 13 and 21 December 2006.

Subspecies and environment comparisons in adults

The subspecies *C. c. islandica* and *C. c. canutus* were classified based on resightings in other parts of the flyway (B. Spaans unpublished data), and on wing moult and body mass criteria outlined in Nebel et al. (2000). Both moulting and non-moulting *C. c. islandica* are found in the Wadden Sea; however, *C. c. canutus* use the area as a stopover on the way to wintering grounds and are not in wing moult. Thus, to examine subspecies effects we compared only non-moulting *C. c. islandica* adults (n = 27) with *C. c. canutus* adults (n = 21). To examine environmental effects we compared *C. c. canutus* adults captured in Banc d'Arguin (n = 62) with both *C. c. islandica* and *C. c. canutus* in the Wadden Sea. First year birds were not compared because we only had two C. c. canutus first year birds in the Wadden Sea.

Age comparisons in the Wadden Sea

We examined age effects in the Wadden Sea for *C. c. islandica* only. First year birds, second year birds and adults (older than 2 years) use the Wadden Sea during fall migration, but they follow different schedules. Adults arrive in the area and begin prebasic and wing moult earlier than first year birds (Davidson and Wilson 1992). Second year birds generally do not travel to the breeding grounds and follow an "over-summering" annual cycle in which they are in prebasic and wing moult when adults arrive on fall migration (T. Piersma pers. obs.). As a result, no first year birds, but all second year birds were moulting in our sample. Thus, to examine age effects in birds with similar moult status, we compared first year birds (n = 15,) with non-moulting adults (n = 27), and second year birds (n = 71) with moulting adults (n = 49).

Age and habitat quality comparisons in Banc d'Arguin

We captured first year and adult *C. c. canutus* at the high tide roosts in Ebelk Aiznay (first year n = 3, adults n = 28) and in Baie d'Aouatif (first year n = 15, adults n = 34; see map in Fig. 11.3). We combined birds caught on the sandbank Zira with the sample from the high tide roost at Baie d'Aouatif. Resighting data indicated that 57% of birds resighted in a different location than where captured had moved between Zira and Baie d'Aouatif (or vice versa; J. Leyrer, unpublished data), while only 14% had moved between Zira and Ebelk Aiznay. A detailed description of the study area in Banc d'Arguin is given in Leyrer et al. (2006a).

Blood Sampling

We collected 200 to 400 μl of blood into heparinized capillary tubes (Fisher Emergo) after sterilizing the area around the brachial vein with 70% ethanol. Blood samples used for leukocyte count analysis were taken immediately after the birds were removed from the mist nets. We checked the nets every five to 25 minutes meaning that the absolute longest a bird could hang in a net was 25 minutes, thus all samples were taken within 30 minutes of the bird hitting the net (mean ± s.d. = 14.2 ± 4.6 min). Time-

series experiments show no change in leukocyte counts within 30 min of capture (Buehler et al. 2008c). Immediately after sampling, we made two blood smears and the remainder of the blood was stored in eppendorf tubes on ice and transported to the laboratory to be used in other assays. Blood samples not used for leukocyte concentrations were taken within two hours of capture (86.6 ± 54.8 min). Complement and natural antibody titres are insensitive to capture and handling times up to two hours (Buehler et al. 2008c). Because the sensitivity of haptoglobin has not been tested, we included time between capture and sampling as a covariate in all statistics with haptoglobin as the response variable. Plasma was obtained by centrifuging blood samples for 10 min at 12000 x g and was stored at -20°C.

Immune Assays

Leukocyte concentrations

After staining (Giemsa Stain, Sigma-Aldrich, Germany) blood smears were examined at 1000X magnification under oil immersion and the first 100 leukocytes were counted and classified as heterophils, eosinophils, lymphocytes or monocytes. Basophils were extremely rare (< 0.5%) and were therefore not included in the counts. Eosinophils were included in the counts, but because they had a high proportion of zero values were excluded from further analysis. While counting the first 100 leukocytes, thrombocytes were also recorded as an estimate of the relative number of thrombocytes per leukocyte. Blood smears were randomized and counted blind to age and environment using the criteria in Campbell (1995) by a single observer (DMB). Total leukocyte concentrations were obtained in combination with the blood smears using the indirect eosinophil Unopette method (Campbell 1995) following the manufacturers instructions (No. 5877; Becton Dickinson). Sample sizes for total leukocyte concentrations are smaller than for other assays due to the need to sample birds within 30 minutes of capture.

Hemolysis-hemagglutination assay

We performed the assay on blood plasma as described by Matson et al. (2005). Complement action was assayed via the amount of haemoglobin released from lysed rabbit red blood cells and natural antibodies were assayed via the agglutination of rabbit red blood cells. Lysis and agglutination were quantified by serial dilution. We placed 25 μl of plasma in the first and second rows of a 96-well plate and then from the second to the eleventh rows we performed ten 1:2 dilutions using Dulbecco's PBS (Mauck et al. 2005). We then added 25 μl of 1% of rabbit red blood cell suspension to each well, and incubated the plates at 37°C for 90 minutes. After incubation plates were tilted at a 45° angle and then digitally scanned (Epson Perfection 4990 scanner) for agglutination after 20 minutes and lysis after 90 minutes. The scans were randomized with respect to sample origin, plate, and location within the plate and were scored blindly by a single researcher (DMB) using the criteria outlined in Matson et al. (2005).

Haptoglobin assay

Haptoglobin concentration in mg/ml was quantified from blood plasma following the 'manual method' instructions provided with a commercially available assay kit

(#TP801; Tri-Delta Diagnostics, Inc., Morris Plains, NJ). Sample sizes for haptoglobin concentrations are smaller than for complement and natural antibodies because we did not have enough plasma to conduct the assay on every bird.

Statistics

We used general linear models to examine the main effects of age (first year, second year or adult), subspecies (*C. c. canutus* or *C. c. islandica*), location (Wadden Sea or Banc d'Arguin), and habitat quality (high or low in Banc d'Arguin). We included sex in our models as a co-factor (sex was never statistically significant and models including and excluding sex produced the same result). We also included body mass as a covariate where it varied significantly between groups and may have contributed to immune effects. Finally, we included time between capture and blood sampling as a covariate for haptoglobin concentrations. Co-factors and covariates were removed from models where $P > 0.05$. All data and residuals of parametric models were tested for normality using 1-sample Kolmogorov–Smirnov tests and histograms were examined visually. Leukocyte and haptoglobin concentrations were logarithmically (base 10) transformed. Agglutination was not normally distributed and transformation did not improve the situation, thus we present the results of both parametric and non parametric models. We used SPSS 14.0 for all statistical comparisons.

RESULTS

Subspecies and environment comparisons in adults

To verify our classification of *C. c. islandica* and *C. c. canutus* in the Wadden Sea, we first examined bill length. Validating our classification, *C. c. islandica* bills were significantly shorter than *C. c. canutus* bills in both the Wadden Sea and Banc d'Arguin (Fig. 11.1A, $F_{1,109} = 10.9$, $P < 0.001$; Nebel et al. 2000). Because *C. c. canutus* are refuelling in the Wadden Sea, we also examined body mass and found that *C. c. canutus* in the Wadden Sea were significantly heavier than *C. c. islandica* or *C. c. canutus* in Banc d'Arguin (Fig. 11.1B, $F_{1,109} = 22.2$, $P < 0.001$). Body mass was therefore included as a covariate in the immune comparisons between these groups.

Both natural antibody mediated agglutination ($F_{1,109} = 4.94$, $P = 0.009$; Kruskal Wallis Test, Chi-square = 10.7, df = 2, $P = 0.005$) and complement mediated lysis ($F_{1,109} = 4.76$, $P = 0.01$) differed significantly between the three groups, but only between environments within *C. c. canutus*, not between subspecies in the Wadden Sea. Agglutination was higher and lysis was lower in *C. c. canutus* in Banc d'Arguin versus *C. c. canutus* and *C. c. islandica* in the Wadden Sea (Fig. 11.1 C and D). Sample sizes were not large enough for comparisons of haptoglobin or total leukocyte concentrations.

Age comparisons in the Wadden Sea: *C. c. islandica*

Natural antibody mediated agglutination was lower in first year birds than in adults (Fig. 11.2A, Table 11.1; Mann-Whitney U = 82, Wilcoxon W = 202, z = -3.31, $P = 0.001$), but complement mediated lysis did not differ (Fig. 11.2B, Table 11.1). Second year and

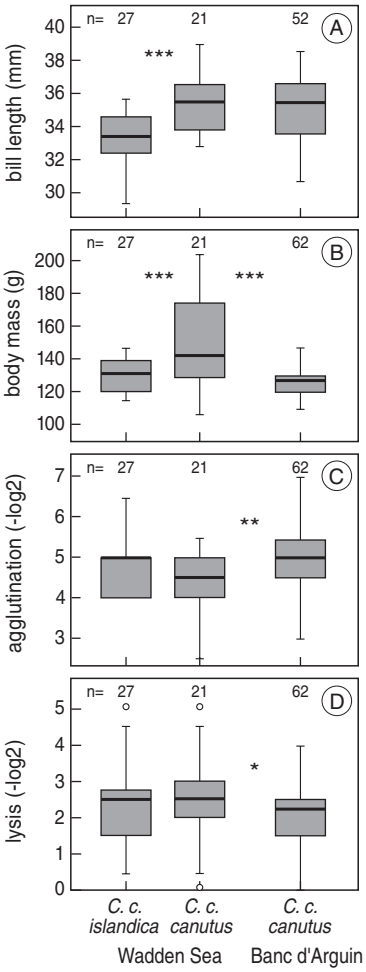

Figure 11.1. The relationship between constitutive immune indices for non-moulting adult *C. c. islandica* and *C. c. canutus* in the Wadden Sea and *C. c. canutus* in Banc d'Aguin: Bill length (A), body mass (B), agglutination (C) and lysis (D). Sample sizes were not large enough for comparisons of haptoglobin or total leukocyte concentrations. Box plots show the median (thick line), interquartile range (boxes), range (whiskers), outliers (open dots) and extremes (black dots). Statistical significance determined by Tukey post-hoc tests is indicated by *** for $P < 0.001$, ** for $P < 0.01$ and * for $P < 0.05$. See Table 11.1 for full statistics.

Table 11.1. Statistical tests of differences in immune indices between *C. c. islandica* age classes (first year versus non-moulting adults, second year birds versus moulting adults) in the Wadden Sea. Statistically significant results are shown in bold.

Response	First Year vs. Adults			Second Year vs. Adults		
	df	F	*P*	df	F	*P*
Hemagglutination (-log2)	1,41	11.9	**0.003**	1,119	3.93	0.14
Hemolysis (-log2)	1,41	0.29	0.59	1,119	0.14	0.23
Haptoglobin (mg/ml)	samples sizes too small			1,41	0.98	0.33
Total leukocytes (per μl)				1,23	2.26	0.15
Heterophils (per μl)				1,23	2.77	0.11
Lymphocytes (per μl)				1,23	2.83	0.11
Monocytes (per μl)				1,23	0.01	0.91
Thrombocytes (per μl)				1,22	0.51	0.48

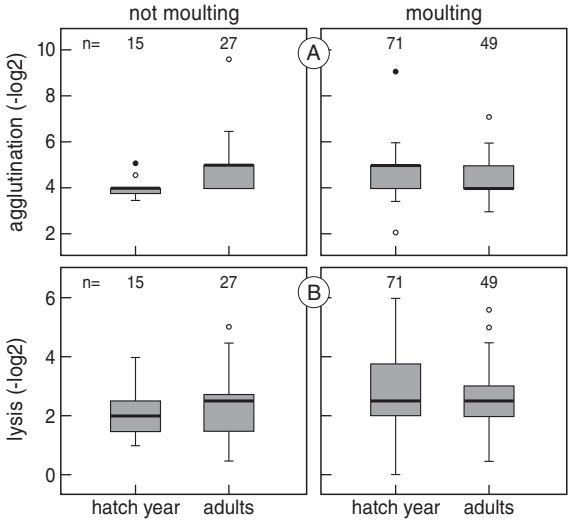

Figure 11.2. The relationship between constitutive immune indices and age for *C. c. islandica* in the Wadden Sea: Agglutination (A) and lysis (B). Box plots show the median (thick line), interquartile range (boxes), range (whiskers), outliers (open dots) and extremes (black dots). Statistical significance at $P < 0.01$ is indicated by **. See Table 11.1 for full statistics.

Table 11.2. Descriptive statistics for immune indices in second year and adult *C. c. islandica* in the Wadden Sea. See Table 11.1 for the results of statistical tests.

Response	Second Year (moulting)					
	n	Median	Mean	SD	Min	Max
Hemagglutination (-log2)	71	5.0	4.7	0.9	2.0	9.0
Hemolysis (-log2)	71	2.5	2.8	1.4	0.0	6.0
Haptoglobin (mg/ml)	25	0.13	0.20	0.19	0.03	0.76
Total leukocytes (per μl)	12	20640.0	20711.5	6595.0	10756.0	33440.0
Heterophils (per μl)	12	2165.7	2361.7	1529.0	668.8	5958.9
Lymphocytes (per μl)	12	15848.2	16982.3	6206.0	9680.0	30430.0
Monocytes (per μl)	12	594.8	703.5	422.2	107.6	1468.3
Thrombocytes (per μl)	11	12078.0	13199.1	9193.0	896.0	36250.0

Response	Adults (moulting)					
	n	Median	Mean	SD	Min	Max
Hemagglutination (-log2)	49	4.0	4.3	0.8	3.0	7.0
Hemolysis (-log2)	49	2.5	2.6	1.2	0.5	5.5
Haptoglobin (mg/ml)	17	0.11	0.12	0.1	0.02	0.38
Total leukocytes (per μl)	12	15805.1	16950.5	7723.0	7040.0	29920.0
Heterophils (per μl)	12	1784.4	2137.1	951.1	889.8	3991.4
Lymphocytes (per μl)	12	11531.3	13304.4	7047.5	5222.5	26329.6
Monocytes (per μl)	12	700.9	827.1	619.7	96.3	1809.3
Thrombocytes (per μl)	12	11810.6	11751.3	8072.3	1564.4	29920.0

adult *C. c. islandica* did not differ in any of the indices that we measured (Tables 11.1 and 11.2).

Age and habitat quality comparisons in Banc d'Arguin: *C. c. canutus*

There were no age or habitat main effects or interactions for natural antibody mediated agglutination, complement mediated lysis or haptoglobin (Fig. 11.3A to C, Table 11.3). However, first year birds had higher total leukocyte concentrations in Baie d'Aouatif than in Ebelk Aiznay (Fig. 11.3D, Table 11.3). This pattern was based mainly on heterophils and lymphocytes (Fig. 11.3E and F); whereas little effect was seen in monocytes or thrombocytes (Fig. 11.3G and H). First year birds showed a trend for higher monocytes irrespective of habitat (Table 11.3).

DISCUSSION

Subspecies and environment effects on immune indices

We found differences in immune indices between environments within *C. c. canutus*, but no differences between *C. c. islandica* and *C. c. canutus* while their flyways overlapped in the Wadden Sea (Fig. 11.1 C and D). This result suggests that constitutive immunity responds more strongly to differences in a bird's immediate environment than to genetic differences between the subspecies or to prior events. This result is not surprising given that knot subspecies are genetically similar (at least in terms of mitochondrial DNA; Buehler and Baker 2005) and that *C. c. islandica* and *C. c. canutus* breed and migrate in very similar environments prior to arrival in the Wadden Sea (Piersma et al. 2005). However, after the period of overlap in the Wadden Sea, *C. c. islandica* begin prebasic and wing moult, while *C. c. canutus* continue migration. Thus our results also suggest that *C. c. canutus* do not adjust immune indices in anticipation of future challenges (i.e. migration or different pathogen pressures in West Africa) while still in the Wadden Sea.

Table 11.3. Statistical tests of main effects and interactions for immune indices between first year and adult *C. c. canutus* in low and high quality habitats in Banc d'Arguin. Significance at the $P < 0.05$ level is shown in bold and trends where $0.1 > P > 0.05$ are shown in italics

Response	Habitat			Age			Age*Habitat		
	df	F	*P*	df	F	*P*	df	F	*P*
Hemagglutination (-log2)	1,79	0.85	0.36	1,79	1.36	0.25	1,79		
Hemolysis (-log2)	1,79	0.08	0.78	1,79	0.65	0.42	1,79	not in model	
Haptoglobin (mg/ml)	1,76	1.28	0.26	1,76	1.07	0.31	1,76		
Total leukocytes (per μl)	1,34			1,34			1,34	6.45	**0.02**
Heterophils (per μl)	1,34	see interaction		1,34	see interaction		1,34	7.38	**0.01**
Lymphocytes (per μl)	1,34			1,34			1,34	3.50	***0.07***
Monocytes (per μl)	1,34	0.56	0.46	1,34	3.91	***0.06***	1,34	not in model	
Thrombocytes (per μl)	1,34	0.82	0.37	1,34	0.10	0.76	1,34		

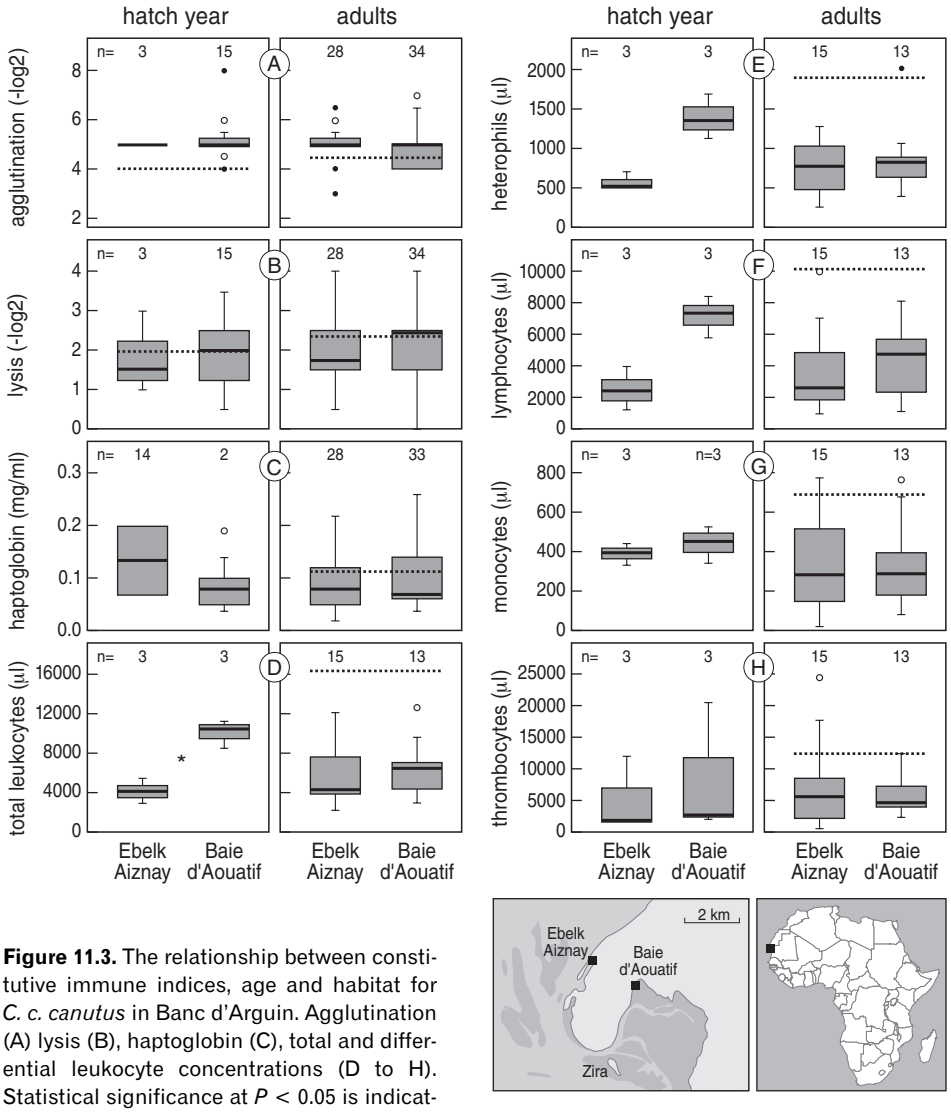

Figure 11.3. The relationship between constitutive immune indices, age and habitat for *C. c. canutus* in Banc d'Arguin. Agglutination (A) lysis (B), haptoglobin (C), total and differential leukocyte concentrations (D to H). Statistical significance at $P < 0.05$ is indicated by *. See Table 11.3 for full statistics. Box plots show the median (thick line), interquartile range (boxes), range (whiskers), outliers (open dots) and extremes (black dots). Dashed lines indicate median values for *C. c. islandica* in the Wadden Sea. Map details the Banc d'Arguin study site and its location in Africa.

Comparing between environments within *C. c. canutus,* we found higher natural antibodies and lower complement in Banc d'Arguin than in the Wadden Sea. However, limiting the comparison to *C. c. canutus* meant that we were not able to look at haptoglobin or total leukocyte concentrations due to low sample sizes. These indices can be examined if we compare *C. c. canutus* in Banc d'Arguin with moulting *C. c. islandica* in the Wadden Sea. Admittedly, these two groups differ in terms of subspecies and moult

as well as immediate environment. However, we argue that any trends are likely due to environmental effects given that *C. c. canutus* and *C. c. islandica* did not differ in natural antibodies or complement while in the same environment (Fig. 11.1), and that moulting and non-moulting *C. c. islandica* did not differ in natural antibodies (Fig. 11.2A; $F_{1,75} = 0.03$, $P = 0.86$; Mann-Whitney U = 160.5, Wilcoxon W = 538.5, z=-0.047, $P = 0.964$), complement (Fig. 11.2B; $F_{1,75} = 0.30$, $P = 0.59$) or haptoglobin ($F_{1,18} = 0.06$, $P = 0.81$). Comparing *C. c. islandica* in the Wadden Sea with *C. c. canutus* in Banc d'Arguin, we find that, as seen for complement, birds in the Banc d'Arguin show slightly lower haptoglobin (Fig. 11.3C; dashed line represents median for *C. c. islandica* in the Wadden Sea) and considerably lower leukocyte concentrations (Fig. 11.3D to H) than birds in the Wadden Sea. Thus overall, we see a trend for lower immune indices in Banc d'Arguin than in the Wadden Sea, with the exception of natural antibodies.

Natural antibody levels were higher in Banc d'Arguin than in the Wadden Sea. Natural antibodies are at the interface between innate and acquired immunity (Ochsenbein and Zinkernagel 2000). They differ from acquired antibodies in that they are present in the absence of exogenous antigenic stimulation (Ochsenbein et al. 1999), they have broad specificity (are able to bind to more than one antigen) but low affinity to antigens, they are secreted by B-1 rather than B-2 cells (Baumgarth et al. 2005) and they appear to confer little or no immunological memory (Janeway et al. 2004). Furthermore, natural antibody levels cannot be tied to current infection status as can leukocyte counts or haptoglobin (Matson et al. 2005), and they are unique among other indices measured in this study in that they are not plastic over the annual cycle (Buehler et al. 2008a). Therefore, after initial development, a natural antibody repertoire likely characterizes an individual. Furthermore, it is now thought that birds using the Wadden Sea are only a subset of the total *C. c. canutus* population wintering in Banc d'Arguin (T. Piersma et al. unpublished data). Taken together this suggests that the difference in natural antibody levels we find may represent differences among individuals measured in Banc d'Arguin and the Wadden Sea rather than adjustments within individuals to the environment. In contrast, the other indices likely represent a combination of among individual differences and within individual adjustments to the environment (see below).

Age effects on immune indices in *C. c. islandica* in the Wadden Sea?

First year birds had significantly lower natural antibody levels than adults (Fig. 11.2A). Like acquired antibody repertoires, natural antibody repertoires are developed early in life (Baumgarth et al. 2005; Janeway et al. 2004). Our results suggest that first year birds, which are only about two months old, are still developing their natural antibody repertoire. In chickens natural antibody levels increase rapidly between 20 days and 12 weeks of age (Matson et al. 2005; Seto and Henderson 1968). We also found that second year birds no longer differed from adults (Fig. 11.2A), which suggests that in red knots natural antibody development is completed within the first year of life. Indeed, natural antibody repertoire may be developed by about six months in red knots, since *C. c. canutus* first year birds did not differ from adults in Banc d'Arguin and their titres were higher than those of two month old *C. c. islandica*, but similar to those of adult

C. c. islandica (Fig. 11.3A). In contrast, complement requires no early repertoire development (Janeway et al. 2004), therefore, it is not surprising that we did not find differences complement between first year birds and adults.

The lack of differences in immune indices between second year birds, which oversummer in the Wadden Sea, and adults, which have just migrated, indicates that recent migratory flights may have little effect on immune function, at least after the birds have landed and refuelled.

Habitat quality and age effects on immune indices in *C. c. canutus* in Banc d'Arguin Age and environment had interactive effects in Banc d'Arguin. First year birds (but not adults) in Baie d'Aouatif had higher leukocyte concentrations than first year birds or adults in Ebelk Aiznay (Fig. 11.3D). Ebelk Aiznay and Baie d'Aouatif differ in many respects that might explain a difference in immune function between sites (see below). But why were only juveniles affected by these differences? High leukocyte counts may indicate current infection, thus juveniles in Baie d'Aouatif may be fighting disease while adults are not. *C. c. canutus* spend six months of the year in Banc d'Arguin, where they presumably encounter similar pathogens year after year, thus adults might possess immunity (i.e. acquired antibodies) against pathogens that juveniles are encountering for the first time. To test this hypothesis, we would need more information about specific pathogens infecting knots in Banc d'Arguin, antibody assays for those specific antigens and records of infection history.

Contributors to spatial variation in immune indices at the environmental and individual level

This study found significant spatial variation in immune indices (i.e. between the Wadden Sea and Banc d'Arguin, and between high and low quality habitats within Banc d'Arguin). The sites at which we sampled differ along two main lines that could contribute to the patterns we describe: features of the environment itself, and differences among individuals differentially distributed between sites.

Environments at our sampling sites differed in resource availability, resource demands and pathogen pressure, all of which could contribute to a birds' immune profile (Piersma 2006). Differences in food availability or energy demand affect the amount of resources a bird has to invest in their immune system. Food quantity and quality have differed historically in both the Wadden Sea and Banc d'Arguin (Piersma et al. 1993a; Zwarts et al. 1990) and today food is likely more abundant, though not necessarily higher quality, in Banc d'Arguin (T. Piersma unpublished data.). Ambient temperature also differs between the sites, affecting thermoregulatory costs, with birds in Banc d'Arguin spending less on thermoregulation (Wiersma and Piersma1994). The sites also differ in predation risk, affecting the amount of energy and time birds must spend avoiding predators. Though the situation may be changing, there are more predators in Banc d'Arguin (T. Piersma unpublished data). Finally, survival in environments with higher pathogen pressure likely requires a high investment in immune protection. Temperature and salinity differences between the Wadden Sea and Banc d'Arguin (Wolff and Smit 1990) may affect pathogen pressure between the sites; however, this has not yet been studied.

Similar factors likely affect the comparison between Ebelk Aiznay and Baie d'Aouatif within Banc d'Arguin. Food quality appears to be lower in Baie d'Aouatif (J. A. van Gils unpublished data), predation risk appears to be higher in Baie d'Aouatif (P. J. van den Hout and J. A. van Gils unpublished data) and pathogen pressure may differ as well, though that has yet to be studied.

The second major factor affecting immune function between sites is the fact that individuals of differing quality (or age) are differentially distributed between sites. For example, birds sampled in the Wadden Sea may represent only a subset of the total *C. c. canutus* population in Banc d'Arguin, thus differences we find may be affected by the fact that birds sampled in the different locations represent different individuals. Within Banc d'Arguin, lower quality individuals are likely found in the lower quality habitat at Baie d'Aouatif. Birds appear to select their roost site upon arrival in Banc d'Arguin and lower quality birds, which arrive late, likely get the last choice. The age and sex segregation of individuals between the two sites (more females and more adults at Ebelk Aiznay; Leyrer et al. 2006b) supports this idea: females arrive before males, and adults arrive before juveniles (Piersma et al. 1992). Additionally, more aggression between individuals (especially juveniles) occurs in Ebelk Aiznay (P. J. van den Hout unpublished data), supporting the idea that individuals must be of high quality both to obtain and to retain their position in the higher quality habitat. Therefore, the differences we find in immune function may be caused by individual differences, in addition to, and likely interacting with, environmental differences between sites.

It is beyond the scope of this study to say which of these many contributing factors explain the patterns in our data. Indeed, interactions between all of these factors likely contribute to overall investment in immune function or current infection status in wild birds. What is clear from this study is the importance of age and environment on immune function. Future studies, on wild birds, that examine questions of how resource availability, resource demands, predation, pathogen pressure and individual quality affect immune function will be important for our understanding of how animals function in their natural environment.

ACKNOWLEDGEMENTS

We thank A. Castillo for help in the field and lab; Nina Bhola, Daliborka Barjaktarov, François Vézina, Magali Petit, Anne Dekinga, Bernard Spaans and the crew of the H. M. Navicula for support in the Wadden Sea; Bernard Spaans, Jutta Leyrer, Jeroen Reneerkens, Edward Koomson, H. ould M. El Hacen and M. Camara for support in Mauritania; Bernard Spaans and Jutta Leyrer for help identifying *C. c. islandica* and *C. c. canutus* from resight/recapture data; and Dick Visser for finalizing the figures. Funding was provided by the Natural Science and Engineering Research Council of Canada (NSERC) PGSB-267701-2003, a University of Groningen Ubbo Emmius Scholarship, a Netherlands Organization for Scientific Research (NWO) travel grant, and Schure-Beijerinck-Popping Fonds to DMB, NWO and the University of Groningen to BIT and TP, operational grants from the Royal Netherlands Institute for Sea Research (NIOZ) to TP and a MAVA grant to TP and J. Leyrer.

NIOZ and Wadden sea

CHAPTER 12

Synthesis: towards a better understanding of immune responses and disease resistance

Deborah M. Buehler

BOTTLENECKS, BUDGETS AND IMMUNITY IN RED KNOTS

The question of how migrant birds stay healthy while travelling thousands of kilometres, undergoing enormous physical strain, and encountering many different environments with different pathogen challenges is fascinating. The question is especially intriguing because immune function which protects against pathogens also comes with costs that must be balanced against other important aspects of migrant life. This thesis was inspired by this question and its goal is to clarify our understanding of immune function over the annual cycle and in different environments in order to better understand how migrating birds deal with disease threats and balance competing demands for resources. Below I summarize the main findings of this research. Hopefully the data and ideas presented provide a strong foundation for future studies.

Research presented in this thesis focuses mainly on immune function and generally does not address the pathogen pressures that the immune system protects against. Furthermore, I focus on red knots *Calidris canutus* as a model migrant species and consider immune function mainly from the perspective of migrant birds. The aim of this synthesis is to place this thesis into the broader context of understanding immune function in relation to pathogen pressure, not only in red knots but also in other species. I first summarize the results of this thesis, then, based on ideas derived from this thesis and the literature, I introduce a conceptual model which puts ideas about resource allocation and the costs of immunity into the broader context of defence against real pathogens in environments where a myriad of factors change in time and space. I also suggest avenues for further research, which will help to test the model and better link measures of immune function to pathogen pressure and optimal defence against disease.

Summary of research findings

Part I: Study system and predictions

Part I of this thesis (chapters 2 and 3) introduces red knots as a study species. Knots are medium sized shorebirds with six subspecies and a web of migratory routes that spans the globe. This diversity of flyways, occurring within a single species, allows the effects of different living environments and different migration distances to be examined comparatively. Furthermore, knots adapt well to living in captivity making experiments possible. This is important for studies of immune function where many potentially significant factors must be controlled.

In chapter 2 we combine molecular dating of population divergence times with a review of polar and intertidal palaeovegetation to present a hypothesis for the evolution of red knot migratory pathways. This study suggests that all ancestral populations of knots emerged within the last glacial period of the Pleistocene and that their flyways evolved from an ancestral population in Eurasia via an eastward expansion into North America. This implies that the Greenland/Iceland migratory route was established very recently from breeding grounds in the Americas to wintering grounds in Europe.

In chapter 3 we address the question of when the "toughest" times of the year occur for migrant shorebirds such as knots. The red knot annual cycle includes northward migration in the spring, breeding in the Arctic during the summer, and southward

migration to wintering areas, where moult takes place, in the fall. Understanding when knots face "tough times" or bottlenecks during this annual cycle allows predictions about when immune function might be decreased due to trade-offs or increased due to high pathogen pressure. We describe a framework of bottlenecks that constrain knots during their annual cycle. Using the quality of breeding plumage and the timing of moult as bottleneck indicators we conclude that nutritional, energetic, temporal (time-limited) and disease risk bottlenecks vary throughout the year and among red knot subspecies. In general, the longest distance migrating subspecies, *C. c. rogersi* and *C. c. rufa*, show the greatest impact of bottlenecking, and tropical winterers, *C. c. canutus* and *C. c. rogersi*, may face high pathogen pressures in winter. In terms of the annual cycle, bottlenecks overlap during spring migration and arrival on the breeding grounds for all subspecies making this period of the year the "toughest".

We then use the bottleneck framework to make predictions about variation in immune function over the annual cycle. During migration, a time of considerable energetic and temporal bottlenecking for knots, we predict relatively low immune function. However, the fact that knots migrate through a variety of environments harbouring potential pathogens argues for at least some baseline immune protection. Thus, we predict a tendency towards constitutive immunity and antibody-based responses and away from inflammation-based responses during migration (see Box 12.1). During breeding, also a period of energetic and temporal bottlenecking in knots, but when pathogen pressures are relatively low, we predict low investment in immune function. We predict immune investment to be highest during winter when knots are no longer investing nutrients, energy or time in migration or reproduction.

Part II: Assessing immunity and how it responds to different environmental conditions

Part II of this thesis focuses on how immune function varies over the annual cycle and how it responds to different conditions in a controlled environment. In chapter 4 we address the practical question of how soon after capture birds need to be sampled to get reliable baseline immune data. We find that our measures of constitutive immune function (microbial killing, leukocyte concentrations, and complement and natural antibody levels) are not affected by handling stress if blood is taken within 30 minutes of the birds first being captured.

With that practical detail sorted, in chapter 5 we describe variation in immune function (microbial killing, leukocyte concentrations, and complement and natural antibody levels) over an entire annual cycle. We perform monthly immune measurements on captive knots living in controlled conditions and we manipulate thermal regime. We address how immune function varies over the annual cycle, whether birds use different immune strategies during different times of the year, and whether temperature (energy expenditure) affects immune function. We find that immune indices are repeatable and that constitutive immune function is enhanced during mass change (weight gain or loss), a period which coincides with migration and arrival on the breeding grounds in free-living birds. This period is complex in terms of energetic, temporal and disease risk bottlenecks. In free-living birds, limited resources argue for decreased immune function; how-

ever, migration may also expose birds to greater pathogen pressures, arguing for increased immune defence. Captive knots do not experience the same resource limitations or pathogen pressures as do wild birds. Thus the increase in immune function that we find during mass change in captive birds cannot be definitely interpreted; however, it may indicate that captive birds bolster immune function in anticipation of bottlenecks experienced in the wild (like in small mammals; Nelson et al. 2002). We also find co-variation among immune indices at the among- and within-individual levels suggesting birds use different immune strategies during different annual cycle stages (i.e. migration, moult). This finding supports the idea that some immune strategies are more costly than others and that over the annual cycle these strategies will be used only when their benefits outweigh their costs. Finally, we find that experimental manipulation of temperature has little effect on annual variation in immune function. This finding suggests that constitutive immune function is not greatly affected by changes in energy expenditure, and that other environmental factors such as food availability should be examined.

We address the question of food availability in chapters 6 and 7 where we experimentally limit access to food resources. In chapter 6 we establish that limiting knots to 6 hours of food access per day leads to a significant weight loss and increased feeding when food is available. This provides clear evidence that birds in this treatment had to spend more energy than they gained (negative energy balance). Food restricted birds also exhibited a decrease in pectoral muscle thickness and basal metabolic rate in association with weight loss. However, they did not reduce mass-independent basal metabolic rate. With regards to immune function, in chapter 7 we find little effect of food restriction on constitutive immune function, indicating that even under resource limitation, a baseline level of immune function is maintained. We also challenge birds with lipopolysaccharide (LPS) to induce an acute phase response. The acute phase response is considered one of the most costly types of immune function in terms of resource, energy and immunopathology costs (Klasing 2004). We find that birds enduring limited access to food adjust aspects of the acute phase response, suggesting that birds save energy on more costly aspects of immune defence when necessary. The next step will be to simultaneously manipulate energy expenditure and energy intake, an experiment which was not performed due to practical constraints but will be possible in the future.

In chapter 8 we return to the data on annual variation in immune function presented in chapter 5 and combine it with a detailed dataset of annual variation in melatonin to test the winter immunoenhancement hypothesis. This hypothesis associates long winter nights with increased exposure to melatonin and enhanced immune function. Thus, we predict peak exposure to melatonin during the shortest days of the year and positive correlations between melatonin exposure and indices of constitutive immune function. We find that melatonin levels vary significantly over the annual cycle, but that variation is not linked to day length and does not correlate with annual variation in immune function. Thus, we reject the winter immunoenhancement hypothesis for knots. Our findings also question whether the link between short days and increased exposure to melatonin can be generalized to birds and whether the idea that immune function should be bolstered in winter can be generalized to systems where winter is not the toughest time of the year.

Part III: Immune function in free-living birds

Chapter 9 marks a transition between studies of immune variation under controlled conditions in captivity (part II) and studies on free-living birds (part III). In chapter 9 we examine how captivity itself affects immune function. We find that more costly immune indices are lower in captivity. This result does not support the idea that captive birds are released from energetic trade-offs due to benign conditions in captivity. Conversely, we hypothesize that in captivity, where cleaning regimes are likely to decrease pathogen pressure, the costs of certain types of immunity (i.e. immune strategies) might outweigh their benefits. This hypothesis emphasizes the importance of pathogen pressure in shaping a bird's immune profile, a topic that is discussed further below.

In chapters 10 and 11 we begin the process of data collection to test if hypotheses and results from captivity can be corroborated in the field. This process will hopefully cumulate in large scale studies across whole flyways and annual cycles in the future (box 12.1). Specifically, in chapter 10 we address the question of how immune function changes during spring stopover by sampling C. c. rufa knots on stopover in Delaware Bay. We find that immune function is higher in fattening birds than in new arrivals suggesting high pathogen pressure during spring migration as predicted in chapter 3. In chapter 11 we consider how environment, subspecies and age contribute to variation in immune function in the Wadden Sea and Banc d'Arguin. The flyways of *C. c. islandica* and *C. c. canutus* overlap in the Wadden Sea during fall migration allowing subspecies comparisons in a common environment. The Wadden Sea also provides an opportunity to compare different age classes in a common environment. Finally, Banc d'Arguin, the *C. c. canutus* wintering area, contains different age classes as well as high and low quality habitats, providing an opportunity to look at age and environment interactions. We find that *C. c. canutus* in the Wadden Sea differ more from *C. c. canutus* in the Banc d'Arguin than from *C. c. islandica* in the Wadden Sea, emphasizing the importance of environmental factors. Furthermore, first year birds have significantly lower natural antibody levels than adults, but second year birds no longer differ from adults. Finally, first year birds in the low quality habitat in Banc d'Arguin have higher leukocyte concentrations than first year birds or adults in the high quality habitat. Taken together these findings suggest that immune function is determined more by the surrounding environment than by subspecies, that natural antibody repertoires develop at some point in the first year of life, and that variation in immune function in free-living birds likely reflects trade-offs between available resources and defence needs in different environments.

This thesis taken as a whole provides researchers studying migration, annual cycles and ecological immunology with several conclusions. First, migrant birds face bottlenecks or "tough times" during their annual cycle and a framework of these bottlenecks can be used to make predictions about immune function. Second, immune function varies significantly over the annual cycle, even in captive birds, and variation suggests that birds use different "immune strategies" during different annual cycle stages. Third, constitutive immunity persists under conditions that challenge energy balance, suggesting that a baseline level of immune function is compulsory and that birds save energy on more costly aspects of immunity when necessary. Fourth, in addition to

available resources, pathogen pressure in the immediate environment likely shapes the strength and strategy of immune defence. Fifth, variation in melatonin is not linked to day length and does not correlate with immune function in knots. Thus, although melatonin may underlie the mechanism for annual variation in immune function in mammals, this is not likely the case in birds. Finally, in the wild immune function is affected by a myriad of factors including differences in available resources, energy expenditure and pathogen pressure in different environments.

From a methodological standpoint this thesis demonstrates that especially in young fields like ecological immunology detailed and thoughtful observations (chapters 5 and 8) remain scientifically valuable and form the foundation upon which experiments are based (Tinbergen 1963). It also demonstrates the strength of the red knot as a model system and ideas for future research using knots are provided in box 12.1.

BOTTLENECKS, ASSETS AND COSTS: IMMUNE POTENTIAL IN RED KNOTS

One major thread of this thesis is testing predictions based on a series of bottlenecks that describe how resources (nutrients, energy and time) and pathogen pressure vary over the annual cycle (chapter 3). Another major thread is the idea that different types of immune function have different costs and benefits (Lee 2006; Schmid-Hempel and Ebert 2003), thus not only may the strength of an immune response vary in time and space, but also the type of immune response - the immune strategy (chapters 5, 7 and 9). This second point addresses a major short fall which has plagued the field of ecological immunology until recently, namely that "immunocompetence" is not a simple and monolithic entity that can be measured with a single assay (Adamo 2004; Lee 2006; Martin et al. 2006b; Matson et al. 2006a). However, the data and immune strategies presented in this thesis focus mainly within constitutive immunity, whereas predictions made in chapter 3 encompass all branches of immune function. Furthermore, much of the work on annual variation in immune function presented in the literature is based on assays of cell-mediated inflammation (phytohaemagglutinin (PHA) induced wing web swelling; e.g. Greenman et al. 2005; Lozano and Lank 2003; Martin 2005; Martin et al. 2004; Møller et al. 2003; Moreno et al. 2001) and specific antibody responses to non-pathogenic vaccines (e.g. Hasselquist et al. 1999; but see Owen and Moore 2006 and Owen-Ashley and Wingfield 2006 who measure leukocyte counts and the acute phase response respectively). In order to consolidate ideas about resource fluctuation with ideas about immune costs, a model synthesizing the resources limited during bottlenecks and resources needed for immune costs across all branches of immune function is necessary.

Here I use red knots as a model species to introduce such a theoretical framework. First I focus on predicted nutritional, energetic and temporal bottlenecks and then I discuss immune costs and benefits for all branches of the immune system. In a later section of the synthesis I further generalize the model to other hypothetical species and include fluctuation in pathogen pressure.

Nutritional, energetic and temporal bottlenecks address the *assets* of nutrients, energy and time. During a bottleneck these assets are limited and only what is leftover can be used to pay the costs of immunity. Thus *available assets* represent the inverse of the strength of a bottleneck, and all available assets become *capital* for immune investment. This is conceptualized in Figure 2.1A with the contrasting annual cycles of *C. c. islandica*, *C. c. canutus* and *C. c. rufa* shown to highlight the flexibility of the model. Pie diagrams are used to illustrate how nutrients, energy or time might be limiting. In reality these assets may not add up; however, I wish to illustrate that the total amount of capital varies with time and space, and within that total, constraints on each asset also vary. Working our way down the "migration" column illustrates how the overall amount of capital available for investment in immune function (circle size) might vary

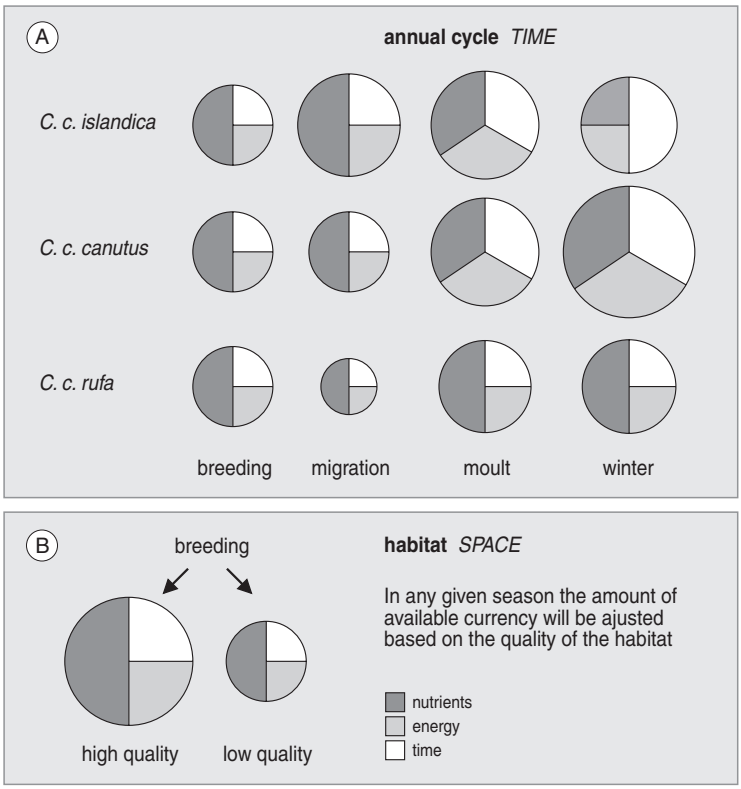

Figure 12.1. A conceptualization of how capital to invest in immune function varies over the annual cycle (A) and in different habitats (B). The sizes of the circles represent the overall amount of available capital and the different assets are shown as proportions (see text and box 2 for details). Note that the circle sizes and proportions are based on predictions discussed in chapter 3; however, empirical data from free-living birds will be needed to verify them. Pie diagrams are used to illustrate how particular nutrients, energy, or time might be limiting. In reality these assets may not add up; however, I wish to illustrate that the total amount of capital varies with time and space and within that total constraints on each asset also vary. The contrasting annual cycles of *C. c. islandica, C. c. canutus* and *C. c. rufa* are shown to highlight how annual cycles can differ.

among the subspecies, assuming that capital must be divided between the demands of immune function and migration. *C. c. islandica* (the shortest distance migrant) will likely have having the most capital to invest and *C. c. rufa* (the longest distance migrant) will likely have the least. Working our way across the *C. c. islandica* row and looking at the asset of time (in white) illustrates how time is most limited during breeding and migration, when something like a sickness response would compromise reproduction or delay migration. The same exercise can be done across any row to examine annual fluctuations within a subspecies and down any column to compare among subspecies. It is important to note that the circle sizes and proportions are not based on data but on predictions discussed in chapter 3. Empirical data from free living birds will be needed to verify them. Figure 12.1B illustrates the fact that habitat quality will adjust the amount of overall capital available in any given season. In a good habitat overall capital is increased, whereas in a poor habitat it is decreased. Theoretically, habitat can also adjust the proportions of available assets; however this is not shown.

From an immune standpoint, available nutrients, energy and time limit the strength and type of immune response based on the costs of immunity. In the introduction I discussed three costs of immunity: *resource cost, immunopathology cost* and *opportunity cost*. These costs can be roughly matched to the bottlenecks introduced in chapter 3. Nutritional bottlenecks and energetic bottlenecks link to resource costs with nutrients and energy as assets. Furthermore, because extreme energy expenditure can increase the risk of immunopathology (Råberg et al. 1998), energetic bottlenecks can also be linked to immunopathological costs. Finally, temporal bottlenecks correspond to opportunity costs with time as the asset. Figure 12.2 illustrates how total costs and individual assets might vary between the different arms of the immune system. The dif-

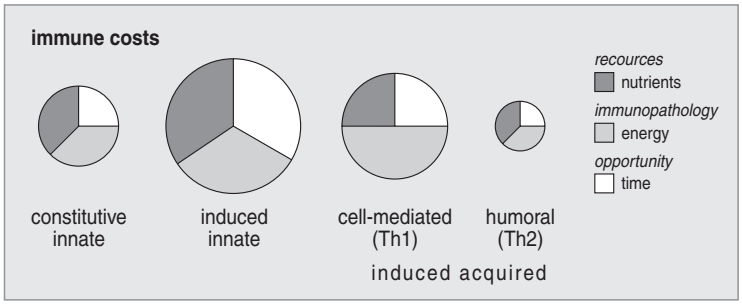

Figure 12.2. An illustration of how the costs of immunity vary between the different arms of the immune system. The different sub-branches of constitutive immunity treated in chapters 5 and 7 are combined for simplicity. Cell-mediated immunity (Th1) refers to induced acquired immunity mediated by Type 1 helper T-cells and associated with inflammation. Humoral immunity (Th2) refers to induced acquired immunity mediated by Type 2 helper T-cells and associated with antibodies. Circle size depicts total cost, and proportions the different assets. Again it is important to note that the circle sizes and proportions are based on ideas in the literature (i.e. Clark 2008; Janeway et al. 2004; Klasing 2004; Martin et al. 2003; Splettstoesser and Schuff-Werner 2002); they remain largely conceptual and empirical data from experiments testing the costs of different types of immunity with respect to different assets will be needed to verify them.

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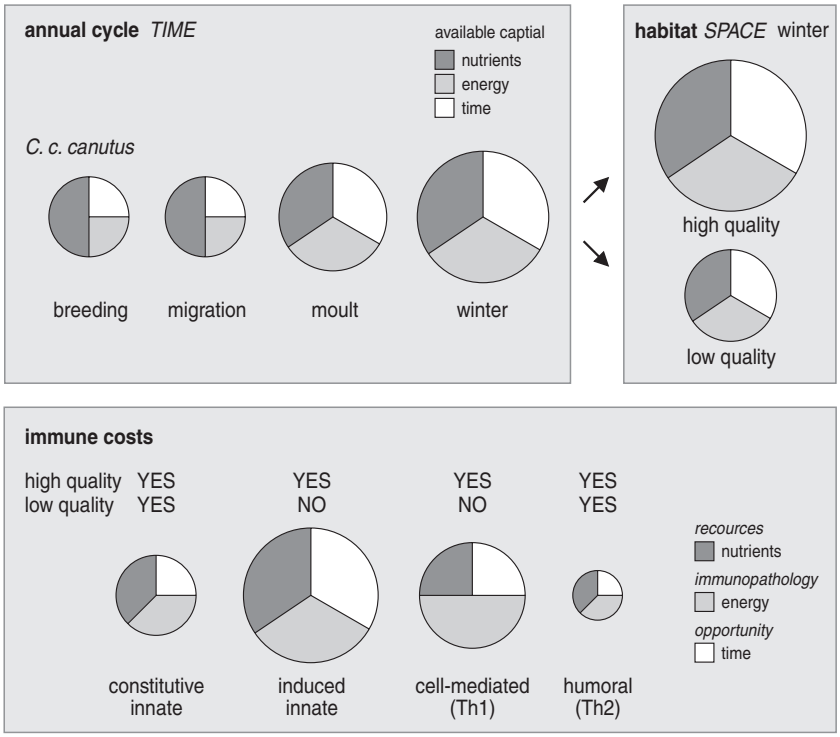

Figure 12.3. A hypothetical example of how annual cycle and habitat circumstances can combine with the costs of immunity to determine an individual's immune potential, which is constrained by the total amount of capital it has to invest. A red knot of the *C. c. canutus* subspecies, wintering in a high quality habitat, will have the potential for the full compliment of immune responses, whereas another individual of the same subspecies wintering in a poor quality habitat may have compromised induced innate and cell-mediated defences.

The idea that capital for immune investment is saved and costs for immunity are paid in similar assets allows us to hypothesize immune potential. *Immune potential* refers to the immune strategies at an animal's disposal given its circumstances at a particular time and place. Immune potential is constrained by bottlenecks which limit the amount of available capital to invest in immune function. Figure 12.3 provides a hypothetical example. A red knot of the *C. c. canutus* subspecies, wintering in a high quality habitat, will have the potential for the full compliment of immune responses, whereas another individual of the same subspecies wintering in a poor quality habitat may have compromised induced innate and cell-mediated defences. This is important because immune systems have evolved and persist to protect their hosts for pathogen invasion and each branch of immune defence has benefits as well as costs (Clark 2008; Janeway et al. 2004).

The benefits of different branches of the immune system are illustrated in figure 12.4. Pathogen threats come in a myriad of forms, but for simplicity the benefits of immunity are depicted in relation to only two pathogen categories. Pathogens are classified as intra and extracellular because defence against these two types of pathogen may require differential activation of more and less costly responses (i.e. Th1 and Th2). Cell-mediated responses (Th1) are most important against intracellular pathogens and humoral responses (Th2) against extracellular pathogens as discussed in chapter 1. In figure 4 benefits against intracellular pathogens are depicted on the left side of the circles and benefits against extracellular pathogens on the right side of the circles.

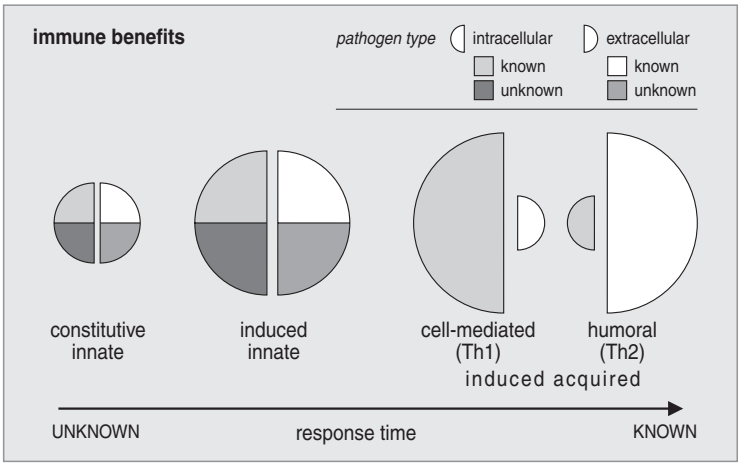

Figure 12.4. An illustration of the benefits of different branches of the immune system. Pathogens are classified as intracellular and extracellular with benefits against intracellular threats depicted on the left side of the circles and benefits against extracellular threats on the right side of the circles. Pathogens are also be classified as known (previously encountered) or unknown (no previous exposure), as depicted by light and dark shading respectively. The different branches of the immune system are shown separately for clarity, but see text for details. The arrow at the base of the figure illustrates the fact that induced acquired responses have a response time lag and the fact that over the course of an immune response, unknown pathogens become known.

Pathogens can also be classified as known (previously encountered pathogens against which the host already possesses acquired immunity) or unknown (no previous exposure), as depicted by light and dark shading respectively. When a pathogen is unknown, initial defence comes entirely from innate immunity. The different branches of the immune system are shown separately for clarity; however, it is important to remember, as described in chapter 1, that during pathogen invasion aspects of constitutive innate, induced innate and acquired immunity work together over the course of the infection. It is also important to acknowledge the fact that induced acquired responses have a response time lag. I have tried to illustrate this using the arrow at the based of the figure. When an unknown pathogen invades, constitutive innate immunity is the first and only defence. If the attack is very strong and spreading, a systematic innate response will be induced (i.e. the acute phase response). After a few days either cell-mediated or humoral acquired immunity will come into play depending on whether the pathogen is intra or extracellular.

Generalizing the model: immune potential, required response and optimal defence

I have now defined bottlenecks that fluctuate in time and space and limit resources used as capital for immune investment. I have also introduced the concept of immune potential which is based on capital available to pay the costs of immunity. Finally, I have discussed the benefits of different immune defences. I would now like to consider how pathogen pressure and the nature of a particular pathogen challenge shape the nature of immune responses. As discussed above different branches of immunity have different benefits, and as such the required response to a challenge (given unlimited immune potential) will depend on the nature of the pathogen. However, immune potential is rarely unlimited, thus optimal immune defence must be approached from two sides. Optimal immune defence (the optimized response given the circumstances) aims for the best possible response against the particular pathogen (required response), but is constrained by the animal's immune potential. This idea is summarized in figure 12.5 and the terms are defined in box 12.2.

To illustrate the model and its potential to be generalized to other species I consider two hypothetical species with contrasting annual cycles. The changes in available capital and pathogen pressures presented are not based on data but are simplified to highlight the flexibility of this model to different circumstances. By approaching immune function from this perspective I hope to highlight the fact that an animal's optimal immune defence is a balance between the capital it has to invest and the costs of immunity on the one hand, and the pathogen threats and the benefits of different aspects of immunity on the other hand. The left side of figure 12.6 shows variability in available capital and pathogen pressure over the annual cycle in two hypothetical species. The first species approximates an open cup nester that migrates to wintering areas in the tropics. The second species approximates a cavity nester that resides year round in the North Temperate Zone. In terms of available capital, again the sizes of the circles represent the overall amount and the different assets are shown as proportions. During breeding, in both species, most of the capital will be invested in reproduction

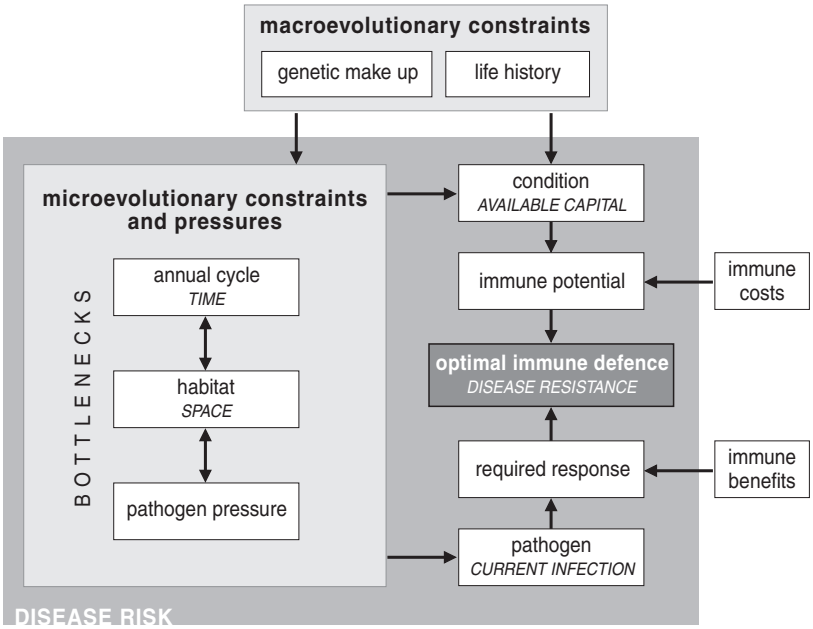

Figure 12.5. The immune potential-required response-optimal defence model. This model highlights the fact that an animal's optimal immune defence is a balance between the capital it has to invest in the costs of immunity on the one hand (immune potential), and the pathogen threats and the benefits of different aspects of immunity on the other hand (required response). Definitions for the terms used in the model are in box 12.2.

(especially energy and time). The same will be true for species 1 during migration. During winter, species 1 in the tropics may have more capital to invest than species 2 in the North Temperate Zone due to lower thermoregulatory costs and a more stable food supply.

In terms of pathogen pressure, again pathogens are classified as intracellular (left side of the circles) and extracellular (right side of the circles) and as known or unknown as depicted by light and dark shading respectively. During breeding the cavity nester (species 2) may face greater pathogen pressure in the form of extracellular brood parasites and the intra and extracellular microorganisms they carry (Møller et al. 2003). The migrant (species 1) may encounter a high proportion of novel pathogens during migration (Møller and Erritzøe 1998) and more extracellular parasites (i.e. ticks) as well as the intra and extracellular microorganisms they carry during the tropical wintering. Conversely, the resident (species 1) may encounter more intracellular pathogens such as viruses than extracellular pathogens such as ticks during the North Temperate winter.

To illustrate how immune potential and required response combine to give optimal defence I focus on an example using the breeding season in species 1 (right side of figure 12.6). Working from the top downwards, we see that the bird would have enough capital to invest in constitutive innate immunity, humoral immunity and an

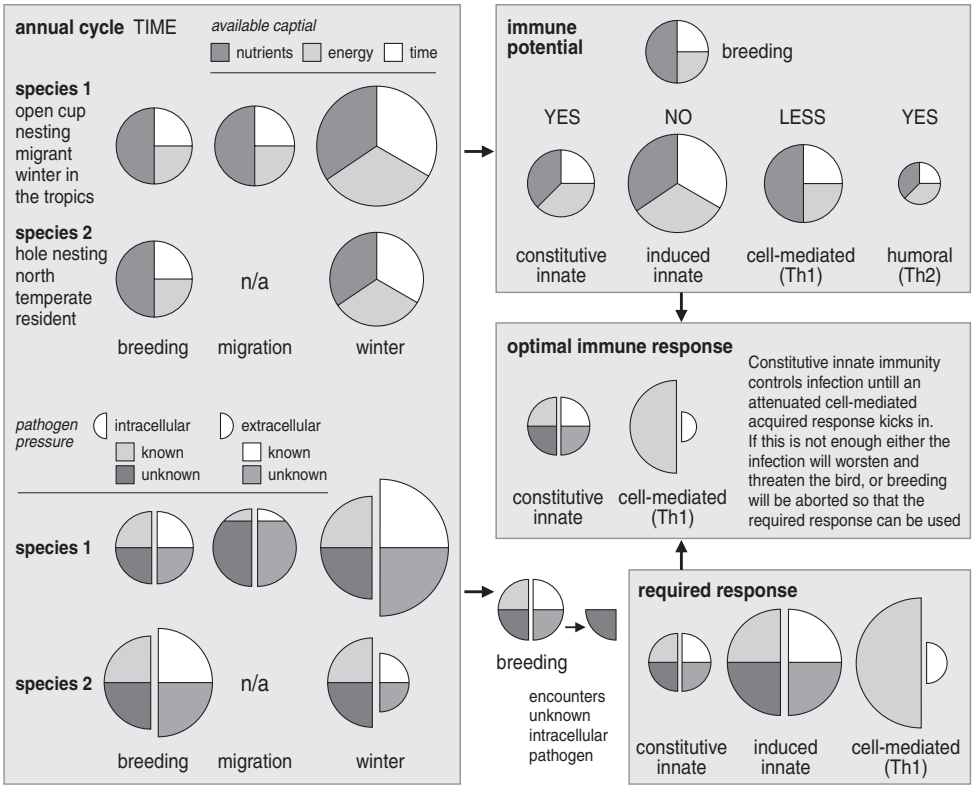

Figure 12.6. An example of the immune potential-required response-optimal defence model in two hypothetical species. The left side of the figure illustrates variation in available capital and pathogen pressure in two contrasting species. Variation is not based on data for real species and is simplified to highlight the flexibility of this model to different circumstances. The first species approximates an open cup nester that migrates to wintering areas in the tropics and the second species approximates a cavity nester that resides year round in the North Temperate Zone. In terms of available capital, again the sizes of the circles represent the overall amount and the different assets are shown as proportions. In terms of pathogen pressure, again pathogens are classified as intracellular (left side of the circles) and extracellular (right side of the circles) and as known or unknown as depicted by light and dark shading respectively. The right side of the figure illustrates how immune potential and required response combine to give optimal defence, using breeding in species 1 as an example (see text for details).

attenuated cell-mediated defence. Working from the bottom upwards we see that the bird has been infected by an intracellular pathogen that it has not previously encountered. The required response for this pathogen would be constitutive innate defence followed by an induced innate defence if the pathogen were aggressive. After a few days a primarily cell-mediated acquired response would kick in and eventually clear the infection. However, working to the middle of the figure we can see that the bird does not have the potential for the required response. Thus the optimal response would be constitutive innate immunity alone to control infection until a somewhat attenuated cell-mediated acquired immunity kicks in. If this optimal defence is not sufficient then

either the infection will worsen and threaten the bird, or breeding will be aborted to free up capital for the induced innate response and a full cell-mediated acquired response.

The examples given here and many aspects of this model are of course a simplification. Animals experience a wider range of circumstances than I have portrayed and are faced with an unimaginable diversity of pathogens. Furthermore, the immune system is more complex than depicted. However, this simplification provides a way to conceptualize how optimal immune defence is shaped in individuals of differing condition, living in different situations, and faced with different pathogen threats.

Towards testing the model: avenues for future research

The model presented above highlights the importance of detailed information on annual cycles, habitats, resource allocation, immune costs, pathogen pressures and an understanding of specific pathogen threats. If this model is to be tested, we must develop ways to assay all branches of the immune system over the course of the annual cycle in free-living environments. We must also more precisely measure the costs of immunity in non-domesticated species and free-living individuals. Finally, we must learn more about pathogen threats and better link measures of immune function to disease.

To really examine trade-offs within the immune system as hypothesized in chapters 5, 7 and 9, and to test the model presented in this synthesis, it will be necessary to concurrently assay constitutive immunity, induced innate immunity, cell-mediated acquired immunity (Th1) and humoral acquired immunity (Th2) in free-living animals. However, from a practical standpoint, this remains difficult because it is still impossible to measure induced innate or acquired immunity from a single blood sample. One approach to this problem, at least for acquired immunity, is to look at gene expression. As discussed in box 12.3, MHC genes code for surface proteins that are essential for the proper functioning of helper T cells. Once activated these T-cells act to determine the cell-mediated (Th1) or humoral (Th2) character of acquired immune responses through the release of cytokines, which then feed back into the response and further adjust MHC gene expression (Clark 2008; Janeway et al. 2004). Th1 responses are associated with MHC class I expression, whereas Th2 responses are associated with MHC class II expression (Clark 2008; Janeway et al. 2004). Gene expression is defined as the translation of the information encoded on a gene into protein or RNA. One way to measure gene expression is to assay the cytokines present in blood challenged with pathogens *in vitro*. Another possibility is to quantify messenger RNA (transcribed DNA) in blood samples using real time polymerase chain reaction (RT-PCR) techniques. Neither of these assays is fully developed yet; however, they represent promising possibilities for the future.

To test ideas presented in the model about resource allocation in terms of assets used to pay for immune investment, we need to measure the costs of immunity more precisely, particularity in non-domesticated species and free-living individuals. Energy costs have been measured for cell-mediated (i.e. Martin et al. 2003) and humoral responses (i.e. Mendes et al. 2006) but are still needed for constitutive and induced innate immunity. Measurements could also be performed in a wider variety of species

to see if costs change with life history traits. The field could also benefit from measuring the nutrient costs of different branches of the immune response (i.e. Klasing 1998) in a variety of non-domesticated species. Finally, we need to develop ways to precisely measure immunopathology costs in different circumstances for all branches of the immune response (i.e. research in humans Clark 2008; Janeway et al. 2004; Smith 2003; Splettstoesser and Schuff-Werner 2002).

With regards to learning more about pathogen threats and better linking measures of immune function to disease, we could begin by addressing the question "What are the diseases that threaten species of interest and how is pathogen pressure distributed over time and space?" In many species, a good starting point would be tapping into large scale screening for wildlife diseases such as avian influenza or malaria. For species amenable to studies in captivity, discovering and developing assays for cryptic pathogens may also be instructive. For example, in red knots a protozoal infection in the endothelial cells of the pulmonary artery was recently discovered (T. Kuiken pers comm). This infection caused absolutely no signs of disease in terms of behavioural change, weight loss or skin lesions in captivity. However, similar protozoal diseases have had large fitness consequences in the wild (Simpson et al. 1977; Woodard et al. 1977). Developing an assay for this pathogen could provide large scale screening for another potentially important wildlife disease.

Next we could address the question "What is the effect of relevant diseases on immune indices?" Comparing the immune profiles of healthy and sick individuals will help to establish a link between changes in immune indices and current infection, and will help us to understand how the immune system responds to disease. Finally we could address the question "Do high scores on immune indices signify better resistance to disease?" This question must be answered by performing experiments to link individual scores on immune indices with subsequent disease resistance. If high scores (prior to inoculation with the pathogen) result in high resistance then that index is a definitive proxy for resistance against that disease. If no relationship is found then the index can tell us nothing about that disease, but may still be linked to other diseases. If a negative relationship is found then there may be trade-offs within the immune system with resistance to one type of disease causing susceptibility to another type of disease.

In the short term, a better understanding of the links between immune function and disease may help to explain difficult to interpret variation in immune indices in seemingly healthy individuals. For example in chapter 5 we reported significant annual variation of *E. coli* killing and lysis that could not be explained by annual cycle stages experienced by knots in captivity. During the experiment methodological factors were carefully controlled and great care was taken to assess knots for visible signs of disease. However, it remains possible that the birds experienced a microbial challenge which presented no symptoms in the relatively benign conditions of captivity. Better screening for cryptic pathogens (discussed above) might clarify this situation.

In the long term, once we have established that immune index scores correlate with disease resistance, we could study whether increased resistance improves survival and reproduction. Doing this would bring the field a step closer to linking immune function to fitness (in the Darwinian sense Darwin 1859). It would bring us closer to under-

standing how patterns of immune function and disease susceptibility affect population viability in the wild. Finally, we would be in a much better position to study how aspects of immune function and disease affect topics of interest in ecology and evolution (e.g. the maintenance of migratory routes or the evolution of life histories).

Concluding thought

My research does not provide the final answer on how migrating birds deal with disease threats and balance competing demands for resources, but it provides a foundation and it points out areas for further study (see also box 12.3). That, I have discovered, is the joy of science.

BOX 12.1. FUTURE ENDEAVOURS FOR RED KNOTS: WHERE AND WHEN TO SAMPLE

I began this thesis by stating that the research was inspired by a fascination with migration. This fascination has not waned and now that observations and experiments on captive birds have built a foundation for field studies it is time to take the ideas of bottlenecks, budgets and immunity around the world.

To fully use the potential of red knots as a comparative model, sampling will be needed to fill both temporal and spatial gaps. First, measures of immune function need to be taken from free-living *C. c. islandica* along their flyway and throughout their annual cycle. These data could be used to compare patterns of variation between free-living and captive knots and to answer questions about the importance of pathogen pressure and resource-based bottlenecks. For example, would immune function be uniformly higher throughout the annual cycle in free-living birds because pathogen pressure is higher in the wild (as suggested by Chapter 9)? Would high immune index scores during the spring migration and breeding periods be dampened in free-living birds due to energetic and nutritional bottlenecks (as seen in mammals Nelson et al. 2002)? Second, measures of immune function need to be taken along the *C. c. canutus* flyway throughout the annual cycle. These data in conjunction with the data from free-living *C. c. islandica* could be used to test hypotheses outlined in Chapter 3 about differences in pathogen pressure between tropical and temperate winterers. Finally, if data could be obtained from free-living *C. c. rufa* along their flyway, hypotheses about temporal bottlenecks in long distance and shorter distance migrants could be tested.

BOX 12.2. DEFINITIONS FOR THE IMMUNE POTENTIAL - REQUIRED RESPONSE - OPTIMAL DEFENCE MODEL

- *Available capital* refers to resources that vary in time and space, and can be used to pay the costs of immunity.
- *Bottlenecks* refer to periods when nutrients, energy and time are limited due to temporal and spatial circumstances.
- *Current infection* refers to an existing infection by a given pathogen
- *Disease resistance* refers to an animal's ability to resist pathogen challenge
- *Disease risk* refers to the risk of becoming ill and is affected by a myriad of factors including pathogen pressure, the animals' condition at the time of challenge and the type of pathogen challenge.
- *Immune potential* refers to the immune strategies at an animal's disposal given its circumstances at a particular time and place. Immune potential is constrained by bottlenecks which limit the amount of available capital to invest in immune function.
- *Macroevolutionary constraints* are defined as factors which make populations resistant to evolutionary change. The concept is included in Figure 12.4 to illustrate that the model may be expanded to include predictions at the population and species levels where macroevolutionary factors constrain immune function. However this is not discussed further because it is beyond the scope of this synthesis.
- *Microevolutionary constraints* are defined factors which limit resources. For example, nutrients to invest in immune function may be limited in a habitat where food is scarce or difficult to find. These constraints are more plastic than macroevolutionary constraints.
- *Optimal immune defence* refers to the response actually used by an animal given its immune potential and the pathogen at hand. It is this response that will determine the animal's resistance to disease.
- *Pathogen* refers to a disease causing biological agent (including microorganisms and parasites). This may also include commensal organisms because they are kept in check by the immune system. For example, some *E. coli* bacteria are beneficial in the gut but are disease causing if allowed to establish in the bloodstream.
- *Pathogen pressure* refers to the possible pathogens that an animal might encounter at a given time and place.
- *Required Response* refers the best possible response against a given pathogen assuming unlimited capital.

BOX 12.3. LINKING GENETIC PROFILES TO DISEASE RESISTANCE

Another promising avenue for research involves looking at the genetic basis of immune function and linking genetic profiles to aspects of disease susceptibility. The idea that genetic variation is connected to disease susceptibility was suggested over half a century ago (Haldane 1949). Correlations between host genetic variation and pathogen prevalence at the individual (e.g. Acevedo-Whitehouse et al. 2003; Ortego et al. 2007) and population (e.g. Luikart et al. 2008; Meagher 1999; Ross-Gillespie et al. 2007) levels provide indirect evidence that less diverse hosts are more susceptible to many pathogens. However challenge experiments are needed to provide a causal link. For example, inbred house mice *Mus musculus* were more susceptible to experimental infection with *Salmonella* and house finches *Carpodacus mexicanus* with lower heterozygosity at microsatellite loci were more susceptible when challenged with conjunctivitis *Mycoplasma gallisepticum* (Hawley et al. 2005). Inbreeding decreases genetic variation throughout the genome and heterozygosity at selectively neutral microsatellite loci is thought to be correlated with heterozygosity at loci under selection (reviewed in Hansson and Westerberg 2002). However, measures of genetic diversity and allele distribution for genes of known importance to immune function will be needed to provide a more direct link between genes and disease susceptibility.

Major histocompatibility complex (MHC) genes code surface proteins that are essential for the proper functioning of helper T cells (Clark 2008; Janeway et al. 2004). T-cells can not recognize antigens unless they are presented by MHC molecules and the variability of MHC peptide binding regions determines which antigens can be presented. MHC genes and the proteins they code for are highly polymorphic, meaning that there are many different alleles scattered throughout animal populations. Because of this high polymorphism, the chances of individuals inheriting the same combination of MHC alleles are very slim (Clark 2008; Janeway et al. 2004). Individuals with different allele combinations present slightly different antigens for T-cell inspection and thus produce slightly different immune responses. In this way high polymorphism at the population level keeps the group covered against a wide range of pathogens, but some individuals will be more successful at fending off specific pathogens than others (Clark 2008). Specific MHC alleles have been associated with decreased malaria prevalence in house sparrows (*Passer domesticus*; Bonneaud et al. 2006). In the future, studies that combine challenge experiments with measures of MHC diversity, heterozygosity and specific alleles will be important for our understanding of the link between genes and immune defence.

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Nederlandse samenvatting

"Hoe gaan trekvogels om met het risico om ziek te worden en hoe verdelen ze hulpbronnen die nodig zijn voor immuunfunctie gedurende hun drukke jaarcyclus?" Dit proefschrift is geïnspireerd door deze vraag en heeft als doel om ons begrip te vergroten van immuunfunctie gedurende het jaar en in verschillende omgevingen. Tijdens dit onderzoek hebben wegebruik gemaakt van nieuwe technieken om verschillende aspecten van immuunfunctie met behulp van bloedmonsters te meten. Ik heb ook gebruik gemaakt van multivariate statistische technieken om te onderzoeken hoe verschillende aspecten van immuunfunctie te groeperen zijn in "immuunstrategieën". Hieronder geef ik een samenvatting van de belangrijkste bevindingen van dit onderzoek. Box 1 geeft achtergrondinformatie over het immuunsysteem, de kosten van immuniteit en de meetmethodes die gebruikt zijn in dit onderzoek.

DEEL I: STUDIE SYSTEEM EN VOORSPELLINGEN

In deel I van dit proefschrift (hoofdstuk 1 en 2) wordt de kanoetstrandloper *Calidris canutus* geïntroduceerd als modelsoort. Kanoeten zijn middelgrote strandlopers met zes ondersoorten en een web van trekroutes die de hele aarde omvatten (zie figuur 3.1). De diversiteit in trekroutes binnen deze ene soort maakt het mogelijk om de effecten van verschillende leefomgevingen en trekafstanden met elkaar te vergelijken. Het feit dat kanoeten zich gemakkelijk aanpassen aan het leven in gevangenschap is zeer belangrijk voor de bestudering van het immuunsysteem, waar veel mogelijk belangrijke factoren gecontroleerd moeten kunnen worden.

In **hoofdstuk 2** combineren we moleculaire datering van populatie-divergentietijden met een overzicht van de palaeovegetatie van Arctische gebieden en waddengebieden, om zo een hypothese te vormen over de evolutie van de trekroutes van kanoetstrandlopers. Deze studie suggereert dat alle populaties van voorouders van kanoeten tijdens de laatste ijstijd van het pleistoceen ontstonden, en dat hun trekroutes zijn geëvolueerd vanuit een populatie in Eurazië, via een oostelijke uitbreiding naar Noord Amerika (zie figuur 2.2). Dit impliceert dat de trekroute via Groenland / IJsland recent is ontstaan, doordat kanoeten broedden in Noord Amerika en overwinterden in Europa.

In **hoofdstuk 3** richten wij ons op de vraag wanneer de "zware" periode in het jaar voor trekkende strandlopers zoals kanoeten zich voordoet. De jaarcyclus van kanoeten bestaat uit een trek naar het noorden in het voorjaar, het broedseizoen in Arctisch gebied in de zomer en een trek naar de zuidelijke overwinteringsgebieden, waar de rui plaatsvindt in het najaar. Door te bestuderen wanneer kanoeten "zware tijden" of bottlenecks ondervinden gedurende hun jaarcyclus, wordt het mogelijk om voorspellingen te doen wanneer hun immuunfunctie verminderd zou kunnen zijn vanwege trade-offs, of juist versterkt vanwege een hoge pathogenen druk. We beschrijven een kader van bottlenecks die kanoeten zouden kunnen tegenwerken gedurende hun jaarcyclus. Als indicatoren voor bottlenecks gebruiken we de kwaliteit van het broedkleed en de timing van de rui. De conclusie is dat bottlenecks (veroorzaakt door nutriënten, energie, tijdsdruk) en risico om ziek te worden variëren, zowel gedurende het jaar als tussen verschillende soorten kanoeten. In het algemeen geldt dat de soorten die de lang-

ste afstand afleggen tijdens de trek, *C. c. rogersi* en *C. c. rufa*, de grootste impact van bottlenecks kennen, en dat tropische overwinteraars, *C. c. canutus* en *C. c. rufa*, in de winter hoge pathogenen druk zouden kunnen ervaren. Voor alle soorten overlappen de bottlenecks gedurende de voorjaarstrek en de aankomst in de broedgebieden, en dat maakt dit de "zwaarste" periode.

Vervolgens hebben we dit kader van bottlenecks gebruikt om voorspellingen te doen omtrent de variatie in immuunfunctie gedurende de jaarcyclus. Tijdens de trek, een periode met voor kanoeten aanzienlijke energetische en tijdsbottlenecks, voorspellen we een relatief lage immuunfunctie. Echter, het feit dat kanoeten door verschillende omgevingen met verschillende pathogenen trekken, suggereert dat een basale immuunverdediging te allen tijde nodig is. We voorspellen dan ook dat gedurende de trek een trend zich zal voordoen richting constitutieve immuniteit en reacties gebaseerd op antilichamen, en juist niet in de richting van ontstekingsreacties (zie box 1). Tijdens het broedseizoen, voor de kanoeten eveneens een periode met bottlenecks voor wat betreft energie en tijd, maar met relatief lage pathogenen druk, voorspellen we een lage investering in immuunfunctie. Ook voorspellen we dat de investeringen in immuunfunctie het hoogst zijn in de winter, wanneer kanoeten geen voedsel, energie of tijd steken in trek of voortplanting.

DEEL II: HET BEPALEN VAN IMMUNITEIT EN HOE HET REAGEERT OP VERSCHILLENDE OMGEVINGSCONDITIES

Deel II van dit proefschrift concentreert zich op de vraag hoe immuunfunctie varieert gedurende de jaarcyclus en hoe het reageert op verschillende condities in een gecontroleerde omgeving. In **hoofdstuk 4** behandelen we praktische vragen met betrekking tot de snelheid waarmee een bloedmonster verkregen moet worden nadat vogels gevangen zijn om betrouwbare baseline immuundata te verkrijgen. We vinden dat, als het bloed binnen 30 minuten na het vangen van de vogel genomen wordt, onze maten van constitutieve immuun functie (microbial killing en concentraties leukocyten, complement en natuurlijke antilichamen) niet beïnvloed worden door de stress van het hanteren.

Nadat we dit praktische aspect behandeld hebben, beschrijven we in **hoofdstuk 5** variatie in immuunfunctie (microbial killing en concentraties leukocyten, complement en natuurlijke antilichamen) gedurende een hele jaarcyclus. We hebben maandelijks immuunmetingen gedaan bij kanoeten in gevangenschap, die leven onder gecontroleerde condities en waarbij we het temperatuur regiem manipuleerden. We bespreken hoe immuun functie varieert geduurde de jaarcyclus, of vogels verschillende immuunstrategieën gebruiken tijdens verschillende periodes in het jaar en of temperatuur (energieuitgave) invloed heeft op immuunfunctie. We vinden dat immuunmaten herhaalbaar zijn binnen een individu, en dat constitutieve immuunfunctie versterkt wordt tijdens een periode van verandering in gewicht (gewichtsverlies of -toename). Dit is een periode die bij vrij levende vogels samenvalt met de aankomst in de broedgebieden (zie, figuur 5.1). Deze periode blijkt complex voor wat betreft bottlenecks die te maken hebben met energie, tijd en het risico om ziek te worden. Bij vrij levende vogels doet de

beperkte aanwezigheid van hulpbronnen vermoeden dat de immuunfunctie verminderd zou moeten zijn; echter, de trek zou er voor kunnen zorgen dat vogels blootgesteld worden aan meer pathogenen, wat de immuunfunctie juist zou versterken. Echter, kanoeten in gevangenschap worden niet blootgesteld aan dezelfde beperkte hulpbronnen of pathogenen druk als vrij levende vogels. Daarom kan deze toename in immuunfunctie die we zien tijdens de verandering in gewicht niet ondubbelzinnig geïnterpreteerd worden. Het is echter kenmerkend dat de vogels in gevangenschap immuunfunctie versterken in anticipatie op periodes die vrij levende vogels ervaren als bottlenecks. Ook vinden we covariatie tussen immuunmaten zowel tussen individuen als binnen individuen, wat suggereert dat vogels verschillende strategieën gebruiken in verschillende stadia van de jaarcyclus (b.v. trek, rui). Deze bevinding ondersteunt het idee dat sommige immuunstrategieën kostbaarder zijn dan andere, en dat de vogels gedurende de jaarcyclus de kostbare strategieën alleen gebruiken wanneer de baten groter zijn dan de kosten. Tenslotte vinden we dat experimentele manipulatie van de temperatuur weinig effect heeft op de jaarlijkse variatie in immuunfunctie. Deze bevindingen doen vermoeden dat constitutieve immuunfunctie niet sterk wordt beïnvloed door energieverbruik, en dat andere omgevingsfactoren zoals aanwezigheid van voedsel bestudeerd zouden moeten worden.

In hoofdstuk 6 en 7 gaan we in op de vraag hoe aanwezigheid van voedsel de immuunfunctie beïnvloedt, door we de toegang tot voedsel experimenteel beperken. In **hoofdstuk 6** bepalen we dat een een beperkte toegang tot voedsel van slechts 6 uur ervoor zorgt dat kanoeten gewicht verliezen en dat er een toename in foerageren plaatsvindt in de periode dat er wél voedsel aanwezig is. Dit is een duidelijk bewijs dat vogels onder deze behandeling meer energie spendeerden dan ze verkregen hadden (m.a.w., een negatieve energiebalans hadden). Vogels met een voedsel restrictie vertoonden naast het gewichtsverlies ook een afname in pectorale spieren en een laag basaal metabolisme. Het gewichtsspecifiek basaal metabolisme nam echter niet af.

Het effect van voedselrestrictie op immuunfunctie beschrijven we in **hoofdstuk 7**. We vinden weinig effect van voedsel restrictie op constitutieve immuun functie, wat erop wijst dat zelfs in aanwezigheid van beperkt voedsel een basaal niveau van immuunfunctie onderhouden wordt. Ook hebben we lipopolysaccharide (LPS) geïnjecteerd bij vogels om een acutefase-reactie te induceren. De acutefase-reactie wordt gezien als een van de meest kostbare immuunfuncties in termen van voedsel, energie en immunopathologische kosten (zie box 1). Onze vinding dat vogels met beperkte toegang tot voedsel delen van de actutefase-reactie aanpassen, suggereert dat zij wanneer nodig energie besparen met de meer kostbare aspecten van de immuunverdediging. De volgende stap is om tegelijkertijd de energie-uitgave en -opname te manipuleren; een experiment dat vanwege praktische beperkingen helaas niet uitgevoerd is, maar dat in de toekomst wel mogelijk is.

In **hoofdstuk 8** gaan we terug naar de data van de jaarlijkse variatie van immuunfunctie die eerder gepresenteerd werd in hoofdstuk 5, en we combineren deze gegevens met een gedetailleerde dataset van jaarlijkse variatie in melatonine om de "winter immuno-enhancement" hypothese te testen. Deze hypothese associeert lange winternachten met een toename in blootstelling aan melatonine, met als gevolg een verster-

king in immuun functie. Met het oog op deze hypothese voorspellen we dat we gedurende de kortste dagen van het jaar een piek zullen vinden in de blootstelling aan melatonine, en dat er een positieve correlatie tussen melatonine en maten van constitutieve immuun functie zal zijn. We vinden echter dat melatonine niveaus significant variëren gedurende het jaar, maar deze variatie valt niet samen met daglengte en correleert niet met de jaarlijkse variatie in immuunfunctie (zie figuur 8.2). Daarom verwerpen we de "winter immuno-enhacement" hypothese voor kanoeten. Onze bevindingen trekken ook in twijfel of de link tussen korte dagen en toename in blootstelling aan melatonine wel gegeneraliseerd kan worden voor vogels. Daarnaast betwijfelen we of het idee dat immuunfunctie versterkt moet worden in de winter gegeneraliseerd kan worden voor systemen waar de winter niet de zwaarste tijd van het jaar is.

DEEL III: IMMUUNFUNCTIE BIJ VRIJ LEVENDE VOGELS

Hoofdstuk 9 markeert een overgang van studies omtrent immuunfunctie onder de gecontroleerde omstandigheden in gevangenschap (deel II) naar studies in vrij levende vogels (deel III). In hoofdstuk 9 bestuderen we hoe gevangenschap zelf immuunfunctie beïnvloedt. In gevangenschap blijken kostbaardere immuunmaten lager te zijn dan in het wild. Dit resultaat is niet ondersteunend voor het idee dat vogels in gevangenschap door gunstige condities geen energetische trade-offs ervaren. Integendeel, we hebben juist de hypothese dat in gevangenschap, waar het regelmatig schoonmaken van de kooien de aanwezigheid pathogenen allicht vermindert, de kosten van bepaalde soorten immuniteit (immuun strategieën) zwaarder wegen dan de baten. Deze hypothese benadrukt het belang van pathogenen druk in het vormen van het immuunprofiel van een vogel, een onderwerp dat hieronder verder besproken zal worden.

In hoofdstuk 10 en 11 beginnen we met het verzamelen van data om te testen of de hypothesen en resultaten van de studies in gevangenschap bevestigd kunnen worden in het veld. Met name in **hoofdstuk 10** richten we ons op de vraag hoe immuunfunctie verandert tijdens de stop-over in het voorjaar, door *C. c. rufa* te bemonsteren tijdens hun stop-over in Delaware Bay. We vinden dat immuunfunctie hoger is in opvettende vogels dan in vogels die pas zijn gearriveerd, wat suggereert dat er een hoge pathogenen druk is tijdens de voorjaarstrek, zoals voorspeld in hoofdstuk 3. **In hoofdstuk 11** bespreken we hoe omgeving, ondersoort en leeftijd bijdragen aan de variatie in immuunfunctie in de Waddenzee en de Banc d'Arguin. De vliegroutes van *C. c. islandica* en *C. c. canutus* overlappen elkaar in de Waddenzee tijdens de najaarstrek, wat het mogelijk maakt om ondersoorten in dezelfde omgeving met elkaar te vergelijken. De Waddenzee biedt ook de mogelijkheid om verschillende leeftijdsklasses in dezelfde omgeving met elkaar te vergelijken. Tot slot zijn er op de Banc d'Arguin, de overwinteringsplaats voor *C. c. canutus*, verschillende leeftijdscategorieën kanoeten te vinden en bevat het gebied bovendien hoge en lage kwaliteit habitat, wat de mogelijkheid biedt om naar de interactie tussen leeftijd en omgeving te kijken. We vinden dat *C. c. canutus* in de Waddenzee meer verschilt van *C. c. canutus* in de Banc d'Arguin dan van *C. c. islandica* in de Waddenzee, wat het belang van omgevingsfactoren benadrukt. Bovendien hebben

één jaar oude vogels significant minder natuurlijke antilichamen dan adulten, maar dit verschil is weer verdwenen bij twee jaar oude vogels. Ten slotte hebben één jaar oude vogels in een lage kwaliteit habitat in de Banc d'Arguin een hoger aantal leukocyten dan één jaar oude vogels en adulten in een hoge kwaliteit habitat. Samengevat suggereren deze bevindingen (i) dat immuunfunctie sterker bepaald wordt door de omgeving dan door ondersoort; (ii) dat het repertoire aan natuurlijke antilichamen zich ontwikkelt in het eerste jaar en (iii) dat variatie in immuunfunctie in vrij levende vogels waarschijnlijk trade-offs tussen de aanwezige hulpbronnen en de noodzakelijke verdediging in verschillende omgevingen weergeeft.

Het onderzoek dat besproken wordt in dit proefschrift concentreert zich hoofdzakelijk op immuunfunctie, maar behandelt over het algemeen de pathogenen druk waartegen het immuunsysteem verdedigt niet. Bovendien concentreren we ons op de kanoetstrandloper *Calidris canutus* als een model voor trekvogels en bespreken we immuunfunctie voornamelijk gezien vanuit het perspectief van trekvogels. In **hoofdstuk 12** plaats ik dit proefschrift in de bredere context van het begrijpen van immuunfunctie en de verhouding met pathogenen druk, niet alleen voor kanoeten maar ook voor andere soorten. Gebaseerd op ideeën die afgeleid zijn van dit proefschrift en van de literatuur, introduceer ik een conceptueel model dat ideeën over het verdelen van hulpbronnen en de kosten van immuniteit plaatst in een bredere context van verdediging tegen echte pathogenen in omgevingen waar tal van factoren veranderen met de tijd en ruimte. Ik suggereer ook onderzoeksrichtingen, die zullen helpen bij het testen van dit model en die in staat zijn om een verband te leggen tussen immuunfunctie, pathogenen druk en een optimale verdediging tegen ziekte.

CONCLUSIES

Dit proefschrift biedt een aantal conclusies voor onderzoekers die trek, jaarcycli en ecologische immunologie bestuderen. Ten eerste hebben trekvogels te maken met bottlenecks of "zware tijden" tijdens hun jaarcyclus en een netwerk van deze bottlenecks kan gebruikt worden om voorspellingen te doen over immuunfunctie. Ten tweede varieert immuunfunctie significant tijdens de jaarcyclus, zelfs in vogels in gevangenschap, en de variatie suggereert dat vogels verschillende "immuunstrategieën" gebruiken tijdens verschillende stadia in hun jaarcyclus. Ten derde hebben condities die uitdagend zijn voor de energiebalans geen invloed op het constitutieve immuunsysteem, wat er mogelijk op wijst dat een basaal niveau van immuun functie nodig is en dat vogels energie besparen met meer kostbare aspecten van immuniteit. Ten vierde bepaalt, naast de aanwezige hulpbronnen, de pathogenen druk in de onmiddellijke omgeving waarschijnlijk de sterkte en de strategie van de immuun verdediging. Ten vijfde is melatonine niet verbonden met daglengte en correleert het niet met immuun functie in kanoeten. Dus, hoewel melatonine het mechanisme voor jaarlijkse variatie in zoogdieren zou kunnen veroorzaken, is dit waarschijnlijk niet het geval in vogels. Ten slotte wordt immuunfunctie in het wild bepaald door tal van factoren waaronder verschillen in hulpbronnen, energie-uitgave en pathogenen druk in verschillende omgevingen.

BOX 1. HET IMMUUN SYSTEEM, DE KOSTEN VAN IMMUNITEIT EN DE IN DIT ONDERZOEK GEBRUIKTE MEETTECHNIEKEN

Het immuun systeem

Onze wereld bevat een diversiteit aan besmettelijke micro-organismen en het levende lichaam biedt een omgeving die rijk is aan water en nutriënten voor deze indringers. Het wordt bovendien al bevolkt door een florerende populatie commensale micro-organismen die onderhouden moet worden. Vogels en andere vertebraten hebben een complex netwerk van overlappende verdedigingsmechanismen ontwikkeld om het lichaam te beschermen; het immuunsysteem. Immuunreacties zijn complex en zijn op vele manieren beschreven. Een van de meest bondige omschrijvingen stelt voor dat het immuun systeem op twee algemene assen valt. De eerste as verwijst naar de mate van specificiteit van de immuun reactie, met als twee extremen *niet-specifiek* en *specifiek*. De tweede as verwijst naar de tijdsgebonden dynamiek van de immuun reactie en hier zijn de twee extremen *constitutief* en *geïnduceerd*. Constitutieve immuun functie wordt voortdurend onderhouden en biedt zo een algemeen systeem dat surveilleert en repareert. Een geïnduceerde immuunreactie wordt slecht op gang gebracht wanneer een pathogeen zich gevestigd heeft in het lichaam. Over het algemeen is constitutieve immuunfunctie niet-specifiek. Geïnduceerde immuun functie reageert daarentegen op specifieke pathogenen. Deze associatie leidt tot de beschrijving in de meeste immunologische tekstboeken in twee algemene categorieën: aangeboren en verworven immuun functie.

De weg van een pathogeen

Een goede manier om een immuunreactie te begrijpen is door de weg die een hypothetische pathogeen aflegt te volgen. De weg van een pathogeen begint buiten het lichaam en de indringer moet eerst de fysische, chemische en gedragsbarrières (de huid, maagzuur en gedrag zoals poetsen en verzorgen van de vacht) van het lichaam overwinnen. Wanneer de pathogeen het lichaam heeft binnengedrongen komt het surveillance cellen tegen zoals fagocyten, cytotoxische T-cellen en natural killer cellen. Deze cellen fagocyteren de indringers en laten stoffen los die meer fagocyten en dendritische cellen naar de plek van de infectie brengen. Veel pathogenen eindigen hier en de niet-specifieke cellen en eiwitten kunnen het lichaam in een paar uur van pathogenen vrij maken. Als de pathogeen echter stand houdt laten macrofagen cytokinen los die een acutefase-reactie op gang brengen. Gedurende de acutefase-reactie produceert de lever acutefase-eiwitten en de gastheer voelt zich moe, heeft minder eetlust en koorts. Tegelijkertijd bewegen de fagocyten die de pathogenen gefagocyteerd hebben zich naar de lymfknopen of de milt om daar de peptiden die kenmerkend zijn voor de pathogeen aan de T-cel lymfocyten te tonen. Gedurende de volgende paar dagen zullen T-cellen zich vermeerderen en, afhankelijk van het type pathogeen, zullen ze een reactie gekoppeld aan

de ontsteking en T-cellen (intracellulaire pathogenen) of een reactie gekoppeld aan antilichamen en B-cellen (extracellulaire pathogenen) teweegbrengen. Cytokinen en antilichamen zullen feedback geven aan niet-specifieke surveillance cellen, wat de efficiëntie van fagocyten verhoogt doordat de pathogenen op een specifieke manier gemarkeerd worden voor de vernietiging. Nadat de infectie is opgeruimd zorgen geheugencellen (B- en T-cellen) er voor dat er een snelle, specifieke reactie plaatsvindt wanneer dezelfde pathogeen het lichaam opnieuw probeert binnen te dringen. Alle takken van het immuun systeem werken samen gedurende een immuunreactie, met constitutieve aangeboren immuniteit als de eerste verdedigingslinie en geïnduceerde verkregen immuniteit die de efficiëntie van aangeboren schakels in het immuunsysteem gedurende de latere stadia van de reactie verhogen.

De kosten van immuniteit

Het hebben van een immuunsysteem gaat samen met het voor de hand liggende voordeel van verhoogde resistentie tegen ziektes, maar het brengt ook kosten met zich mee. De meest basale prijs die verbonden is met het immuunsysteem is een evolutionaire prijs, die zich uit wanneer het immuun systeem zich ontwikkelt ten koste van een andere eigenschap. Vanuit een ecologisch standpunt zijn er ten eerste bronnen-kosten, die betaald worden in de vorm van gelimiteerde hulpbronnen (energie of nutriënten) die belangrijk zijn voor zowel immuunfunctie als voor andere aspecten van het leven van de gastheer (b.v. broeden, trek). Ten tweede zijn er immunopathologische kosten die betaald worden in de vorm van schade aan eigen cellen wanneer het immuun systeem vecht tegen de cellen van de indringer. Ten slotte zijn er kansen-kosten die betaald worden in verloren kansen wanneer tijd gespendeerd wordt aan de ontwikkeling of het gebruik van het immuun systeem in plaats van aan andere belangrijke gebeurtenissen in de levensloop (b.v. broeden, trek). Deze kosten bieden een conceptueel kader en benadrukken het feit dat er, door de complexiteit van het immuunsysteem, geen eenduidige prijs voor immuniteit is.

In dit onderzoek gebruikte immuun assays

We hebben verschillende meetmethodes gebruikt om constitutieve immuniteit te kwantificeren: (1) de microbial killing assay meet de functionele capaciteit om de infectie in te perken. Hogere “killing” betekent een betere verdediging tegen het micro-organisme dat gebruikt is in de assay. (2) Leukocyt concentratie geeft informatie over de circulerende immuun cellen en kan gebruikt worden als een indicator voor de gezondheid. Met name heterofiele en eosinofiele cellen zorgen voor aangeboren immuniteit tegen nieuwe pathogenen en zijn belangrijke fagocyten. Monocyten vormen een verbinding tussen aangeboren en verworven immuniteit en lymfocyten verzorgen pathogenen-specifieke antilichamen en cellulaire afweer. (3) Het hemolyse-hemagglutinatie assay meet de concentraties complement en antilichamen die een eerste verdedigingslinie vormen tegen zich verspreidende

infecties via lyse en agglutinatie van cellen, en die het aangeboren en verworven immuun systeem verbinden. (4) Het haptoglobine assay meet haptoglobine, een acutefase eiwit dat ijzer (heem) bindt zodat het niet meer beschikbaar is voor pathogenen. We kwantificeren geïnduceerde immuniteit door een bacteriële infectie na te bootsen met lipopolysaccharide (LPS) om een acutefase-reactie te induceren en meten vervolgens gewichtsverlies, voedselopname, koorts en gedragingen die duiden op ziekte.

Wadden Sea

Acknowledgements

To my family, *in every sense of the word*

Family: A group of individuals related by blood or marriage or by a feeling of closeness.

I've always been a dreamer, but if someone would have told me when I was 10 that I'd get paid to feed my own curiosity and to travel around the world studying migrating birds, even I might not have believed them. And yet, in many ways that is what I've been privileged enough to do these past four years. But doing a PhD is not all the stuff of dreams, it is hard work and many people have helped me on this journey.

I would like to start by thanking my supervisors – or perhaps "mentor" is a better word. A mentor is defined as "a wise and trusted counselor or teacher" and both Theunis Piersma and Irene Tieleman have been mentors to me and have taught me much about science and about being a scientist. I thank Theunis for always believing in me, even when I couldn't believe in myself. Your guidance and support, from my arrival in the Netherlands to our "Tuesday meetings" while I was writing up, were invaluable. Even the most cryptic of comments ended up making sense in the end. I thank Irene for coming in as a guiding light during the "dark days" of start-up and for keeping me grounded. Your clarity of mind is unlike anything that I have encountered before, thank you for sharing it with me and for teaching me to think and write more clearly. Thank you also for our conversations about ideas and ideals in academia. They were wonderful "career counselling sessions" for me.

This research was conducted both at the University of Groningen and at the Royal Netherlands Institute for Sea Research (NIOZ), and more people than I can mention here helped me in both places. During the bleary-eyed days of our year-long experiment François Vézina was an endless source of inspiration and comic relief. Your humour kept me going and you taught me much about experimental design, bird metabolism, and how to balance being a scientist with having a family. Wouter Vahl was kind enough to offer me his house during the food limitation experiment and was also a wonderful office mate. I enjoyed our discussions about science and life immensely. When I needed "girl power" Jutta Leyrer was there, at NIOZ, on the Navicula and in Mauritania. Thanks for the friendship, conversations, rants and laughs. Jeroen Reneerkens shared his knowledge about food limitation experiments and corticosterone studies at NIOZ, and in Mauritania he taught me much about photography and the art of sanderling catching. All of my experiments at NIOZ and in the Wadden Sea were helped by Maarten Brugge, Anne Dekinga, Bernard Spaans and the crew of the H. M. Navicula who provided excellent technical support in the field and lab, and by Judith van Bleijswijk who generously allowed me to run assays in the ML II lab. I also thank Anneke Bol and Oliver Haddrath (in Toronto) for helping with the molecular sexing of birds when I couldn't be in contact with ethidium bromide. Finally, I thank the whole Department of Marine Ecology at the NIOZ for a stimulating work environment and lots of support (I still owe you all cake).

In Groningen, Julia Schroeder stood by me from start to finish (even now as my paranymph) offering stimulating professional conversation, great food and most importantly friendship. You helped me to stick with this thing through good times and bad, and have been a role model in terms of balancing family and PhD. I think that neither one of my "babies" would be here without your support. Yvonne Verkuil has also been there from start to finish offering support when I first arrived in Groningen and insight with my synthesis figures in Toronto just a few weeks ago. At the halfway point of this work "Team Tieleman" entered the scene and I found myself surrounded by an interna-

tional group of great people all interested in immune function and disease in birds! Nick Horrocks, Arne Hegemann, Katie Hine and Liz Kleynhans thanks for great fun at social gatherings, field sites and in the lab. Kevin Matson and Maaike Versteegh thanks for being great office mates and exercising your uncanny ability to listen to (and even enjoy) my rants. Thanks also for lots of supervision, especially with stats (Kevin) and for being a fellow "everythingologist", document translator, and paranymph (Maaike). From an administrative standpoint, Suus Bakker-Geluk, carole Elling, Joke Nunnink and Joyce Rietveld helped me to navigate the Dutch university system and handled a myriad of organizational tasks. Many more people entered my life at the Biology Centre in Groningen and I thank in particular members of the Animal Ecology Group for a stimulating work environment and members of the Theoretical Biology Group for letting me sit in on a few of their meetings even though most of it sailed right over my head.

Academic jobs are as much about teaching as about research and I thank my students and assistants Daliborka Barjaktarov, Nina Bhola, Francisco Encinas-Viso, Steven Haan, Mieke Lange, Eeke Marks and Magali Petit for their excellent assistance and for helping me to gain the confidence I needed to supervise students. I'll not soon forget the laughter, brain storming sessions, ferry rides, camper living, or bucket carrying ("over the dyke"). I would also like to thank the people in my "adoptive" group in Toronto for providing me with a place to work while finishing the last bits of writing.

Of course I cannot forget the finances. This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC), the University of Groningen (to me in the form of an Ubbo Emmius grant and to my supervisors), Schure-Beijerinck-Popping Fonds, the Netherlands Organization for Scientific Research (NWO) and the Royal Netherlands Institute for Sea Research (NIOZ). It was also *unofficially* supported by the Groningen student's financial "holy trinity": CareX which kept us housed, Adli which kept us fed and Mamamini/ Notabene which kept us clothed and furnished.

PhD researchers are also supported by many people outside of their working groups. I thank all of my friends, close by and far away, for showing an interest in my research and then for helping me to forget about it. Thank you for accepting me as I am and for encouraging me to never be anything less. I also thank my parents who have always supported my "global gallivanting" and my academic endeavours. Your love and support from day one have helped to make this thesis possible, in particular chapters 1, 8 and 12 could not have been written without the excellent childcare provided by "grandma and grandpa" when we moved back to Canada.

My husband Alberto Castillo supported and helped me in so many ways that I don't even know where to begin. This work is just as much yours as it is mine. When I had ideas and goals, you helped me to achieve them as my field technician, my lab technician and my computer technician ... the list goes on and on. When I needed a break you provided the music, the laughter and the love. In the beginning when all was dark, you were my light. And in the end when we produced a baby, you were there to take care of him so that chapters 8, 10 and 11 could be written.

Finally I thank Aramis, my beautiful baby, for giving me the most important personal and professional gift of all – perspective.

Deborah M. Buehler – Toronto, 1 September 2008

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