CHAPTER 12

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

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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

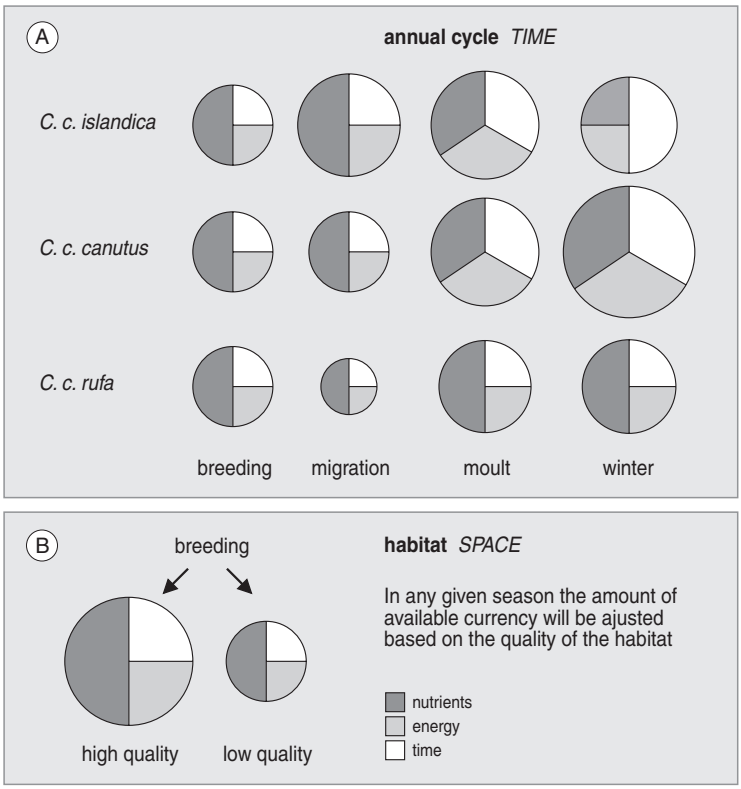

Figure 12.1. A conceptualization of how capital to invest in immune function varies over the annual cycle (A) and in different habitats (B). The sizes of the circles represent the overall amount of available capital and the different assets are shown as proportions (see text and box 2 for details). Note 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-

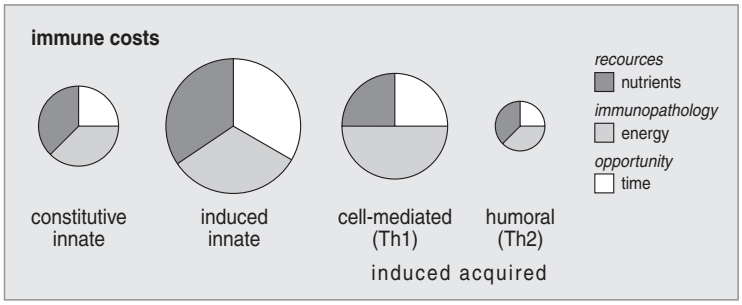

Figure 12.2. An illustration of how the costs of immunity vary between the different arms of the immune system. The different sub-branches of constitutive immunity treated in chapters 5 and 7 are combined for simplicity. Cell-mediated immunity (Th1) refers to induced acquired immunity mediated by Type 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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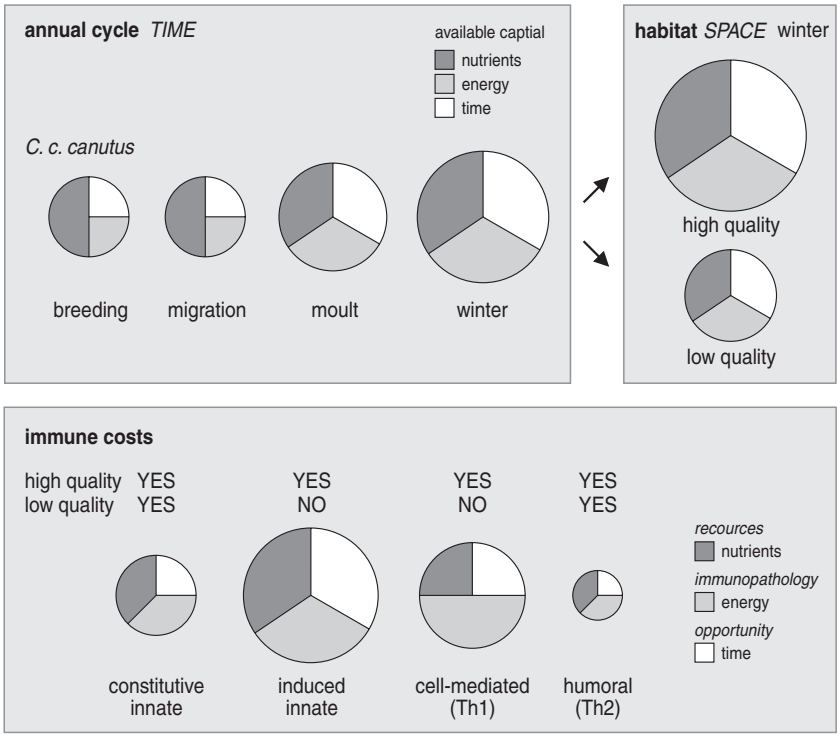

Figure 12.3. A hypothetical example of how annual cycle and habitat circumstances can combine with the costs of immunity to determine an individual's immune potential, which is constrained by the total amount of capital it has to invest. A red knot of the *C. c. canutus* subspecies, wintering in a high 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.

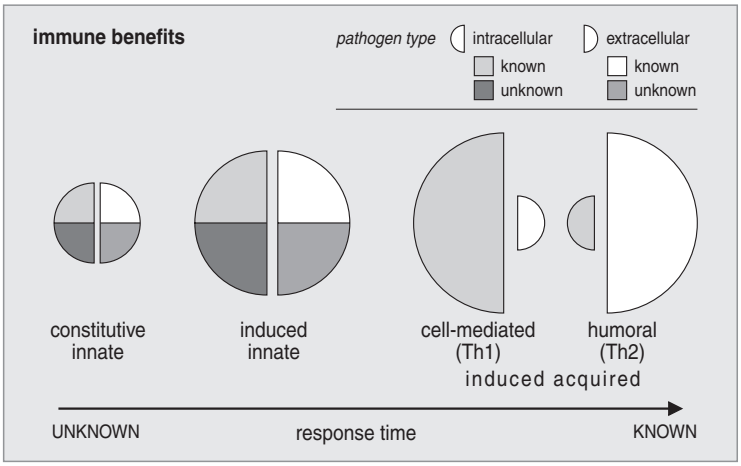

Figure 12.4. An illustration of the benefits of different branches of the immune system. Pathogens are classified as intracellular and extracellular with benefits against intracellular threats depicted on the left side of the circles and benefits against extracellular threats on the right side of the 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

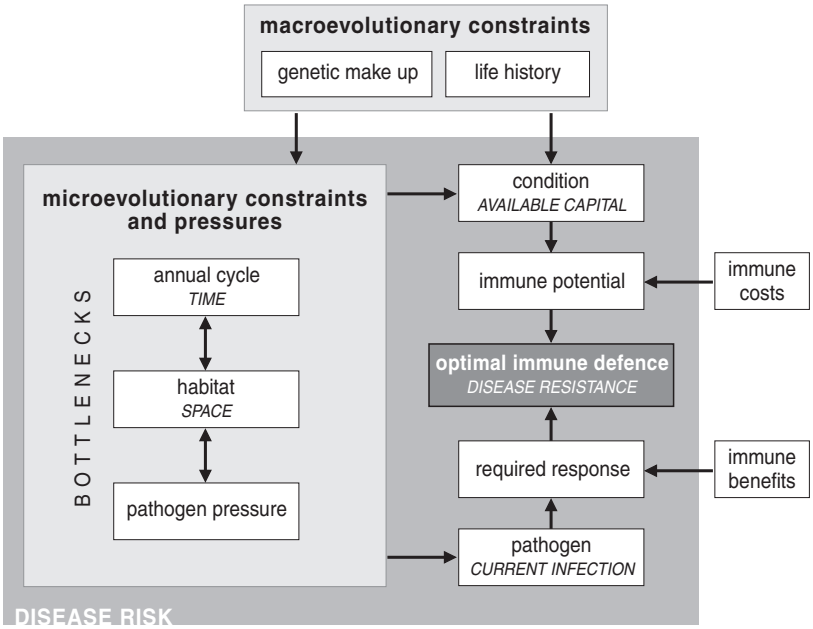

Figure 12.5. The immune potential-required response-optimal defence model. This model highlights the fact that an animal's optimal immune defence is a balance between the capital it has to invest in the costs of immunity on the one hand (immune potential), and the pathogen threats and the benefits of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

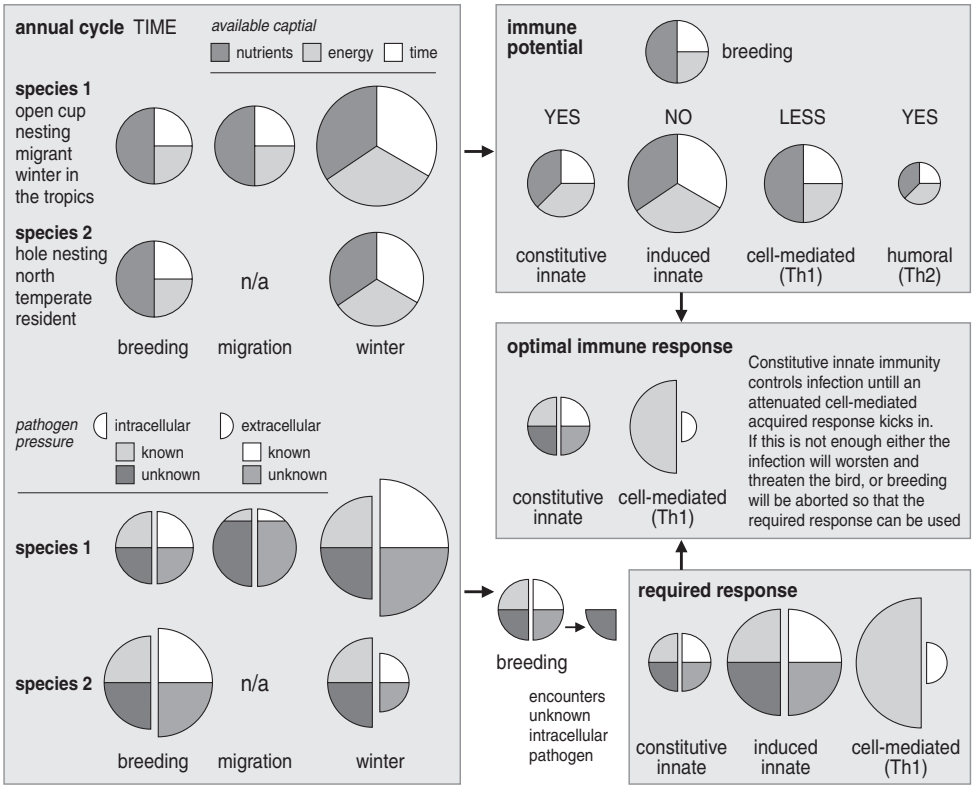

Figure 12.6. An example of the immune potential-required response-optimal defence model in two hypothetical species. The left side of the figure illustrates variation in available capital and pathogen pressure in two contrasting species. Variation is not based on data for real species and is simplified 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.