ENVIRONMENTAL INFLUENCES AND EARLY LIFE EXPERIENCES ON IMMUNE FUNCTION OF THE ZEBRA FINCH (TAENIOPYGIA GUTTATA)

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GENERAL ABSTRACT

Understanding what drives variation in immune function has been a focus of avian eco-immunologists in the past few decades. I conducted several very novel experiments to investigate potential sources of variation. First, I assessed variation in innate and adaptive immunity during nestling rearing in adult zebra finches whilst experimentally manipulating the microbial load of the nesting environment. The primary immune response (adaptive) was much higher in birds attending dirty nests (higher microbial load) than clean nests, suggesting that the microbial environment upregulated the adaptive immune system. Despite differences in nest sanitation, constitutive innate immune function decreased in adults throughout the period of caring for their young, but was not affected by nest microbial load itself. Surprisingly, nest sanitation did not impact upon innate or adaptive immune function or growth in nestling zebra finches. Interestingly, the innate and adaptive immune measures tested were not fully functional at the time of fledging or even at 60 days, which may suggest that young altricial passerines may tolerate rather than defend against potential pathogens at that stage. Secondly, I investigated the development of innate immune function in juveniles that had been subjected to an experimental acoustic playback whilst they were embryos. Eggs exposed to incubation calls were fed more as nestlings and were heavier at 13 days of age. However, as juveniles (around 60 days of age), males were in poorer body condition than females but males had much better constitutive innate immune function, suggesting a trade-off between condition and immune function. Overall, these results collectively suggest that the microbial environment, and acoustic components of the rearing environment, can lead to variation in different facets of immune function. Studies investigating immune function in wild birds should consider these potential causes of variation to get an accurate representation and understanding of bird health and functioning.

DECLARATION

I certify that the work in this thesis entitled "Environmental influences and early life experiences on immune function of the Zebra Finch (*Taeniopygia guttata*)" has not been previously submitted for a degree nor has it been submitted as part of requirements for a degree to any other university or institution other than Macquarie University.

I also certify that the thesis is an original piece of research and it has been written by me. Any help and assistance I have received in my research work and the preparation of this thesis itself have been appropriately acknowledged (see Preface). In addition, I certify that all information sources and literature used are indicated in the thesis.

The research presented in this thesis was approved by Deakin University Animal Ethics Committee (No. G24-2013, G29-2013)

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PREFACE

Chapters three and four of this thesis will be submitted to peer reviewed scientific journals for publication, and as such, have been edited for the intended journal's styles. Chapter two, after some further editing will be sent to the Journal of Avian Biology. Chapter three is currently in review for the Journal of Experimental Biology. Chapter four will be sent to the Journal of Experimental Biology. Chapter five will be sent to the journal Emu.

In chapter two, there is an adapted figure, originally from Klasing and Leschinsky.

My academic supervisors, Katherine Buchanan, Simon Griffith, Kirk Klasing and Mylene Mariette have helped me with the conception, analysis and writing presented in this thesis. Katherine, Simon and Kirk are co-authors of chapters two, three and five, while Katherine, Simon and Mylene are co-authors of chapter four. My personal estimated contribution, and that of other co-authors, to each chapter, is indicated below.

Chapter One: Conception 100%, writing 90%

Chapter Two: Conception 70%, writing 80%

Chapter Three: Conception 80%, data collection 100%, analysis 80%, writing 90%

Chapter Four: Conception 50%, data collection 50%, analysis 50%, writing 80%

Chapter Five: Conception 80%, data collection 100%, analysis 100%, writing 90%

Chapter Six: Conception 100%, writing 90%

CHAPTER ONE

Introduction

Photo Simon C. Griffith

INTRODUCTION

Understanding variation in immune function has been the main objective of ecological immunologists or eco-immunologists in the past few decades (Nelson and Demas, 1996; Norris and Evans, 2000; Schmid-Hempel, 2003). There are two central hypotheses, that are not mutually exclusive, to explain variation in immune function. The first is the antigen exposure hypothesis (Horrocks et al., 2011), which suggests that exposure to antigens in early life shapes investment in immunity in later life. Environments where there is a greater microbial pathogen exposure therefore might select for a stronger immune response (Horrocks et al., 2012a; Horrocks et al., 2011). The second hypothesis is the trade-off hypothesis (Sheldon and Verhulst, 1996), which predicts that consumption of limited resources by the immune system leads to trade-offs between immune function and other resource-demanding activities such as reproduction (Ilmonen et al., 2000; Lochmiller and Deerenberg, 2000; Norris and Evans, 2000; Sheldon and Verhulst, 1996). However, other factors such as seasonality (Hegemann et al., 2012; Nelson and Demas, 1996), pathogen and parasite load (De Coster et al., 2010; Grindstaff et al., 2006; Horrocks et al., 2011; Råberg et al., 2009; Sorci, 2013), and development (Ardia, 2005; Ardia et al., 2010; Arriero, 2009; De Coster et al., 2011; Martin et al., 2011; Palacios and Martin, 2006; Pap et al., 2015; Saino et al., 2003) can contribute to variation in immune function.

Birds and microorganisms

Birds have coevolved with microorganisms (McFall-Ngai et al., 2013), since birds first appeared in the Mesozoic era (Benson et al., 2014). Thus, it is expected that microbes have played a significant role in shaping the behaviour, physiology and

immune function of birds. However, this complex, yet important relationship has only gained serious attention in the last decade (Archie and Theis, 2011; Bäckhed et al., 2005; Hooper et al., 2012; Lee and Mazmanian, 2010; McFall-Ngai et al., 2013). As a result, there are many gaps in our understanding of bacterial-immune relationships, particularly in non-production birds and natural ecologically-relevant systems.

The avian immune system

In determining how microorganisms may impact upon immune function, we must understand how the immune system functions and fights off bacteria and pathogens. The immune system is complex and highly evolved structure, used as a defence to identify and destroy invading foreign molecules not recognised as "self" in an attempt to prevent disease (Fairbrother et al., 2004; Farmer et al., 1986). There are two arms of the immune system: the innate and adaptive. The innate immune system is the first line of defence and is rapid, non-specific and has no cell memory, so does not need prior experience of a pathogen to mount an attack (Sharma, 1991). The innate system relies on many different cells to initiate these responses including phagocytes (macrophages and neutrophils), mast and natural killer (NK) cells (Fairbrother et al., 2004) as well as antimicrobial proteins that circulate the body as part of the complement system. Constitutive immunity is not induced, it is a response that is always present and capable of defence without previous exposure (Schmid-Hempel and Ebert, 2003). Measures of non-specific constitutive innate immune function include microbial killing assays, which use whole blood to demonstrate the killing efficiency of known strains of microorganism. Depending on the microorganisms chosen, the test can measure both humoral (complement) and

cellular (phagocytosis) constitutive immune function. The other arm of the immune system, the adaptive (also known as acquired or specific) system relies on memory and is specific to foreign antigens. The adaptive immune response has two parts: humoral (antibody) mediated immunity and cell mediated immunity, however, this thesis focuses only on humoral (antibody) immunity. Humoral immunity uses antibodies which are produced by B cells to specifically fight one type of antigen. IgM is the first isotype produced in the primary response when a novel antigen is encountered (Davison et al., 2008). After exposure to an antigen, antibody switching occurs (after specific signaling molecules cause this change) and memory B cells are produced in the form of IgY, which is the main immunoglobulin present in a secondary response.

Microbial-immune relationships in birds

My aim has been to address the gaps in current research focussing on microbial-immune and ecological relationships in birds, in particular, how microbial exposure influences both immune function development and growth in chicks and also the immune function of adult birds. The literature review highlights areas about what is currently known and areas for future research on how bacteria and microorganisms affect birds. The review served to identify when bacteria might have important effects on avian fitness and life history stages. To test microbial immune relationships, it is important to test both adult and nestling immune function in relation to experimentally manipulated microbial exposure levels. Testing both adults and nestlings is important because in offspring of both germ free mice and poultry, it has been demonstrated that bacteria are necessary for the development of a competent immune system (Berg, 1996; Gabriel et al., 2006; Lee and Mazmanian,

2010; Rhee et al., 2004; Thorbecke et al., 1957). However, we do not know whether variation in microbial exposure in the environment (and outside of germ free environments) will have a similar effect on birds. Although not much work has been conducted on adult microbial-immune relationships outside of pathogen and disease studies, there is some evidence to suggest that there is covariation between microbial exposure and certain measures of innate immunity (Horrocks et al., 2012a; Horrocks et al., 2012b; Soler et al., 2011). The antigen exposure hypothesis suggests that exposure to antigens in the environment shapes investment in immunity (Horrocks et al., 2012a; Horrocks et al., 2011). Environments where there is a greater amount of microbial pathogen exposure therefore might select for a stronger immune response (Horrocks et al., 2012a; Horrocks et al., 2011). Environmental influences on immune system development through microbial exposure has been demonstrated previously in chickens and mice, where offspring raised in germ free environments have a reduced functional immune capacity into adulthood, compared with offspring raised in conventional environments (Bedrani et al., 2013; Lee and Mazmanian, 2010; Lundin et al., 2008; Olszak et al., 2012; Pels and Denotter, 1979; Pollard and Sharon, 1970). To some extent, this has also been found in wild birds (red knots (*Calidris canutus*), lark species (*Aluadidae*)), where microbial exposure have positively correlated with various measures of the innate immune response (Buehler et al., 2008b; Horrocks et al., 2012a; Horrocks et al., 2012b; Soler et al., 2011). Both the innate and adaptive immune systems have not been tested at the same time in relation to environmental microorganisms and there are a lack of studies that have experimentally manipulated the microbial environment (outside of germ free environments in poultry and mice). Horrocks et al. (2012a) explored differences in environmental bacteria in the wild by testing immune function in seven species of lark between temperate and desert environments that are known to vary in microbial diversity, but only tested innate mechanisms. The microbial environment differs

between different climatic regions (Horrocks et al., 2012a), between nest types and between nests that are new and reused (Godard et al., 2007; Singleton and Harper, 1998; Walls et al., 2012). Nest sanitation also varies greatly between and within bird species (as reviewed by Guigueno and Sealy, 2012) contributing to microbial diversity and abundance, which could then explain variation in immune function. Potentially, the nesting environment could be a driver of immune function, given that nestlings spend all of their time in the nest during a crucial and sensitive period of early development until fledging and adults spend a considerable amount of time in the nest caring for their eggs and offspring. Nestlings are inoculated with microorganisms from the nest lining, saliva and food from parents after hatching (Benskin et al., 2009; Berger et al., 2003; Kyle and Kyle, 1993; Mills et al., 1999; Singleton and Harper, 1998) and thus the nest environment should play an active role in shaping immune function.

Trade-offs with immune function and other competing physiological demands

Trade-offs are common in animals, particularly since there is a limited amount of resources that are required to fuel many different physiological functions. Sheldon and Verhulst (1996) proposed that the main cause of variation in immune function is a trade-off between immune function and other competing costly physiological functions. Although many studies of birds have focused on trade-offs between reproduction and immune function (Deerenberg et al., 1997; Ilmonen et al., 2000), very few have identified condition immune trade-offs or the relevant situations that occur naturally that would contribute to this variation. It has also been suggested that microbial exposure in the environment during life events could also contribute to variation in immune function (Buehler et al., 2008a; Evans et al., 2015), however,

testing trade-offs and manipulating microbial exposure has not been previously tested to my knowledge.

Incubation calls during embryonic development and growth-immune trade-offs

Incubation calls have recently been identified as a key factor determining the growth, development and behaviour of young nestling birds (Colombelli-Négrel et al., 2012). In this thesis, I have aimed to address the current gaps in knowledge about conditions that occur during embryonic development which lead to trade-offs in growth and immune function in nestlings and juvenile zebra finches. Since incubation calls have been identified as a potential mechanism to increase feed rates and growth, this could also have implications and knock on effects to other important functions such as immunity (Fargallo et al., 2002; Hõrak et al., 1999; Martin-Galvez et al., 2011; Moreno-Rueda and Redondo, 2012; Saino et al., 2001). Incubation calls are uttered when a partner is alone in the nest during incubation, are simple in structure, being dominated by a single frequency. Tet and whine calls however, are uttered by the parents in the nest when one partner comes back to the nest to relay its partner for incubating the eggs (Elie et al. 2008). Whine calls are used exclusively in the nest, whereas tet calls are a contact call used in a variety of contexts, including at the nest. They both are complex calls modulated in frequency. Although the literature on the purpose of incubation calls in birds is fairly limited, it has been found that hearing incubation calls of parents during the last week of embryonic development increased pecking behaviour and showed stronger response to feeding and alarm calls in ring necked pheasants (*Phasianus colchicus*) (Bailey, 1983), although controls were exposed to no noise or calls. More recent work on superb fairy-wren (*Malurus cyaneus*) incubation calls has suggested that at hatching,

nestlings produce begging calls with key elements from the mothers incubation calls (Colombelli-Négrel et al., 2012), in turn causing nestlings producing this element to be fed at higher rates. There is the potential that listening to incubation calls in the egg can increase communication between nestling and parent, increasing food intake and growth, with the potential of a growth immune trade-off occurring. However, because incubation calls have been little studied, it is unknown as to whether growth conditions or immunity may differ in those that are exposed to these calls during incubation.

Ontogeny of immune function

Age related changes to immune function in nestlings have been well established in poultry, however, an understanding of both innate and adaptive immune changes is lacking in non-domesticated birds. Therefore, another important aspect of this thesis is to document age related changes in a captive model species - the zebra finch. Understanding whether innate immune defence is fully developed prior to fledging is of particular interest, given that adaptive immune function seems to develop quite late in finches (Killpack and Karasov, 2012), and it could be predicted that finches rely on rapid innate development prior to fledging for protection against pathogens they might encounter outside of the nest. Also of interest is the exact timings of adaptive immune development of zebra finches which is thought to occur somewhere between 7 and 14 days (Killpack and Karasov, 2012).

Alternative hypotheses for variation in immune function

Although not fully considered within this thesis, there are many other factors that contribute to variability in the functioning of the immune system. Seasonal

variation in immune function is thought to occur due to a reallocation of resources and linked to certain seasonal events (Hegemann et al., 2012; Nelson and Demas, 1996). Events such as reproduction, migration, moult, temperature/seasons have all been examined when assessing variability in immune function. Nelson and Demas (1996) hypothesise that mechanisms have evolved in some animals to combat seasonal immunosuppression by responding to seasonal cues. For example, winter is an energetically costly period for animals. It is known that animals upregulate the immune response during winter periods using cues such as photoperiod and day length, to promote survival (Nelson and Demas, 1996). Handling stress has also been shown to introduce variability in immune measures, caused by the stress induced hormone corticosterone (Buehler et al., 2008). Many studies have shown significant differences in immune function from birds measured at 3 mins and then at 30 mins (Buehler et al., 2008, Matson et al., 2006, Millet et al., 2007). However, chronic stress has also been suggested to be immunosuppressive (Råberg et al., 2008). When examining variation in immune function between species, there are noticeable differences and strategies. One hypothesis examining this variation is the pace of life hypothesis which suggests species with a fast pace of life (short development, high energy turnover) will invest more in growth and reproduction, with little investment in immune function. Slow paced species, however, will invest in immune function and will develop more slowly. For example, Pap et al. (2015) found basal metabolic rate correlated negatively with immune measures in 63 species and also found species with long incubation periods invested more in constitutive innate immune function. Immune function has also been shown to vary due to pathogen and parasite load (De Coster et al., 2010; Grindstaff et al., 2006; Horrocks et al., 2011; Råberg et al., 2009; Sorci, 2013), and development (Ardia, 2005; Ardia et al., 2010; Arriero, 2009; De Coster et al., 2011; Martin et al., 2011; Palacios and Martin, 2006; Pap et al., 2015; Saino et al., 2003). However, hypotheses around variation of immune

function are not limited to these few examples, and of course, many are not mutually exclusive.

My approach and methodological considerations

Overall, this thesis has the aim of testing and manipulating the environment (microbial exposure and parental calling) and documenting developmental changes in embryos, nestlings, juveniles and adults that lead to variation in immune function. The antigen-exposure hypothesis will be tested in relation to manipulated microbial load of nest and the immune response of adults and nestlings to this manipulation. The trade-off hypothesis will be tested in adults from microbial manipulated nests during nestling rearing to reveal whether energetically expensive periods such as parental care and trade-offs with immune function, or microbial exposure, is more important in determining variation in multiple facets of immune function. Similarly, I will test the trade-off hypothesis when testing growth conditions and immune function in nestling and juvenile zebra finches that have different parental calls (incubation or tet calls) played during embryonic development.

To test microbial-immune interactions in nestlings and adults, I need to experimentally manipulate the nesting environment and test across both the innate and adaptive immune system. Experimental manipulation of the bacterial content of the nest will be conducted by cleaning (sterilisation and replacing nest material) and dirtying of the nest (adding novel sources of bacteria by using chicken faeces as well as accumulation of nestling faeces). Testing immune function of adults between clean and dirty environments during breeding will also help understand whether

some aspects of immune function, such as microbial killing are more affected by a trade-off between immune function and other physiologically demanding life events or shaped more by microbial risk. These possibilities have previously not been separated in other wild bird studies (Buehler et al., 2008a; Evans et al., 2015). This in turn will test the trade-off and antigen exposure hypothesis. Understanding microbial-immune relationships and how exposure to microbes in the nest affects different aspects of immune development in nestlings, and general functioning in adults is important and highly novel. Understanding these relationships with help us further understand the importance of microbial exposure in shaping avian physiology and behaviour and give insight into which immune mechanisms are most affected by microorganisms in the environment. This will in turn test whether exposure to bacteria in the environment increases the strength of immune function.

To test the role of the incubation call on growth and immune function, experimental testing of incubation or tet calls being played to eggs during the last 5 days of embryonic development in artificial incubators and being placed back to the nest to assess growth and feeding is necessary. Testing innate immune function in sexually mature juveniles will help assess whether growth conditions during nestling development and after fledging have an impact on immunity at later stages of life, therefore testing the trade-off hypothesis. Testing carry over effects of immune function from incubation to later stages of life has been tested previously (Ardia et al., 2010), however, testing the role of parental communication and incubation calls on growth and immune function is highly novel and is yet to be tested.

To test ontogeny of immune function, I will assess both innate and adaptive immune function before and after fledging and at sexual maturity. This will be to test the developmental timeline of immune function, the age in which adaptive immunity starts to develop as well as which mechanisms are relied upon after the bird leaves the nest. Development and maturation of immune function has been little studied outside of precocial birds and so investigating this development in altricial passerines is extremely important in understanding immune function and variation in immune function in non-production birds.

The laboratory provides a good consistent environment where experimental manipulation can be carried out, particularly in terms of manipulating incubation conditions and microorganism content of the nest. Experimental manipulation of bacterial load in the nest is necessary to test the direct effect on the immune system. Testing both the innate and adaptive immune systems has been a considerable problem in wild studies investigating immune-bacterial relationships because of the issue of recapturing birds. Recapture is necessary in testing adaptive immunity due to giving of multiple injections and sampling blood across different time points. This is where the laboratory environment is critical in understanding direct impacts on experimental manipulation of nest contents on innate and adaptive immunity. To understand the role of the incubation call on growth and immune function at later stages of life, it is important to be tested in the laboratory. This is so that playback of either incubation or tet calls is consistent across treatments and the direct effects of the calls can be tested on growth and immune measures.

To investigate constitutive innate immunity, I used a microbial killing assay (Buehler et al., 2008a; Buehler et al., 2008b; Keusch et al., 1975; Matson et al., 2006; Millet et al., 2007; Tieleman et al., 2005), which tests the ability of whole blood to kill microorganisms. I used two microbial strains to test humoral (complement) and cellular (phagocytosis) innate immune function. To test adaptive immunity, I used an agglutination assay (Deerenberg et al., 1997) with sheep red blood cells (SRBC) as the antigen.

I used the zebra finch (*Taeniopygia guttata*) for this work because its ecology, physiology and behaviour are well studied (Gil et al., 1999; Lemon, 1991; Scharff and Nottebohm, 1991; Zann, 1996), they are well adapted to captivity and are abundant in the wild across different climatic regions of Australia (Zann, 1996), thus a good model to study environmental influences on immune function. In the zebra finch, nest sanitation is quite poor, with faeces in the nest continuously building up as nestlings develop and defecate around the nest cup. This exposes the nestlings and adults to faecal bacteria (Benskin et al., 2009); as well as other sources of bacteria being present within the nest. Also being an altricial passerine, communication through song is extremely important and because this species produces an incubation call, I am able to test the effects of this call on growth and immune function.

Aims of this thesis

The aims of this thesis are to:

1. Determine whether the adult innate and adaptive immune system varies in relation to experimental manipulation of microbial load in the breeding nest.

2. Determine whether exposure to microorganisms in the nest leads to the development of a stronger immune defence in nestlings.
3. Determine innate immune variation during breeding in adult zebra finches, assessing potential trade-offs with reproduction and immune function.
4. Determine whether the incubation call heard during embryonic development has an impact on immune function of juvenile zebra finches due to trade-offs with growth
5. Determine age related changes in the innate and adaptive immune system of nestling zebra finches.

Outline of chapters

Chapter two forms a comprehensive review of the literature in relation to the impact of bacteria at the egg, nestling and adult stage of birds and how this has impacted on the evolution of the immune system, defence and behaviour. In this review we aim to assess how and when bacteria are likely to impact birds and the future work needed to answer these questions. Our intention is to publish this article in the *Journal of Avian Biology* after final editing.

Chapter three addresses aims 1, 2 and 3 specifically, testing the innate and adaptive immune systems in relation to nest sanitation (by experimental manipulation) and testing differences in adult immunity during breeding. This research is currently in review in *Journal of Experimental Biology*, and thus has been edited to meet the style of the journal.

Chapter four addresses aim 4 - to test whether the incubation environment of eggs and in particular, incubation calls heard during embryonic development, has carry-

over effects which impact upon innate immunity in juvenile zebra finches through potential trade-offs with growth. We also intend to submit this paper to the *Journal of Experimental Biology*.

Chapter five addresses aim 5 – to understand age related changes in the adaptive and innate immune system of zebra, particularly the age in which zebra finches can mount an antibody response, as well as to understand whether innate immunity is developed before fledging in this fast growing species. We intend to submit this paper to the journal *Emu*.

Chapter six forms an integrated synthesis of my work and highlights the strengths and weaknesses of my approach. It seeks to draw overall conclusions and make proposals for how this area can develop in the future.

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CHAPTER TWO

Review: What role do bacteria play in determining avian life history evolution?

What role do bacteria play in determining avian life history evolution?

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Abstract

Over the past few decades, there has been increasing interest in the bacterial world and the influence this has on animal ecology, evolution, behaviour and development. This interest in part has been driven by the discovery of bacterial-human relationships and the hygiene hypothesis which indicated that exposure to bacteria during development have positive influences on immune function of adults. However, birds have a different set of challenges, particularly at the egg stage. Protection of the egg against infection from bacteria cannot be ascertained in the same manner as placental animals, which is heavily reliant on the internal state of immunity of the mother. In birds, embryonic growth depends on protection against pathogenic bacteria but then exposure to a diverse range of bacteria to diversify and develop immune functions. We highlight how bacteria have influenced the evolution of defences in birds, from the physiological to the behavioural, at the egg, nestling and adult stages of life. Specifically, we discuss chemical and structural defences of the egg and parental behaviours that protect their developing embryos from bacterial

infection and death. This has potential avenues for future research to answer fundamental questions such as how the diversity of bacteria across climates might have changed the structural components and defences of eggs within and between species, as well as how risk of infection influences parental behaviour. At the nestling and adult stages, we highlight currently what is known about bacterial-immune relationships, the impact on life history traits and areas for future research in the field of ecoimmunology using molecular based methods for bacterial identification. As investigations into this field continue and these fundamental questions are answered, there will be a greater appreciation and understanding of the microbial world and the mechanisms that drive behaviour, evolution and ecology of birds.

Introduction

Since birds first appeared in the Mesozoic era (Benson et al., 2014), they have coexisted with Bacteria, which have been potentially influential throughout avian evolution, affecting avian ontogeny, behaviour, physiology and immune function. To put the importance of bacteria into perspective: the microbial community inhabiting the human gut (10^{14}cells) is estimated to outnumber the somatic and germ cells of the body (10^{13}cells) (Savage, 1977) and en masse are thought to exhibit a range of metabolic activity which exceeds that of the human liver (Berg, 1996). As such, the bacterial community of vertebrates can be envisaged as a significant biological entity that has the capacity to impose significant selection pressures on their hosts. The interactions between bacteria and their hosts are potentially complex and commonly involve mutualism, commensalism and pathogenesis (Ventura et al., 2009), whilst the nature of the interaction may change according to conditions imposed on either the host or the bacteria (Sorci, 2013). Pathogenic bacterial infections can have

detrimental effects on the health and well-being of their host, causing fitness costs. In contrast, commensal bacteria live off host waste-products without a cost to the host, but the host does not benefit from the interaction. Finally, mutualistic associations involve benefit to both bacteria and their host, as would be seen in the beneficial associations of gut bacteria in vertebrate hosts which allow the breakdown of food components that are refractory to avian digestive enzymes and provide nutrients to their host (Backhed et al., 2005). Defining the exact nature of the interaction is potentially problematic as it requires identifying the cost: benefit of harbouring the bacterial community which can change suddenly from commensal to pathogenic with changing conditions. As fitness impacts are often difficult to estimate, unless the costs and benefits of microbial presence are obvious, the impact of bacteria is unknown.

Since the first birds evolved, bacteria have been shaping avian life history by influencing development, immune function, physiology and reproduction. The nature of these impacts will be different according to the physiological impact on the bird however, both commensal and pathogenic bacteria are likely to have had the greatest impact on traits linked to immune function (as reviewed by Hooper and Gordon, 2001; Lochmiller and Deerenberg, 2000; Norris and Evans, 2000). However, as well as the obvious pathogenic effects exerted directly on immune traits, bacteria can also impair the development, growth and reproduction of birds (Soler et al., 2003).

In this article we review the potential beneficial and detrimental effects bacteria may exert on wild birds and highlight their potential to influence life-history evolution at many life stages. We separate the effects into discrete stages for sake of clarity, but it is important to acknowledge the impact of bacterial associations crosses

life stages. We highlight the importance of beneficial bacterial and the interesting associations identified by researchers in recent years, and identify some key areas for future research in this area.

Bacteria and the avian egg: how, when and where?

Birds compartmentalise their reproductive investment by laying eggs that develop externally to the body. Thus eggs require intrinsic means of bacterial resistance, attained through barrier structures and antibacterial compounds. When it comes to understanding how bacteria might impact birds and their eggs, most of our understanding comes from studies of poultry (Bain et al., 2013; Board and Halls, 1973; De Reu et al., 2006; Sparks and Board, 1985). Although we might assume that the same general principles apply to wild birds, interspecific variation in the bacterial resistance of eggs has been little investigated. From an evolutionary standpoint, species which are prone to bacterial infections due to the nature of the incubation period or the environment would be predicted to have more efficient bacterial defences, but only a handful of studies have attempted to quantify this variation (Cook et al., 2005a; D'Alba et al., 2014; Godard et al., 2007; Javurkova et al., 2014; Lee et al., 2014; Peralta-Sanchez et al., 2014; Ruiz-de-Castaneda et al., 2011b; Shawkey et al., 2009; Wang et al., 2011).

As microorganisms are ubiquitous, living in the lining of the nest (Singleton and Harper, 1998) and on the skin of parents, they are able to colonise the egg shell surface and potentially egg contents through direct contact. It has been suggested that microbial infection of egg contents and high microbial loads on eggshells are the primary causes of embryonic death in birds (Peralta-Sanchez et al., 2012; Pinowski

et al., 1994). Penetration of the egg or trans-shell infection is thought to be determined by the climatic conditions that dictate microbial growth on the egg shell, nutrients, as well as the presence of water which facilitates the transfer of microbes to the interior of the egg (Board and Halls, 1973; Cook et al., 2005b). Limiting the number of microorganisms on the shell surface decreases the risk of hatching failure and embryonic death in domestic chicken (*Gallus gallus domesticus*) and pearly eyed thrasher (*Margarops fuscatus*) eggs (Cook et al., 2005a; Cook et al., 2003), as well as decrease bacterial contents of the egg. Various methods of commercial washing of chicken eggs such as the use of quaternary ammonium compound and sodium hypochlorite chemicals have been found to decrease contamination of egg contents (Wang and Slavik, 1998). However, other methods of washing eggs such as sodium carbonate treatment or the use of water have been shown to damage the eggshell cuticle during washing (Wang and Slavik, 1998), which increases bacterial proliferation (Braun et al., 2011). Field studies have also shown that cleaning of the eggs can decrease contamination such as Cook et al (2003) who found that cleaning domestic chicken eggs with 70% ethanol increased hatching success from 20% to 75% in a nest box in the field of a cool humid location, however only marginally increased hatching success in a warm less humid site. Under the same conditions, cleaning pearly eyed thrasher (*Margarops fuscatus*) eggs with ethanol decreased rate of egg infection of internal contents of the egg (either the yolk or albumen) from 90% to 15% at the cool, humid site, and 28% to 10% infected at the warm site. In this study, cleaned eggs were exposed for 5 days without incubation and were then parentally incubated. Of the clean eggs, 40% were found to hatch successfully; however, eggs which were not cleaned with ethanol did not hatch. These data suggest that incubation (see below) may play an important role in limiting bacterial penetration (Cook et al., 2005b). It is important to note that temperature has been found to play a role in the extent of contamination in both poultry literature (Park et

al., 2003) and wild studies (Cook et al., 2003; Cook et al., 2005b), and may act independently on bacterial growth and proliferation of the egg. However, Wang et al (2011) tested egg viability and hatching success in a temperate environment (average temperature 16.7 ± 3.2ºC and humidity 56.7 ± 12.2%) and found no difference in microbial presence on eggs (from Western Bluebird (*Sialia mexicana*), Tree Swallow (*Tachycineta bicolor*) and Violet-green Swallow (*Tachycineta thalassina*)) with twice daily cleaning and found that temperature did not significantly relate to hatching success (Wang et al., 2011). These studies tentatively suggest that extended periods of microbial exposure may be more detrimental to hatching success in tropical environments, than in temperate environments.

The egg has two main defences against pathogenic bacteria: the calcium carbonate egg shell along with the cuticle which covers the shell act as a physical barrier to the environment, and the chemical defences within the egg (Fig 1.). These chemical defences are in the form of antibacterial proteins, glycoproteins and proteoglycans (Wellman-Labadie *et al.* 2008) as well as maternally derived IgM and IgA, which are mainly in the egg white, and maternal IgY located in the egg yolk (Bedrani et al., 2013; King et al., 2010) and small amounts in egg white, to protect the developing embryo. The outermost layer of the egg which coats the calcium carbonate eggshell, is the eggshell cuticle (Fig. 1) which is composed of hydroxyapatite crystals, polysaccharides, lipids and glycoproteins (Fernandez et al., 2001; Wellman-Labadie et al., 2010). This serves as a waterproofing agent (Board and Halls, 1973) by covering and filling the shell pores (Figure 1.), allowing gaseous exchange but preventing bacterial penetration into the egg. A study by Board and Halls (1973) showed that removal of the cuticle from the egg shell of commercial laying chickens, increased the penetration of microorganisms into the egg contents

(Board and Halls, 1973). The cuticle has also been found to vary in terms of thickness and structure between species, presumably driven by the environment that the egg will be exposed to (D'Alba et al., 2014; Kusuda et al., 2011). For example, the Australian Brush Turkey (*Alectura lathami*) does not parentally incubate its eggs, but utilises compost mounds with heat (necessary for embryo growth) provided by microbial decomposition of organic matter (D'Alba et al., 2014). Brush turkey eggs have a modified cuticle that is composed of nanometre-sized calcite spheres (D'Alba et al., 2014). These spheres cover the egg shell and plug many of the pores on the shell (D'Alba et al., 2014). This cuticle allows eggs to be incubated in compost mounds that are humid and have high bacterial abundance and diversity with only around 9% of eggs being infected by microorganisms (D'Alba et al., 2014; Jones, 1988). In comparison, egg mortality can be over 50% in hole nesting passerine species such as house sparrows (*Passer domesticus*) and tree sparrows (*Passer montanus*) (Pinowski et al., 1994). It is possible that the modified cuticle of the brush turkey plays an important role developing viable eggs, particularly in such a damp environment. Birds which nest in damp environments, (e.g. waterfowl or mound builders from the family Megapodiidae, which do not parentally incubate their eggs) are under strong selection to have much thicker cuticles than birds which live in drier conditions (Bain et al., 2013). A study by Kusuda et al (2011), found that egg cuticle thickness differs greatly between species, which could potentially relate to either the conditions of the nesting environment or living in more microbiologically challenging environments, such as wet or damp environments (Kusuda et al., 2011). Species such as the Humboldt Penguin (*Spheniscus humboldti*), the Greater Flamingo (*Phoenicopterus ruber roseus*) and the White Pelican (*Pelecanus onocrotalus*) have cuticle thickness of 45µm, 110µm and130µm respectively (Kusuda et al., 2011) which could either be based on the rocky shorelines that the penguin nest on or potentially damp and microbiologically challenging mud nest built by flamingos.

Red Junglefowl (*Gallus gallus*) and Japanese quail (*Coturnix japonica*) have cuticle thickness of 1μm and 10 μm respectively, potentially relating to the softer environments of the jungle floor and the lack of need for a strong cuticle. However, it is clear that the cuticle thickness differs greatly between species, and whether microorganisms or other factors are driving this difference remains unclear. In addition to cuticle thickness, cuticle deposition (coverage), which is not uniform across the whole eggshell, has been found to be extremely important in deterring penetration of microorganisms through the shell. Studies by De Reu et al (2006) and Bain et al (2013) found chickens had significantly higher bacterial penetration to the egg contents when cuticle deposition was lower (lower % coverage of the shell) (Bain et al., 2013; De Reu et al., 2006). Thus, a well formed cuticle is important to discourage bacterial penetration of egg contents.

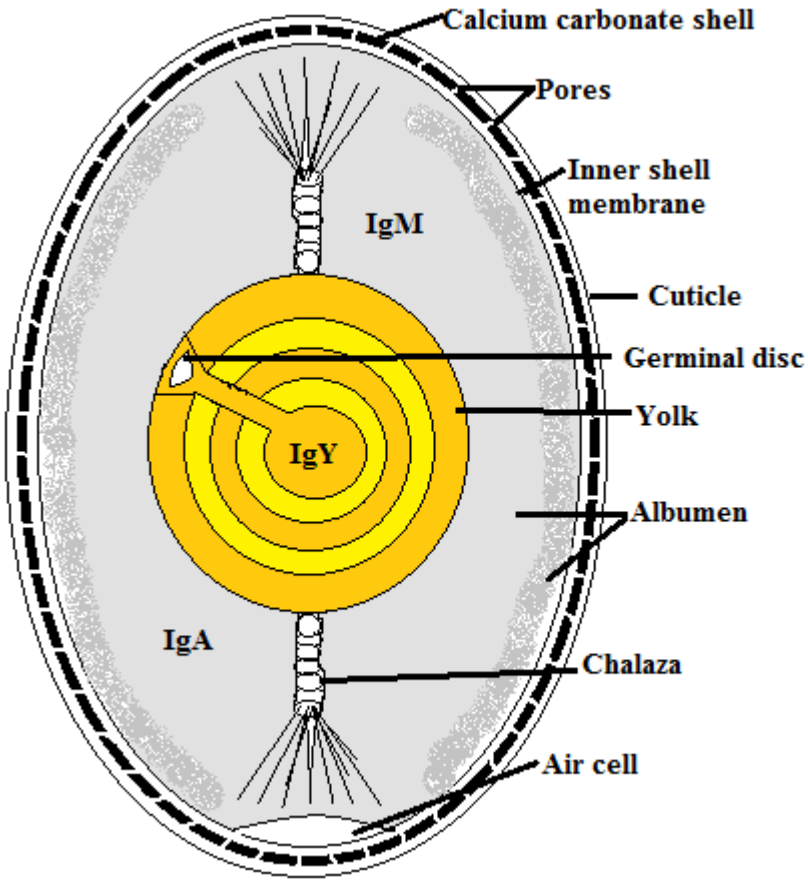

Figure 1. Structure and bacterial defence in the avian egg

Figure 1 depicts the structure of the avian egg. The cuticle, which is the outermost layer, provides a waterproofing agent to inhibit bacterial proliferation to the egg contents through the shell pores. Within the cuticle, antimicrobial peptides, such as C-type lysozyme, ovotransferrin and ovocalyxin-32-like protein inhibit bacterial proliferation. In the albumen, antimicrobial peptides are activated when temperatures exceed 27ºC. Incubation of eggs ensures that temperatures are around 38ºC. The egg yolk contains IgY, which has antibacterial properties and can inhibit the growth of microorganisms.

As well as helping plug pores and regulating water exchange, the cuticle is also known to contain antimicrobial proteins to defend against bacterial invasion through the shell (Wellman-Labadie et al., 2008). A range of antimicrobial proteins such as

C-type lysozyme, ovotransferrin and ovocalyxin-32-like protein have been found in the cuticle and outer shell of water birds such as the wood duck (*Aix sponsa*), hooded merganser (*Lophodytes cucullatus*), Canada goose (*Branta canadensis*) and mute swan (*Cygnus olor*) (Wellman-Labadie et al., 2008). These proteins inhibit the growth of *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa and Bacillus subtilis* (Wellman-Labadie et al., 2008). The C-type lysozyme, or chicken/conventional type lysozyme is one of three main lysozymes found in the animal kingdom (Callewaert and Michiels, 2010). This lysozyme has the ability to cleave the beta-(1-4)-glycosidic bond in peptidoglycan, which form the major layer outside the plasma membrane of gram positive bacteria (Callewaert and Michiels, 2010). However, peptidoglycan are not as important in the structure for gram negative bacteria, thus mostly gram positive bacteria are susceptible to lysozyme. Ovotransferrin is a glycoprotein which can be found in plasma, egg white and the shell cuticle in birds (D'Alba et al., 2010b; Giansanti et al., 2012; Wellman-Labadie et al., 2008) and is reliant on binding and sequestering iron (Fe^{3+}) for antibacterial activity (Giansanti et al., 2012; Valenti et al., 1982). Ovocalyxin-32-like protein shares properties of latexin which inhibits microbial proteases (Wellman-Labadie et al., 2007) and has antimicrobial activity against *Bacillus subtilis* (Wellman-Labadie et al., 2008) Therefore, the egg cuticle plays a major role in both a physical and chemical defence system against bacterial penetration of the egg, and the exact structure of the cuticle is likely to be under selective pressure, according to the risk of bacterial proliferation in the environment.

To test the significance of ecological pressure on the evolution of microbial defence of eggs, it would be beneficial to test microbial defences and eggshell cuticle physiology/structure in a single species of bird that occupies a broad set of ecological

niches. Species which span for example, tropical, temperate and arid conditions could be used to demonstrate the pressures on egg structure and defence strategies. Although there are a handful of studies that have compared egg bacterial relationships across species in different incubation environments such as open and cavity nests (Godard et al., 2007), as well as birds who reuse their nests (discussed further below)(Walls et al., 2012; Wang et al., 2011), addressing egg defence by testing differences in cuticle thickness and deposition along with behavioural mechanisms (such as incubation behaviour) will also be beneficial in understanding the selective pressures that bacteria have on egg defence.

The role of incubation in pathogen control

Incubation provides a mechanism for protection against egg mortality from pathogens and the elements (Shawkey et al., 2009). Studies have found that intensive incubation decreases bacterial load on eggshells (Ruiz-de-Castaneda et al., 2012), with decreases in microorganisms of up to 87% found in a study of wood duck eggs (*Aix sponsa*) (Walls et al., 2012). Incubation provides heat to the egg with optimal incubation temperature set at around 36-38ºC (Wilson, 1991). This initiates non-specific antimicrobial enzymes inside the albumen of the egg (activated when temperatures are over 27ºC (Wilson, 1991)). These enzymes protect the egg from microbes invading through the shell. Importantly, incubation also protects the egg from becoming damp, reducing trans-shell infection by microbes via water (Ruiz-De-Castaneda et al., 2011a). In a study by Ruiz-De-Castaneda et al. (2011), incubation was negatively associated with surface egg shell bacterial loads, and relative humidity was positively associated with egg shell bacteria, indicating that early incubation causes a drying effect of the eggs which inhibits the growth of microbes. Experiments by D'alba et al (2010) of adding water to unincubated eggs in nests and

eggs which have been partially incubated in natural nests indicated that only unincubated eggs had high microbial growth, compared to eggs that had been partially incubated (D'Alba et al., 2010a). In the absence of incubation, wet eggs had higher bacterial growth than eggs that were not experimentally wet (D'Alba et al., 2010a). The level of egg dampness varies in the wild and is exacerbated in nest types that are exposed to rain. Nests exposed to rain such as open cup nests have higher numbers of bacteria and a higher incidence of egg mortality than nests which are not exposed to the elements, such as those in cavity nests (Godard et al., 2007). Furthermore, incubation protects eggs from microbes with the use of preen oil which kills bacteria that may penetrate the egg (Martin-Vivaldi et al., 2010). Antimicrobial chemicals produced by symbiotic bacteria have been identified in the preen secretions in the European hoopoe (*Upupa epops*) and green woodhoopoe (*Phoeniculus purpureus*) (Martin-Vivaldi et al., 2010). These are thought to be produced to kill off potential pathogens on the skin of birds, therefore allowing only beneficial bacteria to remain. Thus, incubation, or more precisely, the intimate contact between the incubating adult and the egg, is extremely important for egg survival by providing protection from damp conditions, providing heat, and production of antimicrobial secretions all of which protect the egg from microbial penetration. Yet to be investigated is whether the process of egg turning has an adaptive role of limiting bacterial growth on the side of the egg that touches the nest material. It is widely accepted that egg turning is necessary to prevent premature adhesion of the chorion to the inner shell membrane (Deeming, 1991), yet whether it discourages bacterial proliferation is unknown.

Recent advances in molecular technology have given researchers the ability to gain a thorough understanding of bacterial diversity and abundance on eggshells using 16s rRNA, rather than relying on culturing techniques which only identify a

small proportion of bacteria. The 16s rRNA gene is 1550 base pairs long (Brosius et al., 1978), however, sequencing of around 400bp of the gene is enough to determine which species of bacteria is in the environmental sample (Clarridge, 2004). These sequences are entered into a database, which holds the full length 16s rRNA sequence of all identified species of bacteria which allows accurate and rapid identification of the species of bacteria in the sample. A recent study by Grizard et al. (2014) determined bacterial and fungal loads on the outer surface of eggshells of the homing pigeon (*Columba livia*) during incubation using 16s rRNA. Bacterial and fungal diversity was found to decrease during the late stages of incubation, however, the abundance of bacteria was found to increase, whereas fungal abundance decreased (Grizard et al., 2014). Similarly, Lee et al (2014) found that incubation limits bacterial diversity, however, harmless and potentially beneficial bacteria increased (Lee et al., 2014). This may fit with the hypothesis that beneficial bacteria are promoted during incubation and pathogenic bacteria are eliminated (Cook et al., 2005b; Shawkey et al., 2009). However, Shawkey et al. (2009) found that incubation did inhibit growth of pathogenic bacteria on the eggshells, however did not promote growth of beneficial bacteria.

Adult behavioural mechanisms to fight pathogens: nest building for bacterial defence

Behaviours such as the placement of antibacterial material in the nest can be extremely important for egg viability (Peralta-Sanchez et al., 2012). A comparative analysis of eggshell bacteria and avian life history traits across 24 bird species found

the use of bacteria degrading materials, such as feathers and green plant material decreased the bacterial density on eggs in the wild (Peralta-Sanchez et al., 2012). The use of feathers in nests by birds has been found to be important because of their antimicrobial properties (Peralta-Sanchez et al., 2012). Feather-degrading bacteria, are speculated to compete for space with other bacterial pathogens on the surface of eggs and may produce antimicrobial substances to kill them (Peralta-Sanchez et al., 2010; Soler et al., 2010). Feather colour may increase the growth of feather degrading bacteria, in particular, *Bacillus licheniformis* growth (Goldstein et al., 2004; Grande et al., 2004; Gunderson et al., 2008). *Bacillus licheniformis* releases antimicrobial substances which may kill other microbes in the nest (Soler et al., 2010). Peralta-Sanchez et al (2010) found that the use of white feathers in barn swallow (*Hirundo rustica*) nests decreased the bacterial load on egg shells whereas nests with black feathers did not decrease bacteria. The authors speculate that black and white feathers may harbour different types of bacteria, as well as levels of *B. lichenformis*, which interact with bacteria on eggshells and either destroy or outcompete bacteria often found on eggs.

Apart from feathers, some species of birds use aromatic herbs that increase volatiles which serve to fumigate their nests (particularly reused nests) and have a toxic effect on bacterial pathogens (Clark and Mason, 1985; Dorman and Deans, 2000). Volatile oils from herbs, not limited to thyme (*Thymus vulgaris*), oregano (*Origanum vulgare*) and clove (*Syzygium aromaticum*), have all been shown to have antibacterial properties (Dorman and Deans, 2000). The blue tit (*Cyanistes caeruleus*) uses aromatic plants in the nest, which decrease cultivable bacterial richness in the nest and on nestlings, but did not decrease richness on adults (Mennerat et al., 2009). The authors speculate that the lack of change in richness of

bacteria on adults is likely because they are away from the nest a majority of the time. Starling (*Sturnus vulgaris*) nests have a decreased bacterial load when aromatic herbs are used in the nest (Clark and Mason, 1985; Gwinner and Berger, 2005). Similarly, the use of the plant *Artemisia maritime*, known for its antimicrobial oil extracts, reduced pathogenic *Staphylococcus* on chicken eggs in a field experiment (Møller et al., 2013). In the same experiment, it was also found that pairs of sandwich terns (*Sterna sandvicensis*) breeding in the area with the *Artemisia maritime* had 18% higher recruitment compared to those without the plant, suggesting that the use of plants may increase reproductive success by eliminating pathogens (Møller et al., 2013). Thus, the use of herbs and plants that contain volatile oils may be an antimicrobial strategy used by birds for protection of eggs in the nest.

Egg mortality rates may be linked to the environment as previously mentioned, but might also differ in regards to nest type. In species where nest reuse is common, it could be expected that there would be greater exposure to microorganisms. In a study of nest reuse of wood ducks by Walls et al. (2012), it was found that reused nests had 2.2 times the bacterial density on eggs than the eggs in cleaned nests. Similarly, Wang et al (2011) found an increase in bacteria on eggs in old nests compared with new nests (Wang et al., 2011). However, in both studies, hatching success did not seem to be affected, possibly due to both studies being conducted in a temperate environment. The effects of exposure to microbes on hatchability between different nest types were investigated in a study by Godard et al. (2007). Using chicken eggs, they found that eggs in open cup nests had lower hatching success and higher rates of trans-shell infection than those in cavity nests (Godard et al., 2007). As the authors found that cavity nests maintained higher temperatures over 27ºC (the point at which enzymatic activity in the egg albumen is activated) compared to open

nests, they suggest that this increased hatching success is due enzymatic protection of the embryo. Also, eggs in open nests had higher numbers of bacteria than eggs in cavity nests, even when the eggs were cleaned with alcohol. The authors hypothesised that open nests are more exposed to elements such as rain, which may facilitate bacterial growth and trans-shell infection of eggs (Godard et al., 2007). Also, eggs in closed nests will undergo less cooling and incubation variability, thus having more consistently functional anti-microbial compounds. Therefore, species which occupy exposed nests that are at greater risk of infection by microorganisms will likely need to use other mechanisms or incubate more intensively to ensure embryonic development.

Nestling defence against bacterial attack

Bacteria are hypothesised to affect the short intense period of nestling growth prior to fledging and there are some convincing studies demonstrating their potential importance. However, the role of bacterial populations is complex, as some studies show adverse effects on growth (Berger et al., 2003; Gonzalez-Braojos et al., 2012; Kozlowski et al., 1991), whilst there is evidence to suggest that there are beneficial probiotics in wild birds, which can promote growth and development via unlocking nutrition from food and by suppression of pathogenic bacteria (Moreno et al., 2003). One such example of probiotics in wild birds was found in pied flycatchers (*Ficedula hypoleuca*), where cloacal bacteria, and more specifically the presence of *Enterococcus faecium*, was found to relate positively to tarsus size and mass in nestlings (Moreno et al., 2003). *E. faecium* has been found to be a growth promoter for chicks and a probiotic in birds (Cao et al., 2013), thought to be due to bacteriocin production and the antimicrobial peptides produced by this bacteria which may

destroy potential bacterial pathogens (Audisio et al., 2000; Guillot, 1998; Moreno et al., 2003). However, other examples can be found in domestic poultry, but clearly demonstrate that bacteria can have a diverse range of effects on their hosts. For example, bacteria in the gastrointestinal tract of chickens can decrease lipid digestion and modify carbohydrate and protein digestion, which may increase energy and amino acid requirements as well as outcompete pathogenic bacteria. Germ free studies of chickens have also demonstrated some of the positive effects of bacteria on the host, for example, a study of dietary urea fed to germ free and conventional chicks demonstrated that conventional chicks had better growth and feed conversion (Okumura et al., 1976). The authors suggested that microorganisms in the gut are responsible for the positive growth and feed conversion effects of urea, possibly through release of ammonia by bacteria and incorporation into amino acids (Okumura et al., 1976). However, bacterial relationships in nestlings are not always positive. Gonzalez-Braojos et al (2012) found increased bacterial loads on the skin of pied flycatcher chicks (*Ficedula hypoleuca*) in nests with reused material compared with those using only new nesting material (Gonzalez-Braojos et al., 2012). Chicks from reused nests tended to have smaller wing lengths when controlling for hatch date and brood size (Gonzalez-Braojos et al. 2012). However, they did not find any difference in nestling weight or tarsus length between reused/new nests (Gonzalez-Braojos et al. 2012). Similarly, Mills et al (1999) found that cloacal gram negative enteric fermenter bacterial load calculated using plate scores correlate positively with degree of wing asymmetry in tree swallow nestlings, which is hypothesised to impact upon fledging survival as wing asymmetry impacts upon flying ability (Mills et al., 1999). *Escherichia coli* has been found to be negatively associated with the growth rate of sparrow nestlings (Berger et al., 2003; Kozlowski et al., 1991), and cause egg mortality and death of sparrow nestlings (Pinowski et al., 1994). However, Berger et al. (2010) found no relationship between the bacteria load of nests and fledging

success of starlings, which is likely dependent on the species of bacteria in the nest and whether they are pathogenic (Berger et al., 2003).

To understand relationships between immune function and bacteria, it is important to understand how immune function develops in birds and at what time periods have the highest risk of infection (see figure 2.). Developing chicks in the egg are protected from bacteria, fungi, parasites and viruses by the egg shell and maternal antibodies that are transferred through the yolk sac, and from numerous peptides and proteins circulating around the egg that have antimicrobial properties (Bedrani et al., 2013; Fellah et al., 2008). This maternal protection is the most important during the first three weeks of life (Smith and Beal, 2008). Unlike mammals, which obtain maternal antibodies across the placenta and through colostrum after birth, birds only receive maternal antibodies from within the egg yolk (King et al., 2010; Smith and Beal, 2008). Acquired humoral or B-cell immunity largely develops in the days after hatching, and exposure to microorganisms and pathogens during the first few days is important for the development and diversification of B cells (Addison et al., 2010; Baumgarth et al., 2005). Chicks rely on innate immune function in the first instance, provided maternally in the egg in the form of anti-microbial peptides and phagocytic cells such as macrophages and heterophils which are important in protecting the embryo and nestling (Ardia et al., 2011). In birds, the only maternal antibodies transferred through the egg sac to the yolk are IgY (IgG in mammals) (Bedrani et al., 2013; King et al., 2010). IgM is not present in the yolk, due to a receptor in the follicle that is specific for IgY and does not bind to IgM, however IgM is found in the albumen (Demas and Nelson, 2012), along with IgA which is also found in the albumen (King et al., 2010). Hormones and nutrients are also supplied maternally to aid in the development of immune cells (Ardia et al., 2011). The amount of maternally delivered antibodies is dependent on

the mothers' physiological condition (Ardia et al., 2011) and differs by life history traits of the species (Addison et al., 2009). Although there is great variation among species of birds, maternal antibodies usually last in circulation between 5-14 days, by which time the nestling will start producing its own antibodies at 10-14 days post-hatch (Grindstaff et al., 2006; Hasselquist and Nilsson, 2009). Studies have investigated further the effect of bacteria on immune function and survival. For example experimental injection of lipopolysaccharides (LPS) has resulted in a decrease in survival of fledging Eurasian collared dove nestlings (*Streptopelia decaocto*), with 72% of controls fledging successfully compared to only 44% experimental birds fledging (Eraud et al., 2009). It was found that birds injected with LPS which mimics infection had lower survival rates after fledging, compared to controls (Eraud et al., 2009). The authors speculate that LPS- injected birds were thought to succumb to predation, possibly due to an inflammatory response, including the release of cytokines which are known to cause behavioural symptoms of sickness such as excessive sleeping (Eraud et al, 2009; Dantzer et al, 1998). To further understand bacteria in the nest, testing bacterial load in a nest manipulation alongside the measurement of immune function would give a clearer indication of immune status in response to reduced bacterial load. It would also be of benefit to test the developmental response of nestlings in relation to ecological setting, to further understand whether the rearing environment contributes to greater variation in immune function and impacts upon survival. One specific question that could be asked is whether nestlings exposed to environments with a diverse range of bacteria strengthen the immune response in adulthood and in turn chance of survival in the wild. Furthermore, to understand bacteria and the effect they have on the immune system in nestlings, future studies need to investigate both the innate and adaptive arms of the immune system and particularly whether certain types of bacteria may

have an effect or whether more antigens increase the diversity and capability of immune function.

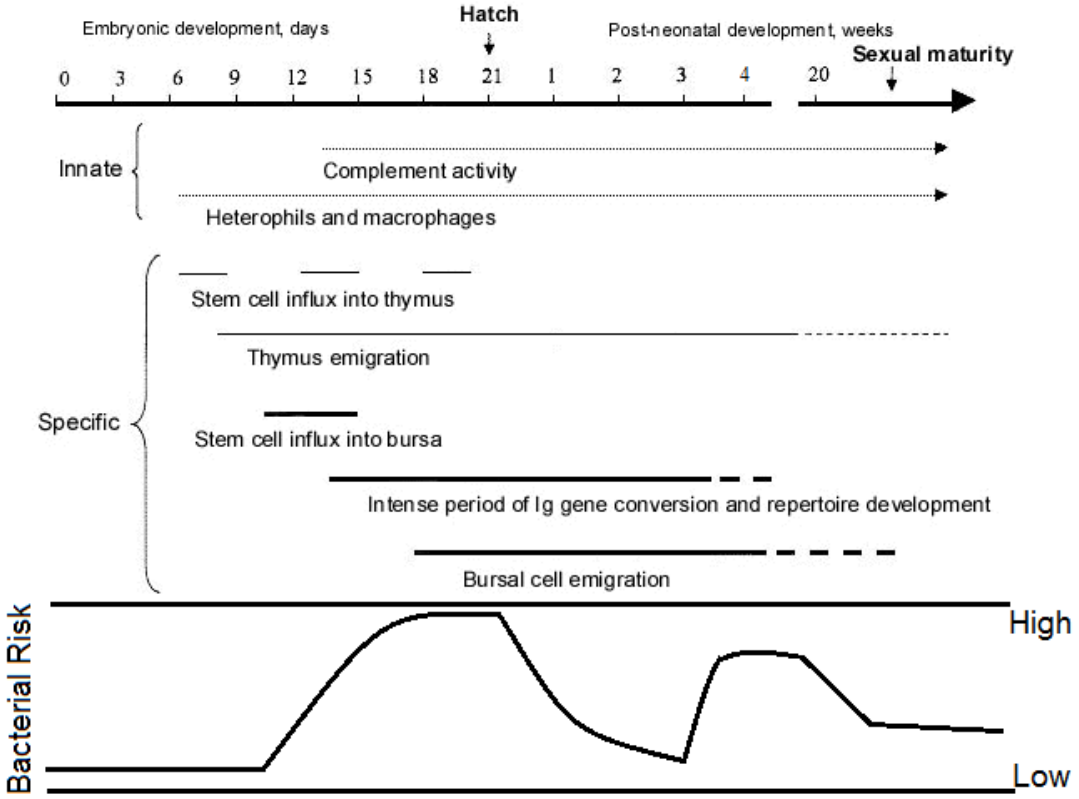

Fig. 2. *The relationship between chronological age and important developmental events for specific and non-specific immunity in the chicken with speculated bacterial risk throughout development (adapted from Klasing and Leshchinsky (1999)).*

Bacterial risk is fairly low during the first 12 days of embryonic development in the chicken, given that incubation is provided by the parent. If incubation by the parent does not occur, the embryo will usually die. However, between day 12 and 15 the chorioallantoic membrane (CAM) fuses with the eggshell membrane and digests eggshell to make bone. During this time, there is a high risk of bacterial penetration through the pores and CAM. At day 17-18, the immune system is activated from this bacterial exposure. Once the chick hatches at day 21, bacterial risk decreases, and although exposure to diverse microorganisms is high, circulating maternal antibodies help protect the chick from infection. Bacterial risk increases once again at 3-4 weeks of age as maternal antibodies are no longer present. However, during this time, the chick relies heavily on the innate immune system. Bacterial risk steadily decreases as specific immune defences develop and Ig gene conversion takes place.

Adult defence against bacterial infection: life stage and selection

The avian immune system is an evolutionary product of the selection pressures exerted by natural pathogens and antigens. The primary role of the immune system is defence against pathogens, and the extent to which an organism can defend itself against these pathogens prevents disease (Horrocks et al., 2011). Generally, scientists have focussed on pathogens or pathogen pressure (Horrocks et al., 2011). However, the immune system need not always eliminate the pathogens (resistance), there may be tolerance for high pathogen loads without impairing host fitness because the negative impacts of the infection are reduced (Medzhitov et al., 2012). For example, the host may concentrate on tissue repair and detoxifying pathogen by-products, rather than killing the pathogen (Medzhitov et al., 2012; Read et al., 2008). Tolerance has been explored extensively in plants and recently in laboratory mice (Råberg et al., 2009). However, there are a few examples of tolerance studies in wild birds, although some have not previously been interpreted in this light. For example, metabolic, locomotor and reproductive responses to inflammatory challenges of lipopolysaccharide (LPS) and phytohemagglutinin (PHA) were measured in invasive house sparrows and their less invasive relative, the tree sparrow (Lee et al., 2005). Challenged tree sparrows had lower metabolic rates, decreased locomotor activity and decreased egg production compared to controls. However, there was no difference in metabolic, locomotor or reproductive activity between challenged and control house sparrows (Lee et al., 2005). This work suggests that house sparrows largely tolerate rather than react to some immune challenges. Similarly, work on domestic Japanese quail have shown that repeated exposure to LPS led to a reduced and then non-existent fever response by the third dose (Koutsos and Klasing, 2001). However, there is recent work directly investigating tolerance in wild caught house finches with different histories of exposure to *Mycoplasma gallisepticum*. Birds with

a long history of exposure to mycoplasma had lower inflammatory cytokine signalling and lower fever, suggesting birds with extended exposure may have developed more tolerance to withstand the negative impact of inflammatory defence on tissues (Adelman et al., 2013).

But is there any evidence to suggest that microorganisms in the environment can shape immune function in animals? The human hygiene hypothesis (Strachan, 1989) is based on the premise that an increase is hygienic practices has decreased exposure to bacteria and other antigens, resulting in an increased prevalence of autoimmune diseases (Strachan, 1989). Similarly, the antigen exposure hypothesis (Piersma 1997; Møller 1998; Matson 2006; Spottiswoode 2008; Horrocks et al. 2012a) posits that exposure to antigens in the environment shapes investment in immunity, whereby environments with an increased number of antigens selects for a strong immune response, and environments with little to no exposure to antigens selects for a weaker immune response (Horrocks et al., 2012a). This has been demonstrated previously in chickens and mice where it was found that offspring raised in germ free environments had a less competent and functional immune system into adulthood compared with offspring raised in environments where they are exposed to antigens (Bedrani et al., 2013; Lee and Mazmanian, 2010; Lundin et al., 2008; Olszak et al., 2012; Pels and Denotter, 1979; Pollard and Sharon, 1970). Having a competent immune system in adulthood is extremely important to destroy invading pathogens, not only for host survival, but for the survival of future offspring. Research has suggested that individuals with a less efficient immune system, have a higher pathogen load, and can vertically transmit pathogens to offspring when forming and laying eggs (Soler et al., 2011). Vertical transmission of bacteria to eggs can occur either in the ovary, oviduct or cloaca (Barrow, 1994). Also, a competent immune system in adults can ensure that immunological factors

that fight infections can be passed to offspring (Soler et al., 2011). A study of germ free, specific pathogen free and conventional hens were used to test whether there were differences in innate immune defences transferred to the eggs during laying in different microbial environments (Bedrani et al., 2013). It was found that hens raised in a conventional and specific pathogen free environment had significantly lower growth of *Staphylococcus aureus* and *Streptococcus uberis* in egg whites compared to germ free chickens (Bedrani et al., 2013). They found no difference in levels of egg white lysozyme, however did find a trend with higher anti-chymotrypsin (an anti-protease which impairs bacterial invasion) activity in the conventional and specific pathogen free group compared to the germ free group (Bedrani et al., 2013). The hens raised in the three different environments were found to vary in immunological status, having increased interleukin 1 beta, interleukin 8 and toll like receptor 4 in the conventional group compared to the germ free chickens, and in some instances, the specific pathogen free group. Interleukin 1 beta and interleukin 8 are markers of inflammation, while toll like receptor 4 detects gram negative bacteria through their lipopolysaccharide and elicits an innate immune attack on invading bacteria (Bedrani et al., 2013). Therefore, bacteria in the environment can be important in development of immune function and defence. Unfortunately there has been little similar work investigating the relevance of this work for wild birds.

There have been a small number of studies investigating environmental impacts and bacterial exposure on immune function in wild birds, but due to the limited number of immune tests suitable for field studies, these studies only give a small snapshot into immune bacterial relationships. For example, Horrocks et al (2012b) found that 5 species of lark had lysis titres that positively and significantly correlated with the number of bacteria shed from the birds captured with an air bacterial sampling device (Horrocks et al., 2012b). Soler et al (2011) found in the 29

birds species sampled, natural antibodies were higher in individuals that had lower density of bacteria on eggshells (Soler et al., 2011). Birds of the lark family (*Alaudidae*) also have differences in immune function according to the type of habitat that the bird occupies and bacterial exposure. It has been found in larks that lower levels of immune defences, more specifically haptoglobin and lysis, were found in birds from desert like environments compared with larks in temperate environments (Horrocks et al., 2012a). Using bacterial air borne sampling, it was found desert larks encountered fewer bacteria than temperate larks due to deserts being inhospitable for microbial growth because of low rainfall, high temperatures and high solar radiation. Therefore, Horrocks et al (2012a) suggest that higher levels of immune defence found in temperate larks might be in line with the antigen exposure hypothesis, which proposes that increased exposure to antigens are linked to more active immune systems (Horrocks et al., 2012a). Further studies could also investigate relationships between environmental bacteria either in air, food sources or in the nest; plumage bacteria and immune function across different environments (temperate, tropical and arid) in the same species of bird to ensure that potential differences may relate to the environment rather than the species itself. Testing this might also help us understand how bacteria may shape immune function.

Future Directions

To date, our understanding of the ecological relevance of the avian immune system in response to microbial exposure is impaired by the limited number of studies addressing these parameters in wild birds. Studies of mice and poultry in germ free environments have greatly helped shape our understanding of the development of immune function (Bedrani et al., 2013; Olszak et al., 2012), however it is now important to bring that knowledge out into the wild in order to understand how evolution has shaped the immune system of birds. The first step to

understanding immune function in wild animals is to identify the most important environmental microorganisms and to test their impact on both immune function and fitness. It is also most important to test bacteria between different environments for example, tropical and temperate; as well as looking at these communities during different times of the year, for example during summer and winter, during incubation and chick rearing. This in turn will give a greater understanding of immune function, and the selective pressures that shape immune function and strategies of wild animals (Horrocks et al., 2011). Technology has come a long way in the past few decades, with sequenced-based methods for identification of microorganisms playing a pivotal role in understanding the ecology and evolution of Bacteria and Archaea (Voelkerding et al., 2009). Next generation sequencing can quantify every microorganism in any environment being tested, which gives this method an advantage over microbiological methods which can only quantify very few types of microorganisms in an environment due to a lack of suitable growth conditions. However, although NGS will allow for identification of the pathogens, it won't allow for determination of their significance for the host. Other disadvantages of next generation sequencing also include the inability to distinguish between alive and dead bacteria which could cause problems when trying to understand the effect of bacteria on the immune system of the host. There is also the problem of contamination which is a common problem amongst bacterial identification methods. At this point in time, a combination of different bacterial identification methods may be necessary. Ecologists studying immune function in wild birds could also incorporate simple techniques into their sampling regime such as taking bacterial swabs and processing these either through commercial companies or using other identification methods such as PCR. This would allow an indication of bacterial presence within the environment and potentially be able to identify relationships with immune functioning throughout seasons. The future will benefit from integrated

studies that not only identify key pathogens but quantify their potential impact on the host at different life stages. Using an integrated approach it will be possible to test for example if prior experience to bacterial pathogens in the nest equips adult birds with more effective immune responses in later life and reduces the chance of vertical transmission to their own offspring. Similarly, we can look further into immunocompetence differences in siblings from the same nest and try to ascertain the drivers of this difference in immune function.

As discussed throughout, understanding how microorganisms impact life history traits such as reproduction, growth, immune function and survival, is crucial in gaining a full understanding of how bacteria has shaped evolution in wild birds. An integrative approach, testing across environments, and adding bacterial identification and abundance techniques to ecological and evolutionary field studies is easy and necessary. Studies that test between and across environments can give a full picture of whether egg structure and defence is driven by bacterial exposure, and can test whether growth, immune indices and survival are stronger in certain environments based upon this exposure. It is most important to understand when bacteria have the biggest impact on avian fitness and what aspects of avian defence are most important in determining fitness. There are so many unanswered questions in the animal-bacterial world and the role in evolution, however, with culture independent techniques widely accessible; these questions should soon be answered.

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CHAPTER THREE

Impact of nest sanitation on the immune system of parents and nestlings in a passerine bird

Impact of nest sanitation on the immune system of parents and nestlings in a passerine bird.

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ABSTRACT

Bacterial communities are thought to have fundamental effects on the growth and development of nestling birds. The antigen exposure hypothesis suggests that, for both nestlings and adult birds, exposure to a diverse range of bacteria would select for stronger immune defences. However, there are relatively few studies that have tested the immune/bacterial relationships outside of domestic poultry. We therefore sought to examine indices of immunity (microbial killing ability in naïve birds, which is a measure of innate immunity and the antibody response to sheep red blood cells, which measures adaptive immunity) in both adult and nestling zebra finches (*Taeniopygia guttata*). We did this throughout breeding and between reproductive attempts in nests that were experimentally manipulated to change the intensity of bacterial exposure. Our results suggest that nest sanitation and bacterial load affected measures of the adaptive immune system, but not the innate immune parameters tested. Adult finches breeding in clean nests had a lower primary antibody response to sheep red blood cells (SRBC), particularly males, and a greater difference between primary and secondary responses. Adult microbial killing of *E.coli* decreased as parents moved from incubation to nestling rearing for both nest treatments; however, killing of *C.albicans* remained consistent throughout. In nestlings, both innate microbial killing and the adaptive antibody response did not differ between nest environments. Together, these results suggest that the exposure to microorganisms in the environment affect the adaptive immune system in nesting birds, with exposure upregulating the antibody response in adult birds.

Keywords: humoral, cell-mediated, microorganism, sheep red blood cell, avian immunity, microbiome

Summary statement

Nest sanitation primes the adaptive immune response of adult birds, but not necessarily the immune response of their nestlings. Adult constitutive immune response also decreases throughout nestling rearing.

INTRODUCTION

Bacteria have had a profound effect on the evolution of animals, impacting ontogeny, behaviour, physiology and immune function (Archie and Theis, 2011; Bäckhed et al., 2005; Lee and Mazmanian, 2010; McFall-Ngai et al., 2013). There are certain stages during the annual life cycle of birds where they may be exposed to greater bacterial diversity and abundance. For example, breeding birds could be exposed to a greater abundance and diversity of bacteria, since nests are likely to contain a diverse and abundant community of bacteria. This may affect adults and perhaps nestlings, particularly in altricial species, which spend the period of early development in this environment. However, exposure to both abundance and diversity of bacteria could vary depending on the sanitation practices between and within species, as well as factors such as nest type and nest reuse (Berger et al., 2003; Brandl et al., 2014; Godard et al., 2007; Gwinner and Berger, 2005; Peralta-Sanchez et al., 2014; Singleton and Harper, 1998). Therefore, exposure to bacteria in the nest environment could be an important driver of variation in immune function (Horrocks et al., 2011) for both adults and nestlings. Nestlings are inoculated with microorganisms from the nest lining as well as through saliva and the delivery of food by parents which begins soon after hatching (Benskin et al., 2009; Berger et al., 2003; Kyle and Kyle, 1993; Mills et al., 1999; Singleton and Harper, 1998). In some species, such as the zebra finch (*Taeniopygia guttata*), faeces continuously builds up in the nest as nestlings grow and defecate, exposing them continuously to faecal bacteria (Benskin et al., 2009) and the community of bacteria that presumably builds up in the nest over the period that it is used. It would be expected that nestlings raised in nests that are not regularly cleaned would have developed stronger immune defences since bacteria drive immune development (Hooper et al., 2012; Lee and Mazmanian, 2010). Similarly, we would expect adult birds that are exposed to additional bacteria in the

nest would upregulate immune defences to cope with an elevated exposure to bacteria.

The primary role of the immune system is defence against pathogens, and the avian immune system is an evolutionary product of the selection pressures exerted by commensal bacteria and natural pathogens. Immune function is adaptively developed and regulated, and variation in immune defence in birds has been found between seasons (as reviewed by Nelson and Demas, 1996), life stages (Evans et al., 2015; Hegemann et al., 2012; Pap et al., 2010) and environments (Buehler et al., 2008b; Buehler et al., 2009). One particular hypothesis to explain variation in immune function is the antigen exposure hypothesis, which suggests that exposure to antigens in the environment shapes investment in immunity (Horrocks et al., 2012a; Horrocks et al., 2011). Environments where there is a greater amount of microbial pathogen exposure therefore might select for a stronger immune response (Horrocks et al., 2012a; Horrocks et al., 2011). Environmental influences on immune system development through microbial exposure has been demonstrated previously in chickens and mice, where offspring raised in germ free environments have a reduced functional immune capacity into adulthood, compared with offspring raised in conventional environments (Bedrani et al., 2013; Lee and Mazmanian, 2010; Lundin et al., 2008; Olszak et al., 2012; Pels and Denotter, 1979; Pollard and Sharon, 1970). To some extent, this has also been found in wild birds (red knots (*Calidris canutus*), lark species (*Aluadidae*)), where microbial exposure have positively correlated with various measures of the innate immune response (Buehler et al., 2008b; Horrocks et al., 2012a; Horrocks et al., 2012b; Soler et al., 2011). The mechanisms behind this cause and effect remain unclear and experimental studies outside of domestic poultry are certainly lacking and necessary to determine the nature of causality in the data supporting this hypothesis.

A second explanation for variation in immune function is the trade-off hypothesis (Sheldon and Verhulst, 1996) which predicts that consumption of limited resources by the immune system leads to trade-offs between immune function and other resource-demanding activities such as reproduction (Ilmonen et al., 2000; Lochmiller and Deerenberg, 2000; Norris and Evans, 2000; Sheldon and Verhulst, 1996). Production of acute phase proteins and lymphocytes can be nutritionally costly (Lee and Klasing, 2004). However, hypertrophy of the liver which rapidly produces acute phase proteins is the biggest cost of the acute phase response (Iseri and Klasing, 2014). Decreased intake of food and inefficient digestion are also important consequences of a robust immune response (Iseri and Klasing, 2014). Examples of a trade-off between immune function and other physiological processes include an increase in metabolic energy expenditure following an experimental challenge by the injection of sheep red blood cell in great tits (*Parus major*) (Ots et al., 2001) and increases in metabolic rate following injection of phytohaemagglutinin (PHA) in sparrows (*Passer domesticus*) (Martin et al., 2003). Experimental tests of this proposed trade-off between immune function and reproduction have found that, in support of the hypothesis, antibody titres decreased with increasing number of offspring in nesting zebra finches (Deerenberg et al., 1997). Similarly, basophilic inflammation in response to PHA results in slower growth rates of nestlings (Soler et al., 2003). Thus, investment in either immune function or other physiologically demanding processes may compete for resources.

Birds differ significantly in the level of nest sanitation across species, with some species in which parents actively remove excrement from the nest, some in which young will deliberately defecate over the side of the nest, and many species where neither behaviour is observed and excrement accumulates over the course of the

breeding attempt (as reviewed by Guigueno and Sealy, 2012). Nest sanitation can be further hindered by species that reuse their nests, which can increase bacterial exposure to eggs and nestlings, and a community of bacteria will develop over time (Walls et al., 2012; Wang et al., 2011). Despite this variation in nesting environment, the effect of nest cleanliness on the development of the avian immune system and the impact it has on adult immune function remains largely unaddressed.

In this study, by experimentally manipulating nest bacterial communities and nest hygiene, we sought to determine whether bacterial load within the nesting environment impacts the innate and adaptive immune response in breeding adult zebra finches and their developing nestlings, and also examined potential trade-offs in nestling growth. Together, these data allow a robust experimental test of the antigen hypothesis and the trade-off hypothesis, by testing both the innate (humoral (complement) and cellular (phagocytosis) immunity) and adaptive immune response (with the use of sheep red blood cells) in breeding birds with manipulated nest sanitation. Fundamentally, we sought to test the hypothesis that exposure to microorganisms in the nest environment leads to robust immune defences, with the possibility that nest sanitation behaviour causes variation in the immune response.

MATERIALS AND METHODS

Birds

Adult zebra finches (n = 42) were sourced from local breeders and maintained under standardised conditions of 14 H: 10 H L:D photoperiod, 21-24°C with 40-50% relative humidity. Birds were initially kept in single sex groups for 2 weeks, and then

were able to choose a mate. The pairs were then kept in cages (Terenziani, Montichiari, Italy, 50 cm high x 50 cm wide x 100 cm long) and provided seed (Golden Cobb, Victoria), cucumber, eggs, shell grit and water *ad libitum*. Pairs were provided with nest boxes and nesting material at time of pairing. Nests were monitored daily after the onset of egg laying to determine hatch date of nestlings and post-hatch timings. Nestlings were marked with individual coloured non-toxic nail varnish on the toe nail for identification. There were 10 pairs (20 birds) in the clean nest treatment and 11 pairs (22 birds) in the dirty treatment. To ensure adequate power, we used sample sizes similar to that of previous studies that have either assessed adaptive immunity in zebra finches (Deerenberg et al., 1997) or microbial killing in birds (Evans et al., 2015).

Manipulation of nest hygiene

Nests were randomly allocated to two treatment groups that differed in their level of nest hygiene. For 'clean nests', nesting material was replaced with fresh sterilised (autoclaved) nesting material within two days of hatching, and was continuously replaced with sterilised material twice weekly until nestlings were 30 days of age, by which time the nestlings had completely fledged from the nest (ca. 20 days). In this treatment the replacement of the nest material removed all nestling faeces at least twice weekly so that the nests remained clean. For 'dirty nests' 5 g of chicken faeces were added on the interior surface of the nest cup within two days of hatching, and 2 g of chicken faeces (from free-ranging chickens) were further added twice weekly for 30 days. This was in addition to the natural finch faeces deposited in the nest. Chicken faeces was pulverised and mixed with distilled water to create a thick paste for spreading around the nest cup. Dirty nests were also sham cleaned so that

nestlings were disturbed for the same time period as nestlings from clean nests and the nesting cup was moved gently around the box. Gloves were used in the handling of clean and dirty nest material, nestlings and adults. Bacterial abundance within the nests was assessed using bacterial agar paddles (Hycheck paddles, Micromedia: Moe, Victoria, Australia) the day after nesting material changes (clean) or the adding of chicken faeces (dirty) at around day 7 after hatching.

Blood sampling and microbial killing assay

Adult birds were bled at three different time periods and assayed for microbial killing capacity: i) during incubation (ca. 10 days after clutch completion); ii) 10 days after nestlings hatched; and iii) 20 days after nestlings hatched (See Fig. 1). Comparing across these periods gave an indication of how innate immune function changes across reproductive stages. In addition, blood samples from adults were taken at 27 days and 34 days post hatch for the purpose of antibody detection (after sheep blood cell injection at 20 days).

Two separate groups of offspring were assayed for microbial killing. One group (GR1, Fig. 1) were tested as nestlings, before fledging (18-20 days of age) and after fledging (25-27 days of age). The second group (GR2, Fig. 1) were assayed as sexually mature birds, after 60 days of age to test whether rearing environment had an impact on innate immune function into adulthood (See Fig. 1). Each offspring had three blood samples taken, where blood was used for testing microbial killing and antibody detection, but not all samples were used for microbial killing (if the blood collected was not adequate to test both immune measures for each sampling). Therefore, nestlings were blood sampled and injected at either 11-13 days (20 nestlings total, 10 in dirty nests, 10 in clean treatment) or 60 days (12 birds, 6 per

treatment) with sheep red blood cells, with two blood samples occurring over the following fortnight. Only primary (day 7 after injection) and secondary (day 14 after injection) blood samples were used to assess microbial killing. Offspring in the nest were randomly allocated an age group of either 9 days (results not reported – collected for the purpose a parallel study on age related changes), 11 days, 13 days or 60 days.

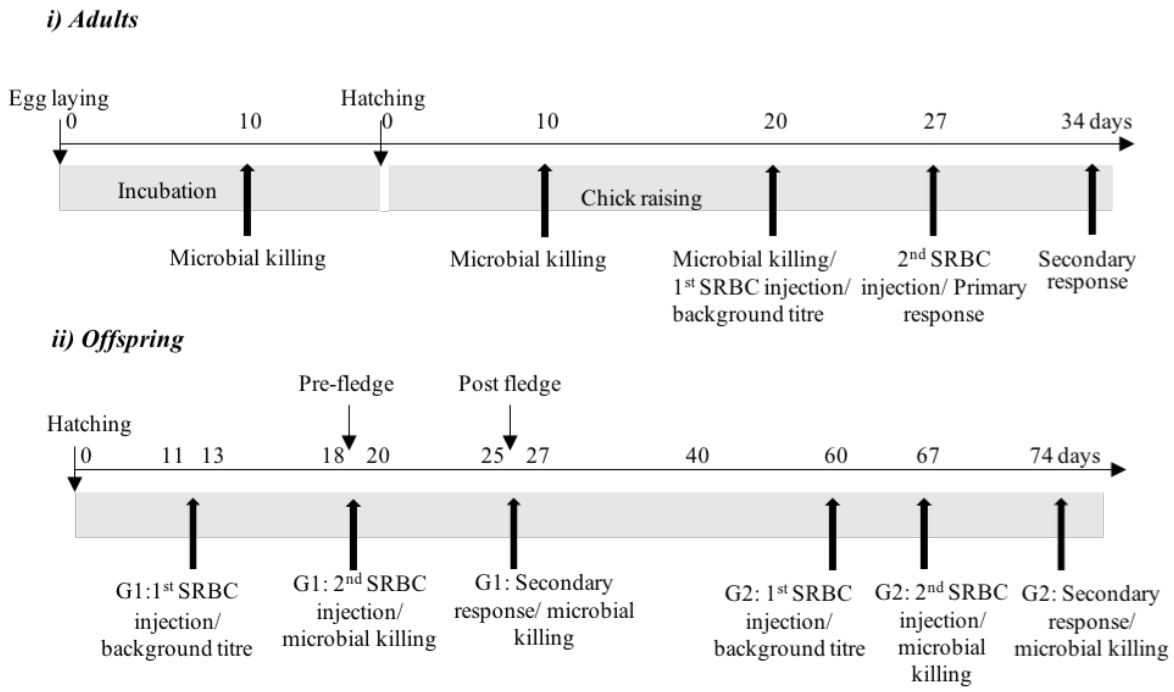

Figure 1. Timeline of immune measurements in adults and their offspring.

Timeline of blood sampling and immune measurements including microbial killing and secondary responses to sheep red blood cell (SRBC) injections in i) adults and ii) offspring. The bold arrows along the timeline indicate a blood sample being taken and immune measurements at a given stage (offspring have 3 blood samples taken in total, adults have 5). The timings for adults were before offspring hatched (incubation) and after hatching (from day 0). Offspring timings were from hatching (day 0). Offspring were sampled at different ages in two groups (as nestlings or juveniles of 60 days) and are indicated as group 1 (G1) and group 2 (G2).

For both adults and their offspring, blood samples were collected in the morning between 9 and 11am and within 10 min of the experimenter entering the room. Blood was collected from the brachial vein and no more than 200 µl was taken at a time (<2% body mass). Blood for the use of microbial killing was tested within 1 h of sampling.

To assess the variation in microbial killing ability, we followed the procedure of Millet et al. (2007) and Tieleman et al. (2005), under sterile working conditions. We used 2 different microbial strains: *Escherichia coli* (ATCC 8739) and *Candida albicans* (ATCC 10231). The use of *E.coli* in this assay tests killing that is complement dependent (Demas et al., 2011), involving circulating proteins in the blood that kill bacteria, and is a measure of humoral immune function. *C.albicans* tests the ability of blood to kill via phagocytosis, a measure of cellular immune function. We reconstituted the lyophilized pellet (Microbiologics: St.Cloud, Minnesota, United States) in sterile PBS and made a working solution of approximately 300 colony forming units (CFU) per 100 µl (or 3000 CFU per mL). The working solution was made fresh daily. A control to test CFU was incubated with each batch of test plates. A negative control plate with only whole blood was also made for each bird to ensure sterility was maintained whilst sampling. Whole blood was diluted (1:10) in CO_2 – independent medium (Gibco, Life Technologies: New York, United States) containing 4 mM L-glutamine (Sigma Aldrich: Castle Hill, New South Wales, Australia) and the test microbe. Diluted blood was incubated for 30 min (for *E.coli*) and 2 h (for *C.albicans*) at 41°C. Following incubation, 50 µl of diluted blood was spread on soy tryptic agar in duplicate (total 100 µl) using sterile spreaders. The plates were then inverted and incubated at 37°C overnight (*E.coli*) or for around 48 h (*C.albicans*) and the colonies were then counted visually across the whole plate (blindly). All plates, except for negative controls, were run in duplicate.

For both strains, we calculated the proportion killed as the average number of colonies on experimental plates relative to the average number of colonies on the CFU control plates ((1 – (average number of CFU on experimental plate / average number of CFU on control plate)) x 100). No negative controls contained CFUs.

Vaccination

To test the adaptive antibody response, we used sheep red blood cells (SRBC), which possess a variety of antigens that are not considered pathogenic. The antibody response to SRBC estimates the ability of the humoral immune system to mount a response to foreign antigens (Bacon, 1992; Deerenberg et al., 1997). To make the vaccine of sheep red blood cells, sheep blood in Alsever's solution was spun at 2000 rpm (295 x g) for 15 min and the Alsever's solution discarded. The blood cells were washed 3 times in 1x phosphate buffered saline (PBS), and then prepared as 40% sheep red blood cells by volume in PBS. The preparation was made fresh daily. Twenty days after their offspring had hatched, adults were blood sampled to assess the initial level of natural antibodies to sheep red blood cell and part of the blood sampled at this time was used for microbial killing. The two offspring groups also had background antibodies assessed at 11-13 days or 60 days. The blood collected from adults and nestlings for all background, primary and secondary responses were spun at 14000 rpm in a benchtop centrifuge for 15 min with the plasma stored at -80°C for later analysis. Adults and offspring were weighed prior to each vaccination to enable administration of the correct dose (50 µl of 40% SRBC per 13 g bird). At the time of blood sampling, the vaccination was administered intramuscularly into the left breast. Seven days after the first vaccination, blood was taken for analysis of the primary immune response and the plasma stored. However, nestlings sampled at

this time (18-20 days) for the primary response did not show any titre when analysed and therefore only the secondary response is mentioned for nestlings throughout the rest of the paper. The second vaccination of 40% SRBC was then administered into the right breast. A blood sample to analyse the secondary immune response was taken seven days after the second vaccination (blood sample at 34 days post hatch for adults, 25-27 days of age for nestlings, 74 days of age for sexually mature offspring). The blood was spun and the plasma stored at -80°C. Plasma from each blood sample (initial, primary response, secondary response) was then analysed using an agglutination assay.

Agglutination assay

The agglutination assay was conducted to test the humoral component of the immune system and antibody response and followed previous protocols (Deerenberg et al., 1997), although we used 15 µl of unheated plasma for each sample serially diluted in PBS. Fifteen microliters of 2% SRBC were added to each well, the plate gently tapped, covered with clingfilm and incubated in a waterbath for 1 h at 37°C. Agglutination was scored twice, blind to the sample identity by the same person. Plates were then scanned on an Epson flatbed scanner (model V700). Lysis scores were recorded 1 h after incubation. The visualisation of lysis (cloudiness in wells) indicates the ability of complement to lyse the red blood cells.

Statistics

Data was analysed using SPSS (version 22). Normality of the data was assessed using Shapiro-Wilk tests. To test the effect of nest treatment on humoral and cellular

innate immune measures across the breeding period, microbial killing data of *E.coli* and *C.albicans* were analysed separately using general linear mixed models (with restricted maximum likelihood (REML) estimation) where Bird ID was used to account for repeated measures. Fixed factors in the model included treatment, stage of breeding and sex. Potential nest effects that may influence condition were included as covariates, such as number of nestlings per nest and body mass. For all models described, all two-way interaction terms and single variables were included initially in each model and then interactions removed sequentially by highest p-value for those interactions with $p>0.10$. Repeatability of microbial killing was also tested. Repeatability was calculated with the equation (between individual variance) / (within individual variance + between individual variance). Models with both random intercept, and random intercept with random slope were calculated and the best model was selected based on the AIC and degrees of freedom. A lower AIC indicated a better fit for the data. Random intercept was chosen as the best model for the adult data. The estimated means (± s.e.m.) are presented throughout the results (unless otherwise indicated) and actual means can be found in Table 2. Note that there are 4 microbial killing samples (2 nests) and 1 additional sample (in *C.albicans* killing only) missing across nest treatments during incubation. This was either due to the unavailability of microbes (for a week during the work) or our failure to collect enough blood to measure all functions (the additional bird). To test the relatedness, relationship and immune strategies between the innate immune measures during breeding, a Pearson's product moment correlation between the killing of both microbes for both adults was assessed at incubation, 10 days post hatch and 20 days post hatch. To test the effect of the nest treatment on the primary and secondary immune response, agglutination was assessed using general linear mixed model (with restricted maximum likelihood (REML) estimation) with nest ID used as a random effect to account for non-independence of data and potential nest effects. For both

the primary and secondary immune response (and lysis), all sets of analysis was run with main effects of treatment and sex, with number of offspring and mass as covariates. Interactions were dealt with in the same manner as above. For the assessment of nest treatment on immune function of offspring, a general linear mixed model was used. To account for non-independence of data and potential nest effects, nest ID was included as a random factor in all linear mixed models. Model parameters used to assess whether there were differences in agglutination and microbial killing included treatment, sex, mass and number of nestlings. Sexually mature (60 day) offspring were tested separately using the same parameters. Growth rate was calculated by calculating the percentage increase in mass between day 5 and day 10 ((day 10 mass- day 5 mass/ day 5 mass) x 100). When testing relationships between growth rate and microbial killing, only the final sample of blood (day 25-27) was used as this was an age where the birds were big enough to take a sufficient amount of blood for testing microbial killing of every individual. Parent offspring immune relationships were assessed by taking the nest average of nestlings (aged 25-27 days) for microbial killing or agglutination (secondary response) and running a correlation between the father, mother or midparent (average of the mother and father) 20 day microbial killing data or secondary agglutination scores.

Ethics statement

All experimental procedures were approved by the Deakin University Animal Ethics Committee (permit no. G40-2013).

RESULTS

Nest treatment

Data from the agar paddles confirmed that the experimental treatment increased the abundance of bacteria in dirty nests: there was a significantly higher number of colonies collected with agar paddles swabbing dirty nests compared with clean nests (independent samples t-test; $t = 12.91$, $df = 19$, $P < 0.001$) with dirty nests having a mean of 155.64 ± 10.22 and clean 14.6 ± 2.09 colonies per $7cm^2$.

Adult Immune Function

Microbial killing

Microbial killing of E.coli

The microbial killing capacity via complement proteins in the whole blood of adult zebra finch against *E.coli* was significantly repeatable across individuals and across reproductive stages ($r = 0.42 \pm 0.056$). However, microbial killing of *E.coli* in adults attending clean and dirty nests at different stages was not significantly different (mean $56.01\% \pm 6.07$ vs $57.35\% \pm 5.72$; See Tables 1, 2), suggesting circulating proteins involved in complement-mediated immunity did not differ in birds attending nests with varying levels of nest sanitation. Despite the repeatability of microbial killing measures within individuals over time, there was a decrease in microbial killing capacity from incubation throughout nestling rearing (Tables 1 - 2, Fig. 2). Post hoc tests indicated that microbial killing of *E.coli* was significantly higher at incubation compared to 20 days after their offspring hatched ($P < 0.001$) and *E.coli* killing 10 days after their offspring hatched was significantly higher than at 20 days after their offspring hatched ($P = 0.02$). There was no significant difference in the

microbial killing of *E.coli* in adult males and females and it was not related to an individual's mass or the number of offspring they were rearing (Mean number of offspring = 3.14 ± 0.48; clean = 2.70 ± 0.29, dirty = 3.55 ± 0.28).

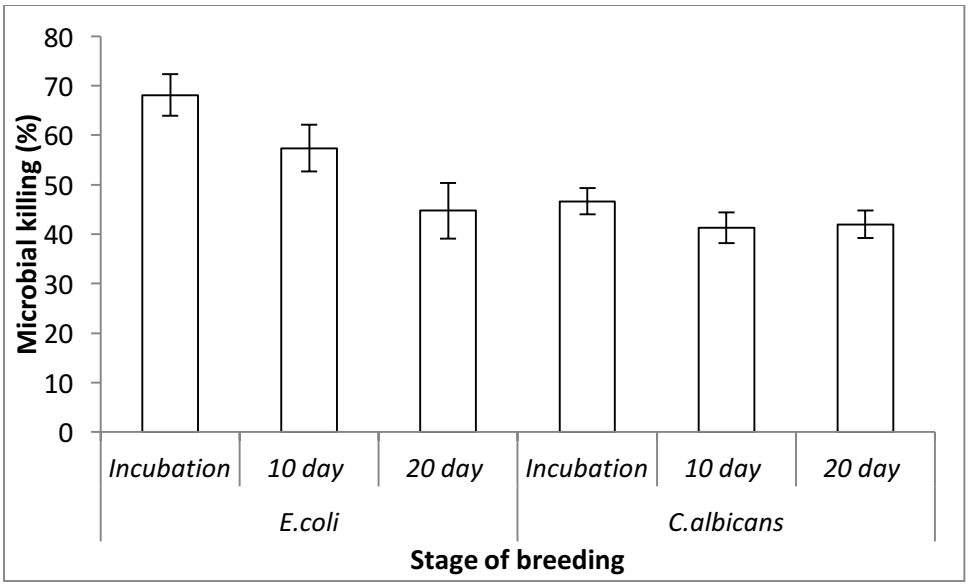

Figure 2. Adult microbial killing during breeding and nestling rearing (Mean ± s.e.m.).
Breeding stages for each microorganism were: Incubation (*E.coli*: *n* = 38, *C.albicans* = 37); 10 days after offspring hatched (*n* = 42); and 20 days after offspring hatched (*n* = 42).

Microbial killing of C.albicans

The microbial killing capacity and level of phagocytosis of adult zebra finch blood against *C.albicans* was also significantly repeatable in an individual's blood test across stages of incubation, 10 days after offspring hatched and 20 days after offspring hatched (r = 0.35 ± 0.059 (S.E)). However, there was no significant treatment effect, with no difference in killing of *C.albicans* in adults attending clean versus dirty nests (Tables 1 - 2, Fig. 2; means of 42.86% ± 3.13 vs 44.38% ± 2.94). This suggests that mechanisms inducing phagocytosis in adults may not be stimulated by variation in bacteria in the nesting environment. Furthermore, the killing capacity of adults against *C.albicans* did not differ between incubation, 10

days post hatch or 20 days post hatch (Tables 1 - 2, Fig. 2). Overall, there were no significant differences in *C.albicans* killing between males and females, and killing was not related to the number of nestlings that they were rearing or adult body mass.

Table 1. Statistics for adult immune measures

	F	df	P
Adult			
Killing of E.coli			
Nest treatment	0.28	1,121	0.87
Stage (incubn, 10d, 20d)	10.38	2,121	<0.001
Sex	0.29	1,121	0.59
Mass	0.15	1,121	0.70
Number of offspring	0.82	1,121	0.37
Killing of C.albicans			
Nest treatment	0.15	1,120	0.70
Stage (incubation, 10d, 20d)	1.70	2,120	0.19
Sex	2.54	1,120	0.12
Mass	1.72	1,120	0.20
Number of offspring	0.83	1,120	0.37
Primary response			
Nest treatment	4.24	1,40	0.05
Sex	0.002	1,40	0.97
Mass	1.48	1,40	0.25
Number of offspring	0.000	1,40	0.997
Treatment*sex	4.32	1,40	0.05
Secondary response			
Nest treatment	0.001	1,40	0.98
Sex	2.65	1,40	0.12
Mass	0.17	1,40	0.90
Number of offspring	0.15	1,40	0.71
Treatment*sex	5.48	1,40	0.03
Lysis			
Nest treatment	1.15	1,40	0.29
Sex	0.08	1,40	0.78
Mass	0.01	1,40	0.91
Number of offspring	0.22	1,40	0.64

Table 2. Mean (± s.e.m.) microbial killing (%) of *E.coli* and *C.albicans* during breeding

	Escherichia coli						*Candida albicans*					
	Clean nest			Dirty nest			Clean nest			Dirty nest		
Stage	Male	Female	Total	Male	Female	Total	Male	Female	Total	Male	Female	Total
Incubation	76.5±8.5 (n=9)	65.6±8.7 (n=9)	71.1±6.0 (n=18)	66.7±7.3 (n=10)	64.0±9.5 (n=10)	65.3±5.9 (n=20)	40.6±7.7 (n=8)	51.6±4.1 (n=9)	46.4±4.3 (n=17)	44.3±5.2 (n=10)	50.1±4.1 (n=10)	47.2±3.38 (n=20)
10 days post hatch	60.1±11.5 (n=10)	55.0 ± 9.5 (n=10)	57.5±7.3 (n=20)	55.7±7.0 (n=11)	58.7±10.5 (n=11)	57.3±6.2 (n=22)	38.0±3.5 (n=10)	38.6±6.1 (n=10)	38.3±3.4 (n=20)	38.8±7.5 (n=11)	50.2±6.2 (n=11)	44.5±4.9 (n=22)
20 days post hatch	56.3±12.7 (n=10)	35.2±11.3 (n=10)	45.7±8.6 (n=20)	41.6±12.4 (n=11)	45.7±8.7 (n=11)	43.8±7.3 (n=22)	34.3±6.0 (n=10)	47.1±2.9 (n=10)	40.7±3.6 (n=20)	45.8±4.7 (n=11)	41.3±6.8 (n=11)	43.5±4.1 (n=22)

Relationship between E.coli and C.albicans

There was no significant relationship between killing of *E.coli* and *C.albicans* at incubation (r = -0.14, n = 37, P= 0.41) or at 10 days after their offspring hatched (r = 0.26, n = 42, P= 0.09). However, at 20 days after their offspring hatched, there was a significant positive relationship in adults between the ability of their blood to kill both *E.coli* and *C.albicans* (r = 0.35, n = 42, P= 0.02).

Agglutination

Background analysis

All adults and offspring had a zero agglutination score.

Primary response

Nest treatment (clean/dirty nests) had a significant effect on agglutination score (Table 1, Fig. 3). Agglutination scores were higher in adults attending dirty nests (Fig. 3, mean = 3.21 ± 0.48) compared to clean nests (mean = 1.52 ± 0.48), indicating that antibody titres were higher in adults from dirty nests. There was also an interaction between sex and nest treatment. Further analysis suggested that titres were higher in males from dirty compared to clean nests (Fig. 3, $F_{1,20}$=7.61, P=0.01). There were no significant differences between females from different treatments ($F_{1,20}$=0.46, P=0.51), or sexes within each treatment (clean: $F_{1,20}$=1.60, P=0.23; dirty: $F_{1,20}$=3.03, P=0.12). There was no significant effect of adult body mass, the number of nestlings they were rearing or overall sex differences.

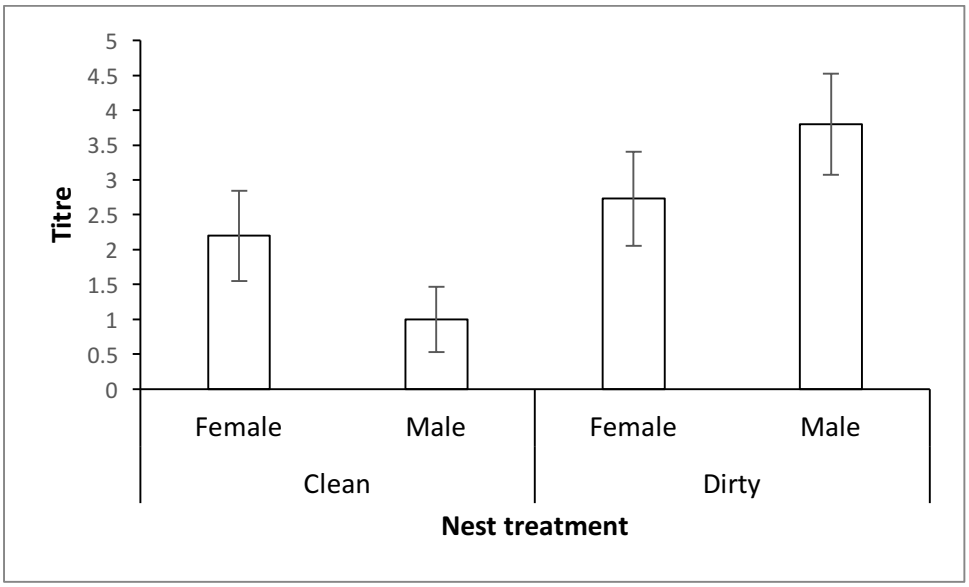

Figure 3. Adult primary agglutination titres between nest treatments (Mean ± s.e.m.).

Clean treatment includes female titres (*n* = 10), male titres (*n* = 10). The dirty treatment includes female titres (*n* = 11), male titres (*n* = 11).

Secondary response

Agglutination titres for secondary response were not significantly different between nest treatments (Table 1, Fig. 4) with agglutination score means of 6.48 ± 0.57 and 6.05 ± 0.56 for adults attending clean and dirty nests respectively. There was no significant relationship between agglutination score and adult sex, body mass and the number of nestlings that they were rearing. There was a significant interaction between sex and treatment (Table 1), with further analysis indicating that males had significantly lower agglutination than females in the clean treatment ($F_{1,20}$=10.79, P=0.01). However, there were no sex differences within the dirty treatment ($F_{1,20}$=0.25, P=0.63), no differences in males between treatments ($F_{1,20}$=0.69, P=0.42)

or between females ($F_{1,20}$=0.77, P=0.39). There was a significant positive relationship between agglutination and lysis ($F_{1,40}$ = 7.39, P = 0.01), therefore indicating a relationship between antibody titres and complement. Of 41 birds given a second challenge with SRBC, only 1 female was a non-responder (agglutination titres were zero). Of these, 15 did not show any lysis. However, there was no significant relationship found between lysis and nest treatment, sex, mass or the number of offspring they were rearing.

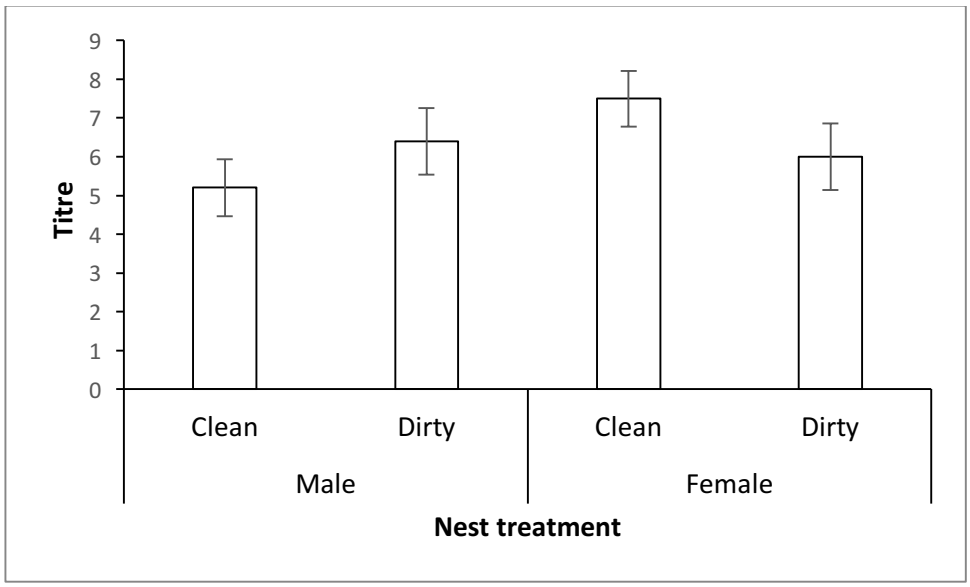

Figure 4. Adult secondary agglutination titres between nest treatments (Mean ± s.e.m.). Clean treatment includes female titres (*n* = 10), male titres (*n* = 10). The dirty treatment includes female titres (*n* = 11), male titres (*n* = 11).

Difference in primary and secondary response

There was a correlation between the primary and secondary immune response to SRBCs (Pearson correlation 0.78, $P < 0.001$, $n = 21$) in adults attending dirty nests, however there was no such relationship within clean nests (Pearson correlation -0.13, $P = 0.96$, $n = 20$). This indicates that adults attending dirty nests with lower titres in the primary response usually had lower titres in the secondary response. However, birds attending clean nests that had low (or zero) titres in the primary response did not have low titres in the secondary response, and matched the titre scores of those in dirty nests. The relative upregulation of the secondary response was calculated as the primary titre - the secondary titre score. There was a significant difference in upregulation of the secondary response between clean and dirty nests ($F_{1,40} = 5.3$, $P = 0.026$, Fig. 5), with adults attending clean nests having a greater titre difference (4.75 ± 0.7) compared to those attending dirty nests (2.95 ± 0.4). There were no differences between males and females in the agglutination titre differences ($F_{1,40} = 1.3$, $P = 0.27$).

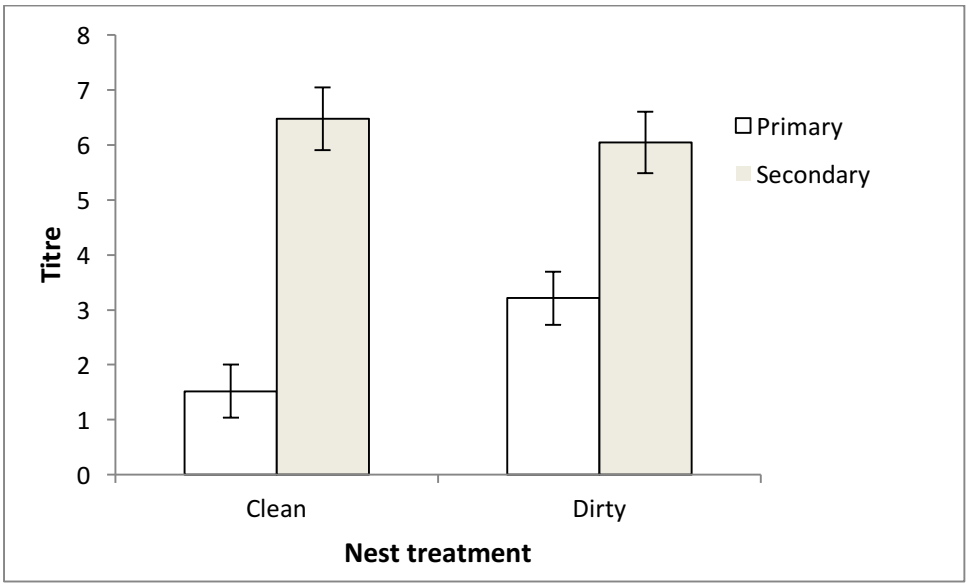

Figure 5. Differences in adult primary and secondary responses between nest treatments (Mean ± s.e.m.). The titre difference are analysed between clean (*n* = 20) and dirty (*n* = 22) nests.

Offspring immune function

Microbial killing of E.coli

In nestlings, microbial killing was tested before fledging (18-20 days) and after fledging (25-27 days). Before fledging, nestlings did not differ in their ability to kill *E.coli* between nest treatments (Table 3), with estimated means of birds from clean nests -7.7 ± 5.7 and dirty nests 3.9 ± 5.5. There was no difference in microbial killing between males and females and microbial killing was not affected by body mass or the number of siblings a nestling had. After fledging, there was also no significant difference between the immune response of nestlings from clean and dirty nests (Table 3) (killing % clean 2.58 ± 9.31; dirty 12.59 ± 9.83), and there were no differences across the two sexes (Table 3). There was no significant effect of body mass on nestling microbial killing or the number of siblings in the nest.

Table 3. Statistics for offspring immune measures

	F	df	P
Offspring			
Before Fledging - microbial killing			
Nest treatment	1.78	1,19	0.23
Mass	1.22	1,19	0.30
Sex	0.91	1,19	0.36
Number of siblings	0.32	1,19	0.59
After Fledging – microbial killing			
Nest treatment	0.03	1,15	0.86
Mass	2.70	1,15	0.15
Sex	3.36	1,15	0.13
Number of siblings	1.17	1,15	0.32
Secondary response (agglutination)			
Nest treatment	0.06	1,20	0.82
Sex	0.66	2,20	0.55
Number of siblings	0.09	1,20	0.78
Mass	0.06	1,20	0.81
60 day old offspring			
Nest treatment (*killing E.coli*)	1.2	1,12	0.30
Nest treatment (*killing C.albicans*)	0.39	1,12	0.55
Nest treatment (secondary response)	0.18	1,12	0.68

Agglutination response

The secondary response of nestlings (secondary response at 25-27 days, n = 20), showed no significant difference in agglutination titres between nest treatments (Table 3, Fig. 6). There were no differences in titres between males and females, or with the number of siblings or mass at the time of sampling (Table 3).

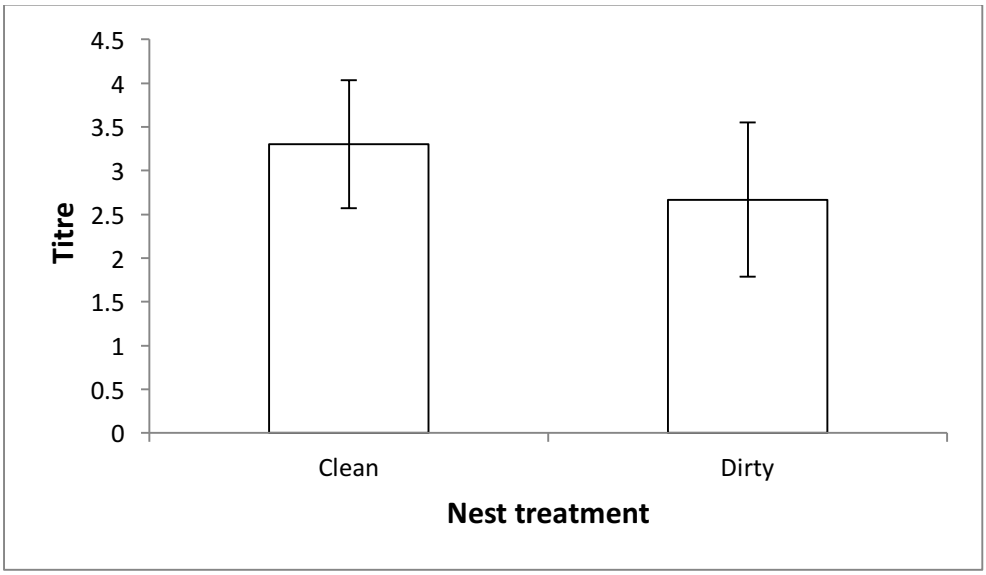

Figure 6. Nestling secondary total agglutination titres between nest treatments (Mean ± s.e.m.). (*n* = 11 birds per nest treatment)

Growth rates

The growth rate of nestlings was not affected by nest treatment ($F_{1,23}$ = 0.53, P = 0.48), sex ($F_{2,23}$ = 2.36, P = 0.13) or number of siblings ($F_{1,23}$ = 3.50, P = 0.08). No relationship was found between growth rate and microbial killing of *E.coli* ($F_{1,23}$ = 1.88, P = 0.19) or between growth rate and agglutination score ($F_{1,23}$ = 0.70, P = 0.80).

Immune function of offspring at 60 days of age

Nest treatment did not impact killing of *E.coli* in the 60 day age group (Table 3). Similarly, microbial killing of *C.albicans* was not affected by nest treatment (Table 3). There was no significant difference between offspring that were tested in adulthood from the two nest treatment groups with respect to the agglutination of SRBC (Table 3) with birds from clean nests having mean agglutination to SRBC of 5 ± 1.45 and birds from dirty nests having agglutination titres of 5.75 ± 1.02.

Parent and offspring relationships

Whilst controlling for treatment group, we found no evidence of any correlation between nestling innate immune function (with *E.coli* killing at 23-27 days of age) and the immune function of their parents (at 20 days after offspring hatching) (See supplementary material – Table 1). To compare adaptive immune function, we tested the average secondary titres of offspring from the same nest and parental secondary response, and found no correlation between mother, father or the mid-parent value (i.e. the average of the parental values). Similarly, there were no relationships between the offspring values at 60 days old and parental killing of *E.coli*, *C.albicans* or agglutination (see supplementary material, Table 1).

DISCUSSION

We found that increasing nest bacterial load up-regulated the adaptive humoral response in adults that attended dirty nests, but only the primary response, which can be attributed to the difference in males between treatments. However, manipulation of nest bacteria did not impact either the complement / humoral components of immunity (as tested by the killing of *E.coli*) or cellular component (phagocytosis) (as

tested by the killing of *C.albicans*) in either adults or their offspring. Therefore, our results suggest that nest sanitation affects aspects of adaptive immunity, but not the innate immune measures taken in our study of adult zebra finches. Previous studies testing the impact of environmental pathogens, including bacteria, have mostly focused on innate mechanisms, rather than adaptive immunity (Horrocks et al., 2012a; Horrocks et al., 2012b). However, despite these findings in adults, we found no difference in the adaptive immunity of nestlings as a result of our experimental nest treatments. This latter result is interesting and somewhat surprising, as it would be presumed that nestlings should be impacted by microbes in the nest, where they spend more time in the nest than adults. Also surprising is that our measures of innate immunity was not impacted by the nest manipulations in either adults or nestlings. Manipulating the hygiene of the nest, and in turn increasing birds exposure to higher abundance of presumably novel bacteria, has been hypothesised to up-regulate microbial killing mechanisms such as phagocytosis and complement proteins, which are relevant for non-specific defence against microorganisms and are mechanisms that should be flexible in varying conditions (Buehler et al., 2008b; Millet et al., 2007).

Although not expected, in adults there was a significant difference between those in the clean and dirty nest treatments for the primary immune response, but not the secondary response, although, this result can be largely attributed to the low titre of males in clean nests. In the secondary response, males also had lower titre than females, but only in the clean treatment. This result could indicate that the adaptive immune system was upregulated in adults, particularly males, attending dirty nests compared to clean nests. This also suggests that males may be more immunologically affected by the nest treatment than females. This could be potentially due to

differences in incubation, or nest cleaning behaviours and the amount of time spent on the nest, however, this was not measured. A previous study of zebra finches by (McGraw and Ardia, 2005) found that males had a significantly higher humoral response to sheep red blood cells, but females had a higher cell-mediated immune response. However, the nest cleaning treatment seems to have had the opposite effect in our study. To account for individual differences in adaptive immune function, we tested the difference in titre scores between the primary and secondary antibody response. We found that adults with clean nests had a greater relative secondary response than those with dirty nests. Because the absolute level of the secondary response did not differ across treatment, the relatively greater secondary response in adults with clean nests can be attributed to low initial titre scores in the primary response. It is possible that the primary immune response in adults exposed to dirty nests was higher due to upregulation of immune response to defend against more frequent microbial challenges (priming effect), whereas birds from clean nests had fewer challenges and consequently less priming. The lack of an effect of nest sanitation on the secondary response was not expected, but indicates that the primary exposure resulted in similar rates of class switching and production of memory B cells, even though the initial rate of IgM production was low in adults attending clean nests. It is possible that increased titre indicates that the adults were sick from introduced pathogenic bacteria, however, it would be expected that titre would be higher in the background agglutination analysis, and they were not. This could also be the case for nestlings, however, the lack of difference in immune measures, as well as growth rate, may suggest otherwise. It should also be noted that in one treatment we changed the nest material but not in the other and that may have affected the results. Although we tried to control for this by sham cleaning the dirty nest by moving the nest cup around and handling nestlings for the same period of time, it may be a confounding factor.

Microbial killing of *E.coli* decreased in adults throughout nestling rearing in this study, regardless of nest treatment. This finding was consistent with a previous study by Evans et al. (2015) that demonstrated a decrease in microbial killing of *E.coli* between incubation and nestling-rearing in little penguins (*Eudyptula minor*). In a broader context, Pap et al. (2010) found that microbial killing of *E.coli* was at its lowest during breeding compared to the rest of the annual cycle in house sparrows (*Passer domesticus*). Since there was no difference in microbial killing due to nest treatments in our current study, it is possible that the decrease is due to a physiological trade-off or potentially a decrease in nutritional intake as a result of the challenge of supporting growing nestlings. It has been previously demonstrated that there is a decrease in constitutive innate immunity during energy demanding periods (De Coster et al., 2010; Nebel et al., 2012; Pap et al., 2010). Breeding and care of offspring requires a reallocation of resources, due to these tasks being nutritionally demanding (Moreno, 2004) and is one of the most energetically expensive events for birds (Tinbergen and Williams, 2002). The microbial killing of *C.albicans* did not follow the same trend as the killing of *E.coli*, and remained constant throughout nestling rearing. This result was not expected as other studies have found variation in *C.albicans* killing according to their environment (Buehler et al. (2008b). It is possible that the chicken faeces added to the nest did not contain novel bacteria and thus did not result in challenges that triggered an immune response. However, this seems unlikely since chicken faeces came from free-ranging chickens raised in a mixed eucalypt forest, fed a variety of pellets and vegetables, and thus represents a very different environment to that which our captive zebra finches had been exposed. Interestingly, the killing of *E.coli* and *C.albicans* correlated in adults after 20 days post hatch and there was a trend at 10 days post hatch, but not during incubation. The

correlation between the killing of *E.coli* and *C.albicans* was observed previously outside of the breeding period (Versteegh et al., 2012). Potentially, the differences in this correlation between incubation and nestling rearing could be attributed to differing immune strategies and balancing between nestling rearing, potential stressors (recapture/nest manipulations/frequent blood samples) and pathogen pressure. Future studies should measure indices of greater activation of immune system due to microbial manipulation by examining levels of pro-inflammatory cytokines and of Ig specific to the microbes added.

Our experimental manipulations of nest sanitation had little impact on either the innate or adaptive immune measures of nestlings taken in our study despite agar paddles indicating higher bacterial loads in dirty nests. Exposure to a diverse range of microorganisms after hatching is important for the maturation of immune defence, particularly in the first few weeks of development. In the first few weeks after hatching, nestlings rely on innate immune function and maternal antibodies for protection against pathogens, during which time the adaptive immune response matures. The adaptive response begins to become functional at 11-14 days and can take around 6 weeks to mature in many species (Klasing and Leshchinsky, 1999). Furthermore, the nest treatment had no observed effects on mature offspring. There are a number of possible interpretations. It is possible that the immune system does not require much microbial exposure to maximise the development of the immune system, given that exposure to only a few bacteria is able to fully populate the skin and intestinal mucosa. This is a possibility since we chose chicken faeces to introduce novel bacteria into the nest. However, exposure to diverse ranges of microbiota in early life has been shown to increase activation of immune related genes in a wide variety of animals (Hooper et al., 2012; Mazmanian et al., 2005;

Mulder et al., 2011). Potentially the number of bacteria being sampled by the bursa for driving the diversification of the B cells is sufficient to drive lymphocyte differentiation in both clean and dirty nests. Alternately, other sources of bacteria from the skin and saliva of parents (Kyle and Kyle, 1993; Mills et al., 1999) could be important in driving this diversification. There is also the possibility that small sample sizes may have contributed to a lack of difference between the nest treatments. Finally, it is possible that offspring primary response in relation to nest hygiene was similar to that of adults (primary response), however, because of the age of nestling, we were unable to obtain that measurement and as such, future studies could test this possibility.

We did not find any evidence of a relationship between the immune response in adults and that in their nestlings, unlike an earlier study that had found a link with the mothers immune function (Stambaugh et al., 2011). However, a more comprehensive and experimental study by Pitala et al. (2007) found that the immune response was not heritable in 1626 collared flycatcher (Ficedula albicollis) nestlings that had mostly been cross fostered. Evidence of variation in heritability exists in tree swallows (*Tachycineta bicolor*), where out of three different geographic locations, only one location showed heritability of immune response to PHA (Ardia and Rice, 2006). Our sample size and approach was rather small to address this question, and the source of variation in offspring immunity remains to be determined.

In conclusion, manipulation of bacterial load and sanitation in the nest did not impact measures of the innate immune function of either nestlings or adults. But increasing the microbial load of the nest increased the relative magnitude of the secondary

immune response above the primary response to sheep red blood cells in adults. However, nestling immune function was not influenced by nest sanitation and future studies investigating bacterial impact on immune function should test this further. Decreases in microbial killing of *E.coli*, a functional test of complement activity, during the nutritionally expensive period of nestling rearing may provide evidence for the trade-off hypothesis, however, a long term study of birds, both in captivity and the wild is necessary to confirm this observation. Future studies should include analysis of the types of bacteria in the nest paired with immune function tests to get a further understanding of what might affect variation in immune function.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

J.E. designed the study, carried out all experiments, analysed the data, interpreted the findings and wrote the manuscript. K.K., S.G. and K.B. designed the study, interpreted the findings and contributed to the writing of the manuscript.

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Supplementary material

There was no correlation found between nestlings and parents for killing of *E.coli* (average of nest for microbial killing between ages 25 and 27 days). Similarly, there was no correlation found between parents and offspring for agglutination of SRBC with all non responders excluded. There was also no correlation between parents and 60 day old offspring with agglutination, killing of *E.coli* or killing of *C.albicans*.

Supplementary material Table 1. Adult and offspring immune relationships

		r	P
	E.coli		
	Midparent	-0.06	0.83
	Mother	0.18	0.52
Nestlings	Father	-0.20	0.47
	Agglutination		
	Midparent	-0.29	0.42
	Mother	-0.36	0.3
	Father	-0.11	0.77
	E.coli		
	Midparent	-0.1	0.79
	Mother	-0.09	0.8
	Father	-0.05	0.89
60 day old offspring	***C.albicans***		
	Midparent	0.12	0.76
	Mother	-0.05	0.91
	Father	0.28	0.47
	Agglutination		
	Midparent	-0.29	0.42
	Mother	-0.36	0.3
	Father	-0.11	0.77

CHAPTER FOUR

Effects of early developmental priming on adult immune function

Photo Simon C. Griffith

Effects of early developmental priming on adult immune function

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Abstract

Organisms program their developmental trajectories according to their expectations about environmental conditions. Parental effects can play a role in developmental trajectories, with the potential to manipulate offspring through physiological or behavioural signals. These signals can prime offspring for optimal developmental pathways, according to the environmental conditions experienced. We investigated whether differences in parent to egg vocal communication during incubation had an impact on the growth of nestlings and the innate immune mechanisms of juvenile zebra finches. Eggs were artificially incubated and listened to playback of either incubation (n=35 eggs) or tet calls (n=50 eggs) during the last 5 days of incubation. We found that embryos that listened to incubation calls, were fed more as nestlings and were heavier at 13 days of age. However, males in the incubation call group were in poorer body condition as juveniles (around 50 days of age) than females in the same group. Humoral innate immunity, assessed with the microbial killing of *E.coli*, was higher, but not significant, in juvenile males compared to females in the incubation call group only. Cellular innate immunity, as assessed by the killing of

C.albicans, and humoral innate immunity (killing of *E.coli*) was more efficient at higher temperatures. Microbial killing of *C.albicans* was higher for chicks hatched later in the breeding season. This study could suggest that epigenetic effects can impact upon growth, which in turn could potentially impact physiological systems such as immunity. The results suggest that parental-embryo communication could play an important role in fitness and adaptive changes in development.

Key words: epigenetics, immune function, trade-off, development, communication, maternal effects

INTRODUCTION

Early life experiences can influence patterns of development which can have profound effects on the expression of traits in later stages of life (Bredy et al., 2004; Meaney, 2001; Mousseau and Fox, 1998). These experiences can potentially affect the physiology (Ho and Burggren, 2010), behaviour (Crews et al., 2007) and health of an animal (Skinner et al., 2010). Epigenetic effects, that is, changes in gene and phenotypic expression which are not caused by changes in DNA sequence (Bird, 2007; Bossdorf et al., 2008) have been found throughout the animal and plant kingdom. Parental effects, both maternal and paternal, are one type of epigenetic change, where change in offspring phenotype is due to parental influence (Ho and Burggren, 2010; Mousseau and Fox, 1998). The conditions and variation in abiotic and biotic factors in the environment experienced by the parent can result in transgenerational phenotypic plasticity. For example, in waterfleas (*Daphnia cucullata*) and wild radish plants (*Raphanus raphanistrum*), exposure of the mother to a predator leads to increased defence mechanisms in offspring (Agrawal et al., 1999). However, one of the most convincing examples of behaviour of parents and

parental care resulting in transgenerational phenotypic plasticity has been in rats. Increased pup licking and grooming, and arched-back nursing by mother rats found offspring to have altered DNA methylation, related to glucocorticoid receptor gene promoter in the hippocampus, which persisted into adulthood (Weaver et al., 2004). This provided a genetic explanation for the altered behavioural responses of adult rats, where increases in this type of maternal care produced offspring that were less fearful and had more modest HPA responses to stress (Stern, 1997). Importantly, their study showed that behavioural programming and maternal care could alter genes, physiology and behaviour of offspring. Thus, conditions experienced during development, particularly those that are manipulated by the parent, can have an impact on important traits and behaviours expressed in later life. However, the behaviour of parents and the impact this has on the expression of traits in offspring have been little studied in birds.

The environment in which avian eggs develop can have significant carry over effects in later stages of life (Ardia et al., 2010; Colombelli-Négrel et al., 2012; Martin et al., 2011). At the incubation stage, parents have control over the success of their offspring and can potentially change physiology and behaviour. One way in which parents can change offspring development during the egg stage is through vocal communication. An excellent example of this has been shown in the superb fairy-wren (*Malurus cyaneus*). The female superb fairy-wren calls to her eggs during incubation, and at hatching, nestlings produce begging calls with key elements from the mothers incubation calls (Colombelli-Négrel et al., 2012). This in turn causes chicks that produce begging calls with these key elements to be fed at higher rates. This form of communication and learning is likely adaptive because it ensures the success of offspring in a system where brood parasitism is a high probability

(Langmore et al., 2003). Thus, parents can manipulate outcomes based on the conditions experienced (likelihood of parasitism), to ensure the success of their offspring. As well as parents being able to communicate with the embryo, eggs can also communicate with each other. Egg- egg communication has importantly been shown to change the development times of eggs during incubation. This communication is evolutionary adaptive as offspring can compete against each other for food with those hatching later having a decreased chance at survival (Tong et al., 2013). The ability of embryos to recognise, learn and respond to vocal communication (which influences faster development, hatching times and increases feeding rates) could have knock on effects on other important physiological functions such as immunity (Fargallo et al., 2002; Hõrak et al., 1999; Martin-Galvez et al., 2011; Moreno-Rueda and Redondo, 2012; Saino et al., 2001). To date there have been few tests of the long terms effects of parental communication with their offspring prior to hatching and the potential for this behaviour to affect reproductive outcomes. Potentially, in a variable environment, parents could manipulate development of offspring through prenatal signalling, communicating the optimal development pathway to their offspring.

Early life experiences and environmental effects can adaptively change adult immune function. In situations where embryonic development can be changed or manipulated, there may be a carry-over effect on immune variables as nestlings or even into adulthood. For example, experimental cooling of the nest in tree swallows during parental incubation caused differences in embryonic development times, impacting upon nestling humoral innate immune function as tested with the microbial killing of *Escherichia coli* (Ardia et al., 2010). Similarly, immune function in birds has been shown to vary later in life, due to experiences at the egg stage

including incubation behaviour of parents (DuRant et al., 2012), differences in development times (Martin et al., 2011), and in ovo exposure to hormones (Groothuis et al., 2005; Müller et al., 2005), bacteria (Noor et al., 1995), and vitamins (Gore and Qureshi, 1997). After hatching, other factors can influence immune function including growth (Soler et al., 2003; van der Most et al., 2011), food restriction (Alonso-Alvarez and Tella, 2001) and environmental conditions (Ardia, 2005; Arriero, 2009; Benito et al., 2014; De Coster et al., 2011; Dubiec and Cicho, 2005; Forsman et al., 2010). The range of conditions experienced that cause a change in one aspect of physiology can then impact on another. The trade-off hypothesis (Sheldon and Verhulst, 1996) predicts that consumption of limited resources by one physiological process leads to trade-offs between other resource-demanding activities, for example trade-offs between growth and immune function, or immune function and reproduction (Ilmonen et al., 2000; Lochmiller and Deerenberg, 2000; Norris and Evans, 2000; Sheldon and Verhulst, 1996). Thus, there is potential for experiences in the nest and during development which could impact growth and immune function, ultimately affecting success, health and survival.

Epigenetic effects have the ability to speed up evolutionary change, and if parental vocal communication is able to signal current and changing environmental conditions, offspring could develop according to the optimal developmental pathway for a given environment, communicated by the parents. However there have been no experimental tests of the impact of vocal communication on changes to growth and immune function in birds.

Here, we tested the hypothesis that the incubation environment, in particular, parental communication in the form of incubation and tet calls, could explain some of the variation in juvenile growth. The changes in growth could then potentially

have knock on effects on other physiological processes such as constitutive innate immune function. We played incubation and whine calls (experimental treatment), or tet and whine calls (used as a control), to developing embryos in the last five days before hatching. Tet and whine calls are uttered by the parents in the nest when one partner comes back to the nest to relay its partner for incubating the eggs (Elie et al. 2008). Whine calls are used exclusively in the nest whereas tet calls are a contact call used in a variety of contexts, including at the nest. They both are complex calls modulated in frequency. Incubation calls on the other hand are uttered when a partner is alone in the nest during incubation, and are simpler in structure, being dominated by a single frequency. This is potentially a form of parental-embryo communication to signal certain conditions as it is not often displayed. We took growth and body condition measurements as nestlings and juveniles and tested immune function at the juvenile stage (around 50 days of age). We tested innate immune function using a microbial killing assay, which tests the ability to defend against bacterial invasion. We tested this relationship in the zebra finch (*Taeniopygia guttata*) and predicted that vocal communication in the form of incubation calls would alter development in nestlings, in turn impacting upon immune function in juveniles.

Materials and Methods

Breeding experiment and monitoring of birds

Sixty male and 60 female zebra finches were allowed to pair and breed freely in outdoor aviaries (8 x 5 x 2.5m) from December 2013 to March 2014, at Deakin University Geelong, Victoria, Australia. These birds were derived from wild zebra finches from Northern Victoria that were bred in captivity for approximately 7 to 9 generations. They were of mixed age (1-5 years old) and with different levels of breeding experience. All birds were fitted with a black plastic ring with a unique identity number and two color rings making a unique color combination.

Birds were supplied with *ad libitum* seed (Golden Cob finch mix), water, grit, cuttlefish bone and nest boxes/nesting material; cucumber and water baths were refreshed daily; and cooked whole egg was provided once a week. Nest boxes were checked every morning to record the dates of laying, hatching and fledging. The identity of the parents for each nest was established based on direct observation of nest visits during incubation and chick rearing.

Egg artificial incubation and playback experiment

Eggs were removed from the nest on the day of laying and replaced with dummy eggs. The freshly collected eggs were stored on a rocking platform in a room at 25ºC for up to 4 days to standardise incubation onset and hatching asynchrony. Eggs were then placed in an artificial incubator (Octogon 20 plus, Bransea, Australia) at 38ºC and 60% humidity with constant rocking motion. All eggs were in individual compartments so that they did not touch each other. Eggs spent the first 9 days of their development in a main incubator, and were later moved to one of two different incubators according to the experimental group to which they were allocated. Eggs were swapped between experimental incubators daily to ensure embryonic growth

conditions were similar across experimental groups. Whole clutches were randomly allocated to either control (tet calls) or treatment (incubation calls) playback group. In addition, eggs in both the treatment and control groups also experienced playback of whine calls. All three call-types used in playback were therefore calls that eggs could naturally encounter before hatching. In summary, all eggs were exposed to the most common, complex and nest-specific call (i.e. whine) to ensure normal stimulation of the auditory system of the embryos. In addition, treatment eggs heard incubation calls that are presumably directed to them (because the parent is alone when producing this call), whereas control eggs heard another call (tet) that is presumably directed to the incubating partner rather than to the eggs. Incubation and tet calls were recorded from four different pairs (with a mixture of both male and female calls), and whine calls were recorded from a different set of six pairs.

Playback sequences of 1h15 were played in loops continuously from 9:30am to 6:30pm daily from day 10 of incubation until hatching. Each sequence of 1h15 was composed of randomly alternating sequences of whine calls (2 minutes) and incubation or tet calls (5 minutes) separated by silence (2 to 15 minutes), so that it comprised in total 6 minutes of whine call and 20 minutes of incubation or tet calls. The three whine sequences were separated by 19, 22 and 31 minutes and lasted for 2 minutes each to match the average length of incubation bouts in captive zebra finches and the duration of the vocal exchange during nest relay. Calls were played from a Zoom audio recorder through headphone speakers via a XXX amplifier. All eggs were within 5cm of the speaker and trays were moved within the tray daily to homogenize the position of the eggs relative to the speakers. Eggs in the playback incubators were checked four times a day for hatching and once hatched, nestlings were moved to the nest and a dummy egg removed. Hatched nestlings were returned

to either their parents´ nest (n= 60 chicks) or foster parents´ nest (n=34). In either case, broods contained either only control or treatment nestlings (n=31 control and 18 treatment chicks in non-mixed broods) or a mixture of nestlings from both groups (n=23 control and 22 treatment chicks in mixed broods).

Nestling and juvenile monitoring

At 13 days old, nestlings were weighed (nearest 0.1 g) and tarsus length (nearest 0.01 mm), tail length (nearest 0.01 mm), body condition and the number of seeds in the crop were measured and individuals banded with a black plastic numbered ring. The number of seeds were counted in the crop, visible through the skin. Crop content estimated with this technique explains 90% of the variation in actual seed mass present in the crop in adult zebra finches (Meijer et al., 1996). However, because nestlings with more developed feathers were fed less, we used the residuals of seed count in the crop over the total tail length. This method seeks to standardise the amount of food fed to chicks that are faster at developing than others and to get a true understanding of the feed rate. Throughout the paper, the calculated residuals of seed in the crop over tail length will be referred to as 'amount of food at day 13'. As a measure of body condition, we quantified body mass relative to body size using the scaled mass index (SMI) following Peig and Green (2009). This index adjusts body mass of all individuals by giving all individuals the same body size, therefore giving a consistent index of body condition. Body mass alone is not a good indicator of condition because size/bone structure is unaccounted for. To ascertain the condition of jueveniles and whether they had compensated (catch up growth) for conditions experienced as nestlings, using a reliable measurement of condition like SMI was necessary. We used tarsus length as a measure of body size, and body mass, calculating scaled mass index of birds at day 13 and at the juvenile stage.

After nutritional independence (c.a. 30 days), juveniles were separated from the parents and housed in four separate aviaries, with males and females of similar ages. One to two weeks (11.08 ± 0.39 days) after moving into these groups, the immune function of juveniles was tested.

Blood sampling and microbial killing

To test immune function, juveniles were caught in age related cohorts, with each group housed in a separate outdoor aviary. They were in groups caught at 9am on 14th April (N = 16, age range 52-64 days, mean ± S.E = 57.4±1.0), 15th of April (N = 21, age range 53-64, mean ± S.E = 59.2±0.7), 29th of April (N = 29, age range 46-66, mean ± S.E = 55.4±1.1) and 15th May (N = 19, age range 52-63, mean ± S.E = 58.1±0.9). During each capture event, all birds were sequentially caught and held in cloth bags and processed in order of capture and returned to the outdoor aviary.

Blood was collected from the brachial vein and no more than 2% body weight by volume was taken at a time. Blood was collected using heparinised capillary tubes, a capillary adapter and sterile eppendorf 1.5ml tubes. Birds were then weighed and released back into the aviary. Blood was processed within 1 hour of sampling using the method below. The temperature at the time and day of sampling was 16.5ºC, 16.9ºC, 20.7ºC and 17.5ºC respectively, according to the Bureau of Meteorology website (BOM.gov.au). In total, there were 27 males and 23 females in the control group, and 16 males and 19 females in the experimental group.

To assess the microbial killing abilities of blood, we followed the procedure of Millet et al. (2007) and Tieleman et al. (2005), under sterile working conditions. We used two different microbial strains: *Escherichia coli* (*E.coli*) (ATCC 8739) and *Candida albicans* (*C.albicans*) (ATCC 10231), to test different components of constitutive innate immune function. The use of *E.coli* tests the complement and circulating plasma protein content of the blood (innate humoral immunity), whereas *C.albicans* tests complement-independent mechanisms, such as phagocytosis (cellular immunity) (Buehler et al., 2008b; Demas et al., 2011). We reconstituted the lyophilized pellet (Microbiologics, St.Cloud) in sterile phosphate buffered saline (PBS) and made a working solution of approximately 150 colony forming units (CFU) per plate. The working solution was made fresh daily. Whole blood was diluted in CO_2 – independent medium (Gibco, New York) + 4mM L-glutamine (Sigma Aldrich, Castle Hill) and inserted bacteria (1:10), which was incubated for 30 minutes (for *E.coli*) or 2 hours (for *C.albicans*) at 41 degrees Celsius. The media was taken from the incubator and 50µl was spread on soy tryptic agar in duplicate (total 100µl) using sterile spreaders. The plates were then inverted and incubated at 37 degrees Celsius overnight (*E.coli*) or 2 days (*C.albicans*) and the colonies counted. Control plates to validate CFUs were incubated with each batch of experimental plates using the microorganism and medium alone. A negative control plate with only whole blood and medium was tested for each bird to ensure sterility was maintained whilst sampling. All plates, except for negative controls, were run in duplicate. For both strains, we calculated the proportion killed as the average number of colonies on experimental plates relative to the average number of colonies on the CFU control plates (1 – (average number of CFU on experimental plate / average number of CFU on control plate) x 100). No negative controls contained CFUs.

Statistics

To assess the effect of experimental treatment (playback of incubation and tet calls) on growth and condition of nestlings, we ran two models separately testing the effect of treatment on either the food at day 13 or body mass. Analysis was run using linear mixed effects models (with restricted maximum likelihood (REML) estimation). To account for non-independence of data and potential nest effects, brood was included as a random factor in all linear mixed effects models. Fixed factors in the model included treatment and sex. Potential nest effects that may influence condition were included as covariates, such as number of nestlings per nest and hatch date which controlled for age differences within the nest. For all models described, all two-way interaction terms and single variables were included initially in each model and then removed sequentially by highest p-value for those interactions with $p > 0.20$.

We also tested the effect of incubation treatment on the body mass of juveniles using the same model parameters. This was to test whether the detected effect on nestlings was still visible at the juvenile stage or whether juveniles had compensated for this effect. For this reason, a Pearson's product moment correlation was conducted on body mass between nestlings at 13 days and juveniles. We also wanted to assess body condition using scaled mass index. Scaled mass index was calculated at day 13, but SMI did not differ significantly between treatments at day 13, because both body mass and tarsus were affected by the treatment. Also SMI is not considered by some as a great indicator of condition whilst birds are still growing. However, SMI was calculated for juveniles using all the model parameters listed for the models above.

The effects of incubation treatment and other factors on microbial killing mechanisms of complement (killing of *E.coli*) were assessed using a linear mixed

effect model (with REML estimation). Temperature experienced by birds at the time and day of sampling was included as a potential influencing factor on immune function. Assessment of the best model to explain the data was determined using model selection based on the Akaike Information Criterion (AIC). Nest of origin (genetic parent) or brood (rearing nest) were used as random factors; fixed factors included treatment and sex; and covariates included number of nestlings in the nest, hatch date and temperature. Using model selection, the random effect of brood (AIC = 779.6) was slightly better supported to explain the data than nest of origin (AIC = 781.0), therefore brood was retained as a random effect for all models. All two way interactions and variables were excluded from the reduced model, as described above.

A general linear model was used to examine the factors that affected the microbial killing of *C.albicans*. A linear mixed effects model was initially used with random effects of nest of origin and brood. However, convergence of the model could not be achieved. After further investigation (including checking for brood size bias and sex biases), the model indicated that there was no significant effect of the random factors on the killing of *C.albicans* (estimate of parameter value = 0.001), therefore, a general linear model was a better fit for this data. For the model, fixed factors included treatment and sex; and covariates included number of siblings in the nest, hatch date and temperature at the day and time of immune testing.

Each set of data were checked for potential unbalance or biases. When testing immune function, birds were caught in age related cohorts, across four time periods (ages and sample dates listed in methods above), therefore we tested for potential biases of hatching date and age between treatments or sexes. However, there was no bias in hatching date or age between sexes (hatch date: $T_{1,83} = 0.25$, $P = 0.6$; age:

$T_{1,83} = 0.05$, P = 0.8) or between treatment groups (hatch date: $T_{1,83} = 0.21$, P = 0.6; age: $T_{1,83} = 0.23$, P = 0.6). Since immune testing of juveniles was conducted on four separate occasions over a one month period, we tested whether temperature was a real effect, or if the relationships were based on seasonal change. To do this, we analysed temperature and test day in separate models for the microbial killing of *E.coli* and *C.albicans*, using the same models and parameters listed above. However, in each model, we tested the final reduced model only, exchanging temperature for test day. We tested this further by using a linear mixed effect model (fit with maximum likelihood (ML) to test different fixed terms), conducting model selection using Akaike Information Criterion corrected for small sample sizes (AICc) with a lower AICc indicating a better model (see results section below). Temperature and test date could be distinguished because of an increase in temperature in the middle of the immune testing period (see methods for dates of sampling and corresponding temperatures).

Estimated means (± S.E) are presented throughout the results and all full and reduced models can be found in Table 1, 2 and 3. All statistical analyses were performed in the R computing environment (R 2.11.1 and 2.15.0; R Development Core Team, 2010), using the 'nlme' package.

Results

Feed, growth and scaled mass index

As in the whole dataset (Mariette & Buchanan, unpublished data), treatment nestlings (mean ± S.E = 2.55 ± 3.95) were fed significantly more than controls (mean ± S.E = -4.89 ± 3.43; see table 1, figure 1). While seed count per nestling increased

with number of nestlings in control chicks, it was constant and high in treatment chicks. In addition, body mass on day 13 was also significantly higher in treatment (mean ± S.E = 9.66 ± 0.16) than in control nestlings (mean ± S.E = 9.46 ± 0.14; figure 2), especially later in the season, as body mass decreased with hatching date in control nestlings but remained high in treatment nestlings.

Table 1. Body mass and food at day 13

	T	df	P
Food at day 13			
Full model			
Sex	-0.62	1,39	0.536
Treatment	1.42	1,39	0.163
Number of nestlings	2.29	1,38	0.028
Hatch date	1.16	1,39	0.253
Sex*treatment	1.50	1,39	0.142
Treatment*no. nestlings	-2.08	1,39	0.044
Treatment *hatch date	-0.07	1,39	0.947
Reduced model			
Treatment	2.74	1,43	0.009
Number of nestlings	2.67	1,38	0.011
Treatment*no. nestlings	-2.49	1,43	0.017
Body mass of nestlings			
Full model			
Sex	-0.30	1,39	0.763
Treatment	-2.22	1,39	0.032
Number of nestlings	0.22	1,38	0.824
Hatch date	-3.95	1,39	<0.001
Sex*treatment	0.41	1,39	0.685
Treatment*no. nestlings	-0.43	1,39	0.667
Treatment *hatch date	2.70	1,39	0.010
Reduced model			
Treatment	-2.63	1,42	0.012
Hatch date	-4.21	1,42	<0.001
Treatment*hatch date	2.80	1,42	0.008

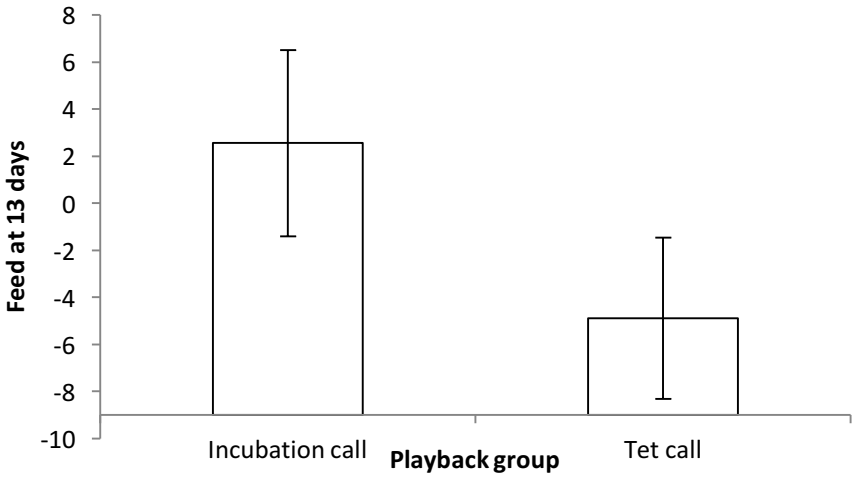

Figure 1. Amount of feed in crop in 13 day old nestlings between playback groups. Playback groups included incubation calls and tet calls

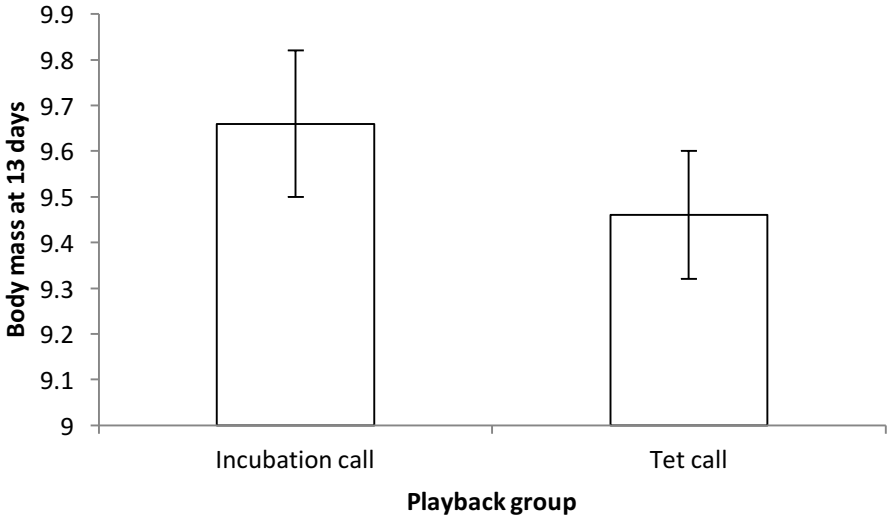

Figure 2. Body mass of nestlings at 13 days of age between playback groups. Playback groups included incubation calls and tet calls

As juveniles, treatment effect was no longer visible on individual weight (means ± S.E, control = 11.72 ± 0.10, treatment = 11.56 ± 0.11), although individuals hatched later in the season were still lighter as juvenile (see table 2). Likewise, juvenile fat score (means ± S.E, control = 3.69 ± 0.18, treatment = 3.17 ± 0.22) and tarsus length did not differ between treatment groups. Individuals had therefore compensated for

early environmental stress and accordingly catch up growth was affected by the same factors as weight on day 13, namely treatment group in interaction with hatching date. Despite this compensatory growth, individuals´ weight as juveniles was strongly correlated to their weight as 13-day nestlings (Pearson's correlation R=0.46, n=85. P<0.001). In summary, individuals´ weight had increased enough since fledging to erase the treatment effect. When taking body condition of juveniles into account, scaling mass for body size (scaled mass index) there was a significant interaction between treatment and sex (see table 2, figure 3), with males having poorer body condition than females in the treatment (mean ± S.E, males=11.40±0.17, females=11.84±0.16) but not control group (males=11.79±0.14, females=11.60±0.15) (see table 2, figure 1).

Table 2. Body mass and scaled mass index (SMI) of juveniles

	T	df	P
Juvenile body mass			
Full model			
Sex	0.15	1,39	0.879
Treatment	-0.80	1,39	0.430
Number of nestlings	-0.18	1,38	0.855
Hatch date	-2.06	1,39	0.046
Sex*treatment	-0.59	1,39	0.559
Treatment*no. nestlings	0.18	1,39	0.859
Treatment *hatch date	0.64	1,39	0.524
Reduced model			
Hatch date	-2.06	1,44	0.045
Scaled mass index (SMI)			
Full model			
Sex	1.16	1,39	0.254
Treatment	-0.24	1,39	0.813
Number of nestlings	-0.35	1,38	0.727
Hatch date	-0.46	1,39	0.651
Sex*treatment	-2.10	1,39	0.043
Treatment*no. nestlings	0.40	1,39	0.691
Treatment *hatch date	0.18	1,39	0.857
Reduced model			
Sex	1.05	1,42	0.300
Treatment	1.14	1,42	0.260
Sex*treatment	-2.16	1,42	0.036

Microbial killing of *E.coli*

The interaction between sex and treatment was not significant, (see table 3, figure 3). There was a trend with males having a higher killing ability than females. Temperature at the time of sampling significantly affected microbial killing of *E.coli*, showing better microbial killing of *E.coli* at higher temperatures. Test date, when analysed in a separate model, did not significantly affect the microbial killing of *E.coli*. When comparing models containing either temperature or test date, we found that temperature (AICc= 812.4) significantly explained the variation in the killing of *E.coli* better than test date (AICc= 817.4). Therefore temperature, not season, had an impact on the microbial killing of *E.coli*.

Table 3. Microbial killing of juveniles

	T	df	P
Killing of *E.coli*			
Full model			
Temperature	2.19	1,35	0.035
Sex	-0.01	1,35	0.996
Treatment	-0.1	1,35	0.921
Number of nestlings	0.87	1,35	0.388
Hatch date	0.40	1,35	0.689
Sex*treatment	1.52	1,35	0.138
Sex*hatch date	0.35	1,35	0.731
Sex*no. nestlings	-0.02	1,35	0.985
Treatment*hatch date	0.89	1,35	0.379
Treatment*no. nestlings	-1.26	1,35	0.217
Hatch date*no. nestlings	-0.73	1,35	0.467
Reduced model			
Treatment	-0.73	1,41	0.472
Sex	1.89	1,41	0.066
Temperature	2.30	1,41	0.027
Sex*treatment	1.73	1,41	0.091
Killing of *C.albicans*			
Full model			
Temperature	3.34	1,73	0.001
Sex	-0.03	1,73	0.787
Treatment	-0.59	1,73	0.561
Number of nestlings	0.60	1,73	0.544
Hatch date	-0.21	1,73	0.836
Sex*treatment	0.26	1,73	0.800
Sex*hatch date	0.72	1,73	0.476
Sex*no. nestlings	-0.51	1,73	0.613
Treatment*hatch date	0.29	1,73	0.772
Treatment*no. nestlings	0.32	1,73	0.748
Hatch date*no. nestlings	-0.42	1,73	0.673
Reduced model			
Temperature	3.42	1,82	0.001
Hatch date	-2.10	1,82	0.039

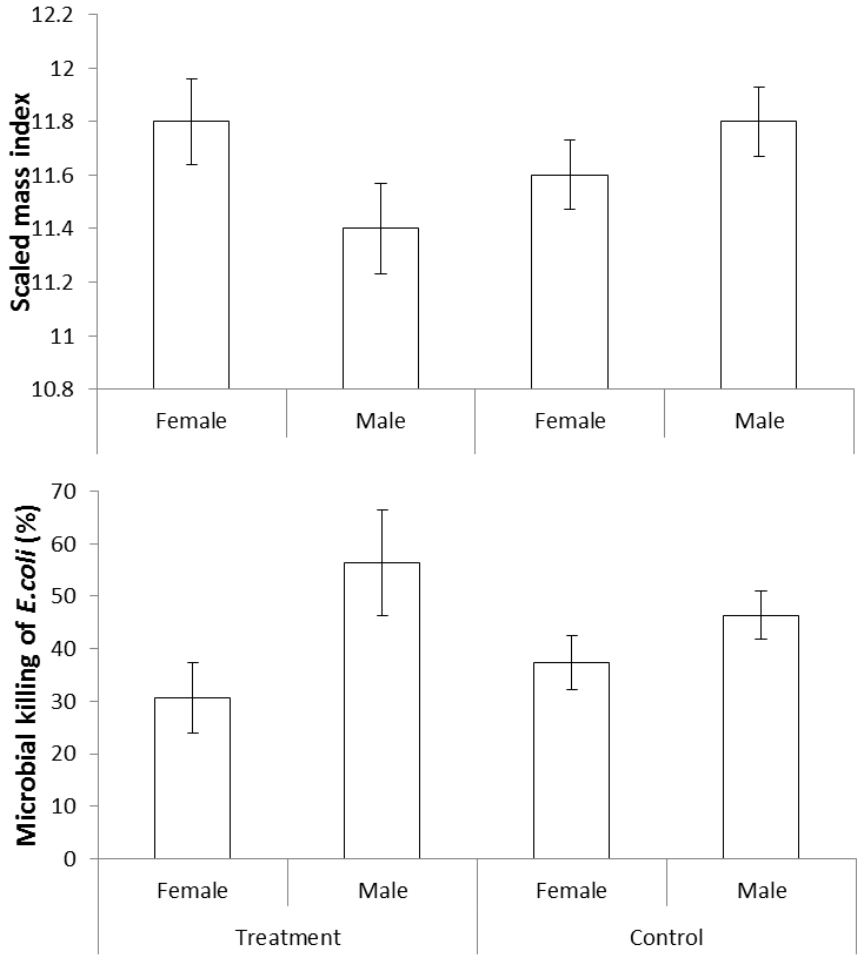

Figure 3. Mean scaled mass index and microbial killing of *E.coli* between playback groups and sexes. Playback groups included incubation (treatment) calls (n males=16, n females=19) and tet (control) calls (males n=27, female n=23)

Microbial killing of *C.albicans*

As with *E.coli*, temperature at the time of sampling had a significant effect on the microbial killing of *C.albicans* (see table 3), showing that there was better killing of *C.albicans* at higher temperatures. Hatch date was also found to significantly affect the killing of *C.albicans*, indicating that birds hatching later in the season had poorer cellular immune function. Test date, when analysed in a separate model, did not significantly affect the microbial killing of *C.albicans*. When comparing models containing either temperature on the day of testing or test date, we found that temperature (AICc= 740.0) significantly explained the variation in the killing of

C.albicans better than test date (AICc= 751.7). We can conclude from this that temperature, not season, had an impact on the microbial killing of *C.albicans*.

Discussion

We found that incubation calls played to eggs led to changes in amount of food and growth in zebra finch nestlings, and condition in juveniles. To the best of our knowledge this is only the second example of parental communication prior to hatching affecting growth and development of offspring (Colombelli-Négrel et al., 2012). Food at day 13 and growth were increased in nestlings that listened to incubation calls as embryos compared to those that heard tet calls as embryos. The body mass of chicks was significantly higher in the treatment group, however, this relationship was no longer present in juveniles, seemingly because birds had compensated and caught up in growth. However, within the group that listened to incubation calls, there were sex differences in body condition of juveniles, with males having reduced condition compared to females. Although not significant, males seemed to have better killing ability of *E.coli* compared with females in the incubation call treatment, however, larger samples sizes are necessary to confirm this. There was no effect of treatment on the microbial killing of *C.albicans*. These data are novel because the effects of incubation calls on early development have only recently been examined (Colombelli-Négrel et al., 2012). Although the exact reason behind the incubation call is not known, these data provide us with good reason to believe that parents are communicating with embryos to influence offspring development, potentially signalling the current environmental conditions. These results suggest physiological changes could be caused by parental signalling at the egg stage. This signalling could also impact upon other physiological processes such as immune function, however, this relationship needs to be tested further.

The results of the present study found nestlings that listened to incubation calls during embryonic development were fed more and were indeed heavier. This was in agreement with the findings of Colombelli-Négrel et al. (2012). They found fairy wren embryos tutored with incubation calls produced begging calls with similar notes to parent incubation calls, which in turn caused nestlings to be fed at a higher rate. This could suggest the incubation call serves as a signal to determine optimal begging effort for a given environment. Whatever the purpose of the incubation call, it has carry over effects on condition. Conditions during incubation and exposure to elements during in ovo, can have carry over effects and impact upon immune function at later stages of life (Ardia et al., 2010; DuRant et al., 2012; Groothuis et al., 2005; Martin et al., 2011; Müller et al., 2005; Noor et al., 1995). Our study did not provide the necessary evidence to suggest that vocal communication and parental signalling may impact upon immune function, and although there seemed to be a trend with males having higher killing abilities in the experimental group, further testing is necessary as sample sizes were not adequate to test this relationship. Considering placental animals are able to signal to their offspring about conditions through both direct and indirect effects whilst pregnant (Guerrero-Bosagna et al., 2005), it seems logical that birds, despite the disadvantages of raising embryos externally, are able to communicate messages in a variable environment to increase the chance of survival of their offspring. However, there are many opportunities to study these relationships further, particularly understanding the role of the incubation call specifically and other aspects of behaviour that may signal to offspring during embryonic development.

Body condition was the same in both sexes listening to tet calls, however males in the treatment group which listened to incubation calls were significantly in poorer condition than females. This result is interesting and somewhat surprising since males and females were fed similar amounts of food by their parents in the treatment group. There is the possibility that males begged more aggressively than females, which could lead to an energetic imbalance and decreasing body condition overall. Increased begging has been shown to affect growth rates of birds (Kilner, 2001; Rodriguez-Girones et al., 2001) and aggressive begging has also been linked to reduced immune function (Moreno-Rueda and Redondo, 2012). Sex differences in begging behaviour of zebra finches have been found with males begging for longer periods of time however growth rate was found to increase in males (von Engelhardt et al., 2006). However, we would also expect males to be fed more if they begged more aggressively. Instead, it is possible that a trade-off between body condition and another physiological function has occurred.

Although there were no significant relationships found between microbial killing and incubation calls, there is a very slight trend. It is possible that the trend of higher killing of *E.coli* in males within the incubation call group could be driven by the sex specific differences in body condition, suggesting a possible trade-off between growth/condition and immune function. It has been previously demonstrated that there is a decrease in constitutive innate immunity during energy demanding periods (De Coster et al., 2010; Nebel et al., 2012; Pap et al., 2010). Microbial killing of *E.coli* tests the circulating plasma proteins and complement killing of pathogens by whole blood, which is important for destroying invading pathogens and therefore is important for limiting infection. Males had reduced body condition but increased microbial killing of *E.coli*, whereas females were in better condition, but had reduced

immune function. Potentially, signalling might be important for success of offspring in certain condition. It could be possible that this signalling by the parental incubation call is related to investing in immune function in male offspring, as strong immunocompetence and the ability to fight pathogens and parasites is a sexually selected trait, particularly in an environment where pathogen risk might be increased (Hamilton and Zuk, 1982). However, it could also be equally as important for females to invest in growth and body condition for future reproductive success (Blums et al., 2002; Haywood and Perrins, 1992), rather than immune defence. However, this is speculation only as our data does not fully support this, but this work does warrant future studies to investigate knock on effects of incubation environment and communication, growth conditions and the impact on the immune system. Furthermore, measurement of other immune measures, particularly those that might be susceptible to trade-offs with growth, would give a greater understanding of early life experiences and epigenetic effects on future physiological development and functioning. Since the incubation call is not used all the time and we do not understand its intended purpose, future work could examine the adaptive nature of the call and the environmental circumstances that give rise to it being given. This would give insight into the likely adaptive changes in offspring and when they are likely to occur.

Temperature was found to significantly affect microbial killing of both *E.coli* and *C.albicans*, with higher temperatures correlating with higher killing, in turn suggesting that defence mechanisms such as complement defence (circulating proteins) and phagocytosis are in part determined by ambient temperature. This is not surprising, as previous studies have found an effect of ambient temperature on immune functioning (Svensson et al., 1998), even when controlling for photoperiod

(Demas and Nelson, 1996). However, ambient temperature has not previously been found to affect microbial killing mechanisms specifically. Buehler et al. (2008a) tested the effect of three temperature ranges (cold - 5 ºC, warm - 25 ºC and variable with mean of 15 ºC) on adult red knot (*Calidris canutus*) immune function. They found that seasonal effects were more important than temperature effects. Since our study tests juvenile birds only, it is possible that adults and developing birds are not affected by the same environmental variables. Some birds were not yet displaying adult plumage and the costs of moulting and thermoregulation as well as other developmental costs could impact upon energy expenditure. Documented changes in immune function throughout the yearly cycle in house sparrows found that birds decreased microbial killing of *E.coli* during winter, however, other changes were in line with specific breeding and moult events (Pap et al., 2010). However, to test this more comprehensively, a long term study should be conducted to test both seasonal and temperature impacts across multiple years on immunity would be necessary.

In conclusion, body mass and food at day 13 were increased in nestlings which were experimentally exposed to playback of incubation calls during embryonic development. Immune function could potentially be affected by these relationships however, further investigation is necessary to determine this. Temperature at the time of blood sampling had a significant effect on the killing ability of both *E.coli* and *C.albicans*, suggested that fluctuations in temperature play a role in variation in either immune mechanisms including circulating plasma proteins and complement as well as phagocytosis. This study suggests a potentially important role for parental-embryo communication in the manipulation of offspring developmental trajectories based on the current environmental conditions.

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CHAPTER FIVE

Development of innate and adaptive immune function in zebra finches

Development of innate and adaptive immune function in zebra finches

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Abstract

The vertebrate immune system serves to protect from the risk of infection by pathogens. Precocial birds are known to have a mature immune system within 6 weeks of hatching. Most mammals are known to rely heavily upon innate immunity during the initial stages of development, whilst the adaptive immune system is maturing. However, much less is known about the timing of immune development and immune strategies in altricial birds, despite the fact that they are likely to be subject to a diverse range of pathogens once leaving the nest three weeks after hatching. We assessed development of constitutive innate immunity in the form of complement based microbial killing and adaptive immune development during the nestling, fledging and sexually mature stages in the zebra finch (*Taeniopygia guttata*). The ability of blood to kill *Escherichia coli* (*E.coli*) at the nestling stage was low and not significantly different from that of fledglings. However, the

microbial killing ability of fledglings was significantly lower than that of sexually mature finches at 65 days of age, with an almost 10 fold increase in ability occurring over forty days between these stages. Induced adaptive immunity (assessed with a novel antigen of sheep red blood cells), was tested in zebra finches aged 9, 11, 13 or 60 days. Nestlings were capable of mounting a secondary immune response when primed at 10 days or older. There was a significant increase in antibody titres with age; however, the antibody response was not mature at the time of fledging. These results together suggest that zebra finches do not have mature immune responses at fledging, which is likely a consequence of fast growth and development. This work suggests that young altricial birds may tolerate infections rather than defend against them, with consequences for host pathogen dynamics in wild birds.

Introduction

The aim of ecoimmunology is to understand variation in immune function and immune defense strategies and how this might contribute to life history traits, disease ecology and health of an animal. Although immune strategies and development have been well documented in precocial birds such as chickens and other domestic poultry, this contributes very little to understanding immune variation and strategies in wild birds. Altricial passerines are thought to be one of the fastest developing land animals (Arendt, 1997; Case, 1978), often reaching adult size within a few weeks of hatching but are completely dependent on their parents to feed and care for them until fledging. In comparison, precocial birds have much longer embryonic and chick development, but hatch with the ability to feed themselves. Despite these differences in life histories, most of our understanding of immune ontogeny has come from studies of poultry, which have been artificially selected for fast growth and increased reproduction in meat and egg industries, and therefore, are not an accurate representation of immune function in wild birds. This is particularly important since

a key hypothesis in ecoimmunology is that immune function is costly and therefore may be traded-off for other competing physiological demands such as growth and reproduction (Sheldon and Verhulst, 1996). The immune system has the function of protecting against pathogens and parasites, however, at hatching, both altricial and precocial birds are quite vulnerable to environmental pathogens, as the immune system is not well developed (Apanius, 1998; Glick, 1983a; Glick, 1983b; Rose et al., 1981). In studies of humans and animals, it is known that there is a strong reliance on maternal antibodies (Brambell, 1970) and innate immune function (Levy, 2007) whilst the more energetically costly adaptive immune system develops (Klasing, 2004; Klasing and Leshchinsky, 1999). Precocial birds such as chickens rely on innate immune defences initially after hatching, whilst the adaptive immune system fully develops at around 6 weeks of age (Davison, 2008; Klasing and Leshchinsky, 1999). However, altricial birds often reach adult size and fledge within 2-3 weeks of hatching, but whether innate and adaptive immune development occurs simultaneously and much faster with this rapid development remains to be documented for most wild bird species.

Innate immunity is the first line of defence against pathogens. It is rapid in its protection, as it does not require previous exposure to a pathogen, and is relatively low in cost in terms of energy expenditure both to produce and develop, compared to adaptive defences (Klasing, 2004). In many animals, it is suggested that during development, innate immune function is relied upon in the first instance and quite heavily (Levy, 2007), whilst the adaptive immune system in maturing. However, a range of studies testing rate of development of immune function in birds conflict in terms of their conclusions about development of innate defences. However, they do overall suggest that most innate defences are not at an adult level at the time of

fledging. For example, a study of ontogeny in free living tree swallows (*Tachycineta bicolor*) found that nestling defences such as total lymphocyte concentration, natural antibody titres and complement mediated cell lysis were not fully developed at the time of fledging (Palacios et al., 2009). Similarly, in nestling great tits (*Parus major*) (De Coster et al., 2010) and house sparrows (*Passer domesticus*) (Killpack et al., 2013), complement mediated lysis was not present until after fledging or below the level of adults. However, a study of 12 day old barn swallow (*Hirundo rustica*) nestlings found that complement mediated lysis titres were already indistinguishable from adult (Moller and Haussy, 2007), although natural antibody levels were lower in nestlings than adults (Moller and Haussy, 2007). Other innate measures, such as bacterial killing of *Escherichia coli* have been shown to be below adult levels at the time of fledging in tree swallows (Stambaugh et al., 2011). Overall, it is clear that ontogeny of the avian immune system increases in efficacy over the course of the nestling period (Arriero et al., 2013; Mauck et al., 2005). That said, the functional significance of these changes and the nature of the defences used at the time of fledging in many altricial bird species are poorly understood.

The same pattern might be said for adaptive immunity; that little is known about the rate of maturation of the system and the speed at which altricial nestlings develop the response to novel antigens. In precocial species such as the domestic chicken, it takes 5-7 weeks for the antibody repertoire to be fully developed (Davison, 2008; McCormack et al., 1991). However, immunoglobulin Y (IgY), the main immunoglobulin isotype produced in the secondary response, is present in the spleen two weeks after hatching in chickens, suggesting that some secondary response is possible 1-2 weeks post hatch (Narabara et al., 2009). IgM is the first immunoglobulin isotype produced in the primary response when a novel antigen is

encountered (Davison et al., 2008). After exposure to an antigen, antibody switching occurs (after specific signaling molecules cause this change) from the IgM isotype and memory B cells are produced in the form of IgY, which is the main immunoglobulin present in a secondary response. Antibody class switching is important so that a secondary immune response can be activated with the use of IgY, which is important to destroy invading pathogens. The specific timing of this class switching, however, is largely unknown in altricial species. In the zebra finch (*Taeniopygia guttata*), a secondary antibody response to keyhole limpet hemacyanin (KLH), was found when the priming offspring at 14 days of age, but not if offspring were primed at 7 days (Killpack and Karasov, 2012). This indicates that the ability to mount a secondary response occurs when priming occurs somewhere between 7-14 days. This study also demonstrated that secondary antibody titres increased during the nestling period, however, did not reach adult levels at fledging (Killpack and Karasov, 2012). In the American kestrel (*Falco sparverius*), nestlings have been shown to mount an antibody response as young as 4 days of age, with the strength of the antibody response increasing with development, although adults produced antibody levels four times greater than those of nestlings (Smits and Bortolotti, 2008). However, in many species the timing of development of the secondary response is unclear as well as understanding how much this might vary either within or between species.

The aim of this study was to determine ontogeny of the constitutive innate and induced adaptive immune defence of the altricial passerine, the zebra finch (*Taeniopygia guttata*). In this, we wanted to determine the immune strategies of nestling, fledgling and sexually mature finches, and determine the age in which zebra finches are able to mount a secondary response which enhances the ability to fight

invading pathogens. Zebra finch development is fast in comparison to precocial species, with embryonic development around 13 days and can fledge within 20 days of hatching (Zann, 1996). Given the results of previous published studies, we predicted that constitutive innate immune function would not be at adult level at fledging, but would increase throughout the nestling period. We also predicted that adaptive immunity would increase throughout the nestling period, and that the ability to mount an antibody response would occur between 8 and 14 days. In this, we wish to understand what defences might be relied upon during development and contribute a further understanding to ontogeny of immune function in altricial passerines and the immune strategies used in this species.

Methods

Bird breeding and handling

Adult zebra finches were sourced from local breeders and maintained under standardised conditions of 14h: 10H L:D photoperiod, 21-24 degrees Celsius with 40-50% relative humidity. Birds were initially kept in single sex groups for 2 weeks, and then were able to choose a mate. The pairs were then kept in single cages (Terenziani, Italy, measurements: 50cm high x 50cm wide x 100cm long) and provided seed (Golden Cobb, Victoria), cucumber, eggs, shell grit and water *ad libitum*. Pairs were provided with nest boxes and nesting material at time of pairing. Nests were monitored daily after the onset of egg laying to determine hatch date of nestlings and post-hatch timings. Nestlings were marked with individual coloured non-toxic nail varnish on the toe nail for identification.

Manipulation of nest hygiene

Nests were randomly allocated to two treatment groups that differed in their level of nest hygiene. For 'clean nests', nesting material was replaced with fresh sterilised (autoclaved) nesting material within two days of hatching, and was continuously replaced with sterilised material twice weekly until nestlings were 30 days of age, by which time the nestlings had completely fledged from the nest (ca. 20 days). In this treatment the replacement of the nest material removed all nestling faeces at least twice weekly so that the nests remained clean. For 'dirty nests' 5 g of chicken faeces were added on the interior surface of the nest cup within two days of hatching, and 2 g of chicken faeces (from free-ranging chickens) were further added twice weekly for 30 days. This was in addition to the natural finch faeces deposited in the nest. Chicken faeces was pulverised and mixed with distilled water to create a thick paste for spreading around the nest cup. Dirty nests were also sham cleaned so that nestlings were disturbed for the same time period as nestlings from clean nests and the nesting cup was moved gently around the box. Gloves were used in the handling of clean and dirty nest material, nestlings and adults. Bacterial abundance within the nests was assessed using bacterial agar paddles (Hycheck paddles, Micromedia: Moe, Victoria, Australia) the day after nesting material changes (clean) or the adding of chicken faeces (dirty) at around day 7 after hatching.

Zebra finch nestlings were assigned to one of four age groups and were vaccinated with sheep red blood cells (described below) at the allocated ages of either: 9 days (n= 8), 11 days (n= 12), 13 days (n= 9) or sexually mature 60 days (n=12). Birds were placed in these groups to test developmental changes in immune function, but remained in their original nest (Mean ± S.E. number of siblings = 2.64 ± 0.17, n=42).

The age group allocated for vaccination were randomly allocated for individuals in each nest. Nestlings were also weighed every 2-3 days to assess health and growth.

Vaccination and blood sampling to assess induced adaptive (antibody) immunity

To test the development of the adaptive antibody response, we used sheep red blood cells (SRBC), which are a mix of various antigens that are not considered pathogenic. The antibody response to SRBC estimates the ability of the humoral immune system to mount a response to invading antigens (Bacon, 1992; Deerenberg et al., 1997). This was given on the allocated age of vaccination as well as a booster given with the same dose, seven days after, to elicit a secondary response. To make the SRBC vaccination, sheep blood in Alsevers solution were spun at 2000rpm (295 x g) for 15 minutes and the Alsevers solution was discarded. The blood cells were then washed 3 times in 1x phosphate buffered saline (PBS), and then prepared as 40% SRBC by volume in PBS. To test for background antibodies, birds were bled immediately before vaccination, according to the age group they were assigned to (9, 11, 13 or 60 days of age). Blood was taken using a 26 gauge needle and heparinised capillary tubes and collected into a 1.5ml Eppendorf tube. A total of three blood samples were taken per bird across a period of 14 days (initial sample, primary sample taken 7 days after vaccination and secondary sample taken 14 days after the initial vaccination), and before spinning at 14000rpm (14462 x g) for 15 minutes to collect plasma, a small amount of whole blood (10µl) was used for the microbial killing assay. Plasma was then stored at -80 degrees Celsius until analysis by agglutination assay. Nestlings were also weighed prior to vaccination. The first blood sample taken at the allocated age (9, 11, 13 or 60 days) was to assess the initial level

of natural antibodies to sheep red blood cells. A vaccination of 40% sheep red blood cells (50µl per 13g body mass) was given intramuscularly into the left breast straight after blood sampling. Seven days after each individuals first vaccination, offspring were blood sampled for the primary immune response (and plasma stored as above) and then the second booster vaccination (40% SRBC) was given intramuscularly into the right breast. Seven days after the booster vaccination was given (and 14 days after the initial vaccination), the final blood sample was collected to assess the secondary immune response and was collected as above. Plasma from each blood sample (initial, primary response, secondary response) was then assessed for antibody production against SRBC, using an agglutination assay.

Agglutination assay

The agglutination assay followed a similar protocol to that described by Deerenberg et al. (1997) and Matson et al. (2005). Each plate contained 2 controls (no plasma) and the initial, primary response and secondary response of two different individuals. 15µl of unheated plasma was diluted 1:1 in phosphate buffered saline (PBS) in the first well of a round bottomed 96-well plate, all remaining wells (2-12) contained 15µl of PBS. The plasma was then serially diluted across the plate. Fifteen microliters of 2% SRBC were added to each well, the plate gently tapped, covered and incubated in a waterbath for 1hr at 37 degrees Celsius. Plates were then tilted at 45 degree angle for better visualisation (wells where blood cells have a drip appearance shows no agglutination), and score was recorded 15-20 minutes after, using negative log2 of the last plasma dilution exhibiting agglutination (cell clumping), i.e. where column 5 is the last to show agglutination (clumping of cells)

means that the titre score is 5. Plates were then scanned on an Epson flatbed scanner (model V700).

IgY determination

To determine the age at which class switching from IgM to IgY occurs in nestlings, plasma gathered during the secondary humoral response was treated with 2-mercaptoethanol (2ME) to dissociate polymeric IgM. The dissociation means that only IgY remains, providing evidence that the agglutination exhibited after running the agglutination assay with 2ME treated plasma suggests that IgY is the factor leading to agglutination. Therefore class switching has occurred. Plasma was placed in an eppendorf tube with 0.1M 2ME and incubated in a water bath at 37 degrees Celsius for 30 minutes. The plasma was then analysed with the same agglutination assay as above.

Microbial killing assay to assess constitutive innate immunity

Blood was used from primary samples taken for agglutination assay only and analysed as pre-fledging age of 16-18 days (n=13), fledging age of 20-22 days (n=9) and birds at sexual maturity of 65 days (n=6). To assess microbial killing abilities of whole blood, we followed the procedure of Millet et al. (2007) and Tieleman et al. (2005) (but also see Buehler et al., 2008a; Buehler et al., 2008b; Keusch et al., 1975; Matson et al., 2006), under sterile working conditions. We used the microbial strain *Escherichia coli* (ATCC 8739) which tests killing that is complement dependent (Demas et al., 2011), involving circulating proteins in the blood which kill bacteria, which is a measure of humoral immune function. We reconstituted the lyophilized pellet of *E. coli* (Microbiologics, St.Cloud) in sterile PBS and made a working

solution of approximately 300 colony forming units (CFU) per 100µl (or 3000 CFU per mL). A control to test CFU was incubated with each batch of test plates. Whole blood was diluted (1:10) in Co_2 – independent medium (Gibco, New York) containing 4mM L-glutamine (Sigma Aldrich, Castle Hill) and the test microbe and incubated for 30 minutes. Following incubation, 50µl of diluted blood was spread on soy tryptic agar in duplicate (total 100µl) using sterile spreaders. The plates were then inverted and incubated at 37 degrees Celsius overnight and the colonies counted, blind to treatment. To determine the ability of the blood to kill of *E.coli*, we calculated the proportion killed as the average number of colonies on experimental plates relative to the average number of colonies on the CFU control plates (1 – (average number of CFU on experimental plate / average number of CFU on control plate) x 100).

Statistics

Data was analysed using SPSS (version 22). Normality of the data was assessed using histograms and Shapiro Wilks tests. To test the factors affecting agglutination, we used a general linear model (using REML estimation) with age and sex as fixed factors, and number of siblings, mass and nest treatment as covariates. A full model was initially conducted with all interactions present. Any variables over $P>0.2$ were then excluded from the model. To test age related differences in innate immunity, microbial killing of *E.coli* was analysed using a general linear model (using REML estimation). To test the factors that affected microbial killing, we used age and sex as fixed factors, and number of siblings and mass as covariates. Interactions were

excluded if the interactions were not statistically significant at the $p<0.05$ level. The estimated means (± S.E) are presented throughout the results.

Ethics statement

All experimental procedures were approved by the Deakin University Animal Ethics Committee (permit no. G24-2013).

Results

Agglutination

There were no differences in agglutination of secondary antibody titres (Table 1.) between broods of different sizes, between males and females, and were not affected by individual body mass. Agglutination titres increased significantly with age. Pairwise comparisons (adjusted for multiple Bonferroni comparisons) indicated that agglutination titres at 9 days of age (0.38 ± 0.84) were significantly different to those at 60 days (5.5 ± 0.69) ($P<0.001$), and 9 days tended to be different from 13 days (3.33 ± 0.79) ($P=0.08$). Nestlings at 11 days of age (2.25 ± 0.69) also had significantly lower agglutination titres to those at 60 days of age ($P=0.01$). Agglutination titres were not different between birds at 13 days and 60 days ($P=0.28$), or between 11 and 13 days ($P>0.99$) (Figure 1). This suggests that antibody titres increase dramatically with age, with young nestlings having significantly lower antibody titres and therefore secondary responses than sexually mature birds. In support of this, analysis of IgM and IgY in secondary samples suggest that from 11 days onwards, nestlings exhibited an IgY response to the booster vaccination (See Table 2), which increased with age. However, within the 9 day age group, one

nestling (from 8 tested) showed an IgM, but not an IgY response to SRBC injections These results suggest that zebra finch nestlings have the capacity to change from IgM to IgY production when vaccination occurs 11 days post-hatch, but not at 9 days, indicating that the ability to class switch occurs somewhere between 9 and 11 days of age in zebra finches.

Table 1. Agglutination and microbial killing changes across ages

	F	df	P
Agglutination full model			
Age	3.77	3,41	0.02
Sex	0.01	1,41	0.92
Body mass	0.11	1,41	0.75
Number of siblings	0.09	1,41	0.77
Nest treatment	0.07	1,41	0.79
Age * Sex	0.25	3,41	0.86
Agglutination reduced model			
Age	8.33	3,41	<0.001
Microbial killing full model			
Age	4.98	2,28	0.02
Sex	0.77	1,28	0.39
Body mass	0.08	1,28	0.77
Number of siblings	0.66	1,28	0.43
Treatment	0.21	1,28	0.66
Age * sex	1.75	1,28	0.20
Microbial killing reduced model			
Age	19.88	2,28	<0.001

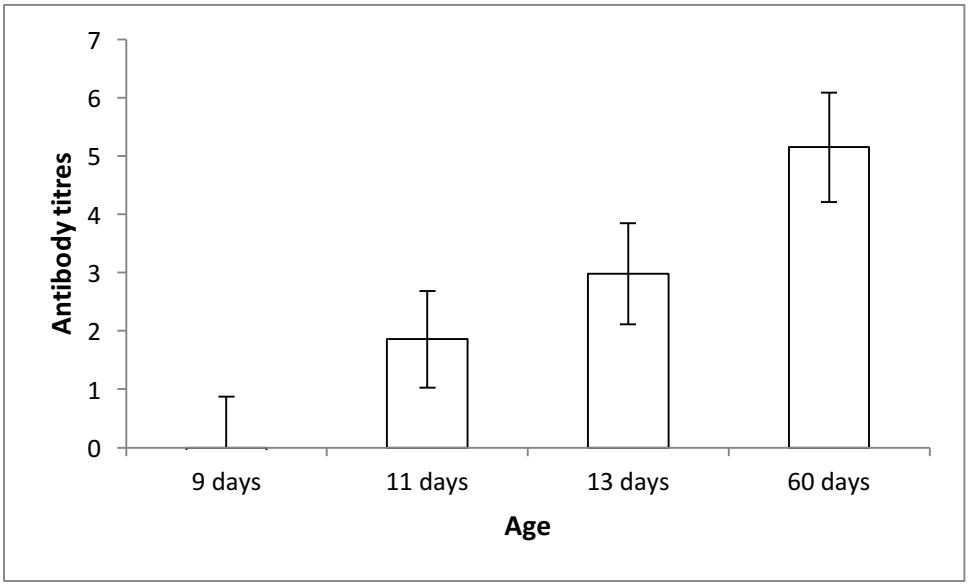

Figure 1. Average secondary antibody titres according to age. Ages included 9 days (n=8), 11 days (n=12), 13 days (n=9) and 60 days (n=12).

Table 2. Nestling agglutination and age group of IgY presence

Age at primary vaccination	Number injected	Total agglutination following booster vaccination	Total number with IgY present	Mean total secondary titre
9 days	8	1	0	-.19±1.06
11 days	11	6	2	1.86±0.83
13 days	12	9	5	2.98±0.87
60 days	6	6	6	5.15±0.94

Microbial killing

The microbial killing ability of blood exposed to *E.coli* significantly differed between age groups (see Figure 2, Table 1.). Pairwise comparisons (Bonferroni adjusted) indicated that microbial killing ability was significantly higher in 65 day old sexually mature birds (estimated mean 52.72 ± 7.63) than post fledging birds of 20-22 days old (estimated mean 1.48± 6.73) ($P<0.001$). Sexually mature birds had significantly better killing ability than pre-fledging nestlings of 16-18 days old (-4.66±5.60) ($P=0.02$). This suggests that the ability of birds to kill invading gram negative bacteria via complement and circulating proteins in the blood increases with age, with nestlings and fledglings having significantly lower complement defence than sexually mature birds. Microbial killing was not different between males and females at any age stage, and was not affected by mass or the number of siblings that the bird had.

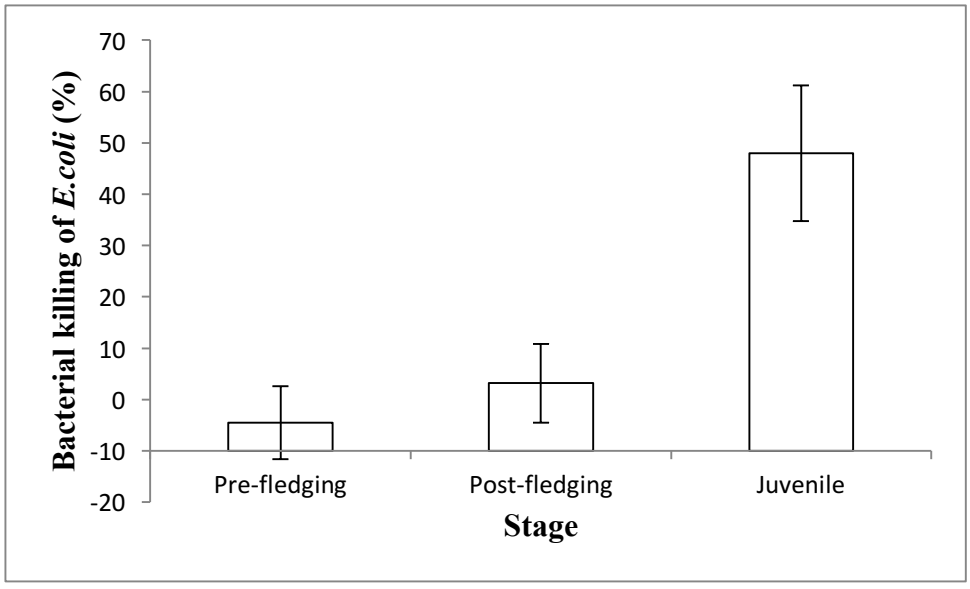

Figure 2. Microbial killing of *E.coli* at between stages of development. Developmental stages included nestling (n=13), fledging (n=9) and sexually mature (n=6) birds.

Discussion

Our study contributes significantly to understanding the timing and rate of development of immune responses and functional immune defence in altricial birds. Our data suggest that not only do the innate immune system and adaptive immune function of zebra finch nestlings significantly increase with age, but their functional defence against bacterial infection also continues to develop post fledging. As predicted, and contrary to domestic animals and humans which rely heavily on innate defences during development (Levy, 2007), the ability to kill gram negative bacteria, more specifically, *E.coli*, via complement, was weak in pre and post fledging birds compared to sexually mature birds. This ability increased however, allowing individuals to kill around 50% of bacteria by 65 days of age. The adaptive immune response was also not mature in post fledging birds.. This suggests that fledgling zebra finches may be vulnerable to infections because their ability to defend against infections and invading pathogens is considerably lower than birds at sexual maturity. This is the first time that development and maturation of the innate and adaptive immune response and the age in which these birds are able to produce a secondary immune response, has been documented together in zebra finches. This is important, as we can enhance our understanding of the life history strategies within and between species, the defences that might be relied upon during development in altricial wild birds, which in turn have important implications for disease ecology in wild avian species.

Nestlings rely on innate immunity provided by protective proteins such as complement and by phagocytic cells as well as by passive immunity provided by yolk IgY (Ardia et al., 2011). Innate immunity is thought to mature quickly in birds and animals, since it should be relied upon as a main defence against pathogens in

the initial stages of development (Levy, 2007). This is whilst the adaptive immune system continues development by diversifying the repertoires of receptor specificities and matures by replicating the specificities (Janeway et al., 1999). However, our results are in line with previous findings that constitutive innate immune defence in the form of complement are immature at fledging in altricial passerines (De Coster et al., 2010; Killpack et al., 2013; Palacios et al., 2009; Stambaugh et al., 2011). Comparisons with adult microbial killing (Evans et al, In press, see chapter 3) indicate that even at 65 days of age, killing ability is not fully developed. This is also supported by findings of the microbial killing capacity of the Stonechat (*Saxicola torquata*), which continually increases capacity between the ages of 1-7 years (Tieleman et al., 2010). Given that zebra finches at the time of fledging have immature adaptive and constitutive immune function it is of interest how a they might cope with infection and disease. It is possible that it may be energetically cheaper for young birds to tolerate infections. Also fast immune maturation may be traded-off for quick development. However, it seems just as likely that reduced immune responses are due to the time taken to develop a fully functioning immune system, which cannot occur before fledging age, and young birds may be more susceptible to infection during this period. To understand ontogeny of immune function, disease ecology and the relationships between life history traits, using the zebra finch as a model to test resilience to pathogens would be beneficial in future studies, firstly to understand whether fledglings are in fact more susceptible to infection, but also because there are very few studies that have tested tolerance in birds (Adelman et al., 2013; Koutsos and Klasing, 2001; Lee et al., 2005).

It has been suggested that immune defence might increase in antigenic environments (Buehler et al., 2008b), potentially influencing rate of immune maturation. Therefore, reduced immune response could be related to reduced bacterial risk. However, since

there was no significant difference in either bacterial killing or the secondary response between birds raised in clean or dirty nests, the results suggest otherwise. It seems likely that the adaptive response and the functionality of B cells naturally takes time to develop, whether or not birds are exposed to diverse antigenic environments. However, further investigation is necessary, particularly across difference species, using different levels of exposure to bacteria and larger sample sizes to determine whether the rate of development different with different levels of bacterial exposure.

Circulating maternal antibodies play an important role in defence against pathogens whilst the adaptive immune system diversifies and matures. However maternal antibodies disappear from circulation within two weeks of hatching (Grindstaff et al., 2003). After this point, a nestling should be able to mount an adaptive immune response. The ability to mount a secondary immune response occurred after priming at 10 days of age and priming at 13 days of age resulted in agglutination titres increasing dramatically within those 2-3 days. However, titres in the 13 day old group were only half that of birds in the 60 day old group. It also seems 60 day old finch antibody titres are below that of adults (Evans et al, In press, see chapter 3). Thus, the antibody response of altricial birds takes much longer to mature than precocial birds and is not mature at fledging, which is in agreement with previous studies of zebra finches (Killpack and Karasov, 2012), American kestrels (*Falco sparverius*) (Smits and Bortolotti, 2008) and tree swallows (Palacios et al., 2009). Thus, the age in which birds had the ability to mount a secondary response, and therefore the ability to class switch, although quite variable between individuals, was displayed post boost in the 11 day age group onwards. It has been found previously that pigeons have the capacity for class switch from IgM to IgY within the first few weeks of hatching (as reviewed in Killpack and Karasov, 2012), and zebra finches

have been found to mount an IgY response to keyhole limpet hemocyanin at 21 days of age (Killpack and Karasov, 2012). However, the results of our study suggest that zebra finches have the ability to respond with IgM which happens quickly between 9 and 11 days of age and have the ability to switch to IgY rather quickly. Compared with domestic poultry, this is quite fast. The ability to mount an IgY response at the time of fledging would be adaptive to enable to defend against potential pathogens that may be encountered when they leave the nest. The variability in the age in which birds were able to mount a secondary immune response is likely due to variation in circulating maternal antibodies, which are known to be differentially deposited according to laying order (Hargitai et al., 2006; Pihlaja et al., 2006). However, this is only possibility as we did not test for maternal antibodies, and should be confirmed in future studies testing immune ontogeny in altricial passerines.

Neither adaptive or innate immune development were impacted by individual mass or by the number of siblings. However, food was provided *ad libitum*, so it is unlikely difference in amount being fed would occur between large and small broods. Other studies investigating the impact of mass on bacterial killing of *E.coli* did not find a relationship, both in wild and captive populations (Ardia et al., 2010; Buehler et al., 2008a; Evans et al., 2015; Morrison et al., 2009; Tieleman et al., 2005) nor a relationship with brood size (Morrison et al., 2009).

In conclusion, our results show that zebra finches develop the ability to recognise and mount an antibody response at 11 days of age. Our results also suggest that both constitutive innate or induced adaptive immunity are not well developed at the time of fledging. Future studies should invest in understanding resilience of disease in altricial wild birds as well as contributing to understanding further how this contributes to the overall life history traits and strategies of wild birds. Further

investigations into immune function development in free living birds will be valuable to the ecoimmunology and comparative avian immunology fields. This will overall contribute to understanding variability in immune function, leading to greater consideration of its role in disease ecology and understanding in relation to life history traits.

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CHAPTER SIX

Discussion

Rationale and approach of research

The factors that contribute to variation in immune function of birds, both wild and production species, have gained considerable interest over the past few decades, particularly in the fields of evolutionary ecology (Nelson and Demas, 1996; Norris and Evans, 2000; Schmid-Hempel, 2003). This in turn has given rise to the field of ecological immunology or ecoimmunology, which aims to understand the variation in the immune response related to life history, ecology and evolution (Demas et al., 2011; Graham et al., 2011; Horrocks et al., 2011; Norris and Evans, 2000; Sheldon and Verhulst, 1996). Within the field of ecoimmunology, there has been significant attention given to factors such as seasonality (Hegemann et al., 2012; Nelson and Demas, 1996), reproduction (Deerenberg et al., 1997; Ots et al., 1998), pathogen and parasite load (De Coster et al., 2010; Grindstaff et al., 2006; Horrocks et al., 2011; Råberg et al., 2009; Sorci, 2013), and development (Ardia, 2005; Ardia et al., 2010; Arriero, 2009; De Coster et al., 2011; Martin et al., 2011; Palacios and Martin, 2006; Pap et al., 2015; Saino et al., 2003) which contribute to the variation in innate and adaptive immune systems. The aim of this thesis was to determine how developmental priming, life changes and environmental conditions lead to variation in immune function in the Australian passerine, the zebra finch (*Taeniopygia guttata*).

In this thesis, there is a focus on microorganism exposure in the nesting environment and how this affects innate and adaptive immunity in parents and offspring. My survey of the literature suggests that there are good reasons to propose bacterial abundance and diversity in the environment might impact on the development and

functioning of the immune system (Archie and Theis, 2011; Hooper et al., 2012; Lee and Mazmanian, 2010; McFall-Ngai et al., 2013; Olszak et al., 2012; Pels and Denotter, 1979; Pollard and Sharon, 1970; Thompson et al., 2008). This is particularly interesting in species that have poor nest sanitation or re-use their nests. However, there are relatively few studies (Buehler et al., 2008b; Horrocks et al., 2012a; Horrocks et al., 2012b) that have examined these relationships outside of domestic poultry and those that have, have not experimentally manipulated the microbial environment or tested both arms of the immune system.

Within this thesis, I examine developmental aspects of immune function including experiences during embryonic development and parental signalling. I also document changes in innate and adaptive immune function of nestlings and juveniles, where there are relatively few studies understanding immune development in both immune systems in altricial passerines (Arriero et al., 2013; Killpack and Karasov, 2012; Killpack et al., 2012; Killpack et al., 2013; Palacios et al., 2009; Smits and Bortolotti, 2008). Finally, changes in immune function during parental care and reproduction in adult birds were explored.

Development of immune function

Development of competent innate and adaptive immune systems is extremely important for protection against the array of pathogens encountered in the environment (Demas et al., 2011). However, there are experiences during development that could influence or lead to variation in immune function. Together chapters four and five provide evidence of developmental changes in immune

function of zebra finches and events during and after hatching that influence immune function.

Previous studies have shown that both the innate and adaptive immune systems are not mature at fledging but continue to develop as altricial birds age into adulthood (Arriero et al., 2013; Buehler et al., 2009; Killpack and Karasov, 2012; Killpack et al., 2013; King et al., 2010; Palacios et al., 2009). For example, innate immunity in tree swallow (*Tachycineta bicolor*) fledglings in the form of PHA response (T-lymphocyte activation), total lymphocytes, natural antibodies, complement mediated cell lysis (Palacios et al., 2009) and bacterial killing of *Escherichia coli* was below that of adults (Stambaugh et al., 2011). The zebra finches in my study do not have mature bacterial killing at fledging, and this defence is at least five times lower than birds at sexual maturity. Adaptive immunity and the secondary antibody response in the zebra finch (*Taeniopygia guttata*) has previously shown to occur when the priming injection was given at 14 days of age, but did not have a secondary response when primed at 7 days (Killpack and Karasov, 2012). This indicates that the ability to mount a secondary response occurs somewhere between 7-14 days. The results of my study further extend on this knowledge, showing that zebra finches are capable of a response after 11 days of age, although a majority responded of nestlings responded at 13 days of age. Compared to studies investigating maturation of the antibody response of altricial birds (Killpack and Karasov, 2012; Killpack et al., 2013; Smits and Bortolotti, 2008), my results also suggest that antibody response is not fully matured at fledging in zebra finches. Future studies should investigate the ability of fledglings to cope with pathogens and test resilience models since neither microbial killing mechanisms nor adaptive immunity are fully matured at fledging, a time where exposure to new pathogens is likely. Furthermore, the age of

development of other innate immune mechanisms would be beneficial in furthering our understanding of immune system development in altricial passerines. Although age contributes to variation in immune function, events during development can change the course of immune maturation and strength of the immune response. Experiences during embryonic development and throughout growth can have carry over effects that impact upon immune function in later life. In my study (Chapter four), listening to incubation calls during embryonic development resulted in being fed more and increased growth in 13 day old nestlings compared to those that heard tet calls. This also had a carry-over effect on body condition and innate immune function in juveniles, with males having poorer body condition than females, but greater microbial killing ability. This suggests sex-specific allocation of resources to immune function, according to the signals provided before hatching. This result is highly novel, as the role of parent-embryo communication in changing developmental nestling trajectories has not been investigated in terms of immune function. My results consistent with the interpretation that incubation environment affects nestling immune development, for example Ardia et al. (2010), who showed that experimental cooling of the nest during incubation increases development times and reducing innate immune function and growth in nestlings. There is much scope for testing these relationships further. Since I examined birds at sexual maturity only, long term studies are necessary to test whether conditions during embryonic development place animals on different immune trajectories for their whole life, including at the nestling stage and as adults. Furthermore, it is of interest whether males that heard incubation calls with increased immune function, were more successful at attracting females and better fitness, with increased immunity potentially a sexually selected trait. This might show an evolutionary role of the incubation call by increasing success of offspring.

There are two overarching hypotheses that were tested in this thesis to explain variation in immune function of birds. The first is the antigen exposure hypothesis (Horrocks et al., 2011), which suggests that exposure to antigens in early life shapes investment in immunity in later life. Environments where there is a greater microbial pathogen exposure therefore might select for a stronger immune response (Horrocks et al., 2012a; Horrocks et al., 2011). The second hypothesis is the trade-off hypothesis (Sheldon and Verhulst, 1996), which predicts that consumption of limited resources by the immune system leads to trade-offs between immune function and other resource-demanding activities such as reproduction (Ilmonen et al., 2000; Lochmiller and Deerenberg, 2000; Norris and Evans, 2000; Sheldon and Verhulst, 1996). Overall, the data from chapters three and four provide support for the trade-off hypothesis, showing that immune function competes with other physiological demands of reproduction/parental care in adults and growth in nestlings. Chapter three provides support for the antigen exposure hypothesis showing that some but not all facets of immune function are affected by microbial load. Below I discuss each hypothesis in turn and the evidence provided in support of either from my thesis.

i) Trade-offs between immune function and competing physiological demands

Sheldon and Verhulst (1996) proposed that the main cause of variation in immune function is a trade-off between other competing costly physiological functions which require allocation of limited resources. The immune system requires energy to function, which has led immunologists to assess the energetic costs of the immune response (Iseri and Klasing, 2014; Klasing, 2004; Klasing and Calvert, 1999; Klasing

and Leshchinsky, 1999; Lochmiller and Deerenberg, 2000). Chapters three and four provide evidence of trade-offs between parental care/reproduction and between nestling growth, showing that immune function decreases with other energy demanding processes.

In chapter three, I found that parents decreased constitutive innate immune function from incubation through to the fledging of nestlings, likely due to an imbalance in energy. Other studies have come to similar conclusions with microbial killing mechanisms and vary with life history stage, for example, Pap et al. (2010) and Evans et al. (2015) found that microbial killing of *E.coli* was down regulated during energetically expensive periods of breeding and chick-rearing. Other measures of complement, such as lysis, have also been shown to decrease directly after an endurance flight in European starlings (*Sturnus vulgaris*) (Nebel et al., 2012). However, the studies assessing life cycle changes are not able to exclude the role of the environment or microbial exposure, which had previously been suggested, could impact upon microbial killing and other innate measures (Evans et al., 2015; Hegemann et al., 2012; Mortimer and Lill, 2007). The previous studies have also not been able to show differences in immune function through different stages of nestling growth in an environment where other factors such as seasonal changes can be eliminated. From our data, it shows that life stages and energetically costly periods, not microbial exposure (in the form of built up faeces, feathers and dirty in the nest) is likely a major driver of variation in microbial killing of *E.coli*.

Interestingly, complement and the ability of circulating proteins to kill invading bacteria in the body, is not a particularly costly mechanism to maintain (Klasing,

2004). Therefore, it is curious why this mechanism is affected by more energetically costly life stages such as nestling rearing, when other mechanisms, such as more costly phagocytosis (as tested by the killing of *C.albicans*), is unchanged throughout nestling rearing. Future studies could document energy expenditure to get a further understanding of this trade-off and of the microbial killing mechanisms. A diverse range of immune tests could also be used to understand changes and trade-offs during parental care.

The results of chapter four provide some support of a trade-off between body condition and constitutive immune function, although further testing of this relationship is necessary to determine the reliability of this. The results from chapter three also indicate a possible trade-off between energetically demanding activities and mounting an immune response, suggesting that immune function does involve a meaningful biological cost. Consistent with these results, trade-offs between growth and immune function are commonly found in birds (Soler et al., 2003; van der Most et al., 2011). As described above, complement dependent killing of bacteria, as assessed with *E.coli,* seems vulnerable to trade-offs when energy is being spent on other physiologically demanding events (De Coster et al., 2010; Nebel et al., 2012; Pap et al., 2010). Although not significant, there was a very slight trend of males males having higher microbial killing but poorer body condition compared to females. The results could suggest that there may be different investment priorities between the sexes in those that heard incubation calls. This is purely speculation only, but males may be increasing investment in innate immunity, whereas females might concentrate on increasing body condition. There is a possibility that sexual selection could be the evolutionary driver behind this relationship. Male zebra finches start to sing and develop their song repertoire after sexual maturity. The

complexity of male song has been shown to be an honest indicator of male quality, which includes information about immunocompetence and parasite load (Buchanan et al., 1999; Buchanan et al., 2004; Leitner et al., 2005). However, because this trade off only occurred in birds that heard incubation calls during embryonic development, there is the potential that parents use this call to signal about conditions, causing epigenetic changes which put the birds on optimal developmental pathways for the conditions experienced. Future studies could investigate the trade-off in condition and immune function further by testing changes in epigenetics and whether this call is changing developmental pathways.

ii) Pathogens and their influence on variation in immunity

The avian immune system is an evolutionary product of the selection pressures exerted by natural pathogens and antigens. Greater exposure to microbial pathogens should therefore select for a stronger immune response (Horrocks et al., 2012a; Horrocks et al., 2011). Chapter two (review) and chapter three of this thesis concentrate on the microbial influence on immune function of birds, with chapter three testing this relationship directly.

In support of the antigen exposure hypothesis, I found adults from experimental nests, manipulated to increase bacterial load (dirty nests), exhibited a stronger (higher titres) primary immune response than birds in sterilised (clean) nests. The difference between primary and secondary responses were significantly larger in adults from clean nests, showing that while titres were low during the primary response, in the secondary response they were similar to birds from dirty nests. This

suggests that the adaptive immune response is primed by exposure to microorganisms in the nesting environment, where upregulation of the adaptive response occurs due to risk and frequent microbial challenges. This novel finding is the first study outside of a germ free environment and poultry to demonstrate that bacterial abundance co-varies with the adaptive immune system of birds. This relationship was not found in nestlings, however, the age in which birds were tested were too young to show a meaningful primary response.

From an ecological perspective, the nest itself is a highly relevant source of bacterial diversity from sources such as dead chicks, nest reuse, visitors and sanitary practises. Previous studies have focused on mostly innate mechanisms and the effect of bacteria in the environment of wild birds (Horrocks et al., 2012a; Horrocks et al., 2015; Horrocks et al., 2012b); likely because of the difficulty of host recapture which is necessary to assess adaptive immunity. It was expected microbial killing mechanisms would be driven by microorganisms in the environment. However, these mechanisms, particularly killing of *E.coli* (complement mediated), seems affected by energy demanding events (see discussion above).Whilst I highlight the importance of both innate and adaptive immunity in understanding covariation with environmental microorganisms, there are few studies published to support the antigen hypothesis and covariation of microorganisms with innate immunity. For example, humoral innate immunity in the form of natural antibodies, are higher in adults that are incubating eggs with lower density of bacteria on the shell surface (Soler et al., 2011). Also, haptoglobin and lysis have also shown to be covary with microbial risk, showing decreased immune measures with lower CFU (colony forming units) of generalist and gram negative airborne microbes in the environment (Horrocks et al., 2012a). Future studies should use molecular tests for microbial identification in the

environment with a combination of immune tests (both innate and adaptive) to further explore immune bacterial relationships.

Limitations and ideas for future work

There are inevitably limitations from this thesis that need to be addressed and could be tested further to advance the field of ecoimmunology.

Few people have tested whether effects are consistent across different life history stages (Evans et al., 2015; Pap et al., 2010) and my work is no exception to this. My conclusion that parental care and physiologically demanding life events affect microbial killing of *E.coli* is limited by not testing energy expenditure during this time and of course, long term studies across years and life events would be useful in understanding the relationship further.

The conclusion that microbial load affects and co-varies with some measures of immune function, as shown by Horrocks et al. (2012a), is supported by my work, however this conclusion depends on the assumption that experimental manipulations have been effective and biologically meaningful. Further advancement of microbial and immune relationships relies heavily on the use of new technologies to advance our understanding, particularly with the use of culture independent techniques for the identification and prevalence of bacteria. Next generation sequencing and molecular PCR methods have started to be used in this manner for bacterial identification on egg shells (Javurkova et al., 2014; Lee et al., 2014), however should be used in a similar fashion to test relationships between immune function and microorganisms for accuracy in this field. Testing adults and developing nestlings using a variety of

immune tests in conjunction with molecular technologies for identification of microorganisms in wild populations across generations would add to the eco-immunology field and develop our understanding of immune-bacterial relationships further.

The limitations in the field of eco-immunology, particularly the difficulty in testing the adaptive immune system in the wild, mainly due to problems associated with recapture (Norris and Evans, 2000), have led to a focus on testing covariation with innate immunity (Horrocks et al., 2012a; Horrocks et al., 2015; Horrocks et al., 2012b). Although I was able to test adaptive immunity and importantly show covariation with nest bacterial load in captivity, further advancement in this field and understanding immune-bacterial relationships relies upon being able to test this in the wild. To understand the evolutionary impact of bacteria on bird behaviour and physiology, it would be ideal to test egg structure and antimicrobial mechanisms in ovo, between different climatic environments in the same species. This has been somewhat tested by Horrocks et al. (2014) where egg defences of lysozyme and ovotransferrin were tested from birds along an arid-mesic gradient. However, the structural changes in the egg were not measured. Furthermore, and probably of more importance to test the antigen exposure hypothesis and advance the ecoimmunology field, examining bacterial penetration to the egg during chorioallantoic membrane (CAM) attachment (the period of high risk for bacterial egg contamination – see chapter 2) and further testing the carry over effect on immune function in nestlings, juveniles and adults would be beneficial in understanding early microbial exposure and how this shapes immune function. Experimental manipulations in the laboratory and testing across different environments in the field would be of benefit.

From the data on age related changes in immune function, it is not well understood what system is protect fledglings from pathogens since both constitutive innate mechanisms and adaptive immunity are not well developed in altricial birds during this time. Tolerance and resilience models to infections have been investigated in plants and mice (Råberg et al., 2009), but there are very few studies investigating these models in birds and in ecoimmunology in general (Adelman et al., 2013; Graham et al., 2011; Sorci, 2013). Testing tolerance and resilience models in wild birds would further the field of ecoimmunology, understanding altricial immune development and give us a better understanding of immune defences in relation to pathogens.

The data presented here on potential epigenetic changes that occur when listening to parental incubation calls and the impact on condition of juveniles needs further testing, particularly throughout nestling development and into adulthood as well as in other species. Future studies should test epigenetic changes (changes in methylation) in response to incubation calls during embryonic development and test immune function and condition across different stage of life to get a further understanding of the potential changes that occur because of this communication.

Conclusion

In conclusion, this research provides valuable and novel insights into variation of immune function including developmental conditions and the carry over effects as well as bacterial-animal interactions. From this research, we now understand that the

adult adaptive immune system is influenced by nest sanitation and microbial exposure in the environment. There is also potential that microbial killing mechanisms are not driven by microorganisms in the nesting environment but potentially vary due to life events, however future studies should consider testing these relationships further, potentially with the assessment of individual energy budgets. Although this research has suggested that developmental conditions during embryonic development (playback of incubation calls), had an impact on growth, further investigations are necessary to determine both the mechanism and the function of this response and whether there is an impact on the functioning of the immune system.

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