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Avian life in a seasonally arid tropical environment: adaptations and mechanisms in breeding, moult and immune function

Chima Josiah Nwaogu

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Chapter 1

General introduction: Avian life in a seasonally arid tropical environment

Nwaogu Chima Josiah

Keywords: life history strategies, annual cycle, tropical environment, diet, self-maintenance, reproduction, ecological immunology, Common Bulbul

Ecological immunology as a sub-discipline in animal ecology and evolution

"How could animals choose resistant mates? The methods used should have much in common with those of a physician checking eligibility for life insurance. Following this metaphor, the choosing animal should unclothe the subject, weigh, listen, observe vital capacity, and take blood, urine, and fecal samples. General good health and freedom from parasites are often strikingly indicated in plumage and fur, particularly when these are bright rather than dull or cryptic" – Hamilton and Zuk

The quote above by (Hamilton and Zuk 1982) from their very influential paper 'Heritable true fitness and bright birds: the role for parasites' marked the beginning of thoughts that shaped the later development of ecological immunology as a sub-discipline in animal ecology and life history evolution. Unlike classical immunology where physicians seek to prevent infections by boosting immune defence, ecological immunology was built by an interest in the cost of maintaining immunity while showing off costly physical qualities such as sexual ornaments that imply freedom from parasites (Saino and Møller 1996, Saino et al. 1997). Here an animal likened to a 'life insurance physician' inspects and decides whether to bet the survival (or fitness) of its offspring on a potential mate depending on whether that mate has shown honest capacity to bear the cost of parasite defence. Thus, immunology entered animal ecology and life history evolution on the premise that immune function is costly. In fact the cost of immune response was hypothesized to drive the evolution of adaptive immunity (Raberg et al. 1998). As a consequence early studies sought to understand trade-offs between investing in immune function and other life history traits such as reproduction (reviewed by Lochmiller 1996, Sheldon and Verhulst 1996, Lochmiller and Deerenberg 2000, Norris and Evans 2000). These studies viewed immune function as a component of self-maintenance that may be traded-off to improve reproductive performance or suppressed by investing too much in reproduction (Drent and Daan 1980). However, the role of environmental conditions in determining disease risk, resource availability and occurrence of annual cycle stages was rarely considered, and this gap influenced the development of later studies.

Studies within the late 1990s and 2000s focused on the energetics of immune function (Read and Allen 2000, Martin et al. 2003, Demas et al. 2012). Such studies, although within an environmental framework, focused on how environmental conditions determined energy expenditure or pace of life rather than how it influenced disease risk. This gave rise to comparative and experimental studies among species (Martin et al. 2001, Lee et al., 2008, Tieleman et al. 2005), seasons (Nelson and Demas 1996, Nelson et al. 2002, Martin et al. 2008a), annual cycle stages (Hegemann et al. 2012b, Versteegh et al. 2014) and between tropical and temperate animals (Martin et al. 2003, Martin et al. 2004). Evidence supporting the occurrence of energetic trade-offs between immune function and other life history traits remained equivocal: while some found negative correlations between immune function and energy expenditure or traits like clutch size (Tieleman et al. 2005, Martin II et al. 2006, Lee et al. 2008) others did not (Versteegh et al. 2012, Horrocks et al. 2015). Experimental studies testing trade-offs also faced the problem of whether to manipulate the immune system or other life history traits. Studies that manipulated the immune system by inducing inflammatory response during reproduction recorded a decrement in reproductive effort depending on breeding stage. But notable among these studies was (Williams et al. 1999) who injected egg laying European Starlings *Sturnus vulgaris* with sheep red blood cell to stimulate primary antibody production and found no effect of immunization on breeding investment. However, (Råberg et al. 2000) vaccinated Blue tits *Parus caeruleus* with human diphtheria-tetanus vaccine during chick feeding and found that vaccinated females reduced chick feeding rate. The conclusion from these studies was that trade-off between immune investment and reproduction depended on energetic constraint with egg laying being considered less constraining than chick feeding. On the other hand, studies that manipulated reproductive effort also found variable results depending on manipulation type, environment, individual quality or whether manipulated birds were additionally challenged (Ardia 2005a, Ardia 2005b, Tieleman et al. 2008, Hegemann et al. 2013a). It is important to note that most of these studies used either a single or different index of immune function (Box A) and did not measure whether a reduction in immune function was followed by increased susceptibility to infection. This does not consider both the complexity and the ultimate function of the immune system which is enhancing survival (Martin et al. 2006b).

Box A – The immune system and assays to measure immune function

An overview of immune function

Immune function is classified in general (but not in all cases) with respect to how an invader is managed by the immune system (Janeway et al. 2001, Schmid-Hempel 2003). A foreign agent is recognised and eliminated through behavioural, physical and chemical barriers of a host's defence, by either a *constitutive* or an *induced* immune response and this response could be *specific* or *nonspecific* (Figure A1). Constitutive immune responses are maintained even without being triggered by infection, providing surveillance for and early eradication of infectious agents. Induced immune responses on the other hand, is triggered when a pathogen has established itself in an organism's body. If an immune response is influenced by previous exposure to an invader, it is classified as *acquired*. If previous exposure has no effect on response intensity, then immune response is *innate*. These could be *cell mediated* when pathogen destruction is by circulating cells or *humoral* when pathogen destruction is by chemical substances in the serum. Humoral responses target extracellular microbes such as bacteria, fungi and some protozoans while the cell mediated responses target intracellular parasites via phagocytosis of infected cells. Cell mediated responses may be enhanced by humoral responses such as in *opsonisation* - when infected cells are coated with antibodies for ease of recognition and elimination by phagocytic cells (Janeway et al. 2001). In general, constitutive immune responses are nonspecific while induced responses are specific. However, immune responses can fall into an intersection between constitutive-induced and specific-nonspecific responses (Figure A1).

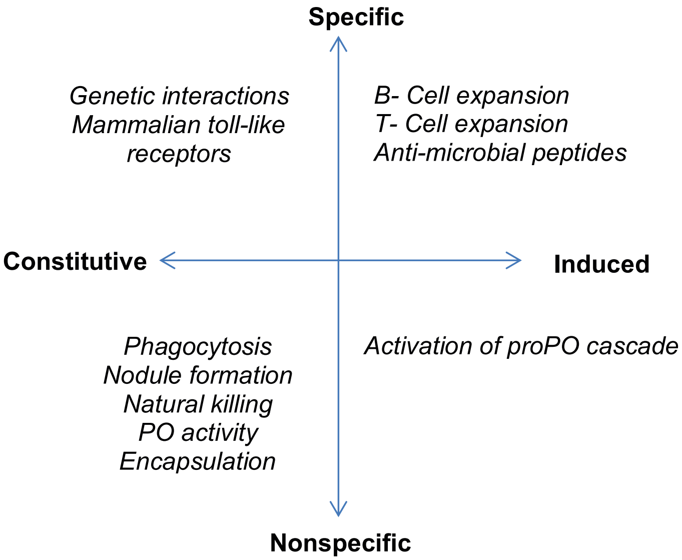

Figure A1: Classification of the immune response based on Schmid-Hempel and Ebert (2003). **Mammalian toll-like receptors** – pattern recognition receptors that initiate key inflammatory response and shape adaptive immunity. **B-cell** – antibody secreting B lymphocytes which function in the humoral immunity component of the specific immune system. **T-cell** – T lymphocytes with specific antigen binding glycoprotein receptors on their cell membrane. **Anti-microbial peptides** – potent broad-spectrum antimicrobial agents. **ProPO** – prophenoloxidase is a modified form of compliment response controlled by the enzyme phenol oxidase. **Phagocytosis** – the ingestion of bacteria by micro and macrophages. **Nodule formation** – enclosing an invading pathogen, allowing its isolation and neutralization (see also **encapsulation**). **Natural killing** – destruction of pathogens in infected cells by cytotoxic lymphocytes. The **constitutive – nonspecific** axis is largely **innate** because responses are not influenced by previous exposure to infection, while the **specific – induced** axis is largely **acquired** because previous exposure effects response intensity. However, **anti-microbial peptides** are specific and induced but innate.

Immune assays

The complexity described above means that understanding variation in immune function requires carefully selected immune indices (Norris and Evans 2000, Adamo 2004). For ecological immunology studies with a life history focus, investigating variation in innate immune indices has become popular because innate immune responses are unaffected by past exposure to specific antigens and largely form the first line of defence to infectious agents (Schmid-Hempel 2003). Hence, innate immune indices should reflect an organism's capacity to destroy an infection, or its response to infection, at a given time. Measuring innate immune indices are also suitable for wild birds that may only be caught once and where sampling yields only limited quantities of blood or plasma. The current practice for ecological immunology is to assess several innate immune indices to gain a broader view of immune function and aid better interpretation of variations. For this project, I considered five innate immune indices: haptoglobin, nitric oxide and ovotransferrin concentrations, and natural antibody-mediated haemagglutination and complement-mediated haemolysis titre. Haptoglobin, ovotransferrin and nitric oxide concentrations are biomarkers of inflammatory response (Jain et al. 2011), while haemagglutination and haemolysis are indices of constitutive innate immunity (Ochsenbein et al. 1999, Matson et al. 2005). Haptoglobin is a positive acute phase protein which circulates in low concentration but increases with inflammation (Jain et al. 2011, Matson et al. 2012). Ovotransferrin on the other hand, is a negative acute phase protein which increases with inflammation but may decrease during high inflammatory response because temporarily high free hormones bind to ovotransferrin. Haptoglobin and ovtransferrin increase during inflammation because they bind to and remove haem from circulation, so that haem becomes unavailable as nutrient to pathogens (Horrocks et al. 2011a). NOx, modulates inflammatory processes, and also participates in the direct killing of parasites and tumor cells (Allen 1997, Sild and Hõrak 2009). Natural antibodies and complement activities neutralize pathogens, activate the complement system and form antigen-antibody complexes that enhance the elimination of infectious agents. Their activity forms a useful link between innate and adaptive immune responses (Panda & Ding 2015). Being largely nonspecific and requiring no previous encounter with invaders (Schmid-Hempel and Ebert 2003), constitutive innate immunity forms a broad spectrum defence against predictable and non-predictable infections. Inferences such as severity of immune challenge can be made when haemaglutination and haemolysis titres are interpreted alongside haptoglobin, ovotransferrin and nitric oxide concentrations which will vary with infection, trauma or inflammation (Jain et al. 2011).

More recent studies provided evidence that environmental condition plays a more crucial role in shaping immune function than life history strategy (Horrocks et al., 2011b, Horrocks et al. 2012b, Horrocks et al. 2015). This is consistent with the idea that the ‘immunobiome’ i.e. the whole suite of pathogenic and commensal organisms in an animal’s environment (Horrocks et al. 2011b) changes in space and time and shapes immune function. Differences in disease risk influenced by the ‘immunobiome’ composition of marine, freshwater, tropical, temperate, mesic or arid environments have been proposed to drive patterns of animal distribution (Piersma 1997, Mendes et al. 2005) and migration (O’Connor et al. 2018). This idea that environmental factors determine disease risk and shape immune function, shifted the focus of ecological immunology from the historic view that immune function was a liability to one that explored the benefit of immune defense to animals in specific environments (Tieleman 2018b). This newer concept proposes ‘operative protection’ - that immune defense should be proportional to immune challenge in specific environmental conditions (Matson 2006, Horrocks et al. 2011b).

But there remains a problem with using environmental condition as a proxy of disease risk. Environmental proxies of disease risk such as aridity, may encapsulate several factors that influence immune function in different ways, and this may operate in sequence, parallel or as a network of processes, making variation in immune indices difficult to interpret (Matson et al.

2006). Although, variation in environmental aridity is hypothesized to predict disease risk (Horrocks et al. 2011b), it also determines resource availability and occurrence of life history events such as breeding, moult and migration. These events may compete for limited resources with immune function (Sheldon and Verhulst 1996, Hegemann et al. 2012b, Hegemann et al. 2013a). Another important component of environmental variation associated with aridity is diet, and diet is likely to affect immune function directly (Klasing 1998, Klasing 2007) or indirectly via effects on other life history traits. Therefore, although the seasonality of environmental conditions and annual life history events are well documented (Wingfield 2008, McNamara et al. 2011), identifying their effects on immune function is difficult because life history events co-vary with each other and with multiple environmental factors, that are themselves correlated in time and space.

Temperate systems are particularly prone to the problem of covariation between environmental factors and life history events because annual life history events such as breeding, moult and migration are strictly seasonal (Baker. 1939). Yet, until recently (Tieleman et al. 2005, Lee et al. 2008, Tieleman et al. 2008 Horrocks et al. 2015, Ndithia et al. 2017a) studies in ecological immunology have focused on temperate systems. This temperate bias has limited the scope of environmental conditions and life history strategies covered in ecological immunology, and this is evident from findings in pioneering studies comparing how life history and environment explain immune variation over a large scale environment (Horrocks et al. 2015). There is therefore a clear need to expand studies to other environments and the diversity of tropical systems presents a unique opportunity for this expansion. Tropical systems are diverse in both life history strategies and environmental conditions, thus the linkage between factors such as environmental aridity, resource availability and occurrence of life history events can be decoupled, by both weak breeding seasonality of tropical birds and the spatio-temporal variation in environmental conditions such as aridity.

This thesis was thus, inspired by the need to understand how variation in immune function and associated life history traits arise in a natural tropical system where the linkage between potential explanatory factors, particularly environment and life history variation being the most prominent, can be decoupled. To tackle this problem, I exploited the wide variation in spatio-temporal arid environmental conditions in Nigeria, and a ubiquitous tropical species, the Common Bulbul *Pycnonotus barbatus*, which breeds and moults across a range of different environmental conditions in time and space throughout the country.

Here, I first introduce the model system in detail, and then outline the specific objectives addressed in the individual chapters of this thesis which together explore how environmental conditions affect immune function and associated life history traits in time and space, and through the alteration of diet composition. Although, the focus of this thesis is the relationship between environment and immune function, my co-authors and I started with studying the natural history of the Common Bulbul in order to gain first-hand insight of the relationship between its life history traits and its immediate environment. This approach is, of course essential for interpreting variation in immune function within its appropriate ecological context (Tieleman 2018b).

A model system – a songbird resident across great variation in spatio-temporal environmental aridity

The spatial environmental aridity gradient in Nigeria

West Africa is bounded to the north by the Sahara Desert and to the south and west by the Atlantic Ocean (Figure 1.1). Across West Africa, there is a single period of precipitation and drought annually - the wet season is later and shorter going from south to north. This difference accounts for much of the physical and biological characteristics that creates a gradient of decreasing temperature and aridity from the dry Sahel zones of northern Nigeria to the wet coastal areas of the Atlantic Ocean in the south. Thus, Nigeria provides a highly varied environment within about 800km of latitudinal distance along which organisms experience different environmental conditions.

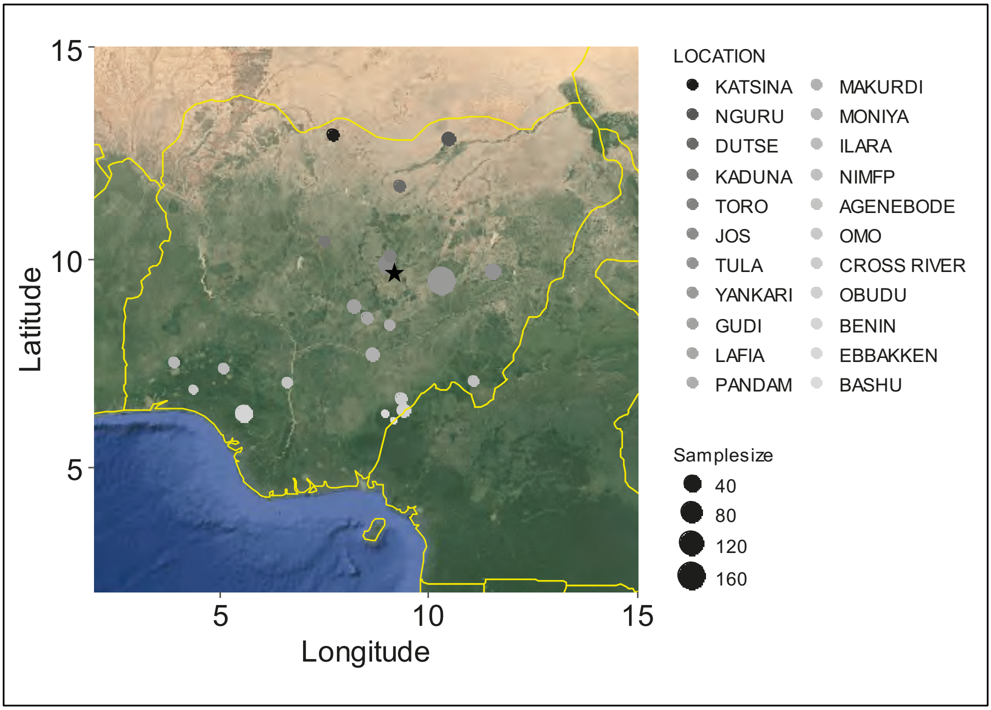

Figure 1.1: Spatial aridity gradient across Nigeria and sites where Common Bulbuls were measured and/or sampled for blood to study the effects of spatial environmental aridity on body size, moult and innate immune function. Grey shading of location points indicates increasing latitude from south to north. * indicates the location of the main study site, the Amurum Forest Reserve where the effect of temporal environmental factors was investigated.

Environmental seasonality: temporal aridity gradient in the Amurum Forest Reserve

Located relatively mid-way along the spatial aridity gradient in Nigeria (Figure 1.1) is the A. P. Leventis Ornithological Research Institute's Amurum Forest Reserve (APLORI - 09°55′N, 08°53′E) – a regenerating savannah wood land on a central highland (c. 1325m asl) in the Guinea savannah zone of Nigeria. There are typically three main habitat types in the Amurum Forest Reserve: savannah woodland, riparian forest fragments and rocky outcrops (Inselbergs), surrounded by farmlands and human settlements. Vegetation is composed of short trees, shrubs and grasses. Like many other parts of Nigeria, the reserve experiences a single wet and dry season annually (Figure 1.2). The wet season usually lasts from April to October while there is usually no rain at all outside these months, forming a clear dry season. Total monthly rainfall in the wet season is usually over 200 mm on average, but it is usually less in April, May and October. This rainfall pattern creates an annually predictable unimodal temporal humidity pattern. Minimum and maximum daily temperatures vary in a bimodal fashion (Figure 1.2) due to increased cloud cover in the wet season and the movement of cold dry north-easterly trade winds from the Sahara to the Gulf of Guinea during the dry season, specifically between November and February. Overall, temperatures are lowest in the wet season between July and August and in the dry season between December and January. Daily temperature range is unimodal and lowest at the peak of the wet season during the months of July and August.

Overall, the effect of rainfall on environmental productivity may vary between seasons, because the onset of the wet season is rapid, and the effects of precipitation are quickly felt, while the commencement of the dry season, and associated drying out of the environment is much more gradual and prolonged. Thus, conditions within the wet season are less variable than within the dry season. Overall, food is usually more abundant in the wet season (Molokwu et al. 2008), but pathogens and disease vectors should also be more abundant due to higher environmental productivity in the wet season. Nonetheless, food, water and diet vary both between and within seasons, and this is partly due to a combination of environmental heterogeneity, plant phenology and distribution. For example, within the Amurum Forest Reserve, gullies in riparian forest fragments retain water in the dry season (Brandt and Cresswell 2008), and these provide nourishment for plants and watering holes for animals for the period when there is no rain. Similarly, some plants flower and fruit during the dry season, providing food for wildlife, including insects, birds and mammals. Such environmental buffering is likely responsible for maintaining the diverse life history patterns in resident savannah birds, including the Common Bulbul (Cox et al. 2011, Cox et al. 2013, Stevens et al. 2013).

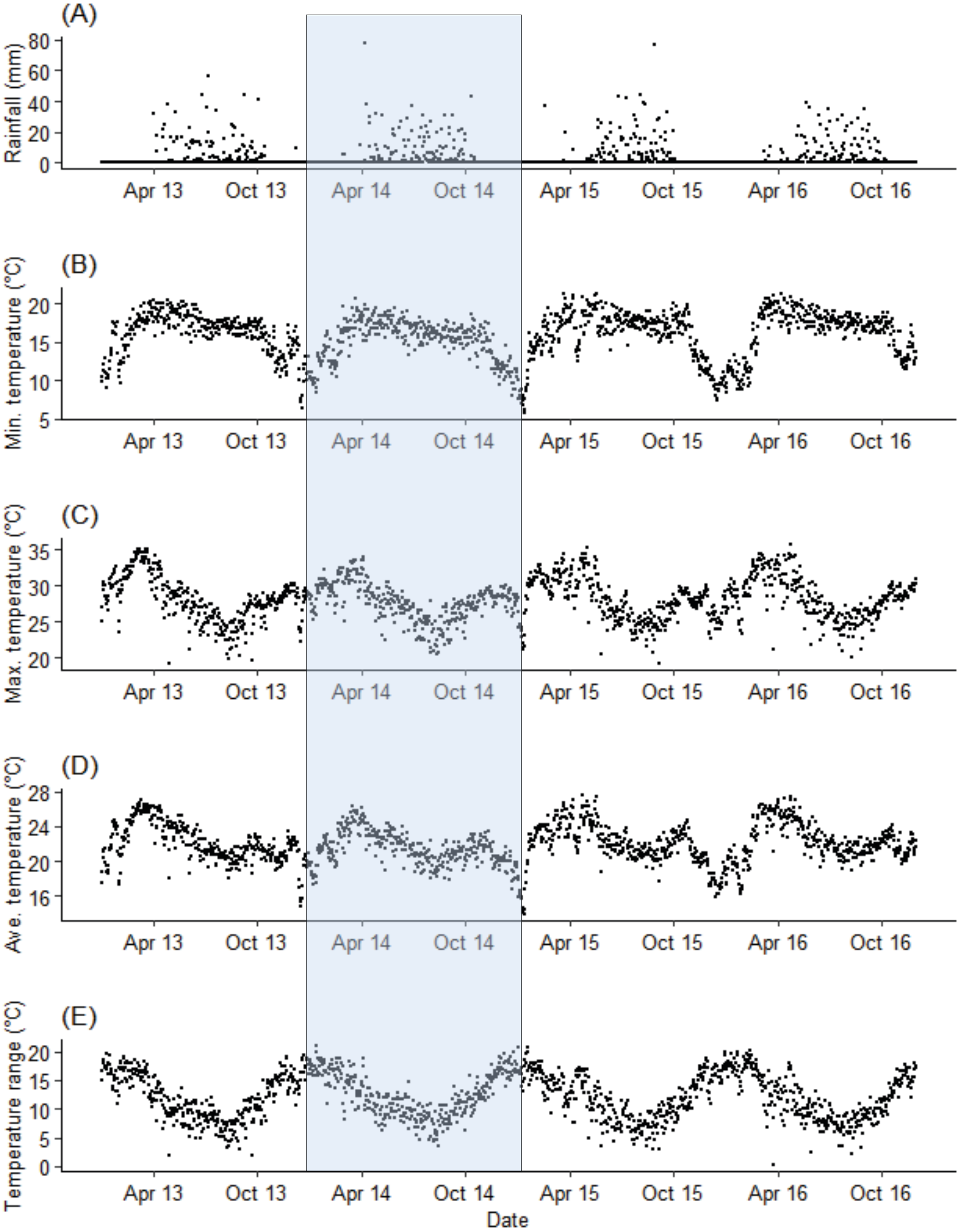

Figure 1.2: Environmental variability in (A) daily rainfall, (B) minimum daily temperature, (C) maximum daily temperature, (D) average daily temperature and (E) daily temperature range over four consecutive cycles of wet and dry seasons from January 2013 to November 2016 in central Nigeria. Highlighted portion indicate one complete annual cycle. Weather data was obtained from the Nigerian Meteorological Agency at Jos Airport, located 26km from the A. P. Leventis Ornithological research Institute.

Seasonality of annual cycle stages in the Common Bulbul

The Common Bulbul is a very widespread tropical songbird. It is a resident breeder throughout its range and is present in all parts of Nigeria. Common Bulbuls are sexually monomorphic (Figure 1.3), although males are slightly larger than females overall. They are usually 9 to 11 cm in length and weigh 25 - 50g. This large body mass range is partly due to a large variation in body size across their range. Common Bulbuls are omnivores, foraging mostly on fruits and insects and occasionally on nectar and seeds (Box B). In the Amurum Forest Reserve, Common Bulbuls are territorial throughout the year but may move up to c. 2km to forage or drink from wet gullies during the dry season (Nwaogu *pers obs*). Common Bulbuls have an annual survival rate of 0.67 ± 0.05 (Stevens et al. 2013). They may breed year round (Figure 1.4) (Cox et al. 2013), building cup shaped nests on small trees or shrubs and laying a typical clutch of two eggs. Nestlings are predominantly fed insects and then fruits later, and these are available year-round, though with some temporal variability. Common Bulbuls moult mostly in the wet season, but some individuals may extend moult into the dry season and some may even overlap moult with breeding (Nwaogu et al. 2018a).

Figure 1.3: Marked free living breeding pair of Common Bulbuls at the Amurum Forest Reserve, Nigeria – Common Bulbuls are sexually monomorphic.

Decoupling environmental seasonality from the seasonality of annual cycle stages

The seasonality of an annual cycle stage can be decoupled from environmental seasonality if the occurrence of the annual cycle stage does not overlap completely with the periodicity of seasonal environmental factors. Thus, Common Bulbul represents a model system to study variation in immune function in a seasonal environment because the occurrence of annual cycle stages are not constrained to any particular season as frequently observed in temperate species or organisms with seasonal annual cycle stages that are completed within a narrow range of environmental conditions (Figure 1.4). However, it is important to note that because moult is seasonal, it should not be decoupled from season if all birds completed moult in the wet season, but because some birds continue to moult after the wet season, moult events are recorded in the wet and early in the dry season, causing occurrence of moult to become decoupled from environmental seasonality. Although, this is less so for moult compared to breeding which non-seasonal. Consequently, cross seasonal breeding, breeding-moult overlap and the extension of moult into the dry season in Common Bulbuls allow the testing of the separate and interactive effects of breeding and moult on immune function in the wet and dry season. In addition, residency allows repeat sampling of individuals between seasons and across annual cycle stages. In the Common Bulbul, temporal increase in resource demands due to the occurrence of breeding and/or moult is decoupled from seasonal environmental conditions, allowing life history and environment effects to be decoupled at both population and individual levels in a natural environment. Furthermore, omnivory allows captive rearing and experimental diet manipulation, so that the effect of diet composition on immune function and other life history traits can be tested explicitly.

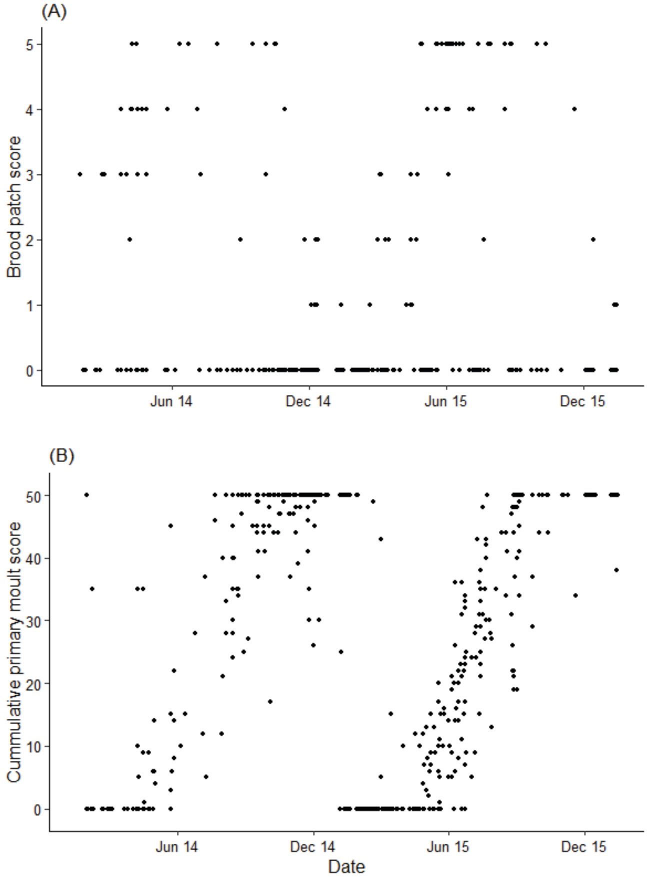

Figure 1.4: Non-seasonal occurrence of breeding and seasonal occurrence of moult in the Common Bulbul *Pycnonotus barbatus* over two annual cycles of wet and dry seasons from January 2014 to February 2016.

Thesis set-up and hypotheses

This thesis is organised into four major parts, followed by a synthesis of the major findings and presentation of ideas for further consideration in a final chapter. It is important to note that each chapter of this thesis was written as a stand-alone article at a different stage in course of the project to address a specific objective or test a hypothesis, hence it reflects a refinement of my thoughts and ideas about the study system which may lead to few inconsistencies in the interpretation of observations. Two major areas where this is notable is in the relationship between the seasonality of breeding and moult, and the seasonal diet variation of the Common Bulbul.

First, I tested how breeding and moult fitted into the annual cycle of the Common Bulbul in relation to the occurrence of wet and dry seasons annually, and then how female Common Bulbuls adjusted their body reserves during breeding in response to differences in foraging conditions in the wet and dry seasons. In Chapter 2 (the first results chapter) I will show that weak breeding seasonality in Common Bulbuls, despite seasonally arid environmental conditions, is due to individual flexibility and strongly seasonal timing of moult. I collected breeding records based on brood patch occurrence (Redfern 2010) in female birds over two annual cycles. I also considered the occurrence of moult in both males and females, since moult is the other main annual life history event for adult Common Bulbuls. Moult may impose constraints on the timing of breeding (Siikamaki et al. 1994) or itself be affected by breeding (Echeverry-Galvis and Hau 2013). I tested whether (1) the relative timing of breeding and moult in the Common Bulbul follow an annual pattern at population level, (2) individual Common Bulbuls breed flexibly as opposed to seasonally within and between years, and (3) the occurrence of breeding and moult are determined by within year variation in rainfall and temperature. In Chapter 3, I will show that breeding limits foraging time and that female Common Bulbuls respond to this limitation more strongly in the dry than the wet season. I tested two hypotheses to find out how female Common Bulbuls respond to limited foraging time and possibly foraging predictability (Macleod and Gosler 2006) imposed during different breeding stages and by breeding in the wet and the dry season. I tested whether: (1) breeding body mass varies in accordance with the amount of time available for foraging at different breeding stages, including, nest building, egg laying, incubation and chick feeding, because engaging in these activities leaves less time for foraging compared to a non-breeding female, thus reducing the probability of finding sufficient food. I predicted that body mass peaks during incubation when foraging time is most limited and (2) breeding body mass varies in accordance with difference in food availability between the wet and dry season. I predicted that breeding females will be heavier when breeding in the dry season because finding sufficient food within limited time will be less probable in the dry season (Ngozi Molokwu et al. 2008).

Secondly, after confirming that seasonality of annual cycle stages (breeding and moult) is decoupled from seasonal environmental conditions (i.e. wet and dry season) for the Common Bulbul, I will show in Chapter 4 that seasonal differences in baseline innate immune function are better explained by environment than annual cycle stage. I tested the main and interactive effects of seasonal environmental conditions (i.e. occurrence of rainfall) and annual cycle stage on innate immune variation by separating Common Bulbuls into annual cycle stages based on occurrence of breeding and moult, and testing differences in innate immune indices between the wet and the dry season at the population and individual level. I expected immune indices to be higher in the wet season compared to the dry season due to the higher environmental productivity associated with the rains (Horrocks et al. 2011b). But I also expected the occurrence of breeding and/or moult to suppress immune function due to competition for limited resources (Sheldon and Verhulst 1996). However, if seasonal variation in immune challenge is the main determinant of immune function, and differences between annual cycle stages and/or food availability are of less importance, then immune indices will be higher in the wet season independent of annual cycle stage. Otherwise, differences between annual cycle stages should be consistent between the wet and dry season depending on immune challenge. But, if both factors affect immune function, then I expected their interactions to yield varying outcomes. For example, I expected immune indices to be lowest for breeding and moulting birds in the dry season (due to an overlap of two resource demanding events during a resource poor period, but with low immune challenge) and highest for non-breeding and non-moulting birds in the wet season. Overall, I expected patterns at the level of the individual to be like patterns at the population level, if the effects of seasonal environmental conditions and/or those of annual cycle stage lead to larger variations within individuals than among individuals. However, if variation in immune function is characteristic of individuals, then patterns will differ between population and individual levels, but with high individual repeatabilities.

Thirdly, I tested how three different traits in the Common Bulbul (body size, moult and innate immune function) vary along the aridity gradient from the north to the south of Nigeria in order to explore patterns of variation in life history traits in space and to confirm if temporal environmental variables operated in a similar manner on a spatial scale. In Chapter 5, I will show that variation in the body size of Common Bulbul along the spatial aridity gradient in Nigeria (Figure 1.1) conforms to Bergmann's rule. I estimated the ratio of squared wing length to body mass and correlated this measure with bioclimatic variables across 22 locations, including seven locations for which data was previously available. According to Bergmann's rule (Salewski and Watt 2017), I predicted that Common Bulbuls in hotter environments will have larger body surface area to mass ratio. Since body surface area is a two-dimensional measure, squared wing length was used a proxy for body surface area and while body mass, a three-dimensional measure was used proxy for volume. In Chapter 6, I then examined whether the timing of rainfall predicted the timing of moult along the aridity gradient, because I had previously found that moult is timed to the wet season within the year in a location mid-way along the aridity gradient (Nwaogu et al. 2018a). I analysed the extent of primary moult at 15 locations along the aridity gradient in Nigeria just before the onset of the rains (Figure 1.1) to verify whether the timing of moult to the wet season is due to seasonal timing of rainfall or due to a periodic occurrence of moult at the same time in all locations along the gradient. Because old feathers are lost and new feather materials are deposited in a fairly regular rate in each bird (Summers 1976), I predicted that the proportion of new primary feather mass grown will be higher in sites where rainfall occurs earlier. In Chapter 7, I tested the hypothesis that immune function decreases with increasing aridity along an environmental gradient due to an expected lower immune challenge and reduced investment in immune function in drier environment (Horrocks et al., 2011, Horrocks et al. 2015). I measured innate immune indices of Common

Bulbuls in 15 locations along the aridity gradient in Nigeria (Figure 1.1) within three months before the onset of the wet season. At this time, I expected differences in aridity to be more pronounced because these locations have experienced varying durations of rainfall and drought over the year due to the contrasting onset and termination of the rains from south to north. Furthermore, I predicted that immune indices will be more variable among locations than within locations because immune response should be similar for individuals experiencing the same environmental conditions, if environmental factors were the key determinants of variation in immune function.

In the fourth part of the thesis, I identified diet as an important component of environmental change (Box B) and experimentally tested its effect on immune function. In Chapter 8, using a balanced parallel and cross over design, I fed 40 wild-caught captive reared Common Bulbuls exclusively on fruits or invertebrates (the main food items of Common Bulbuls in the wild, Box B) for 24 weeks, switching a sub-set of each treatment between treatments after 12 weeks and compared treatment effects on innate immune function, body mass and extent of primary moult. I expected Common Bulbuls on protein-rich invertebrate diet to maintain a more robust innate immune function, to have higher body mass and to moult earlier because fruits may lack essential amino acids that are not synthesised *de novo*. In addition to testing the direct effects of diet, I explored co-variations between immune function, body mass and moult under fruit and invertebrate diet treatments to gain insights on possible diet dependent trade-offs among life history traits. I also performed path analyses - path analyses generate hypotheses about causal connections based on correlative data sets (Shipley 2009) - to generate hypotheses about the mechanistic pathway of diet effects on immune function and body mass. I compared whether the effect of diet on immune function was direct or via a prior effect on body mass because poor nutrition may lead to a deterioration of body reserves, leading to increased susceptibility to infection and increased immune response. Alternatively, poor nutrition may supress mechanisms of prophylaxis within the immune system, leading to infection, increased inflammatory response and loss of body mass.

In Chapter 9, I discuss how environmental conditions affect immune function and associated life history traits in time and space, and through the alteration of diet composition. I point to how understanding the annual cycle and local environment of a species can help the interpretation of variation in immune function as a life history trait. I provide some preliminary interpretations of how immune indices may relate to individual condition and suggest how experimental manipulations may be targeted to test trade-offs. I also discuss the need for quantifying disease risk rather than using proxies of environmental productivity to predict immune function and point to direction for future work within this system.

Box B - Temporal variation in the diet of Common Bulbuls.

This box provides information about temporal variation in the diet of Common Bulbuls based on empirical data collected in the Amurum Forest Reserve using two independent methods – direct foraging observations and analysis of faecal samples. This information formed the basis for the setting up of the aviary experiment (Chapter 8) and for interpreting results from various other chapters. I will refer to these patterns over the course of the thesis.

Prior to this project, I had little information about diet variation or preference over the entire annual cycle of the Common Bulbuls. Studies from other parts of Africa suggested they were omnivores, but predominantly frugivorous (Milla et al. 2005, Okosodo et al. 2016). I expected fruits and invertebrates to be more abundant in the wet season compared to the dry season, but it was unclear how foraging preference will vary between seasons. Nonetheless, I expected less foraging preference and relatively higher diet mixing in the dry season due to expected lower overall food availability.

I analyzed 805 diet records obtained between July 2014 and December 2016 from direct foraging observations (n=657) and analysis of faecal samples (n=148) collected from Common Bulbuls in the wild. I found that Common Bulbuls fed predominantly on fruits and frequently on invertebrates throughout the year (Figure B1). Occasionally I observed them to forage on nectar and seeds. Fruits were from a variety of plants, including *Lantana camara, Phyllanthus muellerianus, Bridelia ferruginea, Harungana madagascariensis, Rhus natalensis, Rhus longipes, Jasminum dichotomum, Santaloides afzeli, Keetia leucantha, Keetia venosa, Keetia cornelia, Ficus spp., Syzygium guineense, Mangifera indica, Saber florida, Psidium guajava, Paulinia pinnata, Uvaria Chamae, Antidesma venosum, Carisa edulis and Ancylobotris amoena.* Insects included termites, dragonflies, butterflies, grasshoppers, mantises and ants. Other items observed were spiders, nectar, fresh maize seeds, tomatoes and cucumber. I summarized diet as fruits, invertebrates and others. However, I present a temporal trend in the occurrence of fruits and invertebrates only, and a seasonal difference in the relative occurrence of fruit and invertebrates in Common Bulbul diet. Fruit and invertebrates make up over 97.7% of the foraging items observed (N = 805). I also report some data on seasonal differences in the occurrence of mixed foraging items in observations.

Overall, Common Bulbuls foraged more frequently on fruits than invertebrates throughout the year (Figure B2), however, fruit occurrence decreased in the wet season while invertebrate occurrence increased (Figure B1), and this was visible for both sampling methods. Based on direct field foraging observations, Common Bulbuls foraged on relatively more invertebrates and fewer fruits in the wet season, and fewer invertebrates and more fruit in the dry season (chi-squared = 21.1, df = 1, p-value < 0.01). The pattern was similar based on analysis of food items in faecal samples, but the difference was only marginally significant (chi-squared = 3.3, df = 1, p-value = 0.07). I also compared the occurrence of fruits and invertebrates in a single sample or observation (Figure B3), but the observed pattern did not differ significantly between the wet and the dry season for both direct observations (chi-squared = 0.001, df = 1, p-value = 0.98) and analysis of faecal samples (chi-squared = 1.2, df = 1, p-value = 0.27).

The mechanism driving the observed patterns of overall more fruit but increased invertebrates in the wet season are not particularly obvious because Common Bulbuls are not seasonal breeders. However, the occurrence of moult largely in the wet season may require increased protein supply from insects. Increase in insects relative to fruits in the diet of tropical birds during the wet season has been previously reported (Poulin et al. 1992). Overall lower water availability during the dry season may also favour the relative higher fruit to invertebrate occurrence in diet during the dry season.

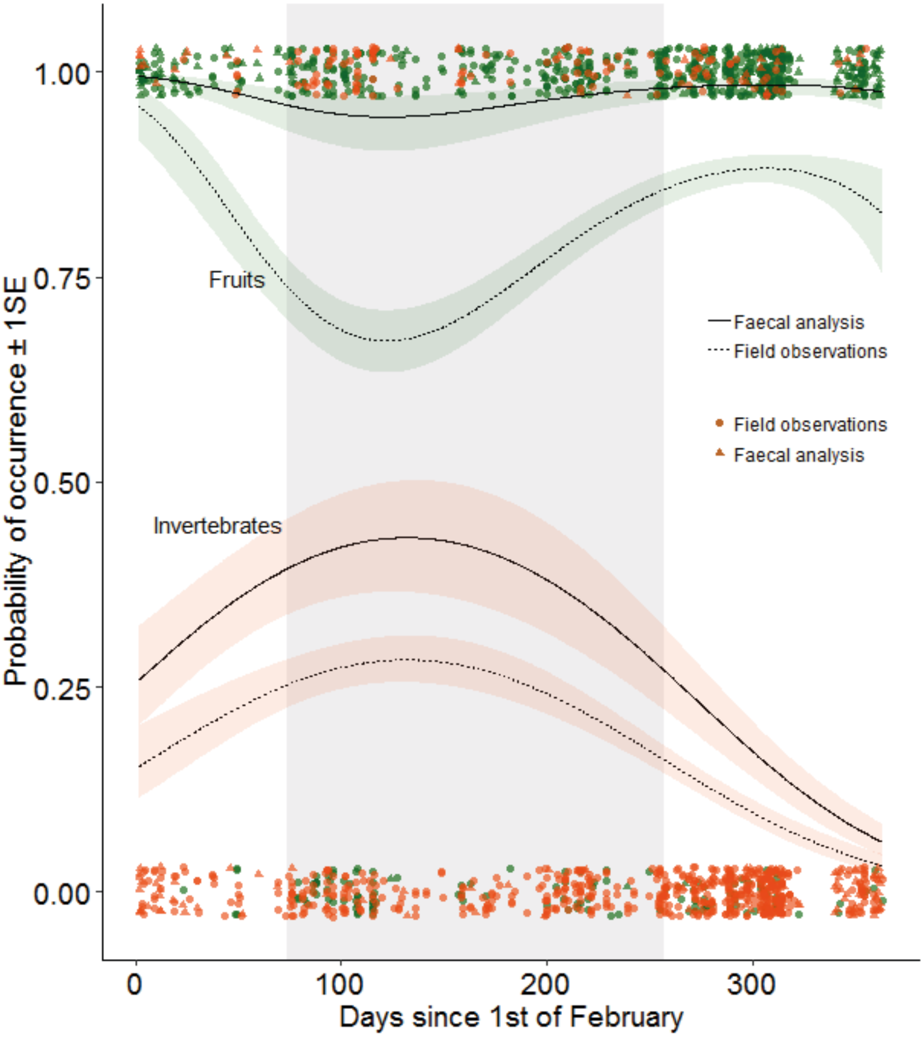

Figure B1: Variation in the occurrence of fruits and invertebrates in the diet of Common Bulbuls based on direct field observations and analyses of faecal samples collected during mist-netting in the Amurum Forest Reserve, Nigeria. Curves and error margins represent pattern of occurrence of fruits and invertebrates in the diet of Common Bulbuls across the year. Grey background represents the annual start to the end of the wet season.

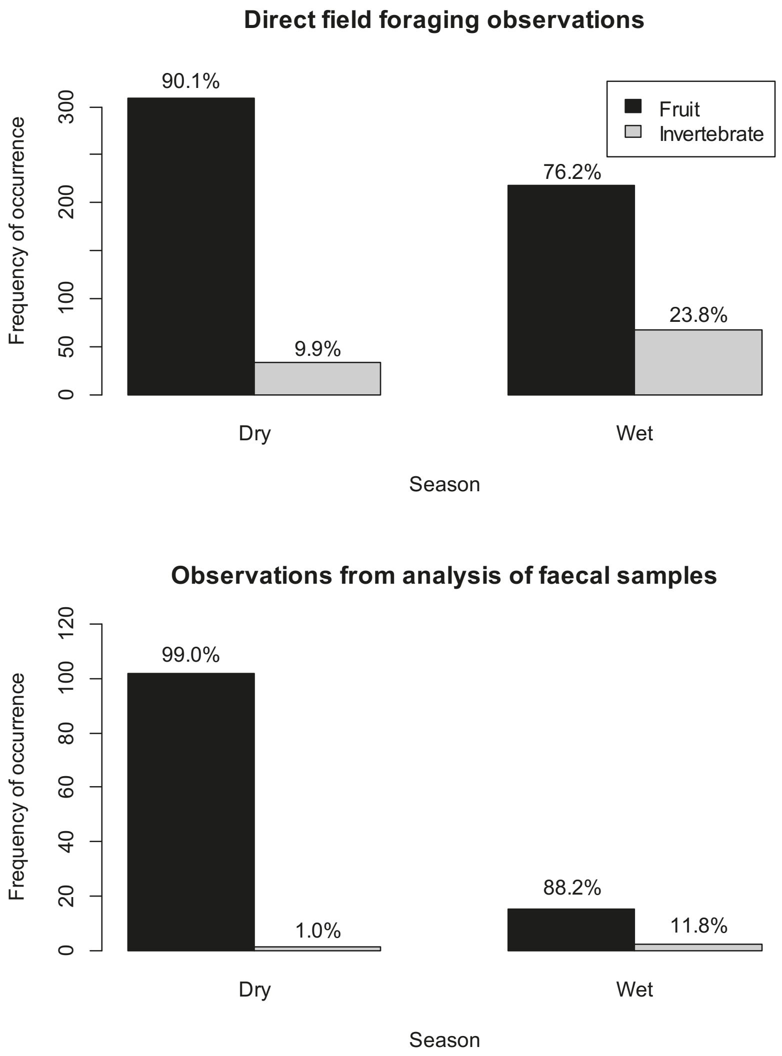

Figure B2: Common Bulbuls forage on relatively more invertebrates and less fruits in the wet season compared to the dry season. Relative occurrence of fruit to invertebrate items in the diet of Common Bulbuls based on data from: top – direct field foraging observations and bottom - analysis of faecal samples. Percentage of fruit and invertebrate in diet is displayed above each bar plot.

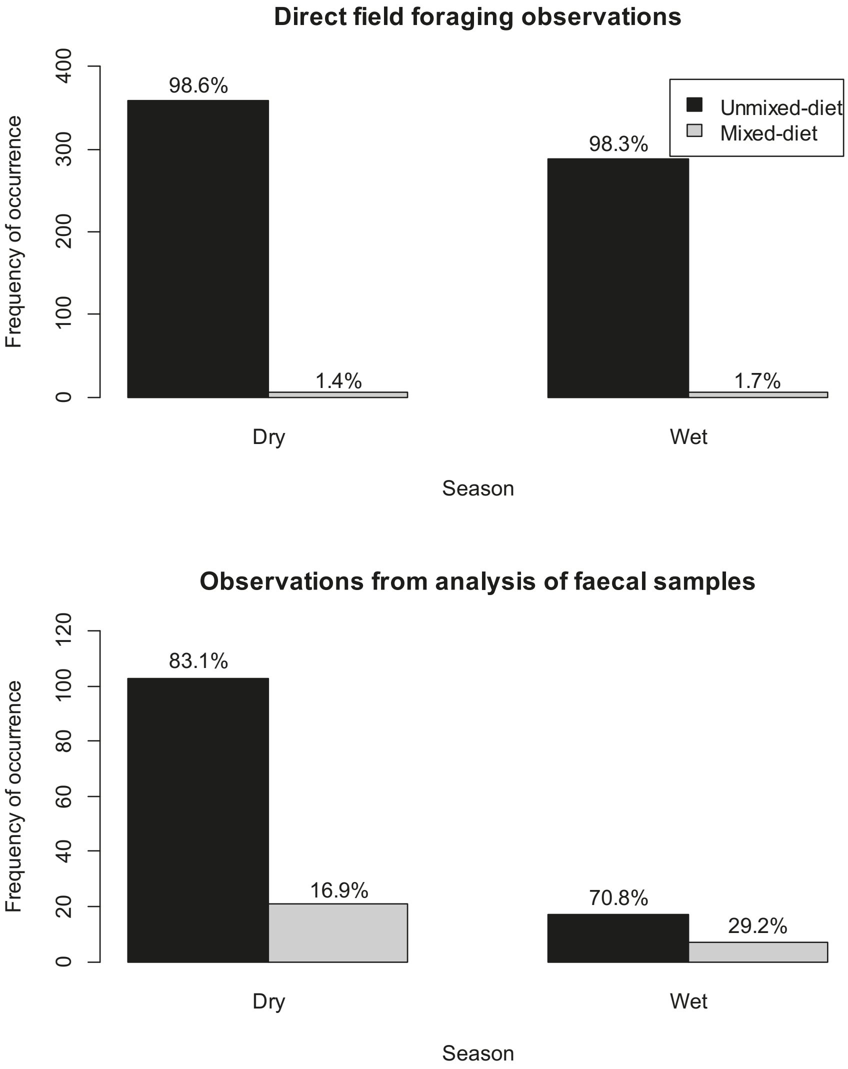

Figure B3: Occurrence of mixed-diet items in the diet of Common Bulbuls does not differ between the wet and dry season. Relative occurrence of fruit and invertebrate items in the diet of Common Bulbuls based on data from: top – direct field foraging observations and bottom – analysis of faecal samples. Percentage of mixed and un-mixed diet observation is displayed above each bar plot.

Part I

Relationship between environmental seasonality and occurrence of annual cycle stages in the Common Bulbul *Pycnonotus barbatus*

Chapter 2

Weak breeding seasonality of a songbird in a seasonally arid tropical environment arises from individual flexibility and strongly seasonal moult

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Abstract

In some tropical birds, breeding seasonality is weak at the population level, even where there are predictable seasonal peaks in environmental conditions. It therefore remains unclear whether individuals are adapted to breeding at specific times of the year or flexible to variable environmental conditions. We tested whether the relative year-round breeding activity of the Common Bulbul *Pycnonotus barbatus* arises due to within-individual variability in breeding dates. We collected data from 827 birds via mist-netting over two years with corresponding local weather data. We used a combination of climate envelope and generalised linear mixed models to explore how the timing of breeding is influenced by time of year, individual variation, rainfall and temperature in a West African savannah where seasonal precipitation determines annual variation in environmental conditions. We also pooled 65 breeding records from 19 individuals recorded between 2006 and 2017 based on brood patch occurrence and behavioural observation to compare within individual and population variability in breeding dates. We show that the breeding dates of individuals may be as variable as the population as a whole. However, we observed a seasonal peak in juvenile occurrence that varies significantly between years. Models suggest no relationship between nesting and moult, and within-year variation in rainfall and temperature, and birds were unlikely to breed during moult but may do afterwards. Moult was very seasonal, correlating strongly with day length. We suggest that because environmental conditions permit year-round breeding, and because reproductive output is subject to high predation risk, there is probably a weak selection for individuals to match breeding with variable peak conditions in the environment. Instead moult, which always occurs annually and successfully, is probably under strong selection to match variable peak conditions in the environment so that long term survival ensures future reproduction.

INTRODUCTION

Optimal fitness outcomes require organisms to match sequences of annual cycle events to the most suitable environmental conditions (Ricklefs and Wikelski 2002, Reed et al. 2009). This may involve control of annual cycle stages by internal biological 'clocks', periodic environmental signals such as day length and locally variable signals such as rainfall, temperature and food availability (Wingfield 2008, Visser et al. 2010, Helm et al. 2013). An individual's ability to assess and determine immediate conditions may also be important for a decision to initiate annual cycle stages, and this may depend on the predictability of its environment (Lisovski et al. 2017b), individual experience, and the degree of environmental limitation on initiation of such stages (Perrins 1970, Hahn 1998, Tieleman *et al.* 2005, Fontaine and Martin 2006, Pimentel and Nilsson 2007, Tökölyi et al. 2012).

Timing of breeding is a crucial component of the annual cycle of relatively short-lived organisms but may be less important for long-lived species where fitness may depend more on survival than annual reproduction. Early ornithological observations outside temperate systems suggested that both the degree of seasonal limitation to the occurrence of breeding and the relative contribution of annual reproduction to overall fitness are low in tropical birds and this was largely attributed to milder food seasonality and high nest predation (Lack 1950a, Lack 1950b, Moreau 1950, Skutch 1950, Thomson 1950, Voous 1950, Ashmole 1963a, Cody 1966). More recent studies (Dittami and Gwinner 1985, Tye 1992), Oschadleus and Underhill 2006, Greeney 2010, Reynolds et al. 2014) revealed however, that breeding strategies are diverse and closely related to species niche, albeit with exceptions such as Ndithia *et al.* (2017) who found no relationship between timing of breeding, invertebrate abundance and weather variables in equatorial larks. Birds in equatorial regions with small variation in day length have sometimes been shown to respond to this minimal environmental variability (Wikelski *et al*. 2000, Quispe et al. 2017, Shaw 2017), so year-round breeding at the population level may not necessarily equate to an absence of breeding seasonality due to weak environmental seasonality.

Breeding seasonality is a function of individual flexibility and environmental constraints. However, the strong correlation between breeding and environmental variables in temperate systems limits the appreciation of individual variability as a possible determinant of breeding seasonality, and results in a focus on factors such as food availability and offspring survival (Lack 1948, Ashmole 1963b, Skutch 1967, Lloyd 1999). In environments where birds have multiple breeding opportunities but experience stochastic nest survival, enhancing adult survival via adequate self-maintenance may be a crucial fitness determinant (Williams 1966, Nilsson and Svensson 1996). Such considerations may explain links between age-related survival, future reproduction, environmental seasonality and iteroparity (Schaffer 1974). The need to enhance adult survival could lead to the prioritization of moult over reproduction in adult birds when moult timing is crucial (Stiles and Wolf 1974), because an unsuccessful moult may have immediate survival consequences for an individual, but failure to breed successfully may not be as costly if there are opportunities to breed in the future. Year-round breeding tropical birds may help us better understand how the timing of annual cycle stages which vary differently within and among individuals interact with environmental seasonality at the population level.

The Common Bulbul *Pycnonotus barbatus* is a long-lived, resident, tropical passerine that breeds non-seasonally in central Nigeria despite clearly seasonal precipitation (Keith *et al.* 2004, Cox *et al*. 2013). However, it is not known whether individual bulbuls breed consistently at different times of the year or flexibly between years in different environmental conditions. It is also not clear how the timing of breeding events and moult interact in a single annual cycle under seasonal variation in precipitation. Residency and longevity of Common Bulbuls therefore permits repeat observations on individually marked birds at different times of year, thus allowing individual breeding patterns to be differentiated from population breeding patterns.

To test whether the breeding seasonality of Common Bulbul populations arise due to within individual variability in breeding dates, we studied the annual cycle of the species in a seasonal West African savannah where a single period of annual precipitation improves foraging conditions in the wet season (Brandt and Cresswell 2008, Ngozi Molokwu et al. 2008), and is likely to determine peak resources for breeding. We also consider the annual timing of moult, the other main annual life history event for adult resident birds, because this may also impose constraints on the timing of breeding (Siikamaki *et al.* 1994) or be the main focus for selection on seasonality. Specifically, we test whether (1) the relative timing of breeding and moult in the Common Bulbul follow an annual pattern at population level, (2) individuals breed flexibly as opposed to seasonally within and between years, and (3) the occurrence of breeding and moult are affected by within year variation in rainfall and temperature.

METHODS

Study site

The study was carried out in the Amurum Forest Reserve (09°52'N, 08°58'E), at the A.P. Leventis Ornithological Research Institute (APLORI), on the Jos Plateau in Nigeria. The Amurum Forest Reserve is a heterogeneous woodland savannah habitat with inselbergs and riparian forests, surrounded by farmlands and human settlements (Nwaogu and Cresswell 2016).

Daily rainfall and temperature data were made available from a weather station located at the APLORI and from the Nigerian Meteorological Agency at the Jos airport, located 26 km from APLORI. However, we used the data from the airport only for modelling the effect of rainfall and temperature on the timing of nesting and moult because there were gaps in data from our local weather station during February 2014 to January 2016, but records from the two weather stations were strongly correlated (daily maximum temperature = 81%, daily minimum temperature = 74% and rainfall = 87%). We obtained sunset and sunrise data from the United States Navy Oceanography Portal http://aa.usno.navy.mil/data/ (USNO 2012), from which we calculated variation in day length across the year in our study area.

There is a single wet and dry season annually, and this influences food availability. Minor precipitation may be observed before the full onset of the wet season in some years, but the annual rainfall and temperature patterns are largely predictable (see Figure 1.2). In APLORI, the wet season usually lasts for approximately six months, between mid-April and mid-October. Total monthly rainfall between June and September is usually over 200 mm but may be less in May and October. The duration of the wet and dry season may vary slightly between years depending on the onset and termination of the rains. Average daily temperatures vary across the year in a bimodal pattern with amplitudes of about 8°C around March and April and 6°C around October and November. Average temperatures are lowest during the peak wet season in July and August and the dry, dusty 'harmattan' periods of December and January (Figure 1.2). Day length varies with an amplitude of 69 minutes across the entire year.

Vegetation is mainly grass, shrubs and small trees, with few large trees around wet gullies which may dry out after the rains. A few of these gullies hold water throughout the dry season depending on the extent of drought and serve as valuable water sources when water is otherwise largely unavailable in the environment. Insects are more abundant in the wet season compared to the dry season with an especially clear peak in numbers of termites and ants at the onset of the rain (Opoku, 2017*,* Nwaogu *pers obs*): observations from insect sampling in our study area from 7 March to 19 May 2017 revealed an overall increase in insect abundance at the start of the wet season, with a greater than 10-fold increase in abundance on 7 April due to eruptions of termites and ants from subterranean castes after the first rain. Seeds are more readily available at the end of the wet season (Brandt and Cresswell 2008) while nectar and fruits may vary according to phenology of flowering and fruiting plants around the reserve (Nwaogu *pers. obs*.). More plants fruit in the wet season and so fruits are abundant and diverse in the wet compared to the dry season, but there is at least one fruiting plant species available at all times of year. Detailed information on variation in food abundance, diet choice and seasonal use of artificial foraging patches by birds around the Amurum Forest Reserve can be found in (Ngozi Molokwu et al. 2008, 2010 & 2011).

Study species

Common Bulbuls are resident, sexually monomorphic passerines, usually 9 - 11 cm long and between 25g and 50g in weight. They have an annual survival probability of 0.67 ± 0.05 (Stevens *et al.* 2013), and individual adults may live for up to 15 years in the wild (unpublished APLORI capture-recapture records). They are territorial throughout the year but may travel up to c. 2km to forage, especially in the dry season when large groups forage on the few available fruiting plants and drink from gullies. They may breed in both the wet and the dry season (Cox *et al.* 2013) and are socially monogamous (Nwaogu *pers. obs.*). They always lay two eggs per clutch, but over 79% (n = 92 nests in 3 years) of these clutches are lost to nest predation, and so pairs may make several repeat breeding attempts in quick succession in the same year. A successful breeding attempt lasts between 30 to 34 days depending on the duration of nest building and whether egg laying is initiated immediately after nest completion. Fledglings leave the nest at 66% of adult body size and attain 92% of adult body size two weeks after fledging; they start a complete post-juvenile moult 6 – 8 weeks after fledging (Nwaogu *pers. obs.)*. Hence the occurrence of young birds is a reliable indication of recently successful breeding events (Ward 1969). Juveniles remain within their family groups, supported by parents for up to 12 weeks after fledging. Common Bulbuls are largely frugivorous, but nestlings are predominantly fed insects and then fruits later. Adult birds also feed on insects, and occasionally on nectar or seeds.

Data collection and determination of variables

We mist-netted 827 Common Bulbuls over two years from February 2014 to January 2016. Mist-netting was carried out daily from February 2014 to January 2016 except when interrupted by unfavourable weather (day 1 = February 1 for data analysis involving calendar date). We caught at least one Common Bulbul on 257 days of two annual cycles (mean = 3.18 bulbuls/day, SD = 2.82, max = 16 in 257 days). We determined the age of all birds based on plumage characteristics or colour of gape and collected blood samples for molecular sexing by gel electrophoresis. DNA extractions followed methods described by (Richardson et al. 2001) and genetic sexing was done using the P2/P8 primers (Griffiths et al. 1996, Nana et al. 2014). For each adult female, we assessed breeding status and stage on the basis of brood patch occurrence (Redfern 2010). Males do not incubate so their breeding status cannot be inferred from brood patch scores. Incubation lasts 13-14 days but starts from the date of laying the first egg. Common Bulbuls typically have a clutch of two eggs laid on consecutive days, so egg laying overlaps with the first two days of incubation. Brood patches at stage 4 and 5 (Redfern 2010) may persist after incubation, hence only females with brood patch scores of 1 - 3 were considered to be in the nesting phase of reproduction (Nwaogu *et al.* 2017).

Moult status and stage was determined based on feather quality (old, moulting or new) and proportion of primary feather material regrown (Underhill & Zucchini 1985), respectively. In the field, birds were grouped into three moult categories: ‘pre-moult’, ‘in moult’ and ‘moult completed’. We further scored each primary feather on the scale of 0 – 5 for use in calculating population moult parameters (duration of primary moult, mean start date of primary moult and standard deviation of start date of primary moult): fully grown new feathers were scored 5 while un-moulted old feathers were scored 0, and feathers at intermediate stages of growth were scored 1 – 4 (Ginn, H.B. & Melville, D.S. 1983). To model seasonality of moult and the effect of weather variables on the occurrence of moult (Objective 1 & 3 respectively), birds ‘in moult’ were scored 1 while birds in the ‘pre-moult’ and ‘moult completed’ categories were scored 0. However, to control for effect of moult stage on breeding when modelling the relationship between occurrence of brood patches and weather variables (Objective 3), all three moult categories were used. We considered females trapped between February 2014 and January 2016 (n= 414) when comparing the occurrence of breeding and moult over the annual cycle but used all 827 records from two years of mist-netting when quantifying the occurrence of juveniles in the annual cycle (Figure 2.1 and Table 2.1).

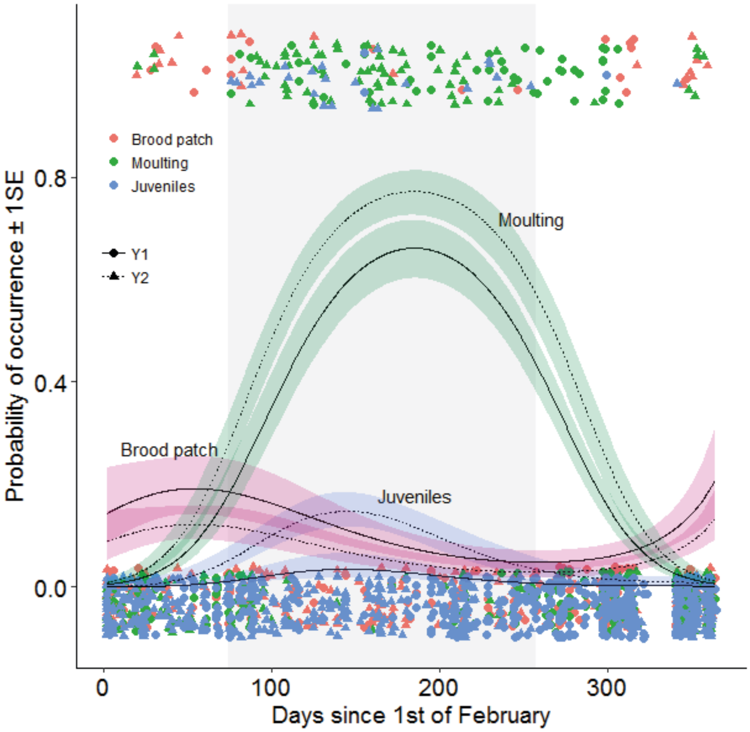

Figure 2.1. Annual occurrence of annual cycle stages: raw data (plot points are jittered around 0 and 1 for clarity) and annual cycle pattern of presence and absence of brood patch, primary moult and juvenile occurrence in the Common Bulbul *Pycnonotus barbatus* in a West African savannah environment over two annual cycles. Occurrence of brood patches and moult was determined from 414 female birds, while for juvenile occurrence, the entire 827 capture records were used. Plot lines are generated from parameter estimates of Generalised Linear Models with binomial error structure (Table 2.1). Colour bands indicate 1 standard error of the probability of occurrence of annual cycle stages. Error bands differ in size due to relative difference in the amplitude of annual peaks between years. Grey background indicates the expected start and end of the wet season. Circles and triangles represent 1st year and 2nd year data points, respectively. Day of year is calculated with reference from 1 February for consistence with models estimating moult parameters (see Table 2.S1).

Table 2.1. Timing of nesting (active brood patch occurrence), moult and juvenile occurrence in the annual cycle of the Common Bulbul *Pycnonotus barbatus*, modelled by a Generalized Linear Model with binomial error structure using two years of capture-mark-recapture data from a West African savannah environment. Occurrence of nesting and moult were modelled from adult female birds only, while juvenile occurrence was modelled using all capture records. Year 2014 is set as intercept in the model. Day of year is included as a continuous variable with day 1 = 1 February annually.

Nesting				
	Estimate	Error	*Z*	*P*
Intercept	-1.82	0.62	-3.0	**0.003**
2015	-0.54	0.37	-1.4	0.152
day	0.02	0.01	1.0	0.305
day^2	-0.0002	0.0001	-1.7	0.080
day^3	0.0000004	0.0000002	2.1	**0.039**
Juveniles				
Intercept	-9.15	2.02	-4.5	**<0.001**
2015	1.58	0.65	2.4	**0.015**
day	0.09	0.04	2.5	**0.014**
day^2	0.0004	0.0002	-2.1	**0.034**
day^3	0.0000006	0.0000004	1.7	0.093
Moulting				
Intercept	-5.42	0.69	-7.8	**<0.001**
2015	0.55	0.29	1.9	0.056
day	0.07	0.01	8.6	**<0.001**
day^2	-0.0002	0.00002	-9.0	**<0.001**

Number of individuals = 539, total number of observations = 827, Number of female observations = 414. Significant p–values are presented in bold.

To estimate within-individual variation in breeding dates, we considered females that were trapped more than once in breeding condition (Figure 2.2) and supplemented these few records from 2014 to 2016 with breeding records of the same individuals from ringing data collected between 2006 and 2013 (Cox and Cresswell 2014), as well as direct nesting observations made between 2014 and 2017. In total, we considered 65 breeding observations of 19 females that were encountered breeding 2 – 9 times between 2006 and 2017 (Figure 2.2).

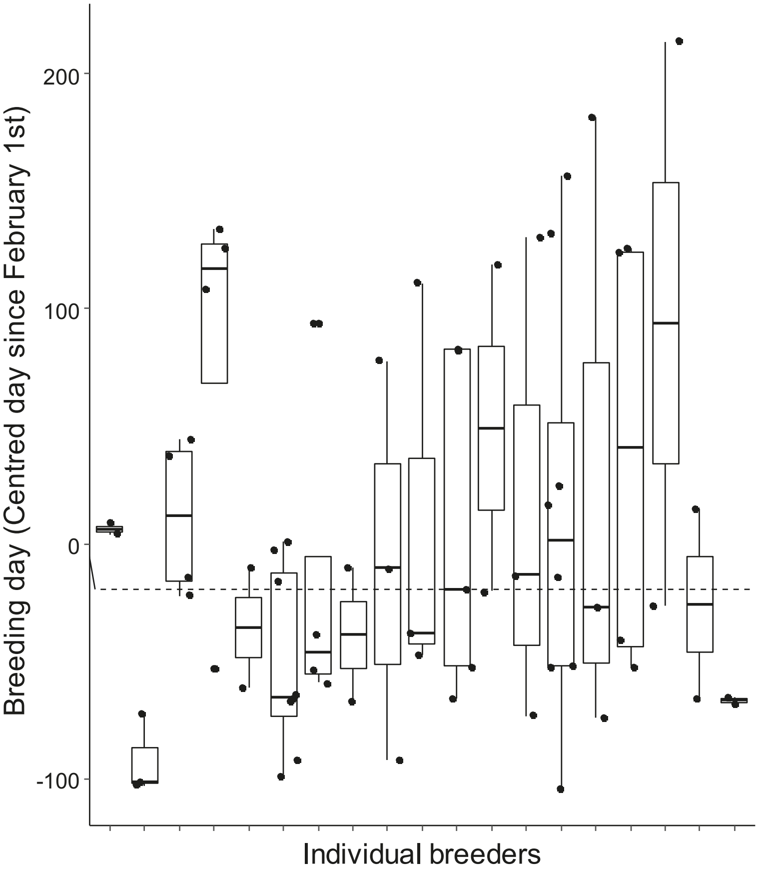

Figure 2.2: High within-individual variability in nesting dates of Common Bulbuls *Pycnonotus barbatus* in a West African Savannah environment depicted from raw data plots of observed breeding dates per individual bird. Within individual (0.49) and population (0.59) coefficient of variation in breeding dates were calculated based on number of days since 1st of February of brood patch occurrence. Dashed line indicates population median date of brood patch occurrence (65 breeding observations in 19 individuals). First eight and last two individuals (left to right) are less variable compared to the population – this would be the case for all birds if individuals bred seasonally despite weak seasonality at population level.

Statistical analyses

Relative timing of breeding, juvenile occurrence and moult

Our first analyses were aimed at predicting the occurrence (binary outcome) of, respectively breeding, juvenile occurrence, and moult, over the year. Each of these three activities were modelled using Generalised Linear Model (GLM) with a binomial error structure, and with day (from 1 February) as a continuous variable and year as categorical predictor variables (Figure 2.1, Table 2.1). We included the quadratic and cubic terms of day in all models where they

were significant. Year was retained in all our models whether it was significant or not, because we intended to compare best estimates of the timing of annual cycle stages between years.
To model the timing of primary moult in more detail, we estimated population-level annual moult parameters using 771 moult records of adult birds trapped between February 2014 and January 2016 (Table 2.S1). Each moulted primary feather was converted to proportion of primary feather mass regrown at the time of scoring (Underhill & Zucchini 1985, Erni *et al.* 2013) using reference feather masses of fully grown individual primary feathers from Museum specimens at the A. P. Leventis Ornithological Research Institute in Nigeria before fitting the moult model (Erni *et al.* 2013).

Within-individual variation in breeding dates

We estimated individual variation in breeding dates. To do this, we calculated the mean within-individual and population level coefficient of variation in breeding dates, using the subset of our data which included females with repeat breeding records. The breeding date was recorded as the number of days from 1 February when an individual was encountered with a brood patch or observed nesting.

Effect of rainfall and temperature on occurrence of breeding and moult

Weather variables may have lag or cumulative effects on life history traits. Hence, we tested the effect of rainfall, minimum temperature, maximum temperature and temperature range on brood patch and moult occurrence over different time windows going back 365 days, using methods described by (van de Pol et al. 2016). We used data of females only so that effects of rainfall and temperature on the occurrence of breeding and moult are tested for the same individuals. We tested several models for different time windows within 365 days and came up with a top model for each weather variable for a linear and quadratic function, each using aggregate measures; slope, sum, mean and maximum of weather variables within the selected time window (see Table 2.S3 & 2.S4). Each model quantifies the additional contribution of a weather variable to the fit of a baseline GLMM with brood patch or moult as response variable, day length as continuous predictor variable and individual identity as random factor (Table 2.2). Top models for each weather variable are those that generate the largest difference in AIC (relative to the baseline model) after the inclusion of the weather variable in the baseline model (Table 2.S3 & 2.S4). We tested the reliability of these top models by comparing their delta AICs to delta AICs of models generated from randomised data, and after only 10 randomisations, we found that the suggested weather signals were all likely to be arrived at by chance: **Breeding**: Rainfall; P = 0.99, Tmax; P = 0.99, Trange; P = 0.97, Tmin; P = 0.96; **Moult**: Rainfall; P = 0.99, Tmax; P = 0.86, Trange; P = 0.92, Tmin; P = 0.98. The effect of rainfall and temperature were thus, not considered further.

Table 2.2. Timing of moult and breeding, measured as occurrence of primary wing moult and active brood patch respectively, in the annual cycle of the Common Bulbul *Pycnonotus barbatus* in a West African savannah. Probability of moult is predicted by day length and breeding status, while probability of breeding is predicted by only moult stage. Moult stage was a categorical variable (pre-moult; in moult; moult completed), with the 'pre moult' stage set as intercept in the model. Breeding status was scored as presence or absence of active brood patch in females. Predictor variables in full models included rainfall, maximum daily temperature, minimum daily temperature and daily temperature range.

Trait	Variable	Estimate	Error	*Z*	*p*
Breeding	Intercept	-1.72	0.27	-6.27	<0.001
	Moult stage: in moult	-1.91	0.65	-2.94	<0.01
	Moult stage: moult completed	-0.60	0.38	-1.59	0.11
Moult	Intercept	-1.75	0.26	-6.75	<0.001
	Day length	0.05	0.01	5.58	<0.001
	Day length^2	<0.01	<0.001	2.67	0.01
	Breeding status-Breeding	-2.00	0.79	-2.54	0.01

N=414, individuals=281, Estimated random effect variance: breeding model = 0.44 ± 0.66, Moult model = 1.22 ± 1.10

All statistical analyses were implemented in R version 3.4.1 (R, Development Core Team 2017).

RESULTS

Nesting and juvenile occurrence peaks were seasonally consistent between years

The timing of moult but not breeding was seasonal in the Common Bulbul (Figure 2.1, Table 2.1). Peak occurrence of brood patch and juveniles followed similar sequence in both 2014 and 2015. Brood patch occurrence peaked weakly prior to the onset of the wet season before the population moult peak, flattened in the wet season during moult, and increased again after the moult peak (Figure 2.1, Table 2.2). Juvenile occurrence peaked within the wet season as did moult, but active brood patches and juveniles still occurred at any time of the year (Figure 2.1). The amplitude of juvenile occurrence peaks differed significantly between 2014 and 2015. More juvenile captures were recorded in 2015, despite similar brood patch peaks in both years (Figure 2.1, Table 2.1). The duration and standard deviation of the start date of wing moult did not differ significantly between years or sexes, but males varied significantly in the start date of moult between years. Moult lasted 138 ± 5 days on average. In 2014, males started moulting on 24 April and females started on 14 May, while in 2015, males started on 16 April and females stated on 13 May (Table 2.S1).

Breeding dates are variable within individual birds

On average, breeding dates were as variable within individual birds as for the population (mean individual CV = 0.49, population CV = 0.59, Table 2.S2). Within-individual coefficient of variation in breeding dates ranges from 0.03 – 0.84. Raw data plots (Figure 2.2) of observed breeding dates per bird reveal that the same individuals nested at different parts of the same or different year(s).

Within-year variability in rainfall and temperature are weakly associated with the timing of nesting and moult

Both the timing of moult and breeding were not predicted by within year local variability in rainfall and temperature, and this was not due to lag effects of weather variables, because we explored the effect of all-weather variables over 365 days before a breeding or moult event. Final models showed that breeding was best predicted by moult ($\chi^2_{2,414}$=-1.91 ± 0.65, P<0.01), and moult was best predicted by day length ($\chi^2_{1,414}$=0.05 ± 0.01, P<0.001) (Table 2.2, Figure 2.3).

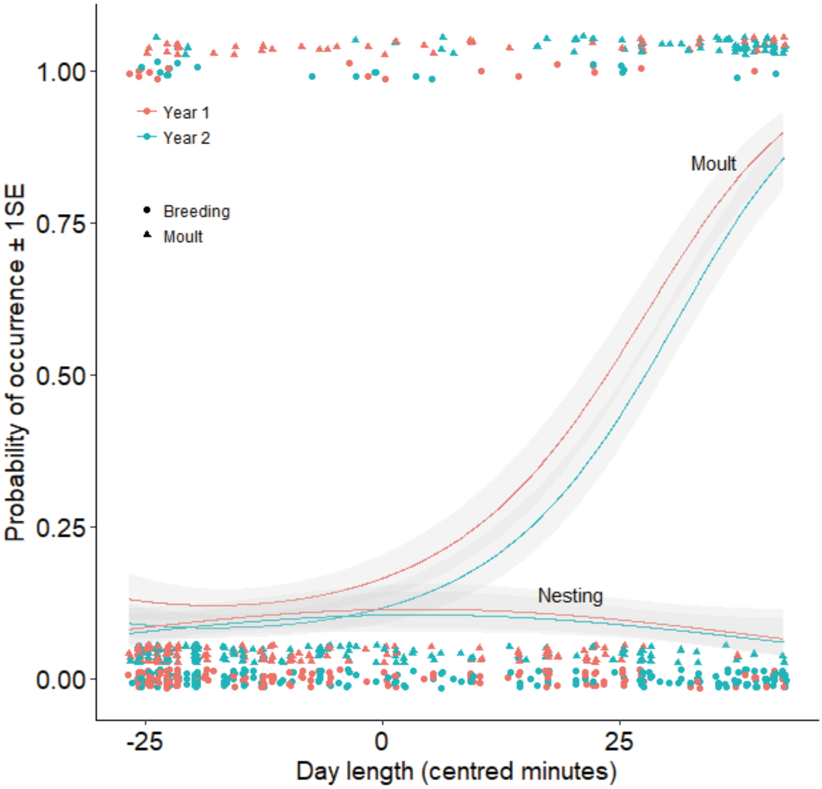

Figure 2.3. Day length predicts moult but not breeding in the Common Bulbul *Pycnonotus barbatus*: relationship between occurrence of moult and brood patches in female birds, and day length in a West African Savannah environment with slight variation in day length and marked seasonal precipitation. Plot lines are generated from predictions based on a generalised linear model fitted for 414 observations of moult and brood patches in 281 female bulbuls in Amurum Forest Reserve, Jos, Nigeria (Table 2.2). Moult points are stacked on breeding points for easy identification. Pattern in both annual cycles are plotted to show consistency between years but

there was no significant difference between annual patterns. Day length varies with amplitude of 69 minutes across the entire year.

DISCUSSION

Common Bulbuls showed weakly seasonal nesting at the population level and this was largely due to individual variability in breeding dates, despite highly seasonal precipitation, and perhaps the need to moult during the wet season (Ward 1969). We found strong evidence for strict seasonal timing of moult based on a correlation of moult with day length. This finding contrasts with the general observation of breeding being timed to match seasonal food abundance, thus in turn driving the start of moult: a notion which prevails in temperate systems (but see Tomotani *et al*. 2017). We discuss these findings in the context of life history adaptation and environmental variation over the annual cycle of the Common Bulbul, paying attention to the benefit of seasonal moult for adult survival and the possible impact of nest predation on re-nesting behaviour.

Seasonality of nesting, juvenile occurrence and moult

The annual cycle of Common Bulbuls suggests weakly seasonal breeding in which peak juvenile occurrence and moult coincide with the start of the wet season even though breeding events were distributed across the annual cycle rather than heavily concentrated in any one part of the year (Figure 2.1). The coincidence of juvenile occurrence and moult with the start of the wet season suggests that the annual cycle is organised such that juveniles and adults' benefit from the relatively higher insect abundance at the start of the rains. A similar synchrony is reported for the Yellow-vented Bulbul *Pycnonotus goiavier* in Singapore (Ward 1969) and the Common Bulbul in East Africa (Moreau et al. 1947). Insects become readily abundant with the first rains, but in contrast to temperate systems, peak insect abundance at the onset of the rains results mostly from termite and ant eruptions (Ward and Jones 1977). For example, in some parts of Africa, winged termites emerge for nuptial flights from subterranean castes during the early rains (van Huis 2017) and this may not require the vegetation regeneration that drives the seasonal peak in abundance of caterpillars in temperate systems. There may be a lag effect of rainfall on the abundance of insect taxa such as Lepidoptera as in other tropical systems (Vasconcellos *et al*. 2010), but this may simply allow insects to be available for longer periods in the year. Common Bulbuls raise young year-round, so seasonal variation in insect abundance is unlikely to be the key factor influencing the timing of nesting. It is therefore, important to note that peak juvenile occurrence is unlikely to be a direct indication of peak nesting period, because the time interval between peak occurrence of brood patch in adult females and the peak of juvenile occurrence is longer than the maximum expected 34-day nesting period. Furthermore, the amplitude of juvenile occurrence peaks varied significantly between years but brood patch peaks did not, so the timing of peak juvenile occurrence may be more strongly related to the likelihood of nesting success, which may be variable but more closely related to annual variation in the timing of rainfall than to the occurrence of breeding. Clearly, an understanding of seasonal adult and nestling diet is crucial for a better understanding of breeding phenology in tropical systems.

The wet season may provide better opportunities for juvenile survival and moult (Moreau 1950, Ward 1969, Chapman 1995), but the dry season still supports successful breeding events (Cox *et al.* 2013). Thus, preparation for nesting and a substantial part of the nesting period takes place outside the rains and this may be supported by the omnivorous foraging behaviour of the Common Bulbul which allows exploitation of multiple food sources. The dry season, although comparatively more impoverished based on evidence from increased foraging from artificial food patches and lower giving-up densities (Molokwu *et al.* 2010), still must provide sufficient food to allow breeding (Perrins 1970). It further implies that breeding by Common Bulbuls is not specifically triggered by rainfall (Hau *et al.* 2004), and that our study area meets the minimum breeding requirement for the Common Bulbul even before the wet season begins, despite about six preceding months of drought (Figure 1.2).

Common Bulbuls were only less likely to breed when in main wing moult and could breed again after moulting (Table 2.2). Nonetheless, some individuals overlapped breeding and moult. Our raw data set (Figure 2.1) has four of 414 (ca. 1%) females with active brood patches while in main wing moult. All four were trapped after April but before September and one of them, nesting in June, was previously caught incubating in March of the same year. Because juveniles remain with their parents for up to 12 weeks after fledging, it is likely that the March breeding attempt was unsuccessful and necessitated replacement clutches. Hence, full overlaps between breeding and moult may be an exception rather than a norm (Foster 1974). We hypothesize that although individuals are capable of breeding year-round, birds which complete a successful first breeding attempt are unlikely to overlap nesting and moult (Jahn *et al.* 2017). A more common scenario in our study area is to find females with brood patch scores of 4 and 5 (post-nesting stages) already starting wing moult or a few pairs initiating breeding attempts after September when moult is largely complete (Figure 2.1). Our observations suggest a tighter schedule for moult compared to breeding at the population level. Main wing moult usually lasts about 138 ± 5 days - a period accounting for roughly 80% of the wet season. This duration may be partly influenced by the 21 days delay and larger variability in the start date of moult in females (Table 2.S1).

Flexible individual timing of breeding

Breeding dates within individuals may be as variable as the population as a whole. Thus, weak breeding seasonality at population level is due to individuals' ability to breed in different environmental conditions within a year rather than being adapted to specific parts of the year (Figure 2.2). Perhaps occurrence of breeding was determined by whether a female was successful or not in a previous attempt to breed and this may be stochastic. A few individuals that successfully bred before moulting have however, been observed breeding again after moult. Hence, a sub-annual periodicity where individuals breed at less than annual intervals (Ashmole 1965), or a bimodal breeding season (Greeney 2010) similar to patterns consistent with the bimodal rainfall pattern in east Africa (Moreau 1950) may be an alternative explanations for weak breeding seasonality. A bimodal breeding season is possible if conditions at the start and end of the wet season are similar, but this may be less apparent for Common Bulbuls because breeding events may be spread out due to replacement clutches on both sides of the single moult peak (Figure 2.1). This may be the reason for conclusions drawn by (Cox et al. 2013) who suggested that the Common Bulbul is an end-of-wet season breeder, based on juvenile occurrence at the start of the dry season from one year of data. Cox *et al.* (2013) on the other hand, suggested a non-seasonal breeding pattern based on similar proportions of brood patch occurrence in four quarters of the year from several years of pooled data.

Only detailed individual observations over several years could confirm individual breeding strategies and allow general conclusions to be drawn about population breeding patterns, because the presence of an active brood patch or nesting event may reflect either an individual's decision to initiate breeding or replace a depredated clutch or brood. We did not consider differences between initial and replacement clutches, and this limits our ability to test whether individuals commence breeding at a similar time in the year. Moreover, a distinction between initial and replacement clutches may be more complicated where birds are capable of breeding year-round. Nonetheless, our conclusion that the environment exerts a weak selection on the timing of breeding for individuals is still valid and opens opportunities for considering alternative explanations for year-round breeding in the Common Bulbul and other tropical species (Ashmole 1965, Moreau 1950).

Variation in temperature and rainfall do not predict the timing of nesting and moult

Variation in rainfall and temperature did not explain the occurrence of breeding and moult (Table 2.S3 & 2.S4). Rather, breeding was best predicted by moult, and moult was best predicted by day length. Thus, the relative timing of breeding and moult suggest that cue(s) other than rainfall and temperature (Lloyd 1999, Hau *et al.* 2004, Houston 2012) are used. Small changes in day length (Wikelski et al. 2000, Quispe et al. 2017) and sun rise time (Shaw 2017) have also been suggested as cues used by tropical birds but we found no support for the former. There was also no evidence that birds avoided nesting in the rains (Thomson 1950, Foster 1974, Shaw 2017). Instead our data reveal a seasonal moult (Figure 2.1, Figure 2.3) and a temporal separation of breeding and moult, albeit with high individual flexibility (Figure 2.2). This synchronisation of moult to the wet season despite flexible individual breeding may be aided by variation in day length, as observed with sunrise and sunset times in African Stonechats *Saxicola torquatus axillaris* (Goymann *et al.* 2012). Common Bulbuls moult during times of above-average day length (Figure 2.3) and can initiate moult in captivity even when breeding does not take place (Nwaogu *pers obs*). Nonetheless, we cannot conclude that day length serves as cue for moult without experiments because moult may as well be controlled by other factors that correlate with day length. In any case, moult does not seem to be casually linked to breeding, thus, early breeding may relax temporal constraints on the timing of moult (Barshep et al. 2013, Tomotani *et al.* 2017).

GENERAL CONCLUSION

We show that year-round breeding by Common Bulbuls probably results from a weak selection for individuals to breed under any specific environmental conditions and perhaps a need to replace stochastically depredated clutches, rather than an individual adaptation to specific conditions at different parts of the year. The small seasonal breeding peak at the population level may be related to the onset of the wet season, but the occurrence of individual breeding events is not necessarily determined by rainfall. In contrast, moult is a more seasonal annual event and if selection acts more strongly on timing of moult then seasonality in breeding may arise because of moult, but not because breeding is timed primarily to specific environmental conditions. Accordingly, we show a temporal separation of breeding and moult with only moult being seasonal. Overall, there appears to be a stronger environmental constraint or a better individual control on the timing of moult compared to breeding in the Common Bulbul. We hypothesize that where environmental conditions allow multiple breeding opportunities year-round, but limited and stochastic annual reproductive output (due to small clutch size and high nest predation respectively), there will be a weak selection for breeding to match peak environmental conditions. In contrast, there will be a stronger selection to organise the annual cycle such that self-maintaining traits (such as moult) are timed to suitable environmental conditions (Stiles and Wolf 1974, Barta et al. 2006, McNamara et al. 2011). This is a testable hypothesis that may apply to comparatively long lived species (Camacho 2013), because an unsuccessful self-maintenance bears immediate survival and thus, long term fitness consequence to an individual, while a failed breeding attempt may be relatively less costly.

SUPPLEMENTARY INFORMATION

Table 2.S1: Annual moult parameters estimated based on proportion of feather material replaced by Common Bulbuls *Pycnonotus barbatus* in a West-African savannah environment calculated based on Underhill and Zucchini primary moult model, using Type II moult data. All estimates are given in days with reference from 1st February annually. Year (though interaction with sex is not significant) is retained in the model to show the consistent difference in mean moult start date of males and females between years.

			Duration	
Parameters	**Estimate**	**Error**		
Intercept	138.30	4.51		
			Mean start date	
			Groups	**Calendar Dates**
Intercept	102.49	5.44	Female 2014	"14 May"
SexM	-19.72	5.92	Male 2014	"24 April"
Year 2015	-0.71	6.19	Female 2015	"13 May"
SexM : Year2015	-7.94	7.93	Male 2015	"16 April"
			Std. in moult start date	
SexF	40.39	9.58		
SexM	29.14	7.39		

Table 2.S2: Coefficient of variation in breeding dates of female Common Bulbuls *Pycnonotus barbatus* within individual birds and among all birds in the population. Breeding date was determined as number of days between the 1st of February and the day of brood patch occurrence or nesting behaviour observation.

Bird	mean	*SD*	*n*	*CV*	meanCV	PopCV
CA01126	169.13	92.53	8	0.55	0.49	0.59
CA01128	150.00	104.84	3	0.70		
CA01433	184.50	98.29	2	0.53		
CA01462	120.75	72.72	4	0.60		
CA01471	143.67	88.74	3	0.62		
CA01561	229.00	169.71	2	0.74		
CA01576	141.50	3.54	2	0.02		
CA02321	195.67	98.80	3	0.50		
CA02326	109.50	57.28	2	0.52		
CA04059	96.50	40.31	2	0.42		
CA04060	99.50	36.06	2	0.36		
CA05306	174.25	99.12	4	0.57		
CC70024	162.00	136.28	3	0.84		
CC73106	43.00	17.35	3	0.40		
CC75168	68.50	2.12	2	0.03		
CV40315	146.75	34.63	4	0.24		
FB23517	127.00	85.02	3	0.67		
RT05761	140.80	72.51	5	0.51		
WWm	84.38	39.58	8	0.47		

meanCV- mean CV of individual breeding dates; popCV - CV of observed breeding dates in the population.

Table 2.S3: Candidate climate models predicting the occurrence of brood patch in female Common Bulbuls *Pycnonotus barbatus* over the annual cycle. Delta AICs relative to baseline model, effect time windows and effect sizes are shown for each model. All models are set at ‘relative’ type to explore time windows within 365 days prior to the observation of active brood patch. Baseline model is a Generalised Linear Mixed-effect Model with day length as main predictor variable and individual identity as random factor. Top climate models for each weather variable are highlighted bold in the table.

Model	climate	Aggregate statistic	function	ΔAICc	Window Open	Window Close	betaL	betaQ
1	**Tmin**	**slope**	**quad**	**-16.28**	**77**	**69**	**1.5**	**2.64**
2	Tmin	slope	lin	-14.85	76	72	1.34	
3	**Trange**	**slope**	**quad**	**-13.07**	**76**	**68**	**0.94**	**3.19**
4	**Tmax**	**slope**	**quad**	**-11.41**	**80**	**77**	**-1.64**	**-0.83**
5	**Rainfall**	**slope**	**quad**	**-10.96**	**73**	**63**	**-2.6**	**-2.73**
6	Rainfall	slope	lin	-10.81	331	317	-1.68	
7	Tmax	slope	lin	-9.72	303	302	0.3	
8	Rainfall	sum	lin	-9.05	365	365	-0.36	
9	Rainfall	mean	lin	-9.05	365	365	-0.36	
10	Rainfall	max	lin	-9.05	365	365	-0.36	
11	Rainfall	sum	quad	-8.88	254	254	0.71	-0.26
12	Rainfall	mean	quad	-8.88	254	254	0.71	-0.26
13	Rainfall	max	quad	-8.88	254	254	0.71	-0.26
14	Trange	slope	lin	-8.42	274	273	0.26	
15	Trange	max	quad	-7.09	3	2	-1	0.04
16	Trange	sum	quad	-6.11	3	2	-0.45	0.01
17	Trange	mean	quad	-6.11	3	2	-0.89	0.04
18	Tmax	max	quad	-5.26	362	33	-52.55	0.77
19	Tmax	max	log	-5.17	167	164	-5.75	
20	Tmax	max	lin	-4.99	167	164	-0.2	
21	Tmin	max	quad	-4.81	263	259	-1.19	0.04
22	Tmax	sum	quad	-4.74	10	7	1.39	-0.01
23	Tmax	mean	quad	-4.74	10	7	5.55	-0.1
24	Tmin	sum	log	-3.35	76	76	2.6	
25	Tmin	mean	log	-3.35	76	76	2.6	
26	Tmin	max	log	-3.35	76	76	2.6	
27	Tmax	sum	log	-3.16	77	77	4.22	
28	Tmax	mean	log	-3.16	77	77	4.22	
29	Tmin	sum	lin	-3.13	76	76	0.17	
30	Tmin	mean	lin	-3.13	76	76	0.17	
31	Tmin	max	lin	-3.13	76	76	0.17	
32	Tmax	sum	lin	-3.02	77	77	0.15	
33	Tmax	mean	lin	-3.02	77	77	0.15	
34	Tmin	sum	quad	-2.62	173	164	-0.19	0
35	Tmin	mean	quad	-2.62	173	164	-1.85	0.06

36	Trange	max	log	-2.43	321	49	-5.94
37	Trange	max	lin	-2.42	321	49	-0.31
38	Trange	sum	log	-2.11	157	157	-0.91
39	Trange	mean	log	-2.11	157	157	-0.91
40	Trange	sum	lin	-1.45	357	357	0.09
41	Trange	mean	lin	-1.45	357	357	0.09

Tmin – Minimum daily temperature, **Tmax** – Maximum daily temperature, **Trange** – daily temperature range, **Rainfall** - total daily rainfall. Number of observations = 414 females. Number of individuals = 281 Best candidate model for each weather variable is indicated boldly.

Table 2.S4: Candidate climate models predicting the occurrence of primary wing moult in female Common Bulbuls *Pycnonotus barbatus* over the annual cycle. Delta AICs relative to baseline model, effect time windows and effect sizes are shown for each model. All models are set at 'relative' type to explore time windows within 365 days prior to the observation of primary feather moult. Baseline model is a Generalised Linear Mixed-effect Model with day length as main predictor variable and individual identity as random factor. Top climate models for each weather variable are highlighted bold in the table.

Model	climate	Aggregate statistic	function	ΔAICc	Window Open	Window Close	betaL	betaQ
1	**Tmax**	**slope**	**quad**	**-19.19**	**317**	**308**	**-1.12**	**-7.44**
2	**Rainfall**	**slope**	**quad**	**-15.42**	**290**	**272**	**-2.2**	**-2.37**
3	**Trange**	**slope**	**quad**	**-14.46**	**90**	**76**	**-1.47**	**-16.4**
4	Tmax	slope	lin	-14.25	188	178	-1.87	
5	Rainfall	slope	lin	-14.06	330	324	-0.35	
6	Rainfall	sum	quad	-13.94	290	288	0.09	0
7	Rainfall	mean	quad	-13.94	290	288	0.28	-0.03
8	Trange	slope	lin	-12.98	179	170	1.94	
9	**Tmin**	**slope**	**quad**	**-12.8**	**333**	**327**	**-1.71**	**-2.28**
10	Tmin	slope	lin	-12.55	338	330	1.71	
11	Rainfall	max	quad	-12.2	291	285	0.06	0
12	Tmin	max	quad	-10.66	128	90	5.56	-0.15
13	Rainfall	max	lin	-9.86	178	176	0.03	
14	Rainfall	sum	lin	-9.14	330	330	-0.08	
15	Rainfall	mean	lin	-9.14	330	330	-0.08	
16	Tmin	sum	quad	-8.58	30	30	0.88	-0.03
17	Tmin	mean	quad	-8.58	30	30	0.88	-0.03
18	Tmax	sum	log	-6.95	146	146	-3.16	
19	Tmax	mean	log	-6.95	146	146	-3.16	
20	Tmax	max	log	-6.95	146	146	-3.16	
21	Tmax	sum	lin	-6.94	146	146	-0.12	
22	Tmax	mean	lin	-6.94	146	146	-0.12	
23	Tmax	max	lin	-6.94	146	146	-0.12	
24	Tmax	max	quad	-6.25	360	48	34.2	-0.5
25	Tmax	sum	quad	-5.98	94	93	1.17	-0.01
26	Tmax	mean	quad	-5.98	94	93	2.34	-0.04
27	Trange	max	quad	-5.83	191	11	5.3	-0.14
28	Tmin	sum	lin	-5.81	330	330	-0.1	
29	Tmin	mean	lin	-5.81	330	330	-0.1	
30	Tmin	max	lin	-5.81	330	330	-0.1	
31	Tmin	sum	log	-5.75	330	330	-1.37	
32	Tmin	mean	log	-5.75	330	330	-1.37	
33	Tmin	max	log	-5.75	330	330	-1.37	
34	Trange	max	log	-5.69	215	214	-1.15	
35	Trange	sum	quad	-4.85	94	94	0.48	-0.02

36	Trange	mean	quad	-4.85	94	94	0.48	-0.02
37	Trange	max	lin	-4.43	215	214	-0.09	
38	Trange	sum	log	-3.3	266	266	0.73	
39	Trange	mean	log	-3.3	266	266	0.73	
40	Trange	sum	lin	-2.84	330	330	0.07	
41	Trange	mean	lin	-2.84	330	330	0.07	

Tmin – Minimum daily temperature, **Tmax** – Maximum daily temperature, **Trange** – daily temperature range, **Rainfall** - total daily rainfall. Number of observations = 414 females. Number of individuals = 281 Best candidate model for each weather variable is indicated boldly.

Chapter 3

Breeding limits foraging time: evidence of interrupted foraging response from body mass variation in a tropical environment

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Keywords: Body reserves, breeding status, food availability, foraging unpredictability, reproduction, starvation-predation risk, tropical seasonal variation, tropical birds.

ABSTRACT

Birds should store body reserves if starvation risk is anticipated; this is known as an ‘interrupted foraging response’. If foraging remains unrestricted, however, body mass should remain low to limit the predation risk that gaining and carrying body reserves entails. In temperate environments mass gain in female birds during breeding is often attributed to egg formation and mass loss after incubation to flight adaptation or the effect of reproductive workload, rather than as a result of an adaptive interrupted foraging response to the limited foraging time or unpredictable foraging conditions that breeding demands. In tropical environments, foraging conditions vary more within the breeding season than in temperate environments, and so studies in tropical environments are more suited to decouple the potentially confounded effects of increase in body reserves versus egg formation on the body mass of breeding birds. In this study, we test whether breeding results in an interrupted foraging response in a tropical savannah system using body mass data collected over a 15-year period from female Common Bulbuls *Pycnonotus barbatus*. This species breeds both in the wet and dry season, despite fewer resources being available in the dry season. Breeding stage predicted female body mass: body mass peaked abruptly during incubation but was not closely associated with the egg-laying stage and declined during brood rearing. Breeding females were heavier in the dry season than in the wet season. In the dry season, heavier birds were more likely to incubate eggs or brood chicks. These observations suggest that increased body reserves are required to buffer the consequence of limited foraging time or impoverished foraging conditions, which may be most pronounced during incubation and in the dry season, respectively. Such mass increases are consistent with an interrupted foraging response, which may apply to temperate zone birds experiencing foraging restrictions during breeding.

INTRODUCTION

Foraging time in breeding birds may be restricted by breeding roles, such as territoriality, mate guarding, nest building, incubation and brood rearing, as well as the constraints of having to forage in a central place and deliver food to a fixed nest site. As a consequence, breeding birds may carry extra body reserves to reduce the risk of starving due to limited foraging time or unpredictable foraging conditions, even though this may increase predation risk (Lima 1986a; Gosler et al. 1995). This body mass gain strategy is called the 'interrupted foraging response' (Lima 1986a; Macleod and Gosler 2006): unpredictable foraging leads to increase mass reserves whether this is, for example, shorter day length in winter or shorter available time to forage during the breeding season.

The interrupted foraging response may then provide a simple universal explanation for body reserves carried by breeding birds beyond the egg laying stage in addition to the unavoidable additional mass that increased size of reproductive organs and egg production entail. For example, body mass gained during breeding has been shown to correlate positively with adult survival probabilities in tropical savannah birds (Cox and Cresswell 2014), which suggests that body reserves carried during breeding decrease starvation risk and increase survival likelihood when foraging opportunities become relatively unpredictable for breeding birds. However, body reserves in birds should reflect a trade-off between their costs and benefits (see Witter & Cuthill 1993). Hence, if starvation risk is low, body mass should remain relatively low even during breeding to limit predation risk (Macleod et al. 2005; Macleod et al. 2008).

Differences in body reserves carried between breeding stages should reflect relative starvation risk between these stages or how available foraging time is constrained by each stage. For example, in the Blue Petrel *Halobaena caerulea*, body mass before and after incubation bouts, and food availability at sea during foraging may predict incubation duration and foraging bouts, likelihood of temporary egg neglect, rate of body mass gain during foraging and overall breeding success (Chaurand and Weimerskirch 1994). Within a breeding attempt, foraging time may be most limited during incubation because incubating birds must spend a fixed amount of time on their eggs for them to develop and hatch as quickly as possible. Larger body reserves may then be particularly valuable if the foraging environment is relatively unpredictable during incubation because birds are less likely to obtain sufficient food in each foraging bout.

Body mass variation in breeding birds has received considerable attention in temperate environments compared with tropical ones. In temperate environments, body mass gain before incubation in female birds has been attributed to the increased size of reproductive organs (Vezina & Salvante 2010), while mass loss after or during incubation is attributed to the effect of reproductive workload or adaptation for better flight performance during nest provisioning (Merkle & Barclay 1996, Neto & Gosler 2010, Boyle, Winkler & Guglielmo 2012). Pre-incubation body mass gain due to increased size of reproductive organs is largely undisputed (see Vezina & Salvante 2010), but the reproductive stress and flight adaptation hypotheses have been tested using experimental food supplementation to temperate breeding birds (Moreno 1989, Merkle and Barclay 1996). However, body mass response to food supplementation in experimental set-ups may be uninformative if non-supplemented control breeding birds do not anticipate any foraging unpredictability. Experimental reduction of food may also be less practical in natural systems where birds move freely and can use other resources. An alternative approach, however, might be to model patterns of breeding body mass from long-term data of individual birds ringed within the same environment with clear resource seasonality for bird species with protracted breeding periods.

Such data are available in a tropical savannah breeding environment for the Common Bulbuls *Pycnonotus barbatus,* which can breed throughout the year (see also Cox *et al.* 2013). By using brood patch presence and development as indicators of breeding occurrence and stage, respectively (Redfern, 2008, Redfern 2010), we assessed how body mass varied within the breeding period and between environmental conditions. The different stages of brood patch development are closely linked to breeding stages in European passerines (Redfern 2010; Myers & Redfern 2011). We assume that savannah bird species show a similar progression of brood-patch development (Table S1). We expect that body reserves will increase with breeding stages that constrain foraging such as incubation but with an interaction with availability of resources. For the Common Bulbuls, the dry season in a tropical savannah is expected to be less favourable for breeding than the wet season because insect abundance is low, and food and water are less widely distributed (Ngozi Molokwu et al. 2008, Molokwu et al. 2010, Brandt and Cresswell 2008). The occurrence of moult may confound the effect of breeding on body mass variation (Gosler 1994) and, therefore, we also consider the possible confounding effects of moult here. We therefore expect that body reserves will offer a greater reproductive advantage under less favourable foraging conditions (Smith & Moore 2003), resulting in more pronounced body mass gain for breeding birds in the dry season in our study area. We, therefore, test two hypotheses:

1. Body mass within a breeding attempt varies in accordance with the level of foraging unpredictability at different breeding stages independently of mass gains due to egg formation. Foraging unpredictability is expected to peak during incubation.
2. Body mass during breeding varies seasonally with peaks in the dry season when foraging is expected to be most unpredictable.

METHODS

Study site

Our study was carried out in Amurum Forest Reserve (09°52’N, 08°58’E), at the A.P. Leventis Ornithological Research Institute (APLORI), on the Jos Plateau in Nigeria. The Amurum Forest Reserve consists of four main habitat types: a regenerating savannah woodland, riparian forests, rocky outcrops (inselbergs) and farmlands. Much of the land surrounding the reserve, like the reserve itself before 2001, is degraded by anthropogenic pressure from farming, bush fires and livestock grazing. There is a single wet and dry season per year lasting about six months each. Generally, the wet season spans from May to mid-October while the dry season spans from the end of October to April. Total monthly rainfall is above 150mm in the wet season except for May and October when it may be lower, while there is almost entirely no rainfall in the dry season. Temperatures vary across the year: temperatures are lowest (8 - 14 ºC) during the dry cold windy periods in December and January when relative humidity may be as low as 10% and also at the peak of the wet season in July and August; temperature maxima (28 - 32 ºC) are in March and April before the start of the wet season (Cox *et al.* 2011, Figure 2.S1).

Study species

The Common Bulbul is widespread throughout Africa. They are sexually monomorphic, usually 9 - 11 cm and weigh 25 - 50g. They have an annual survival probability of 0.67 ± 0.05 (Stevens *et al.* 2013), but some adults can live for up to 15 years in the wild. The Common Bulbul breeds in both the wet and the dry season in our study area with an average clutch size of two. Most breeding attempts fail due to nest predation. Nest building takes 3 - 5 days, egg laying 2 - 3 days, incubation 13 - 14 days, and nestlings leave the nest at 12 days after hatching. Breeding roles are shared by parents. Females build the nest while being guarded by the males. Females incubate the eggs while the male perches close by until both go foraging between incubation bouts. Males feed nestlings during the early nestling phase while females brood chicks. Later, females feed nestlings while males guard the nest from a nearby perch. Both parents continue to feed chicks after fledging and Bulbuls may remain in family groups for 12 weeks after the chicks have left the nest. Bulbuls moult almost exclusively in the wet season, although a few individuals may start before or at the end of the wet season depending on annual rainfall variability. Common Bulbuls are largely frugivorous, but nestlings are predominantly fed insects. Adult birds may also occasionally feed on insects, nectar and seeds. Fruits are available to Bulbuls year-round but from different fruit plants, which vary in fruiting phenology, so that food availability may be constrained by the distribution of a specific fruiting plant. Moreover, insect abundance peaks occur in the wet season while seeds are more readily available at the end of the wet season and start of the dry season.

Data collection and determination of variables

Birds were caught using mist nets from 06:00 to 10:30 hours between 2001 and 2015, inclusively. In the years 2001 and 2013, trapping was mostly concentrated at Constant Effort Sites (CES) during a CES ringing project, which takes place 5 times each year for six consecutive days each. During the CES, 272 meters of nets were used in total. In 2014 and 2015, in addition to the regular CES captures, trapping was carried out repeatedly throughout the entire year with variable lengths of mist nets in addition to the use of playback devices, depending on the trapping area covered.

We trapped 1,422 birds in total, and 271 individuals were trapped repeatedly a total of 673 times. From all birds trapped, there were 135 females with brood patches and 69 identified non-breeding female birds; 50 of these females were caught more than once but at least once with a brood patch. In total these 50 females were caught 147 times. Where possible, caught birds were aged and sexed by the presence of a gape and a brood patch, respectively.

Because only the sexes of breeding female Common Bulbuls are reliably determined by field observations, we considered only adult females for this study. Breeding status and stage were assessed on the basis of brood patch occurrence and development (Table 3.S1). In addition, we verified the relationship between brood patch development and breeding stages in female Common Bulbuls that were caught raising young or incubating eggs. Also note that Common Bulbul females incubating, or brooding chicks have a brood patch score of 3 while those feeding chicks may have a brood patch score of 3 or 4, depending on whether females are still brooding. We are confident that only female Common Bulbuls have brood patches and so birds can be sexed reliably based on brood patch occurrence; however, we verified the sexes of birds with brood patch score of 5 from our molecular sexing data because males undergoing moult may be erroneously scored as having a brood patch of 5.

For each trapped bird, we recorded wing length (± 1mm), moult status, pectoral muscle score, fat score, brood patch score and body mass (± 0.1g, Ohaus Scout). Moult was scored as present or absent: an individual actively replacing main wing or tail feathers was scored 1; otherwise, it was scored as 0 (Svensson 1991, Redfern and Clark, 2001). Birds were labelled as caught during the wet or dry season, based on the usual precipitation in the period when they were caught. The 16th of October to the 30th of April was considered to be dry season, while the 1st of May to the 15th of October was considered the wet season.

Statistical analyses

To test the hypothesis that breeding birds vary body mass in accordance with the level of foraging unpredictability at different breeding stages but controlling for the timing of mass gains due to egg formation, we built a General Linear Mixed Model (GLMM) with body mass as a response variable and brood patch score as a predictor. We controlled for the effect of body size and seasonal variability by including wing length and season of capture in the model, respectively. We also controlled for inter-annual and individual variability between capture years and individuals, respectively, by including year of capture and individual identity in the model as random factors. Individual identity did not significantly improve overall model fit (contribute any additional variance) and so was dropped from the final model.

To test the hypothesis that breeding body mass depends on environmental conditions during breeding, we built a General Linear Mixed Effect Model (GLMM) fitted by Restricted Maximum Likelihood (REML). Only incubating and chick rearing females differed in body mass from non-breeding birds (see results below), thus only these categories were considered as breeding birds for between season comparisons of body mass. Variation in body mass was predicted by season and breeding status. We controlled for the effect of body size and moult on body mass by including wing length and moult status as predictor variables in the model. The effect of moult status on body mass variation was not significant; hence, it was dropped from the final model. We accounted for individual variability and inter-annual variation between capture years by including individual identity and year of capture in the model as random variables.

We relied on actual body mass as a proxy for body reserves because visible fat reserves are rarely observed in tropical savannah birds and pectoral muscle scores mostly vary between scores of 2 and 3. However, we are confident that body mass corrected for size is a reliable measure of relative body reserves size, even in tropical birds based on previous observations of mass gain in intra-African migrants (Nwaogu and Cresswell 2016). Prior to fitting the minimal adequate model, all GLMMs were fitted by Maximum Likelihood (ML) rather than Restricted Maximum Likelihood REML to allow comparison of models with different fixed factors using Akaike Information Criteria (AIC). Normality and homogeneity of variance were tested using the Shapiro–Wilk normality test and residual plots, respectively. The final models were then fitted with REML, which gives a better fit for models with random effects, because we were interested in modelling body mass variation at the individual level. All analyses were carried out in R version 3.1.0 (http://cran.r-project.org/package=nlme, R, Development Core Team 2006).

RESULTS

Body mass variation at different stages of breeding

Body mass was significantly higher in incubating and brood rearing (Brood patch score 3, 4 & 5) compared to non-breeding females (Figure 3.1, Table 3.1). During incubation, breeding birds were 8.1% heavier than non-breeders after controlling for body size, and inter-annual and individual variation (Table 3.1). Body mass peaked during incubation but decreased during the brood-rearing stages (Figure 3.1). The observed pattern is consistent with the assumed available foraging time at the different breeding stages (Table 3.S1). Body mass during egg laying (Brood patch score 2) was low and this did not differ significantly from the non-breeding or post incubation stages (Figure 3.1). Heavier females were more likely to incubate eggs and brood chicks, but this effect was more pronounced in the dry season (Figure 3.2).

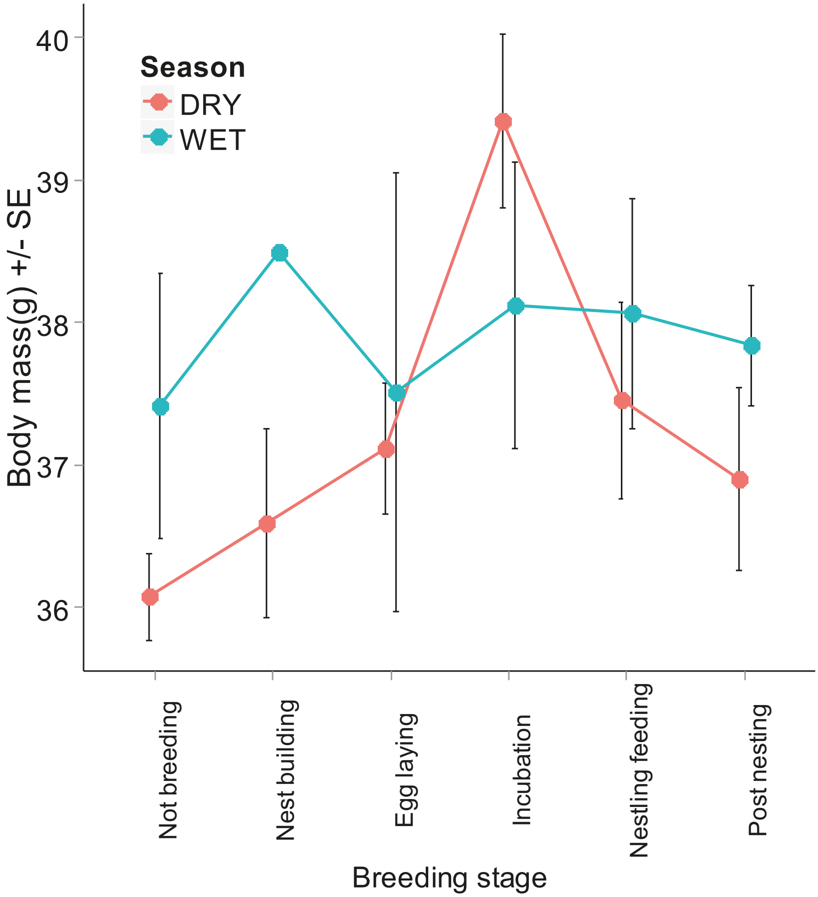

Figure 3.1. Variation in body mass of breeding female Common Bulbuls *Pycnonotus barbatus* at different breeding stages in a tropical savannah environment (determined by brood patch score, see Table 3.S1), means ± SE calculated from raw data used in Linear Mixed-effect Model (Table 3.1). Breeding females were heaviest when incubating but lost body mass as brood rearing progressed.

Table 3.1. Variation in body mass of female Common Bulbuls P*ycnonotus barbatus* between breeding stages in a tropical savannah environment. Variation in body mass was modelled by Linear Mixed-effects Model fit by Restricted Maximum Likelihood. Body mass in breeding females was compared to females with brood patch score of ‘0’ (non-breeding females), set at the intercept in the model. Including season of capture did not improve model fit (AIC 929.55 *Vs* 929.57, L. Ratio = 1.98, p=0.16). Shapiro-Wilk normality test: W = 0.99, p-value = 0.4. Brood patch scores are explained in Table 3.S1.

Parameters	**Estimate**	**Error**	***df***	***t***	***p***
Intercept	36.27	0.31	177	116.8	**<0.001**
Wing length	0.24	0.07	177	3.4	**0.001**
Brood patch score 1	0.60	0.79	177	0.8	0.45
Brood patch score 2	0.76	0.51	177	1.5	0.13
Brood patch score 3	2.94	0.61	177	4.8	**<0.001**
Brood patch score 4	1.81	0.68	177	2.7	**0.01**
Brood patch score 5	1.44	0.52	177	2.8	**0.01**

Random effects: Year (N=15); overall sample size = 196; sample size per brood patch score: 0 = 69, 1 = 12, 2 = 40, 3 =23, 4 = 18, 5 = 37. Significant p–values are given in bold.

Body mass variation when breeding in different seasons

Difference in body mass between breeding (incubating and chick rearing) and non-breeding female birds differed significantly during the dry season (Figure 3.3, Table 3.2). During the dry season, breeding birds were on average 5.7% heavier than non-breeding birds, after controlling for body size and inter-annual and individual variation (Table 3.2). Breeding birds were also on average 2.6% heavier during the dry season than in the wet season. However, in the wet season, breeding and non-breeding females did not differ in body mass (Figure 3.3).

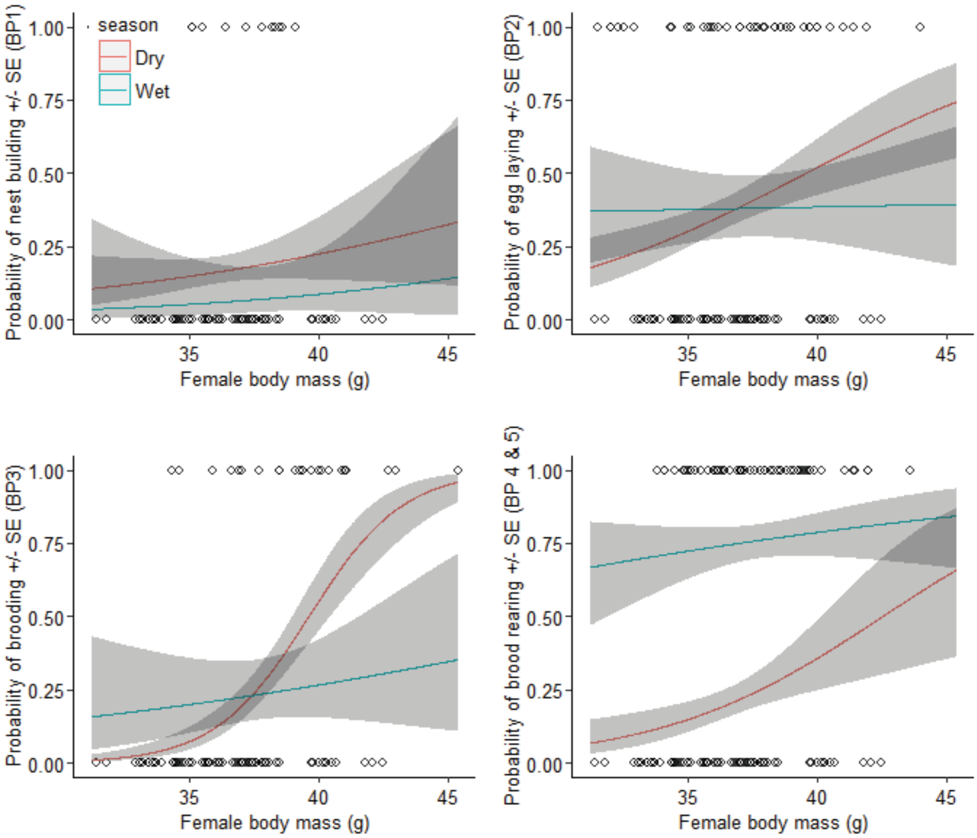

Figure 3.2. In the dry season, there is a high probability that only heavy female Common Bulbuls *Pycnonontus barbatus* are able to incubate eggs or brood chicks. The occurrence of brood patch stages (BP, see Table 3.S1) in female Common Bulbuls predicted by body mass using a logistic regression from a Generalised Linear Model.

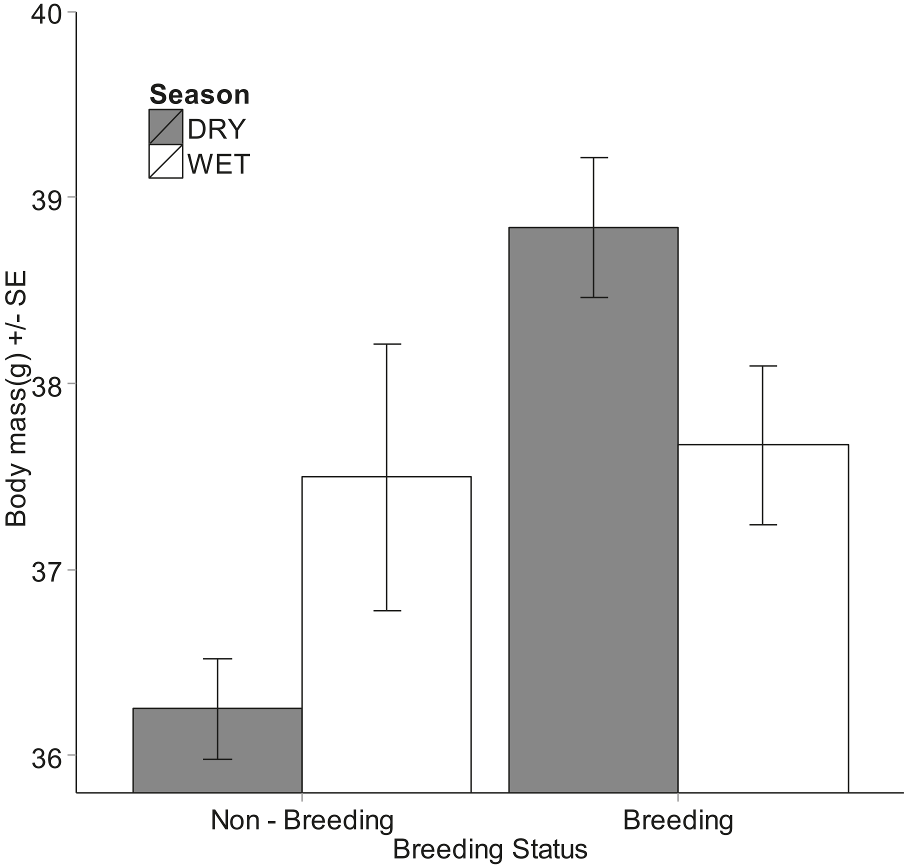

Figure 3.3. Incubating and brood-rearing female Common Bulbuls *Pycnonotus barbatus* were heavier when breeding during the dry season in a tropical savannah environment. Mean body mass ± SE calculated from raw data used in Linear Mixed-effect Model (Table 3.2).

Table 3.2. Seasonal difference in body mass of breeding and non-breeding female Common Bulbuls *Pycnonotus barbatus*, modelled by linear mixed-effects model fit by Restricted Maximum Likelihood. Breeding birds and the dry season were set at the intercept in the model. Shapiro-Wilk normality test: W = 0.99, p-value = 0.5.

Parameters	**Estimate**	**Error**	***df***	***t***	***p***
Intercept	36.30	0.32	93	112.4	**<0.001**
wing length	0.27	0.09	93	3.0	**<0.01**
Season (Wet)	0.59	0.51	93	1.1	0.25
Breeding status (breeding)	2.37	0.45	93	5.3	**<0.001**
Season: Breeding status	-1.54	0.68	93	-2.3	**0.03**

Random effects: Year and individual identity; number of individuals = 50, overall number of observations = 147. Significant p–values are given in bold.

DISCUSSION

Body mass variation in female Common bulbuls was consistent with an interrupted foraging response (Macleod and Gosler 2006). We showed that females maintained body reserves according to the likely availability of foraging time in different breeding stages (Table 3.S1) and with seasonally dependent foraging conditions. Body mass increase was more pronounced during incubation and in the dry season, when foraging time is most limited and less food may be encountered, respectively. In the dry season, only heavy females were likely to incubate eggs or brood chicks (Figure 3.2).

Body mass variation at different stages of breeding

Our results were consistent with the first hypothesis that female birds will maintain a higher body mass during incubation because time available for foraging is most restricted during incubation. Carrying body reserves may be adaptive allowing females to timetable breeding activities as a priority rather than foraging. For example, body reserves may function to keep on/off bouts minimal and sustain longer incubation duration, thus reducing nest visibility to predators by reducing activity around the nest (Basso & Richner 2015). As a consequence, the survival probabilities of incubating birds and nests may be higher because they are less likely to be predated upon (Lima 1986b, Macleod & Gosler 2006, MacLeod *et al.* 2007).

Less pronounced differences between breeding stages in the wet season (Figure 3.1) may result from confounding moult effects (Gosler 1994) or variation in food availability within the wet season. Moulting is largely restricted to the wet season and moulting birds may carry larger body reserves (Gosler 1994). Fluctuations in food availability peaks within the wet season due to inter-annual variation in rainfall pattern may also cause birds to vary body reserves accordingly depending on individual strategies (Cresswell 1998; Babbitt & Frederick 2008; Durant, Hjermann & Handrich 2013).

We did not find any suggestion that body mass variation was due to egg mass or increased size of reproductive organs as previously suggested for temperate birds (Redfern, 2010), which is consistent with our first hypothesis. Our results (Table 3.1) show clearly that the body mass of breeding female Common Bulbuls was significantly higher than non-breeding females only at the onset of incubation. This suggests that while egg-laying females may be as light as non-breeding ones, incubating or chick-rearing females (well after the egg-laying period) are not. It is unclear if the body mass variation observed during the egg phase in the wet season (Figure 3.1) is (partly) due to fluctuating reproductive organ mass. The effect of mass gain due to egg or reproductive organs will likely depend on the timing of weighing, and so body mass is expected to be highly variable during the egg phase (Vezina and Williams 2003) for different individuals weighed before or after laying. Moreover, variation in body mass associated with reproductive organs may also depend on the length of the laying period and the likelihood of successive broods.

In other species male cooperation may compliment a female's foraging effort and allow more efficient foraging within restricted time (Afton 1979; Chaurand & Weimerskirch 1994; Curlee & Beissinger 1995). Males accompany females to forage during bouts between incubation, so this may improve vigilance or search efficiency for females or both. But the role of male Bulbuls is not clear: they mate-guard females during nest building, incubation and brood rearing, and they also assist with nestling feeding, especially when females still brood nestlings, and so we might expect males to also have foraging time constraints.

Females continue brooding chicks after hatching when males feed chicks. This behaviour may be sustained by the body reserves carried during incubation, and females may lose these reserves rapidly before they take over feeding nestlings. This brooding behaviour in the absence of provisioning from their mate may explain body mass loss after incubation (Figure 3.1). By brooding nestlings and losing stored body reserves, females may adapt their body mass for better flight performance during nest provisioning. This post-incubation loss of mass is similar to patterns observed in other species (Moreno 1989; Merkle and Barclay 1996; Neto & Gosler 2010). Thus, our observations suggest that the reproductive stress and flight adaptation hypotheses are not mutually exclusive and probably explain why there is no clear experimental support for each. Moreover, in temperate environments, foraging conditions during breeding may not be sufficiently unpredictable to drive significant changes in body mass between experimentally supplemented and controlled birds in normal years.

Body mass variation when breeding in different seasons

As predicted by the second hypothesis, body mass increases for incubating and brood-rearing female Common Bulbuls were more pronounced in the dry season probably due to higher starvation risk imposed by fewer foraging opportunities (Figure 3.3, Table 3.2). We were able to rule out two potentially confounding factors. There was no indication from the seasonal population body mass pattern (Figure 3.S2) that predation risk varies seasonally in our study area. A difference in predation risk between the wet and the dry season should lead to a difference in population body mass average between seasons depending on the anti-predation strategy employed (see Lima 1986; Gosler, Greenwood & Perrins 1995), which we did not find. Furthermore, Common Bulbuls moult almost entirely in the wet season (Figure 3.S2) so that any increases in body mass during moulting should not be confounded with the observed body mass gain in the dry season.

The difference in food availability between the wet and the dry season is a fundamental assumption in this study. We believe this assumption to be reasonable because it is based on empirical observations from previous studies in the same study area (Molokwu *et al.* 2008, Molokwu *et al.* 2010, Brandt & Cresswell 2008). Furthermore, insect abundance is generally lower in the dry season as in other seasonal tropical environments (Karr 1976, Frith & Frith 1985, Arun & Vijayan 2004, Silva, Frizzas & Oliveira 2011) so that parents may work harder to raise young when they breed during the dry season. Moreover, differences in precipitation (Figure 3.S1) influence productivity in tropical savannah environments and for a largely frugivorous bird like the Common Bulbul, fruits are obviously less readily available in the dry season (Ting, Hartley & Burns 2008, Williams & Middleton 2008).

Our observation of higher body mass during incubation and in the dry season supports observations of higher body mass gain during breeding in species with higher survival probabilities (Cox & Cresswell 2014). Adult survival and, thus, repeat breeding may be the main determinants of fitness in the Common Bulbul because only 1 in 10 nests survive predation on average (*pers. obs.*). Accordingly, breeding pairs make repeated attempts to breed. In this case, selection should favour traits that enhance adult survival during breeding, therefore increasing the likelihood of future reproduction because there is a high chance that any breeding attempt will turn out unsuccessful. For example, incubating Blue Petrels *Halobaena caerulea* were found to temporarily neglect eggs once body mass fell below a threshold (Chaurand & Weimerskirch 1994). Body mass regulation during breeding may help birds manage less favourable foraging conditions in the dry season and still invest in self-maintaining processes.

GENERAL CONCLUSIONS

Intra-individual analyses of females breeding in both the wet and dry season are crucial for understanding whether this behaviour is consistent or flexible in individuals, depending on immediate foraging conditions or trade-offs that may result from other factors. We observed some level of consistency in a few individuals with breeding records between seasons but lack sufficient observations to test this; moreover, breeding records were generally few and repeated individual captures are stochastic.

Understanding body mass variation during breeding as a response to limited foraging time may provide a better appreciation of how birds manage breeding costs, depending on environmental conditions and life-history challenges. Our observations suggest that body reserves are required to buffer the consequence of restricted foraging opportunities during breeding, which may be most pronounced during incubation and in the dry season. Such mass increases during breeding are consistent with an interrupted foraging response, which also seems likely to apply to temperate birds experiencing similarly high restrictions on foraging time. This study, using capture-mark-recapture data from a West African Savannah environment also highlights the relevance of long-term bird ringing operations. It brings to light the added value of tropical seasonality, protracted breeding periods and brood patch scoring to understanding animal ecology (Redfern, 2008, Redfern, 2010).

SUPPLEMENTARY INFORMATION

Table 3.S1. Breeding status and stage was assessed based on the presence and development of brood patches, respectively. Expected sensitivity to flight performance and available foraging time are scored as low, moderate and high across breeding stages, based on flight requirements and restriction to foraging, respectively.

Patch score	Patch development	Breeding stage	Sensitivity to Flight performance	Available foraging time
0	No brood patch	Not breeding	Moderate	High
1	De-feathering breast and belly region	Nest building	High	Moderate
2	Vascularisation of breast and belly region/ loose skin	Egg laying	Moderate	Moderate
3	Moderate and marked oedema/ breast muscle obscure and swollen.	Incubation/hatching/ chick brooding	Low	Low
4	No oedema/ skin stretched with marked wrinkles.	Nestling feeding/ fledging	High	Low
5	Re-feathering (white feathers and pins)/ tighter skin.	“Post nesting phase”	Moderate	Moderate

Scoring of brood patches were carried out using a six-stage scoring system according to Redfern (2008, 2010) with confirmation from field observations on the Common Bulbul *Pycnonotus barbatus.*

Table 3.S2. Pairwise comparison of body mass between breeding stages, based on brood patch occurrence in female Common Bulbuls P*ycnonotus barbatus* in a tropical savannah environment. Variation in body mass was modelled by Linear Mixed-effects Model fit by Restricted Maximum Likelihood (Table 3.1), followed by a multi-comparison of breeding stages using Tukey test from the Multcomp package.

Parameters	Estimate	Error	z	*p*
Brood patch score 1 - Brood patch score 0	0.60	0.79	0.8	0.97
Brood patch score 2 - Brood patch score 0	0.76	0.51	1.5	0.65
Brood patch score 3 - Brood patch score 0	2.94	0.61	4.8	**<0.001**
Brood patch score 4 - Brood patch score 0	1.81	0.68	2.7	0.08
Brood patch score 5- Brood patch score 0	1.44	0.52	2.8	0.06
Brood patch score 2 - Brood patch score 1	0.16	0.83	0.2	1.00
Brood patch score 3 - Brood patch score 1	2.34	0.90	2.6	0.09
Brood patch score 4 - Brood patch score 1	1.21	0.94	1.3	0.78
Brood patch score 5 - Brood patch score 1	0.84	0.84	1.0	0.91
Brood patch score 3 - Brood patch score 2	2.18	0.66	3.3	**0.01**
Brood patch score 4 - Brood patch score 2	1.05	0.73	1.4	0.69
Brood patch score 5 - Brood patch score 2	0.68	0.58	1.2	0.84
Brood patch score 4 - Brood patch score 3	-1.13	0.80	-1.4	0.71
Brood patch score 5 - Brood patch score 3	-1.50	0.67	-2.2	0.21
Brood patch score 5 - Brood patch score 4	-0.37	0.73	-0.5	1.00

Random effects: Year (N=15); overall sample size = 196; sample size per brood patch score: 0 = 69, 1 = 12, 2 = 40, 3 =23, 4 = 18, 5 = 37. Significant p–values are given in bold.

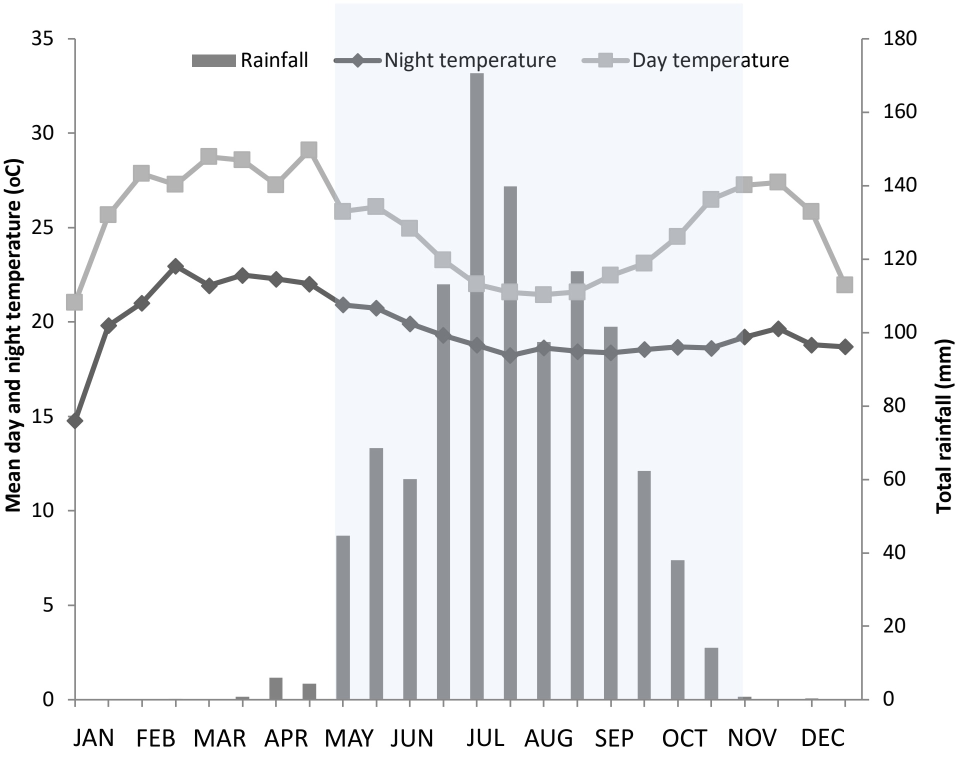

Figure 3.S1. Variation in rainfall and temperature across the annual cycle of the Common Bulbul *Pycnonotus barbatus* in a tropical Savannah environment, calculated from local weather data collected between 2012 and 2015 in the Amurum Forest Reserve, north-central Nigeria. Means per 2-week intervals. Blue shading indicates the wet season.

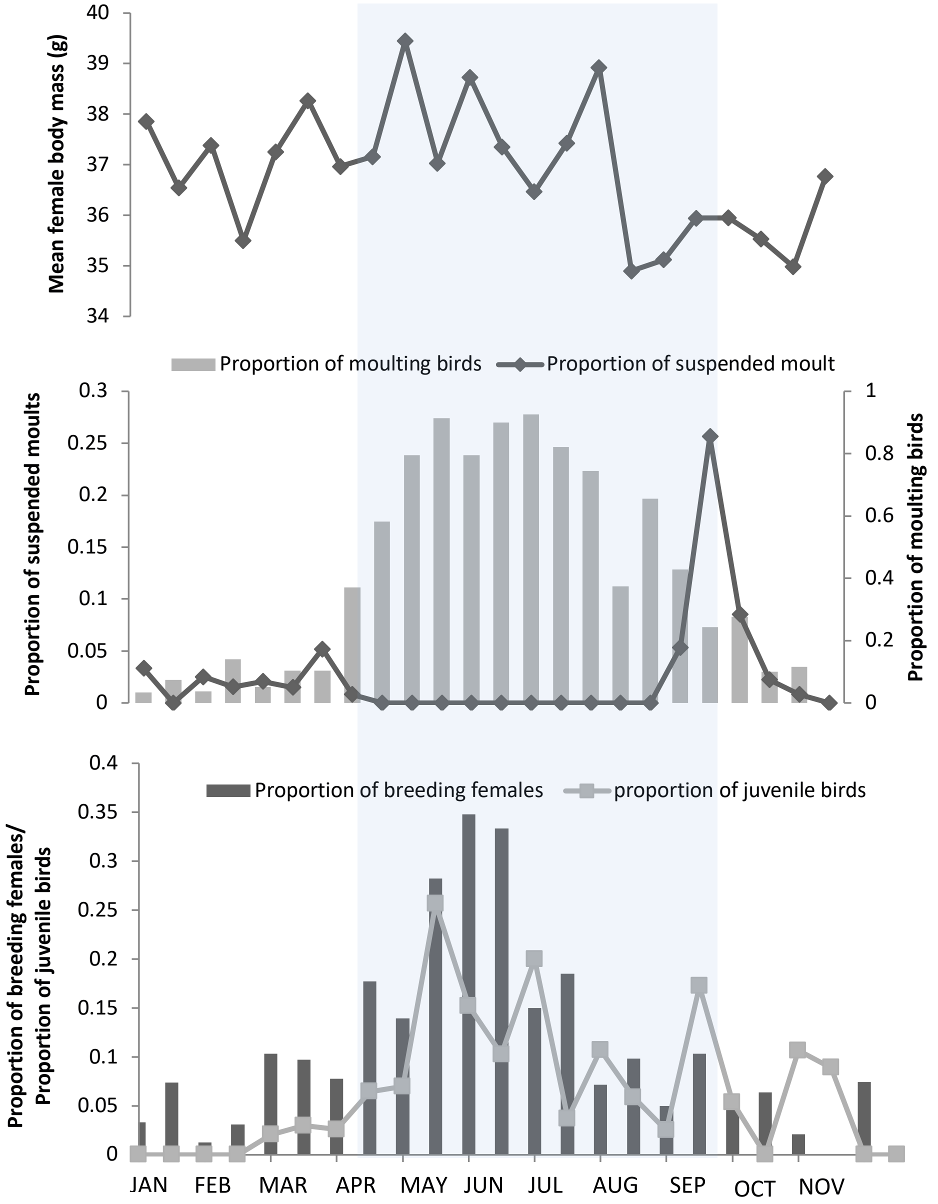

Figure 3.S2. Annual cycle processes in the Common Bulbul *Pycnonotus barbatus* in a tropical savannah environment. Top panel – mean monthly body mass of female Common Bulbuls across the year. Middle panel – mean proportion of suspended moults and proportion of moulting birds per 2-week interval. Bottom panel – mean proportion of breeding females based on occurrence of brood patches and proportion of juvenile birds in total captures per 2-week interval. All data were collected between 2001 and 2015 in the Amurum Forest Reserve, North central Nigeria. The wet season is indicated by the shaded area

Part II

Relationship between environmental seasonality and innate immune function

Chapter 4

Seasonal differences in baseline innate immune function are better explained by environment than annual cycle stage in a year-round breeding tropical songbird.

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Keywords: animal physiology, bird, ecological immunology, environmental change, individual variability, rainfall, seasonality and wild animals.

Abstract

Seasonal variation in innate immunity is often attributed to either temporal environmental variation or to life history trade-offs that arise from specific annual cycle stages but decoupling them is difficult in natural populations. Here, we effectively decouple seasonal environmental variation from annual cycle stage effects by exploiting cross-seasonal breeding and moult in the tropical Common Bulbul *Pycnonotus barbatus.* We test how annual cycle stage interacts with a key seasonal environmental variable, rainfall, to determine immunity at population and individual level. If immune challenge varies with precipitation, we might expect immune function to be higher in the wet season due to increased environmental productivity. If breeding or moult imposes resource constraints on birds, depending on or independent of precipitation, we might expect lower immune indices during breeding or moult. We sampled blood from 818 birds in four annual cycle stage categories: breeding, moult, simultaneous breeding and moulting, or neither. We quantified indices of innate immunity (haptoglobin, nitric oxide (NO_x) and ovotransferrin concentrations, and haemagglutination and haemolysis titres) over two annual cycles of wet and dry seasons. Environment (but not annual cycle stage or interactions between both) explained variation in all immune indices, except NO_x. NO_x concentration differed between annual cycle stages but not between seasons. However, within the wet season, haptoglobin, NO_x, ovotransferrin and haemolysis differed significantly between breeding and non-breeding females. Aside from some recorded inconsistences, population level results were largely similar to results within individuals that were measured repeatedly. Unexpectedly, most immune indices were higher in the dry season and during breeding. Higher immune indices may be explained if fewer or poorer quality resources force birds to increase social contact, thereby exposing individuals to novel antigens and increased infection risk, independently of environmental productivity. Breeding birds may also show higher immunity if less immune-competent and/or infected females omit breeding. We conclude that seasonal environmental variation impacts immunity more directly in natural animal populations than via resource trade-offs. In addition, immune indices were more often variable within than among individuals, but some indices are characteristic of individuals, and so may offer selective advantages if heritable.

INTRODUCTION

Immune function varies seasonally (Nelson and Demas 1996, Dopico et al. 2015) and this is often attributed to either variation in environment (Horrocks et al. 2011b, Tieleman 2018b) or to life history trade-offs that are associated with resource allocation to competing annual cycle stages (Sheldon and Verhulst 1996, Knowles et al. 2009). However, because annual cycle stages often co-vary with environmental factors, seasonal differences in immune function may reflect response to seasonal environmental variation, annual cycle stage or both. The difficulty in decoupling environmental effects (food, diet, vectors or antigenic properties) from life history related effects (resource cost of annual cycle stages or associated effects) sets limitations to interpreting immune variation on the basis of life history trade-offs in natural populations (Ricklefs and Wikelski 2002, Schmid-Hempel 2003, Pedersen and Babayan 2011). Interpreting immune variation on the basis of life history variation at the population level only (Tieleman et al. 2005; Hegemann et al. 2012b) may be problematic if individuals vary consistently in response to environment and/or life history challenges (Ardia 2005) due to differences in personality or quality (Araya-Ajoy et al. 2018). Individuals may vary in exposure or tolerance to infection (Loehle 1995, Lloyd-Smith et al. 2005, Bansal et al. 2007), and in resource acquisition and allocation to immunity and other competing traits (van Noordwijk and de Jong 1986, van de Pol and Wright 2009). These differences may cause relationships between immune function and competing life history traits within individuals to be obscured at the population level by among individual variation (Williams et al. 1999; Merrill et al. 2015). Therefore, understanding variation in immunity on the basis of life history trade-offs requires an ecological context (Tieleman 2018b) where variation in both environment and individual animals are considered using carefully selected immune indices (Norris and Evans 2000).

Seasonally arid tropical environments where resident birds breed and moult across different seasons are suitable for decoupling the effect of seasonal environmental variation from that of annual cycle stages on immune function. In Nigeria, there is a single wet and dry season annually (Figure 1.2), and environmental factors differ between and within these seasons. Food is usually more abundant in the wet season (Ngozi Molokwu et al. 2008; Nwaogu et al. 2017) but pathogens and disease vectors should also be more abundant in the wet season due to high environmental productivity (Young et al. 1993; Pascual et al. 2002). However, social contact may increase with less food and water during the dry season because animals cluster around fewer resources, and this may facilitate disease transmission (Vander Waal et al. 2017), even if pathogen abundance is low. Furthermore, the effect of rainfall on environmental productivity may vary between seasons. For example, while the onset of the wet season is rapid, and the effects of precipitation are quickly felt, the rains do not end as abruptly, and hence the commencement of the dry season, and associated drying out of the environment, is much more prolonged. Thus, conditions within the wet season should be less variable than the dry season. Nonetheless, the resident Common bulbuls *Pycnonotus barbatus* are capable of breeding year-round (Cox et al. 2013, Nwaogu et al. 2018a) and are long-lived (Stevens et al. 2013) in central Nigeria. These characteristics make them ideal for decoupling the effects of environment and annual cycle stage on immune function. Some individuals moult into the dry season or overlap moult with breeding (Nwaogu et al. 2018a), and this allows the effect of moult on immune function to be considered between seasons and breeding states. Residency allows repeat sampling of individuals between seasons and annual cycle stages. Consequently, temporal elevation of resource demands due to the occurrence of breeding and moult (Murphy 1996; Sanz et al. 2004) become effectively decoupled from seasonal environmental variation, allowing life history and environment effects to be decoupled at both population and individual levels.

Nonetheless, despite the large body of literature in ecological immunology, interpreting variation in immune indices is still ambiguous (Matson et al. 2006 Buehler et al. 2011, Versteegh et al. 2012). Depending on the immune index (Demas et al. 2011, Boughton et al. 2011), high values may indicate a well-protected animal capable of destroying invading agents, or a prior poorly protected and now highly challenged animal. From an 'operative protection' point of view, high levels of immune indices should indicate increased investment proportionate to current or perceived infection (Horrocks et al. 2011b), or trade-offs due to increased investment in competing processes within the immune system (Martin et al. 2006a; McDade et al. 2016). Indices such as haemolytic or bacteria killing capacity of blood or plasma reflect ability to destroy foreign cells (Tieleman et al. 2005, Matson et al. 2005), and thus a relative measure of an individual's ability to clear infection. But biomarkers of inflammatory response may have relative interpretations: haptoglobin is a positive acute phase protein which normally circulates in low concentration but increases with inflammation (Jain et al. 2011, Matson et al. 2012, van de Crommenacker et al. 2010); but see Hegemann et al. 2013b). Ovotransferrin on the other hand, is a negative acute phase protein. Both ovotransferrin and haptoglobin increase with inflammation because they bind to and remove haem from circulation during infection, so that haem is unavailable as nutrient to pathogens (Horrocks et al. 2011a). But concentrations of ovotransferrin may decrease with increased inflammation because temporarily high free hormones may bind to ovotransferrin, and other acute phase proteins may be produced at the expense of ovotransferrin by the liver (Gruys et al. 2005, Jain et al. 2011, Giansanti et al. 2012). NOx, modulates inflammatory processes but also participates in the direct killing of parasites and tumor cells (Sild and Hõrak 2009). Overall, combining measures of haemolytic and natural antibody activity with multiple biomarkers of inflammatory response should give a robust assessment of constitutive immune function (Adamo 2004).

In this study we test the main and interactive explanatory power of seasonal environmental variation (i.e. occurrence of rainfall) and annual cycle stage on seasonal immune variation. We separate male and female bulbuls into annual cycle stages based on breeding and moult occurrence and test differences in baseline innate immunity between the wet and dry season and within the wet season at population and individual levels. Specifically, we test: i) the effects of seasonal environmental variation and annual cycle stage on variation in immune function of females and males at the population level. ii) The main and interactive effects of breeding and moult on immune function of females in the wet season (see justification for excluding males and dry season records in method section (below)). iii) Within individuals, the effect of seasonal environmental variation on immune function of non-breeding and non-moulting birds, and the separate effects of breeding and moult on immune function within the wet season. We expect immune indices to be higher in the wet season compared to the dry season due to higher environmental productivity, but we expect the occurrence of breeding and/or moult to lower immune function. However, differences between the wet and the dry season should remain consistent for different annual cycle stages in both sexes, if seasonal environmental variation is a more crucial determinant of seasonal variation in immune function. Otherwise, differences between annual cycle stages should be consistent between the wet and dry season at both population and individual levels. But, if both factors are important, we might expect interactions between them. Under such interaction scenarios, we predict that: i) immune indices should be lowest for breeding or moulting birds in the dry season and highest for non-breeding or non-moulting birds in the wet season. ii) Breeding and moulting females should have lower immune indices compared to non-breeders and non-moulters, especially when breeding and moult overlap. iii) Patterns within individuals, should be similar to patterns at the population level and repeatability should be low, if the effect of seasonal environmental variation and/or annual cycle stage leads to larger variations within than among individuals.

METHODS

Birds, blood sampling and determination of variables

Field work was carried out at the Amurum Forest Reserve (09°52’N, 08°58’E) which is located at the A.P. Leventis Ornithological Research Institute (APLORI) on the Jos Plateau in north central Nigeria (see supplementary information for more details on field site). All field work was approved by the APLORI scientific committee. We collected 818 blood samples from 530 individuals over 256 days between 29th January 2014 and 5th February 2016 (mean=3.2, SD=2.8, min=1, max=16 bulbuls/day, Figure S2). All birds were caught using mist nets between 6:00 and 11:00 hours. On average, birds were bled 7.9 ± 4.3 minutes after capture (Range 2 – 15 mins). Samples were stored on ice in the field until processing in the laboratory to separate plasma from cellular fractions. Plasma was separated and stored frozen at -20° C until immune assays were carried out.

For each bird, we assessed breeding status on the basis of brood patch occurrence (Redfern 2010), and moult status on the basis of feather quality and occurrence of moulting primary wing feathers. Only females incubate eggs, therefore only females could be classified as breeding or not. Since bulbuls are sexually monomorphic, we sexed all birds using gel electrophoresis. DNA extractions followed methods described by Richardson et al. (2001) and amplification was done using the P2/P8 primers (Griffiths et al. 1996).

Immune assays

(i) *Haptoglobin concentration*

We quantified plasma haptoglobin concentration using a functional colorimetric assay which quantifies the haeme-binding capacity of plasma. We followed instructions for the ‘manual method’ provided with a commercially available assay kit (Cat. No.: TP801; Tridelta Development Ltd, Maynooth, Co. Kildere, Ireland). We used 7.5 µl of plasma and standard curves in respective wells. A five-step serial dilution (2.5, 1.25, 0.625, 0.312, 0.156 and 0.08

mg/ml) of haptoglobin standard was used as the standard curve concentrations. We randomised samples before assays. We calculated within-assay variability (n=20 plates, maximum CV = 0.88, minimum CV = 0.31, mean CV = 0.49) and among-assay variability (n=807 samples, CV = 0.58) to verify consistency.

(ii) *Nitric oxide concentration*

We measured nitric oxide concentration by a colorimetric assay described by Sild and Hõrak (2009). This estimates the concentration of nitrate and nitrite in plasma after reducing all nitrate to nitrite using copper-coated cadmium granules. A measurable colour development proportionate to nitric oxide concentration follows reaction with Griess reagent. We used 10 µl of plasma for the nitrate reduction step. A five-step serial dilution (100, 50, 25, 12.5, 6.25, 3.13 and 1.6 µM) of nitrate standard was used as the standard curve concentrations. We randomised samples before assays. We calculated within-assay variability (n=17 plates, maximum CV = 0.94, minimum CV = 0.48, mean CV = 0.70) and among-assay variability (n=667 samples, CV = 0.73) to verify consistency.

(iii) *Ovotransferrin concentration*

Ovotransferrin was quantified by estimating the maximum amount of iron required to saturate all ovotransferrin in a sample. We used methods described by Horrocks et al. (2011); following a three step process involving: saturation of ovotransferrin with ferric iron under alkaline conditions, reduction of excess unbound iron by ascorbic acid, then dissociation of ovotransferrin-iron complex under acidic conditions, leading to colour development whose absorbance is measured by colorimetry. We used 10 µl of plasma and standard conalbumin concentrations (20, 16, 10, 4, 2 and 1 mg/ml) for test and standard curves, respectively. We randomised and analysed each sample in duplicate. We further calculated within-assay variability (n=48 plates, maximum CV = 1.18, minimum CV = 0.21, mean CV = 0.70) and among-assay variability (n=652 samples, CV = 0.53) to verify consistency.

All colorimetric assays (i – iii above) were carried out using the Versamax plate reader (Molecular Devices Sunnyvale, California, US).

(iv) *Haemagglutination/haemolysis titres*

We assessed natural antibody-mediated haemagglutination and complement-mediated haemolysis titres of plasma samples against 1% rabbit red blood cells (Envigo RMS (UK) Ltd.) in phosphate buffered saline as described by Matson et al. (2005). 25 µl of plasma was used as the starting concentration, and subsequent concentrations were obtained by a further 10-step serial dilution of 25 µl plasma in 25 µl of phosphate buffered saline. 25 µl of 1% rabbit red blood cells was incubated in each well. Both haemagglutination and haemolysis titres were recorded as the number of serial dilution steps in which each function was still observable (i.e 1:2 is 2 while 1:1024 is 11) (Matson et al. 2005). We randomised samples before assays. We calculated within-assay variability (n=255 plates, haemagglutination: maximum CV = 1.41, minimum CV = 0, mean CV = 0.43; haemolysis: maximum CV = 2.23, minimum CV = 0, mean CV = 1.48) and among-assay variability (n=801 samples, haemagglutination: CV = 0.51; haemolysis: CV = 2.38) to ensure consistency.

Statistics

We analysed each index separately because immune indices were uncorrelated or at most weakly correlated with each other (All $r^2 < 5\%$), and a principal component analysis did not substantially reduce the number of variables (i.e. the five measured indices clustered into three principal components: PC1 (24%) - NO_x & haemolysis; PC2 (25%) - ovotransferrin and haemaggluttination, and PC3 (21%) - haptoglobin, NOx, ovotransferrin & haemagglutination). First, we tested the effects of environment and annual cycle stage on baseline innate immune function at the population level. We built general linear models for haptoglobin, NO_x, ovotranferrin and haemagglitination, and a generalised linear model for haemolysis. We included season and annual cycle stage, and their interaction as predictor variables. We ran separate analyses for the two sexes because breeding status was only reliably determined for females. We grouped females into breeding (B), moulting (M), and neither (N), and excluded individuals that were breeding and moulting simultaneously (BM) because only 3 records were obtained for this group in the dry season (against 38 in the wet season). We grouped males into non-moulting (NM) and moulting (M). For population level analyses, we used the first capture of an individual if it was caught more than once, to prevent pseudoreplication. Capture date - assigned as number of days from the first day of each season was included in each model to account for seasonal variation within the wet and dry seasons. 1st April was assigned the first day of the wet season while 1st November was assigned the first day of the dry season annually. Body mass was included in all models to account for the possible effect of individual body condition on immune indices. Haemolysis titre was modelled as a binary outcome throughout the study (0 or 1) because this was mostly zero or relatively low for most birds. Because samples were analysed in two batches (one for each year), we included ‘batch’ as a fixed factor in all models. Significant effects of batch may result from natural differences between years and/or differences between the analyses (e.g. reagents, kits).

Secondly, we tested the effects of breeding and moult, and their interaction, on immune function within the wet season for females only. Again, we excluded repeat captures, and included capture date and body mass in all models. We built general linear models for haptoglobin, NO_x, ovotransferrin and haemagglutination, and a generalised linear model for haemolysis.

Finally, we tested whether the population level results occurred in the same way within individual birds, using individuals with multiple sampling records only. We built general linear mixed models for haptoglobin, NO_x, ovotranferrin and haemagglutination, and a generalised linear mixed model for haemolysis, including individual identity as random factor in each model. We modelled within individual variation in each immune index using different data subsets, depending on availability, testing differences between seasons, breeding and moult states, keeping analyses separate for the sexes. To test differences between seasons, we only considered females that were neither breeding nor moulting, and males that were not moulting. To test differences between breeding and non-breeding state and moulting and non-moulting state, we considered individuals caught between these annual cycle stages within the wet season only. Further, we estimated individual repeatability using the package ‘rptR’ (Stoffel et al. 2017), including batch, season, breeding and moult as covariates in each model.

All models were reduced by a stepwise backward elimination, except for the repeatability models in which we retained season, annual cycle stage and batch even if they were not significant. However, we included the non-significant outputs of all eliminated variables in our summary tables to show their performance in each model. We performed posthoc tests implemented by the package 'lsmeans' (Lenth 2016) to determine between group differences where interaction between predictor variables were significant. All statistical analyses were performed in R 3.4.4.

RESULTS

Overall, seasonal differences in immune function were more often explained by season than annual cycle stage (ACS) for all indices, except NOx concentration. The effect of annual cycle stages was largely only visible within the wet season. Aside some inconsistences, results at the population-level were largely to those within individuals that were sampled repeatedly.

Population level differences in immune function

(i) *Between breeding and moult states in the wet and dry seasons*
In female bulbuls, ovotransferrin concentration, and haemolysis titre differed significantly between seasons (Figure 4.1). The effect of season on haptoglobin concentration was influenced by date (Table 4.1): haptoglobin concentration was always higher in the dry than the wet season (Figure 4.1A, post hoc test on dry season – wet season while controlling for date t=4.0, P<0.01), but increased with date through the dry season and remained low through the wet season (Figure 4.S3). The haemagglutination titer did not differ significantly between seasons (Figure 4.1D, Table 4.1). Haptoglobin and ovotransferrin concentration and haemagglutination and haemolysis titre did not differ between annual cycle stages (Table 4.1). In contrast, variation in NO_x concentration was explained by an interaction between season and annual cycle stage (Table 4.1, Figure 4.1B) - NO_x concentration was only significantly higher for breeding females in the wet season (Posthoc test: B – M (t=3.2, P<0.01), B – N (t=2.0, P=0.1), M – N (t=-2.1, P=0.08)), but did not differ between annual cycle stages in the dry season (Posthoc tests: all P>0.94). Body mass did not explain variation in immune indices (Table 4.1).

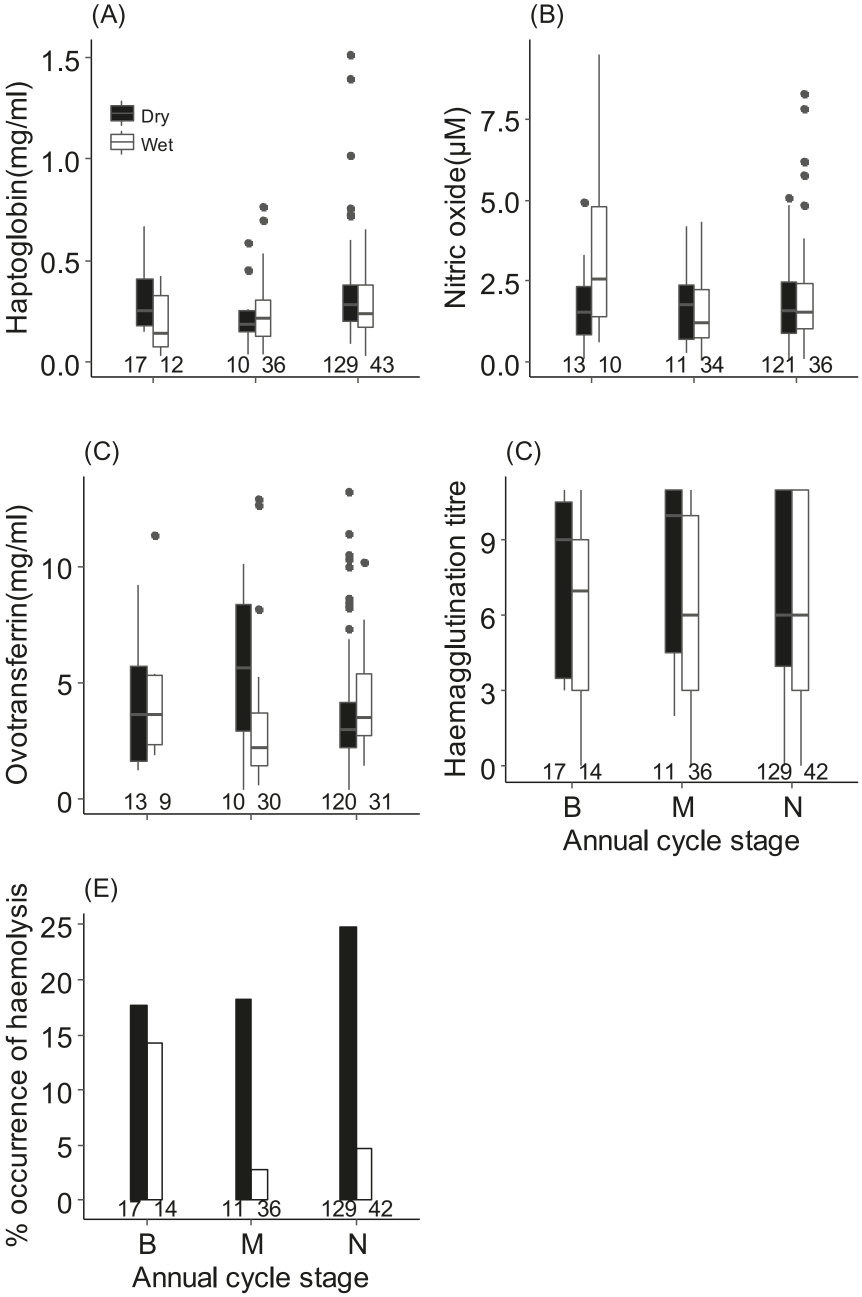

Figure 4.1: Seasonal differences in (A) haptoglobin, (B) nitric oxide, (C) ovotransferrin, (D) haemagglutination titre and (E) % occurrence of haemolysis between annual cycle stages in female common bulbuls, sampled over two annual cycles in Nigeria. Annual cycle stages were determined based on occurrence of brood patch and feather quality: B – breeding; M – moulting and N – non- breeding and non-moulting. Sample sizes are indicated below each box/bar.

Male bulbuls had significantly higher haemagglutination and haemolysis titres in the dry season, and like females, these indices did not differ between annual cycle stages (Figure 4.2,

Table 4.1). Similar to females, the effect of season on haptoglobin concentration was influenced by date (Table 4.1): haptoglobin concentration was higher in the dry season than the wet season (Figure 4.2A, Posthoc test on dry– wet season while controlling for date $t=4.0$, $P<0.01$), but increased significantly through the dry season and remained low throughout the wet season (Figure 4.S4). Similarly, the effect of season on NO_x concentration was influenced by date: the difference between seasons was only significant after controlling for date (post hoc test: dry season – wet season $t=2.7$, $P<0.01$). NO_x increased during the dry season but decreased during the wet season (Figure 4.S4). Unlike in females, NO_x was significantly higher for non-moulting compared to moulting males independent of season (Figure 4.2B, Table 4.1). Ovotransferrin concentration did not differ significantly between seasons and annual cycle stage in male bulbuls (Figure 4.2C, Table 4.1). Unlike females, haemagglutination increased significantly through the wet and dry season. Body mass did not explain variation in immune indices (Table 4.1).

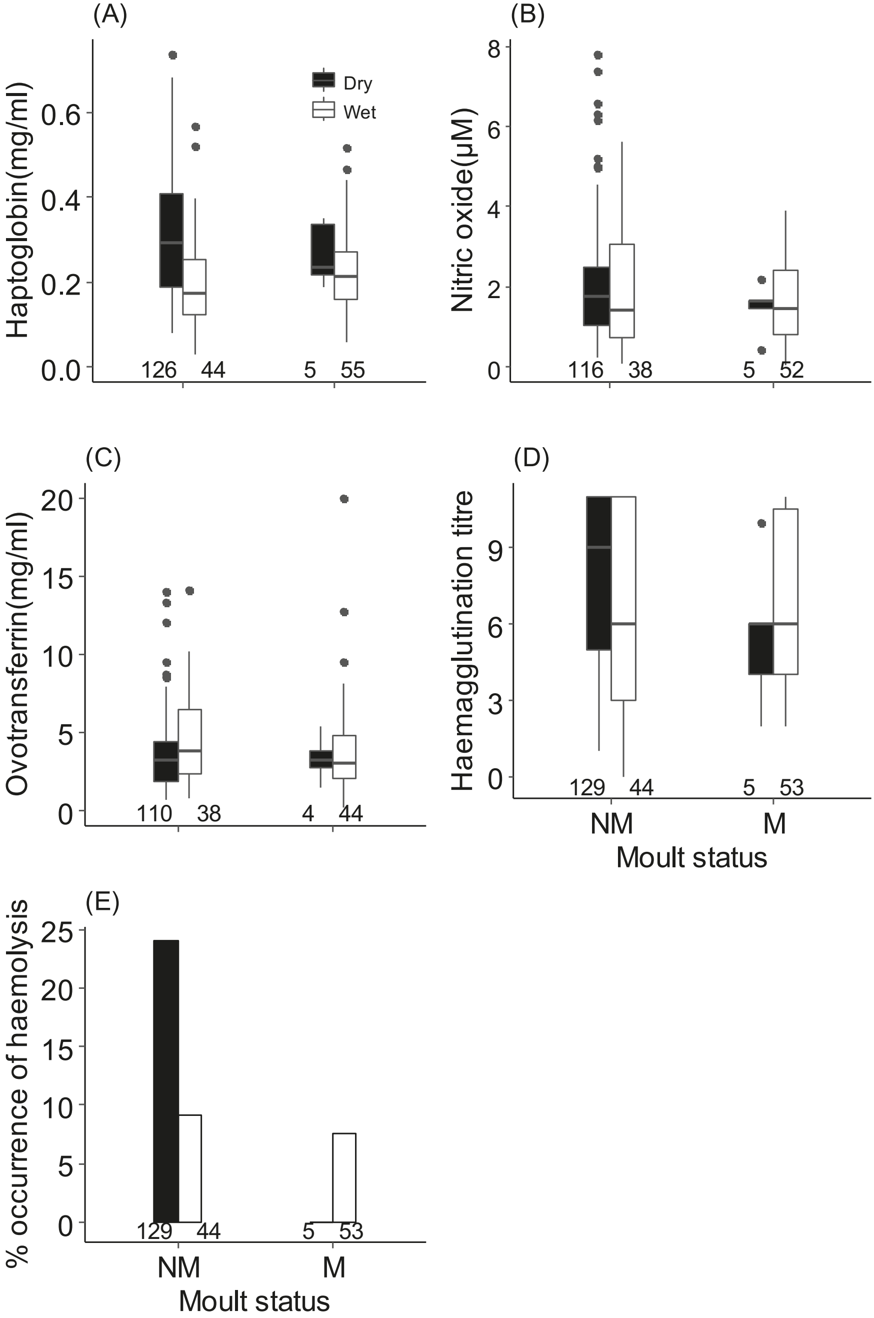

Figure 4.2: Seasonal differences in (A) haptoglobin, (B) nitric oxide, (C) ovotransferrin, (D) haemagglutination titre and (E) % occurrence of haemolysis between moulting and non-moulting male common bulbuls sampled over two annual cycles in Nigeria. Annual cycle stages were determined based on feather quality: NM – non-moulting and M – moulting. Sample sizes are indicated below each box/bar.

Table 4.1: Population level differences in immune indices between annual cycle stages (ACS) between the wet and dry season in female and male common bulbuls *Pycnonotus barbatus*. Birds were sampled over two annual cycles in Nigeria. Sexes were analysed separately. Annual cycle stages were determined based on occurrence of brood patch and feather quality: breeding, moulting and non- breeding and non-moulting birds. Individuals' sampled in both breeding and moult conditions were excluded from analyses. All individuals that were repeat sampled were also excluded. HP – haptoglogbin concentration, NO_x – nitric oxide concentration, OVO – ovotransferrin concentration, AGG – haemagglutination and Lysis – haemolysis.

			HP			NOx			OVO			AGG			Lysis		
	Factor	**Df**	**F**	**P**		**F**	**P**		**F**	**P**		**F**	**P**		**LRT**	**P**	
Females	**Batch**	1	7.46	0.01		0.79	0.38		145.37	<0.01		4.59	0.03		1.15	0.28	
	Body mass	1	3.06	0.08		0.02	0.89		0.55	0.46		0.43	0.51		1.02	0.31	
	Date	1							1.24	0.27		2.68	0.10		2.33	0.13	
	ACS	2	0.70	0.50			†		1.97	0.14		0.53	0.59		0.62	0.73	
	Season	1		†			†		**6.78**	**0.01**	**	1.77	0.18		**11.55**	**<0.01**	***
	Season:ACS	2	0.47	0.62		**3.05**	**0.04**	*	0.70	0.50		1.74	0.18		0.51	0.77	
	Season:Date	1	**15.96**	**<0.01**	***	**8.20**	**<0.01**	*	0.15	0.70		0.00	0.97		0.21	0.64	
Males	**Batch**	1	38.47	<0.01		0.03	0.86		82.80	<0.01		0.77	0.38		1.07	0.30	
	Body mass	1	2.42	0.12		0.04	0.85		0.01	0.91		2.64	0.11		0.01	0.94	
	Date	1							0.80	0.37		**16.92**	**<0.01**	***	0.67	0.41	
	Moult (M)	1	0.33	0.56		**4.41**	**0.04**	*	0.02	0.89		0.26	0.61		1.10	0.29	
	Season	1		†			†		0.13	0.71		**13.78**	**<0.01**	***	**6.81**	**0.01**	**
	M:Season	1	0.25	0.62		0.04	0.84		0.25	0.62		2.06	0.15		1.55	0.21	
	Season:Date	1	**15.69**	**<0.01**	***	7.19	0.01	**	2.25	0.14		0.04	0.85		0.03	0.86	

† Differences determined by posthoc tests due significant interaction between variables

(ii) *Between breeding and moult states in females within the wet season*

Within the wet season, haptoglobin concentration (Figure 4.3A) was significantly lower, while ovotransferrin concentration (Figure 4.3C) and haemolysis titre (Figure 4.3E) were significantly higher in breeding compared to non-breeding females. The moult state of female birds did not influence these immune indices (Table 4.2). The only exception was NO_x concentration (Figure 4.3B), where the interaction between breeding and moult was significant (Table 4.2). NO_x concentration was only significantly higher for breeding females in non-moulting state (Figure 4.3B, Posthoc (NM): NB – B ($t=-2.5$, $P<0.01$)) – during moult, breeding and non-breeding females did not differ in NO_x concentration (Figure 4.3B, Posthoc (M): NB – B ($t=0.1$, $P=0.89$)). Haemagglutination titre did not differ between breeding and non-breeding females in the wet season (Figure 4.3D, Table 4.2). Body mass did not explain variation in immune indices (Table 4.1).

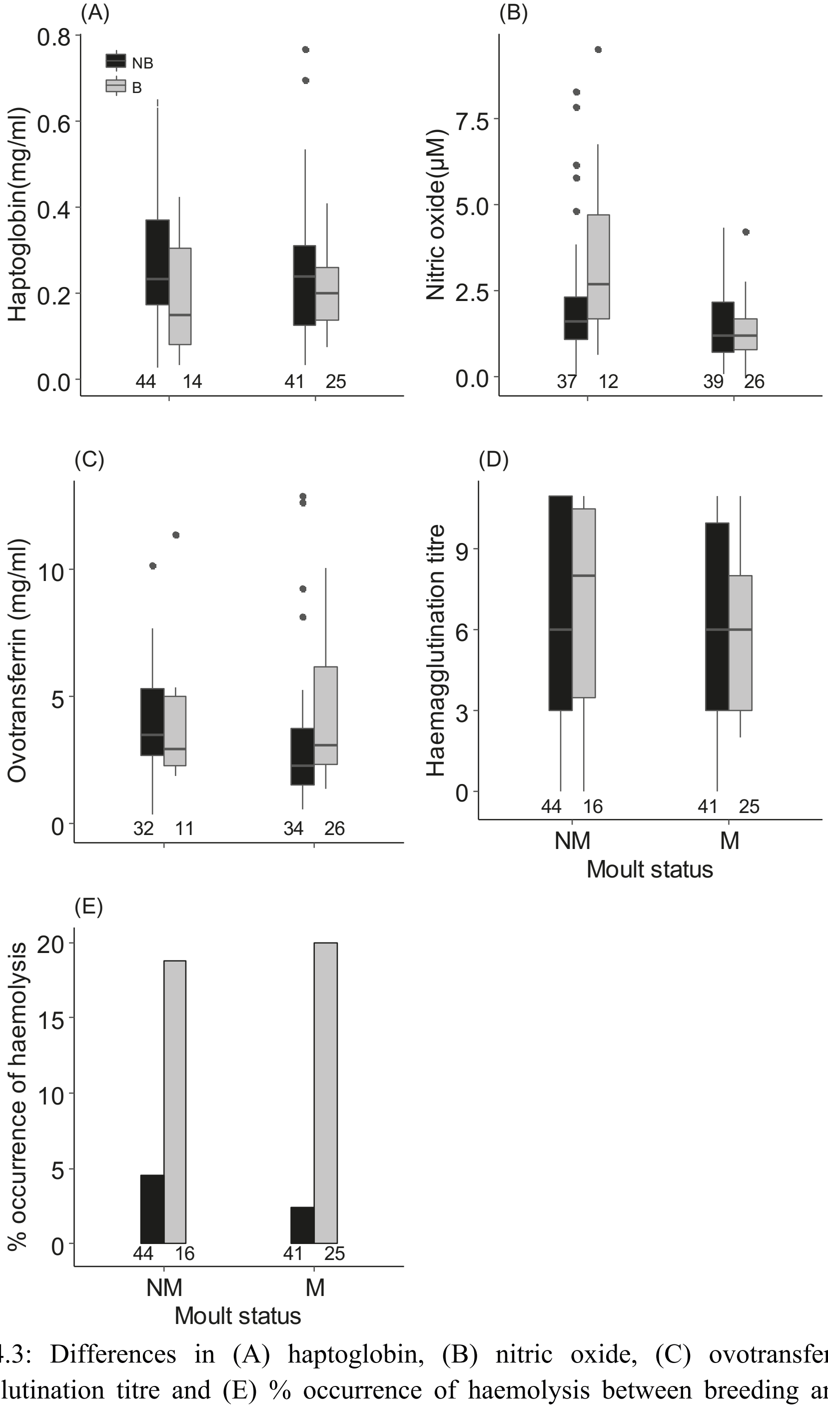

Figure 4.3: Differences in (A) haptoglobin, (B) nitric oxide, (C) ovotransferrin, (D) haemagglutination titre and (E) % occurrence of haemolysis between breeding and moult stages within the wet season in female common bulbuls sampled over two annual cycles in Nigeria. Annual cycle stages were determined based on occurrence of brood patch and feather quality: NM – non-moulting; M – moulting, B - breeding and NB - non-breeding. Sample sizes are indicated below each box/bar.

Table 4.2: Population level differences in immune indices between breeding and moult states in female common bulbuls *Pycnonotus barbatus* in the wet season. Bulbuls were sampled over two annual cycles in Nigeria. Breeding state was determined based on occurrence of brood patch and moult state based on feather quality. All individuals that were repeat sampled were also excluded. HP – haptoglogbin concentration, NO_x – nitric oxide concentration, OVO – ovotransferrin concentration, AGG – haemagglutination and Lysis – haemolysis.

		HP			NO_x			OVO			AGG		Lysis		
Factor	**Df**	**F**	**P**		**F**	**P**		**F**	**P**		**F**	**P**	**LRT**	**P**	
Batch	1	35.65	<0.01		2.17	0.14		39.63	<0.01		3.54	0.06	1.71	0.19	
Body mass	1	0.27	0.60		0.20	0.65		0.01	0.92		0.00	1.00	3.21	0.07	
Date	1	0.16	0.69		3.52	0.06		2.50	0.12		3.42	0.07	0.01	0.91	
Breeding(B)	1	**15.07**	**0.00**	***		†		**6.74**	**0.01**	*	0.28	0.60	**7.36**	**0.01**	**
Moult (M)	1	1.25	0.26			†		0.03	0.86		0.98	0.33	0.48	0.49	
B:M	1	0.35	0.56		**4.46**	**0.04**	*	0.08	0.77		2.25	0.14	0.68	0.41	

† Differences determined by posthoc tests due to significant interaction between variables.

Intra-individual level differences in immune function

(i) *Between seasons in non-breeding and non-moulting females, and non-moulting males*
Within individual females, haptoglobin concentration and haemolysis titre were significantly higher in the dry season, while NOx concentration and haemagglutination titre did not differ between seasons (Figure 4.4, Table 4.3A). In contrast with the population level, ovotransferrin concentration did not differ between seasons (Figure 4.4G, Table 4.3A).

Within individual males, haptoglobin concentration was significantly higher in the dry season, while NO_x and ovotransferrin concentration did not differ between seasons (Figure 4.5, Table 4.3A). Unlike at the population level, haemolysis titre did not differ significantly between seasons, but individuals still tend to have higher haemolysis titre in the dry season than in the wet season (Figure 4.5I, Table 4.3A).

(ii) *Between breeding and non-breeding states in the wet season*
Within individual females, NO_x concentration and haemolysis titre were significantly higher during breeding, while haemagglutination titre did not differ between breeding and non-breeding states (Figure 4.4, Table 4.3A). Unlike at the population level, haptoglobin and ovotransferrin concentration did not differ significantly between breeding and non-breeding states (Figures 4.4B & H, Table 4.3A).

(iii) *Between moulting and non-moulting states in the wet season*
Within individual females, NO_x concentration was significantly higher during moult, while haptoglobin and ovotransferrin concentrations, and haemaglutination and haemolysis titres did not differ between moulting and non-moulting states (Figure 4.4, Table 4.3A).

Within individual males, NO_x concentration was significantly higher during moult, while haptoglobin and ovotransferrin concentrations, and haemolysis titres did not differ between moulting and non-moulting states (Figure 4.5, Table 4.3A). Unlike at the population level, haemagglutination titre was significantly higher during moult (Figure 4.5H, Table 4.3A).

(iv) *Individual repeatability*
Individual repeatability in immune indices was low to modest (0.01 – 0.23) (Table 4.3B). Nonetheless, the repeatabilities for haptoglobin and NO_x concentrations were significant in females, while those of NO_x concentration and haemagglutination titre were significant in males (Table 4.3B).

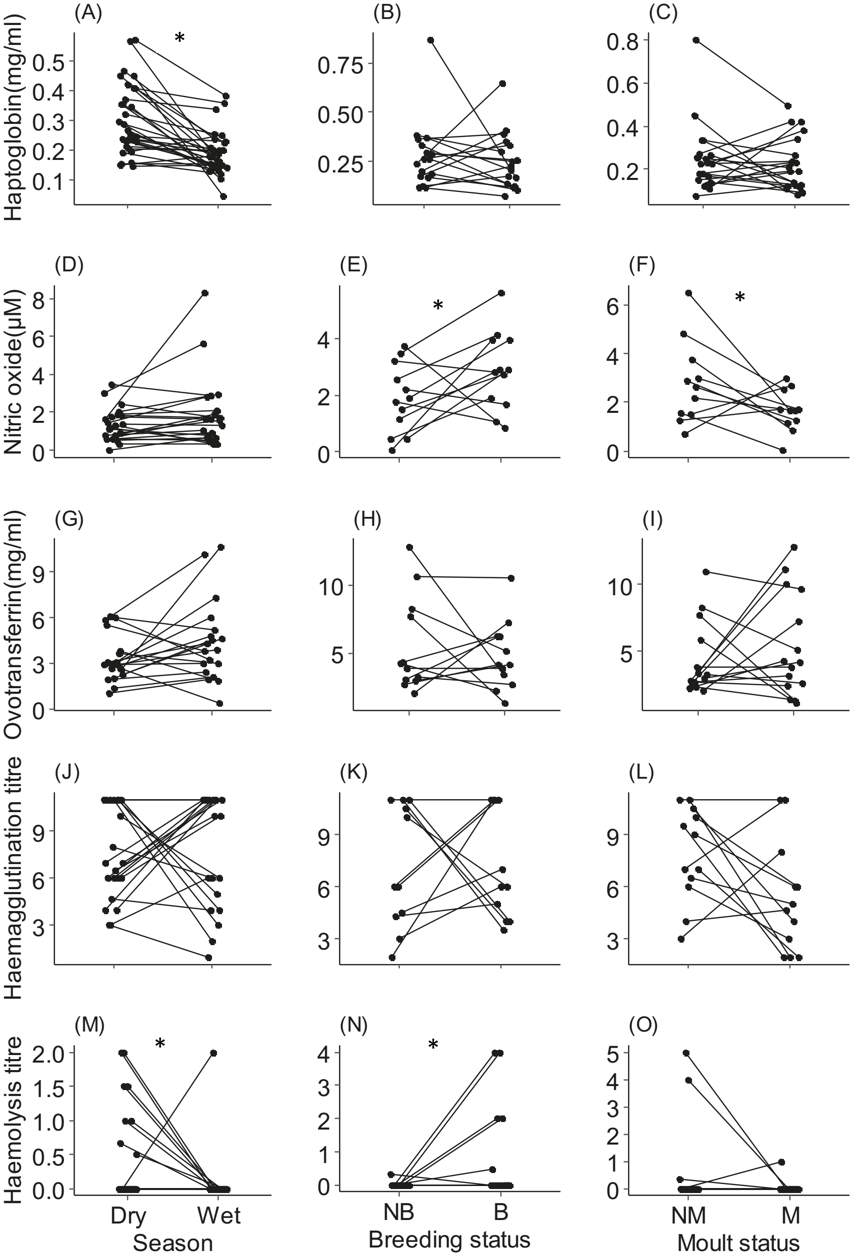

Figure 4.4: Within individual differences in (A-C) haptoglobin, (D-F) nitric oxide, (G-I) ovotransferrin, (J-L) haemagglutination titre and (M-O) haemolysis titre between wet and dry season, breeding and non-breeding, and moulting and non-moulting female common bulbuls. Breeding status was determined on the basis of brood patch occurrence: B - breeding and NB - non-breeding. Moult status was determined based on feather quality: NM – non-moulting and M – moulting. Immune indices with significant pairwise within individual difference are indicated by *.

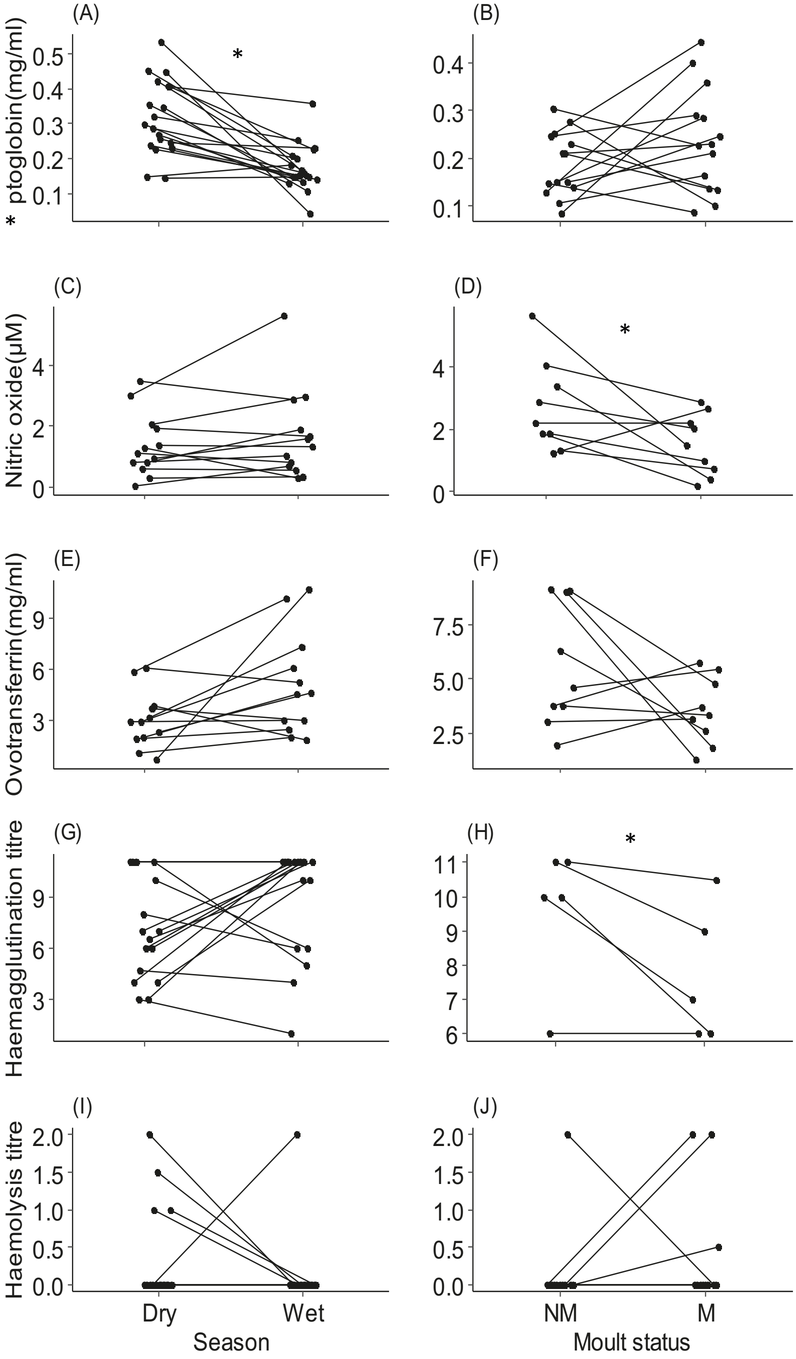

Figure 4.5: Within individual differences in (A-B) haptoglobin, (C-D) nitric oxide, (E-F) ovotransferrin, (G-H) haemagglutination titre and (I-J) haemolysis titre between wet and dry seasons, and moulting and non-moulting in male common bulbuls. Moult status was determined on the basis of feather quality: NM – non-moulting and M – moulting. Immune indices with significant pairwise within individual difference are indicated by *.

Table 4.3: A – Summarised results of pairwise within individual comparison of immune indices in male and female common bulbuls: seasonal differences were compared for non-moulting and non-breeding females, and for non-moulting males. Difference between breeding states was compared for females only in the wet season. Difference between moult states was compared for males and females in the wet season. Each factor was modelled separately using a linear mixed model on each immune index. **Factor**: Group for which within individual's comparison was carried out. **N**: samples size used for analysis - number of individuals with repeat measures and total number of measures from all individuals. **Chisq**: test statistics from glmm. **P**: significance of test statistics (significant P values are highlighted bold). B – Summarised results of individual repeatability in immune indices, estimated for each immune index and sex; controlling for season, breeding, moult state and batch effects. **N**: samples size used for analyses - number of individuals with repeat measures and total number of measures from all individual. **Rpt**: Individual repeatability estimate (significant repeatabilities are highlighted bold). **CI**: upper and lower confidence interval of repeatability estimates. Overall, difference between immune indices within individuals were consistent with differences at population level (Table 1 and 2). HP – haptoglogbin concentration, NO_x – nitric oxide concentration, OVO – ovotransferrin concentration, AGG – haemagglutination and Lysis – haemolysis.

(A)

		HP			**NO_x**			**OVO**			**AGG**			**Lysis**		
	Factor	**N**	**Chisq**	**P**	**N**	**Chisq**	**P**	**N**	**Chisq**	**P**	**N**	**Chisq**	**P**	**N**	**LRT**	**P**
Season	**Females**	31, 79	**7.06**	**0.01**	22, 54	1.02	0.31	19, 46	0.05	0.83	21, 56	0.08	0.78	28, 74	**3.87**	**0.04**
	Males	19, 48	**4.93**	**0.03**	13, 32	0.12	0.73	12, 30	0.08	0.77	16, 40	0.29	0.59	16, 40	0	0.97
Breeding	**Females**	17, 46	1.7	0.19	12, 32	**5.11**	**0.02**	12, 32	0.94	0.33	11, 30	0.04	0.83	16, 45	**7.63**	**0.01**
Moult	**Females**	20, 53	1.18	0.28	11, 31	**4.69**	**0.03**	15, 39	0.48	0.49	12, 34	3.16	0.08	19, 54	1.21	0.27
	Males	14, 36	0.03	0.87	9, 21	**6.25**	**0.01**	9, 21	0	0.98	5, 12	**5.67**	**0.02**	11, 26	1.24	0.26

(B)

	N	**Rpt**	**CI**	**N**	**Rpt**	**CI**	**N**	**Rpt**	**CI**	**N**	**Rpt**	**CI**	**N**	**Rpt**	**CI**
Females	82, 216	**0.12**	0, 0.29	65, 161	**0.17**	0, 0.37	62, 151	0.07	0, 0.29	77, 182	0.03	0, 0.22	77, 204	0.02	0, 0.18
Males	86, 216	0.01	0, 0.16	68, 164	**0.22**	0.04, 0.41	61, 147	0.07	0, 0.30	71, 183	**0.23**	0.04,0.41	84, 209	0.01	0, 0.17

Table 4.4: Summarised population and individual level results showing cells with significant P values shaded grey. WS - indicates analyses within the wet season only. HP – haptoglogbin concentration, NO_x – nitric oxide concentration, OVO – ovotransferrin concentration, AGG – haemagglutination and Lysis – haemolysis.

			HP	NOx	OVO	AGG	Lysis
		Factor	**P**	**P**	**P**	**P**	**P**
Population	**Females**	**ACS**	0.50		0.14	0.59	0.73
		Season			**<0.01**	0.18	**<0.01**
		Season:ACS	0.62	**0.04**	0.70	0.18	0.64
		Season:Date	**<0.01**	**<0.01**			
	Males	**Moult**	0.56	**0.03**	0.89	0.61	0.29
		Season		0.91	0.71	**<0.01**	**0.01**
		Moult:Season	0.83	0.86	0.62	0.85	0.21
		Season:Date	**<0.01**	**<0.01**			
	Females (WS)	**Breeding**	**<0.01**		**0.01**	0.6	**0.01**
		Moult	0.26		0.86	0.33	0.49
		Breeding:Moult	0.56	**0.04**	0.77	0.14	0.41
Individuals	**Female**	**Breeding (WS)**	0.19	**0.02**	0.33	0.83	**0.01**
		Moult (WS)	0.28	**0.03**	0.49	0.08	0.27
		Season	**0.01**	0.31	0.83	0.78	**0.04**
	Males	**Moult (WS)**	0.87	**0.01**	0.98	**0.02**	0.26
		Season	**0.03**	0.73	0.77	0.59	0.97

DISCUSSION

In a system that decouples seasonal environmental variation and annual cycle stage in a tropical songbird, we found that seasonal differences in immune function were more often explained by the environmental variation than annual cycle stage (Table 4.4). Immune indices were generally higher in the dry season than in the wet season, independent of annual cycle stage in both female and male birds. The only exception was NO_x concentration, which was higher for breeding females in the wet season only. We also found that within the wet season, breeding females were more likely to have higher immune indices than non-breeding females, except for haptoglobin concentration which was significantly higher for non-breeding females. Patterns of immune variation at the population-level were largely similar with patterns within individuals that were sampled repeatedly, except for a few inconsistences, suggesting that population level variation reflects similar within-individual variation and not simply among-individual variation in immune function due to personality or quality (see results summarised in Table 4.4). Overall, our results suggest that innate immunity depends largely on the environment because, except for NO_x, annual cycle stages were only significant within the wet season. Immune indices are often more variable within than among individuals as suggested by low repeatability, although some indices were still repeatable.

Our finding of higher immune indices in the dry season independent of annual cycle stage, is contrary to expectations based on lower infection risk with increased aridity (Horrocks et al. 2015, Tieleman et al. *in revision*) and lower investment in immunity under less optimal foraging conditions (French et al. 2007). We found no indication that immune investment in Common Bulbuls was constrained by the occurrence of annual cycle stages - an explanation given for winter immune enhancement in temperate animals (Nelson and Demas 1996). NO_x concentration was the only exception, and it is unlikely that its variation is due to competition for resources, because it was higher for breeding birds and lower for moulting birds. In seasonal environments like central Nigeria, one will predict that natural selection should shape immune function to counter predictable infection risks (Horrocks et al. 2011b), and there are indications that vector and water borne diseases are typically high during tropical wet seasons (Young et al. 1993, Pascual et al. 2002, Altizer et al. 2006). But only few studies have investigated variation in immune function in tropical environments (Tieleman et al. 2005, Lee et al. 2008; (Ndithia et al. 2017a) and none has done so over the entire annual cycle (Figure 4.S1). Our study is also unique in that it takes place in a clearly seasonally arid environment, representative of the west African Savannah – a wide spread habitat where multiple factors may vary between and within seasons (Furley 2006). Our results suggest that smaller scale local epidemiological factors such as seasonal habitat use, social interaction and diet shifts (Loehle 1995, Sah et al. 2018) may have stronger impact on immune variation and possibly infection risk than factors related to general environmental productivity, which has been suggested as a proxy for antigen abundance (Horrocks et al. 2015). In support of this idea, incidences of free range poultry diseases in Africa are more prevalent in the dry season when environmental conditions are presumably harsh, causing birds to range more widely and cluster around fewer resources (Nwanta et al. 2008, Miguel et al. 2013). This may apply to many free living tropical animal species that cluster around resources in times of scarcity (Altizer et al. 2006), and to Common

Bulbuls in particular, who we observe in large mixed species flocks around fruiting plants and water pools retained in gullies during the dry season (Brandt and Cresswell 2008). Clearly, there is need to assess seasonal differences in infection status in addition to immune indices, and to confirm whether enhancement of specific immune indices is in anticipation of, or response to, higher infection risk in the dry season.

Breeding and moult occurrence did not constrain investment in immune function as expected (Sheldon and Verhulst 1996) or alternatively, enhanced immunity did not prevent breeding (Williams et al. 1999). However, the co-occurrence of high haemolysis and NO_x concentrations coupled with lower haptoglobin concentrations in breeding compared with non-breeding females during the wet season (Figures 4.3A, B and E) suggest that immune function may be organised differently for breeding and non-breeding females. It could also mean that breeding females are more immunocompetent and/or less challenged than non-breeding ones, or that challenged females omit breeding. The latter is likely the case if we interpret higher haemolysis titre and NO_x concentrations as enhanced capacity to destroy pathogens, and lower haptoglobin concentration to reflect lower infection. Alternatively, lower haptoglobin concentration in breeding birds may indicate a trade-off between investing in breeding versus immune function (Råberg et al. 2000). But lower haptotoglobin concentration cannot be interpreted as an overall downregulation of immune function in breeding birds, because ovotransferrin and nitric oxide concentrations, and haemolysis titre were higher in breeding birds and haemagglutination titre was not affected by breeding state. Note however, that in a diet manipulation experiment on Common Bulbuls, we observed that low haemolysis titre and high haptoglobin concentration are associated with loss of body mass (Nwaogu et. al *in submission*), and breeding Common Bulbuls are more likely to be in better condition (Nwaogu et al. 2017), so high haemolysis titre and low haptoglobin concentration may indicate less challenge. Similarly incubating Common Eiders *Somateria millissima* in better body condition have higher NO_x concentration (Bourgeon et al. 2007). Perhaps, the immune system is re-organised depending on annual cycle stage rather than out-rightly down or up-regulated (Buehler et al. 2008).

Our observation that moult or its overlap with breeding (Moreno 2004, Sanz et al. 2004) did not explain variation in all measured immune indices (except for NO_x concentration) is largely consistent with the finding of Sandström et al. (2014) where baseline immunity was associated more with date than moult stage in barnacle geese *Branta leucopsis.* But our results contrast those of (Silverin et al. 1999) and (Buehler et al. 2008) who suggested that breaking of feather follicles through the skin creates infected wounds that elevate immune function during moult. Unlike Sandström et al. (2014) we considered the entire moulting period a fixed annual cycle stage, comparable to breeding, and the absence of breeding and moult (see Figure 4.1). We do not expect different outcomes from using moult stage as a fixed or continuous variable, because in Common Bulbuls, moult correlates strongly with sampling date (Nwaogu et al. 2018a), but except for haemagglutination in males, none of the measured immune indices correlated with sampling dates within the wet season when moulting largely takes place (Figure 4.S3). Haemagglutination titre increased with sampling dates in males in both the wet and dry season, so the cause of variation in haemagglutination is not obvious. Overall, the occurrence of breeding and moult did not coincide with lower immune indices within the wet season.

Nonetheless, we cannot conclude that there are no trade-offs between immune function and the occurrence of breeding or moult. Rather we propose that the effect of such trade-offs may depend on environmental conditions (Ardia 2005a), and this may not always lead to lower immune indices. Unfortunately, we could not statistically test the interactive effect of breeding and moult within the dry season because we sampled fewer birds breeding and moulting simultaneously. But plots of available data suggest that there are no differences in immune indices between annual cycle stages in the dry season (Figure 4.S2).

Population and individual level patterns were largely consistent and individual repeatabilities were low to modest. This similarity in trend between population and individual levels coupled with low individual repeatabilities for most immune indices imply that the impact of seasonal environmental variation and that of annual cycle stage within the wet season lead to larger variation within individuals than among individuals in the population (Lessells and Boag 1987). There is no suggestion that population level patterns are resultant of among individual variation in immune function. However, significant individual repeatabilities for haptoglobin and NOx concentrations in females, and NO_x concentration and haemagglutination titre in male Common Bulbuls, suggest that these indices are characteristic of individuals (Sild and Hõrak 2009, Matson et al. 2012, Versteegh et al. 2014). If in addition to being repeatable, they are also heritable, as with other physiological traits like basal metabolic rate (Tieleman et al. 2009), they should be acted upon by natural selection (Falconer et al. 1996), through the selective advantages they may offer individuals depending on environment.

Individual repeatability in some immune indices may account for some of the inconsistences observed between population and individual level patterns, because population level analyses were carried out among individuals. Hence, if individuals are repeatable there is a higher likelihood of finding population level differences even if such differences are not consistent within individuals, and the reverse may be the case if individuals are not repeatable. Nonetheless, some inconsistencies observed may still not be explained by individual repeatability. Patterns of variation in immune function are not always consistent across levels (Matson et al. 2006), and this may be the case between population and individuals. Moreover, immune indices were less variable within the wet season than the dry season (Figures 4.S3 and S4), so wet season values may represent individual baseline. Future studies should therefore, carefully select immune indices and sampling design depending on ecological question (Adamo 2004).

Nitric oxide concentration, varying between annual cycle stages but not between seasons, deviated from the overall pattern observed for other immune indices. Elsewhere, Ndithia et al. (*in submission*) also found higher NO_x concentrations in chick feeding red-capped *Calandrella cinerea* and rufous-naped larks *Mirafra africana* relative to non-breeding ones in Kenya. Baseline NO_x concentrations in bulbuls (0-8 µM) were lower than baseline values in greenfinches *Carduelis chloris* (29-110 µM) (Sild and Hõrak 2009) and red-capped larks (2-26 µM), but similar to those of rufous-naped larks (2-6 µM) (Ndithia et al. *in submission*). NO_x concentration was also repeatable in bulbuls (Rpt = 0.17 (females) and 0.23 (males)) as observed for greenfinches (Rpt=0.35) (Sild and Hõrak 2009). The reason why variation in NO_x concentration deviated from pattern observed for other immune indices in our study is not clear. NO_x is multifunctional, varying with physiological condition, health state, stress and work load

(Bogdan et al. 2000, Bourgeon et al. 2007), hence its concentration may be altered by other physiological processes related to breeding, moult or their interactions with the wet season.

CONCLUSION

By separating among from within individual variation in immune function in a natural study system where annual cycle stage is decoupled from seasonal environmental conditions, this study connects purely controlled laboratory experiments with population level field studies in ecological immunology (Pedersen and Babayan 2011). We show that in a natural population seasonal environmental variation offers better explanation for seasonal differences in immune function than the occurrence of annual cycle stages or their associated costs, because breeding and moult were only significant in explaining immune variation under specific environmental conditions (i.e within the wet season) and variation within individual was larger than among individuals for most indices. We provide evidence that variation in immune function within a single species in a single environment does not follow simple environmental productivity patterns, and this may also apply to disease risk. However, some inconsistencies between population and individual level patterns entail that caution is required when interpreting variation in immune function. A crucial next step is decomposing potential environmental effects into smaller measurable components such as diet, aridity, social contact, vectors and pathogen prevalence, using a combination of spatio-temporal field observations and experiments. We recommend that explanations of seasonal variation in immunity should incorporate epidemiologically relevant ecological factors that reflect infection risk to individual animals under specific environments, and not be restricted to the costs of immune function (Hegemann et al. 2012b; Hegemann et al. 2012a) or the overall productivity of environments (Horrocks et al. 2015).

SUPPLEMENTARY INFORMATION

Study species

Common bulbuls are 25 – 50g sexually monomorphic resident passerines that can be found across Africa. They lay a clutch of typically two eggs. Their nesting period lasts between 30 to 34 days if successful, but fledglings remain in family groups supported by parents for up to 12 weeks post fledging (Nwaogu *pers obs*). Moult is largely seasonal and takes place in the wet season for most individuals in the population but some birds extend moulting into the dry season (Nwaogu et al. 2018a). On average males commence moult 21 days earlier than females at the population level. Common bulbuls are largely frugivorous, but nestlings are predominantly fed insects and then fruits later. Adult birds also feed on insects, and occasionally on nectar or seeds. At the Amurum Forest Reserve in Nigeria, common bulbuls are territorial throughout the year but may move up to c. 2km to forage or drink from gullies in the dry season (Nwaogu *pers obs*).

Study site

The Amurum Forest Reserve (09°52’N, 08°58’E) is located at the A.P. Leventis Ornithological Research Institute on the Jos Plateau in north central Nigeria. It is a heterogeneous woodland savannah habitat with inselbergs and interspersed riparian forests, surrounded by farmlands and human settlements (Nwaogu and Cresswell 2015). It experiences a single wet and dry season annually. The wet season usually lasts from April to October (Figure 1.2). Total monthly rainfall in the wet season is usually over 200 mm, but may be less in April, May and October, creating an annually predictable unimodal temporal pattern in humidity (Figure 1.2). Minimum and maximum daily temperatures vary in a bimodal fashion due to increased cloud cover in the wet season and the movement of cold dry north-easterly trade winds from the Sahara to the Gulf of Guinea during the dry season between November and February. Overall, temperatures are lowest in the wet season between July and August and in the dry season between December and January. Daily temperature range is unimodal and lowest at the peak of the wet season around July and August (Figure 1.2).

Environmental factors such as food, water and diet vary both between and within seasons. This is due to a combination of environmental heterogeneity, plant phenology and distribution, and the lengthy stretch of single wet and dry seasons. In the Amurum Forest Reserve few gullies in riparian forest fragments retain water in the dry season (Brandt and Cresswell 2008), and this usually serve as watering holes for bulbuls and other wildlife. Similarly, some plants flower and fruit during the dry season or in off peak periods of the year, attracting large numbers of insects and birds. Consequently, the number of animals around resources may vary according to the density, distribution or phenology of plants around the reserve, and may lead to a temporal shift in social contact (Nwaogu *pers obs*).

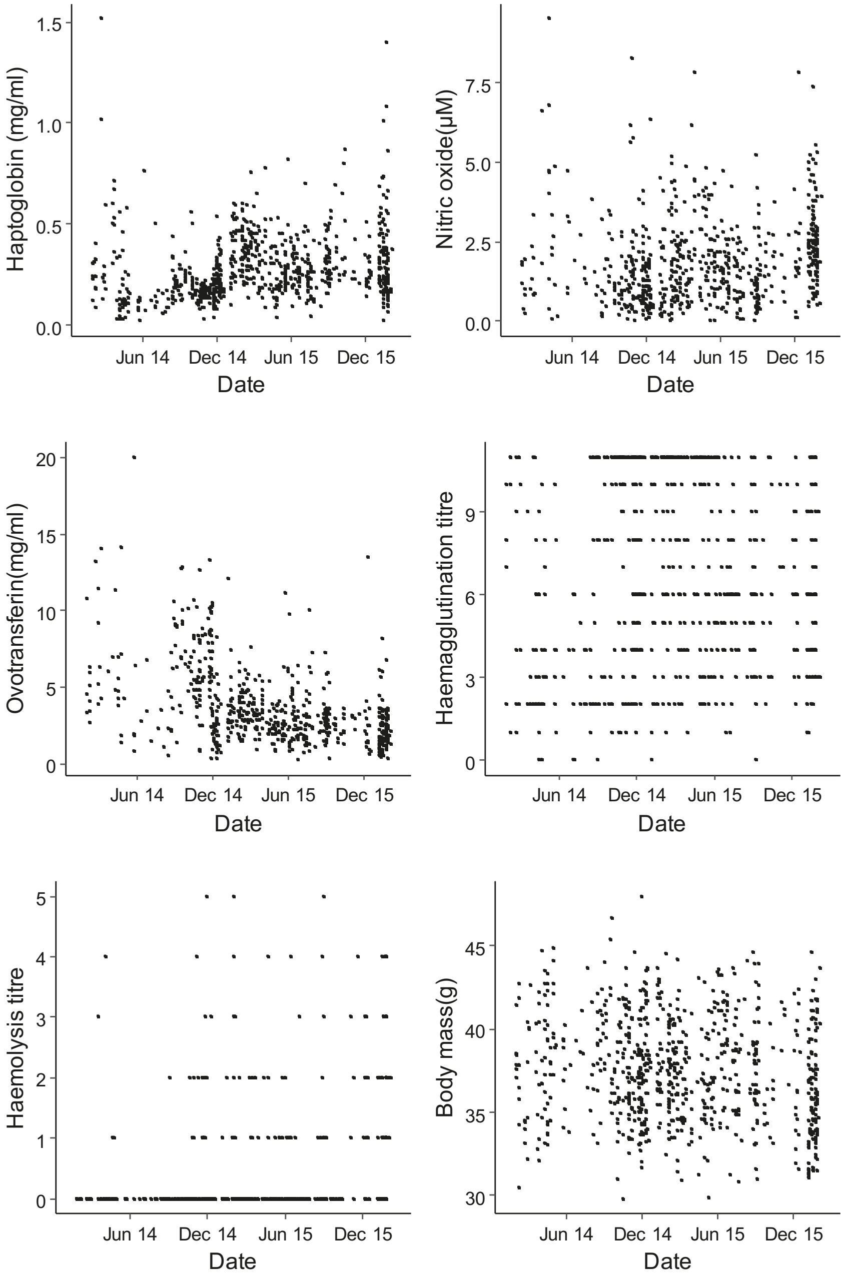

Figure 4.S1: Spread of measured immune indices and body mass from 818 common bulbuls sampled over two annual cycles of wet and dry seasons from January 2014 to February 2016. All samples were collected from the A. P. Leventis Ornithological research Institute and analysed at the University of Groningen the Netherlands.

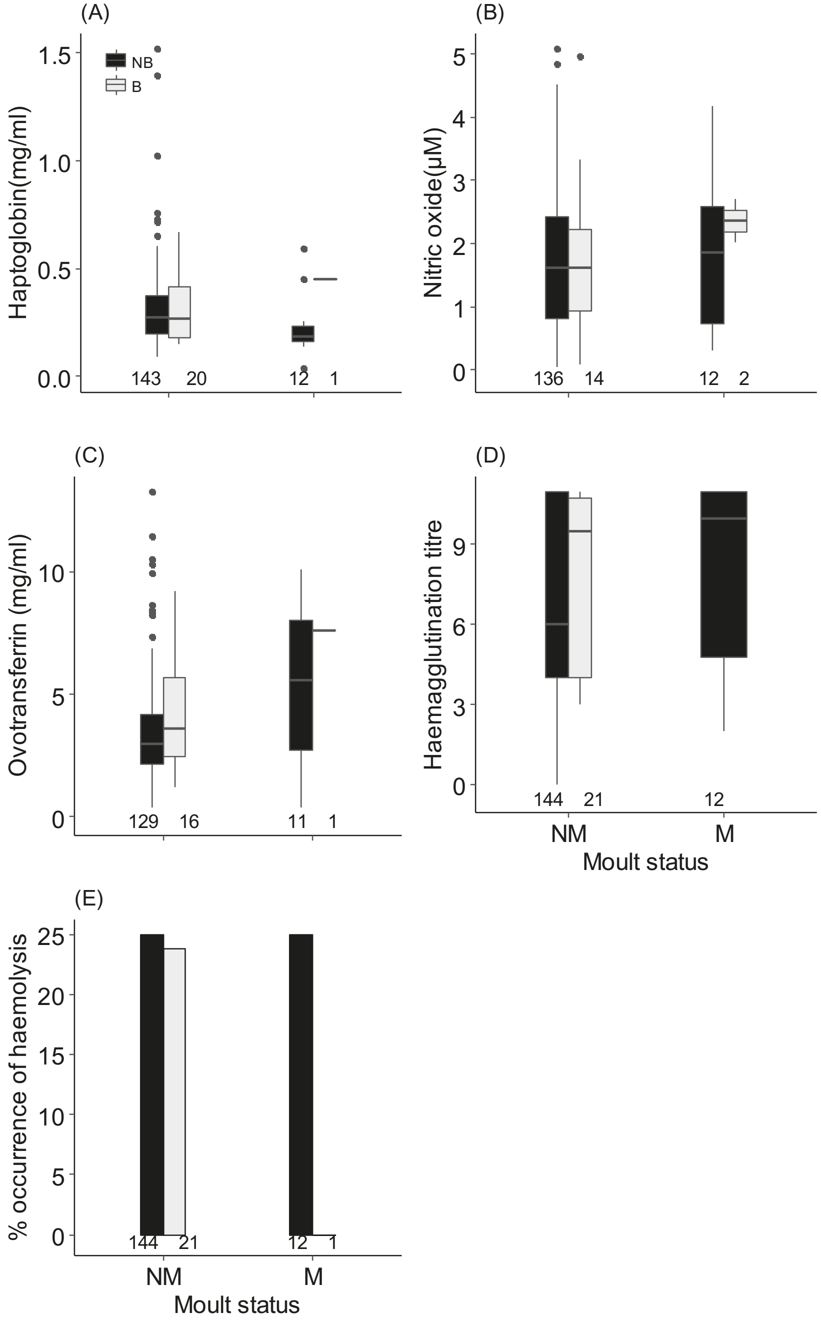

Figure 4.S2: Differences in (A) haptoglobin, (B) nitric oxide, (C) ovotransferrin, (D) haemagglutination titre and (E) % occurrence of haemolysis between breeding and moult stages within the dry season in female common bulbuls sampled over two annual cycles in Nigeria. Annual cycle stages were determined based on occurrence of brood patch and feather quality: NM – non-moulting; M – moulting, B - breeding and NB - non-breeding. Sample sizes are indicated below each box/bar.

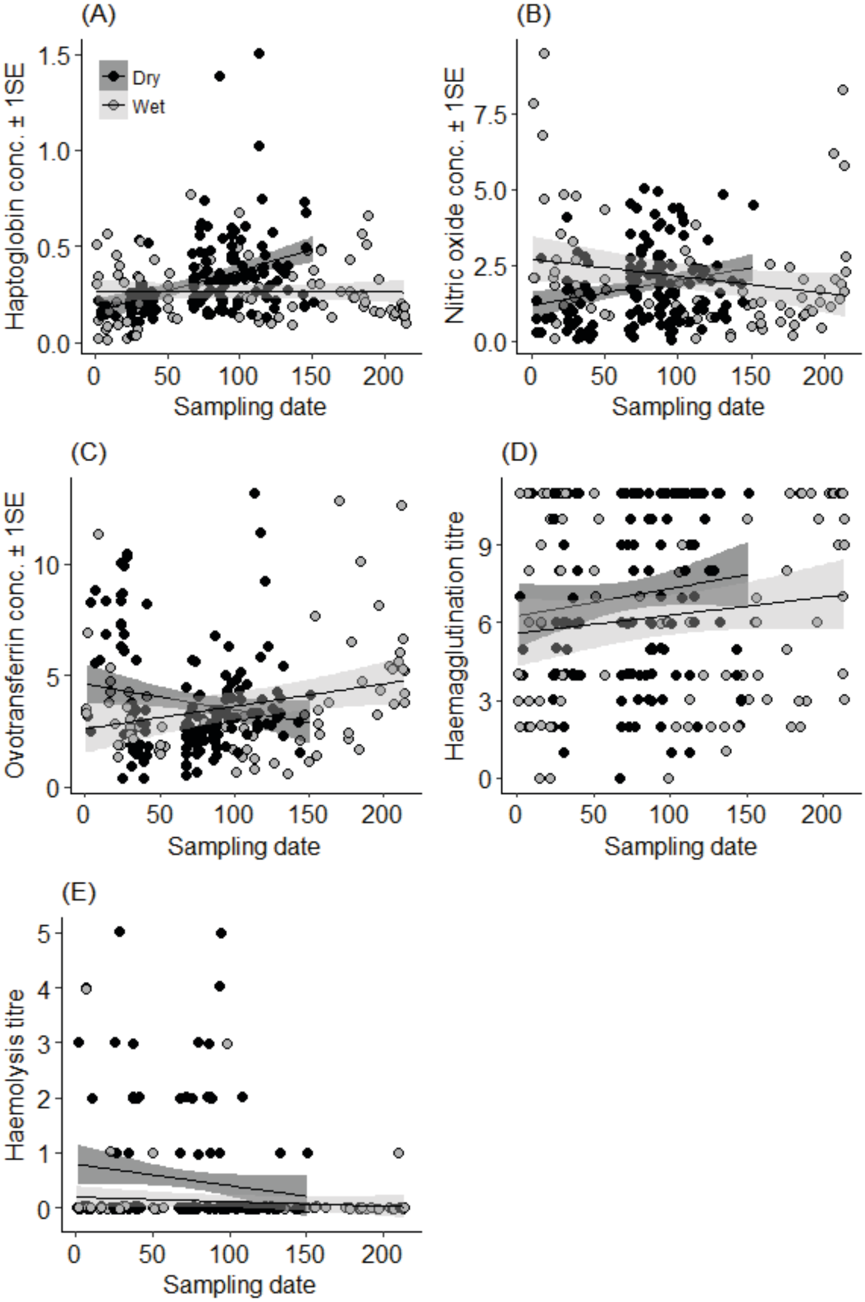

Figure 4.S3: Variation in (A) haptoglobin, (B) nitric oxide, (C) ovotransferrin, (D) haemagglutination titre and (E) haemolysis titre within the wet and dry season in female Common Bulbuls sampled over two annual cycles in Nigeria. 1st April was assigned the first day of the wet season while 1st November was assigned the first day of the dry season annually.

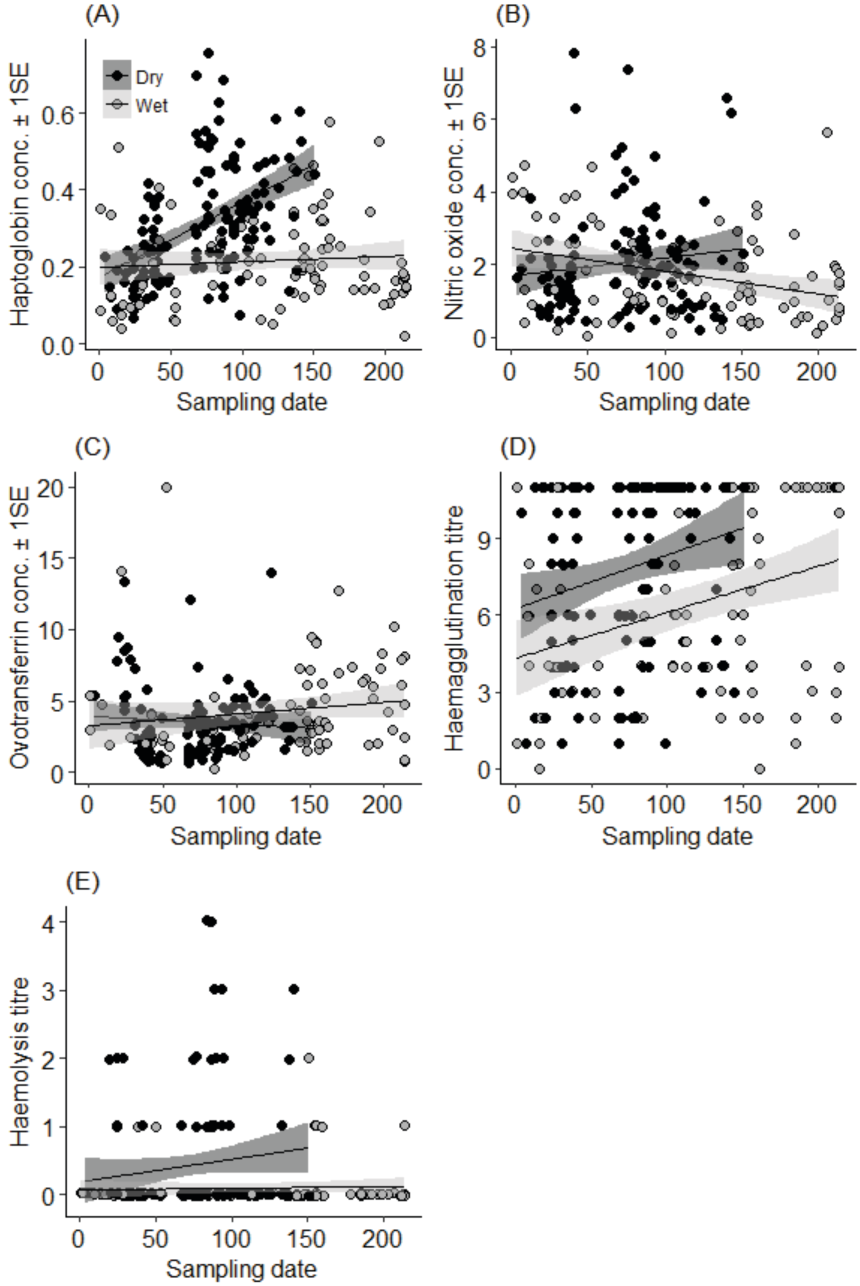

Figure 4.S4: Variation in (A) haptoglobin, (B) nitric oxide, (C) ovotransferrin, (D) haemagglutination titre and (E) haemolysis titre within the wet and dry season in male Common Bulbuls sampled over two annual cycles in Nigeria. 1st April was assigned the first day of the wet season while 1st November was assigned the first day of the dry season annually.

Part III

Relationships between spatial environmental variability and body size, annual cycle stages and innate immune function

Chapter 5

Temperature and aridity determine body size conformity to Bergmann's rule independent of latitudinal differences in a tropical environment.

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Key words: Latitude, thermoregulation, global warming,
birds and tropical environment.

Abstract

Bergmann's rule; the tendency for endotherms to be larger in colder environments, is a biophysical generalization of body size variation that is frequently tested along latitudinal gradients, even though latitude is only a proxy for temperature variation. We test whether variation in temperature and aridity determine avian body size conformity to Bergmann's rule independent of latitude differences, using the ubiquitous Common Bulbul *Pycnonotus barbatus*, along a West African environmental gradient. We trapped 538 birds in 22 locations between latitude 6 and 13^{o}N in Nigeria and estimated average body surface area to mass ratio per location. We then modelled body surface to mass ratio using General Linear Models, with latitude, altitude and one of 19 bioclimatic variables extracted from http://www.world-clim.org/bioclim as predictors. We sequentially dropped latitude and altitude from each model to obtain R squares of resultant models. Finally, we compared R squares of univariate models where bioclimatic variables predicted body surface area to mass ratio significantly (14 of 19) to multivariate models including latitude, altitude and a bioclimatic variable, using Wilcoxon matched pairs test. We found that multivariate models were not better than univariate models with only bioclimatic variables. Six temperature and eight precipitation variables significantly predicted variation in body surface area to mass ratio between locations, in fact, 50% (7 of 14) of these provided better explanation of variation in body surface area to mass ratio than the multivariate models. Birds showed a larger body surface area relative to body mass ratio in hotter environments independent of latitude or altitude and this conforms to Bergmann's rule. Yet, a combination of morphometric analyses and controlled temperature exposure experiments are required to prove the proposed relationship between relative body surface area and thermoregulation in endotherms.

INTRODUCTION

Bergmann's rule, the tendency for endotherms to be larger in colder environments, is a well-known biophysical generalization for thermoregulation along environmental gradients (Salewski and Watt 2017). The rule applies to structural adaptation for thermoregulation in endotherms as observed in other animals (Porter and Kearney 2009, Greenberg et al. 2012, Glanville et al. 2012) rather than simple spatial body size variation, and this distinction is important. Bergmann's rule is based on adaptation to local temperature differences independent of variation across space, although such variation, usually with latitude, is used to test the rule (Gardner et al. 2011).

The usefulness of Bergmann's rule has received renewed attention for assessing the impact of global warming or presenting additional evidence for climate change via variation in animal body size over time (Gardner et al. 2011). But the validity of the rule is largely debated (Scholander 1955, Mayr 1956, Geist 1987, McNab 1971, McNab 2010, Yom-Tov and Geffen 2011) due to inconsistent body size patterns along latitudinal gradients. It seems likely the exploration of absolute body size patterns (Graves 1991, Meiri and Dayan 2003, Ashton 2002) rather than relative body surface area in relation to thermoregulation (Salewski and Watt 2017) and that the use of latitude as surrogate for temperature variation (Meiri and Dayan 2003) are responsible for some of the inconsistencies observed. Apart from Bergmann's rule, there are other ecogeographical hypotheses relating animal body size to environmental conditions, and although these are not mutually exclusive from Bergmann's rule, most address absolute measurements of body parts while Bergmann's rule is based on relative body surface area. Allen's rule postulates that animals should have longer body extremities in warmer environments (Allen 1877), while (Niles 1973) reports larger Horned larks *Eremophila alpestris* in areas of higher environmental productivity and Moreau (Mayr 1957) shows size increase with elevation. Bergmann's rule on the other hand, specifically postulates structural adaptation of the entire animal body to environmental temperature, based on a biophysical principle that more heat is lost to the external environment as body surface area increases relative to volume (Harley et al. 2009, Salewski and Watt 2017). In principle, species that are structurally longer and less compact in warmer environments but relatively plump and compact in cooler ones, consistent with Allen's rule may conform to Bergmann's rule as well because elongated body forms should have relatively larger surface area to volume ratio. Furthermore, variation in the size of body reserves or structural size due to environmental productivity (Madsen and Shine 2000) may also cause variability in surface area to volume ratio. In essence, body surface area to volume ratio should decrease as an animal's environment becomes colder, so that less of its produced heat is lost to its environment. Consequently, when heat loss is required to maintain relatively constant body temperatures in hot environments, we might expect a relatively larger surface area to volume ratio. Bergmann's rule should be interpreted based on two measures of body size: the ratio of an area to a cubic measure of body size.

Latitude encompasses many potentially confounding effects (Meiri and Dayan 2003), such as altitude, aridity, vegetation structure and food availability and all these may affect temperature and body size differently. In birds, measures of body mass combine body size and body reserves (Gosler et al. 1998), and body reserves vary rapidly due to predation or starvation risk (Lima 1986b), so mass alone cannot be used as a measure of body size (Piersma and

Davidson 1991, Cresswell 2009). Physical and biological processes which affect body size or reserves such as, food supply during growth (Madsen and Shine 2000), starvation/predation risk (Cresswell 1998), breeding (Nwaogu et al. 2017) and migration (Åkesson et al. 1992, Nwaogu and Cresswell 2015) may vary with latitude and confound temperature effects. Bergmann's rule is therefore interpreted best based on the local environmental conditions of living animals and not their latitudes, and with live body size measures within a single resident species, so as to eliminate species specific thermoregulatory adaptations (Scholander 1955).

In this study: we test the relationship between body surface area to mass ratio and environmental factors in a West African tropical environment to find out whether environmental temperature variation determine body size conformity to Bergmann's rule independent of latitude. We estimated body surface area as the square of wing length and used body mass as a proxy for volume in the Common bulbul *Pycnonotus barbatus,* a ubiquitous resident tropical songbird. We correlate surface area to mass ratio with 19 climatic variables that explain local environmental conditions. We predict that populations of Common bulbuls in hotter and more arid environments will have larger body surface area to mass ratio independent of latitude. However, since body mass can be highly variable and may result in variation in body surface area to mass ratio even without a variation in body size across latitude, we also modelled variation in body mass and wing length on their own. We provide evidence that wing length but not body mass increases significantly northwards with increasing temperature and aridity, hence patterns of body surface to mass ratio can reliably be interpreted as conformity to Bergmann's rule.

METHOD

Study area

This study was carried out within Nigeria; birds were mist-netted between latitude 6 and 13° N, and 49m to 1716m above sea level. West Africa is bounded to the north by the Sahara Desert and to the south and west by the Atlantic Ocean. This feature creates a gradient of temperature and aridity from the dry edge of the Sahara desert in the north to the wet coastal areas of the Atlantic Ocean in the south of Nigeria, and this contrasts the pattern in the larger scale global gradient of decreasing temperature from the equator to the North Pole. There is also a large variation in altitude and vegetation structure between locations, and this is not entirely consistent with latitudinal differences. Precipitation is seasonal in West Africa, but humidity and environmental temperature do not follow similar patterns across the year. Increase in rainfall is unimodal but temperature variation is bimodal because of low temperatures during the dusty harmattan weather in the dry season and at the peak of the wet season. Differences in humidity due to interactions between temperature and rainfall may affect water balance between organisms and their environment and thus, thermoregulation, and so we also consider the effect of precipitation variables on body surface area to mass ratio. There is usually a single period of rainfall; hence one of drought annually, but the extent of the wet season varies between locations. The wet season is later and shorter in northern latitudes which are more arid compared to southerly ones (or higher altitude locations), and this combines with altitudinal differences and vegetation structure to determine local climates which are largely independent of latitude variation in space.

Study species

The Common Bulbul is widespread and resident throughout Africa. Common bulbuls are sexually monomorphic, usually 9 - 11 cm in body length and weigh 25 - 50g. Adult birds feed on fruits, insects, nectar and seeds. Fruits are generally available to Bulbuls year-round but from different fruit plants that vary in fruiting phenology.

Determination of variables

We obtained body size measurements from a total of 538 Common bulbuls from 22 locations in Nigeria. We trapped 308 of 538 Common bulbuls from 15 locations between 17th January and 8th April 2017, while data for additional 230 birds from seven locations were obtained from our past ringing records archived in the A. P. Leventis Ornithological Research Institute ringing database collected between 2001 and 2016 (Cox et al. 2011). All birds were caught using mist nets from 06:00 to 10:30 hours. For each trapped bird, we recorded wing length (± 1mm), pectoral muscle score, fat score and body mass (± 0.1g, Ohaus Scout) (Svensson 1992, Redfern and Clark, 2001). Tarsus length was also measured for birds trapped in 2017. We estimated average body surface area to mass ratio by dividing the square of wing length by body mass for each individual bird. We extracted 19 local bioclimatic variables for each capture location (Table 5.1), including 11 temperature and eight precipitation variables from http://www.worldclim.org/bioclim, using the 'maptools' and 'raster' packages in R. We relied on wing length and body mass measurements as proxies of body size, because both are more often accurately obtained by ringers (Gosler et al. 1998), although their accuracy as a proxy for body size may vary among species (Rising and Somers 1989, Senar and Pascual 1997). Our method is easily repeatable using records from avian ringing databases for the same species in different locations. However, to validate the reliability of squared wing length as a proxy for body surface area, we correlated squared wing length, and the product of wing and tarsus lengths, since both are linear size measures of the same individual whose product give an area measure similar to the square of wing length ($r = 0.79$, $df = 289$, $p < 0.0001$, see Figure 5.2 (top left)). We used the square of wing length for all our analyses because we only have tarsus length measurements for 15 out of 22 locations. This should be consistent with Bergmann's reference to body surface area to volume ratio (using measures of wingspan as proxy for body size (Salewski and Watt 2017), because wing length is linear and body mass is a similar cubic measure to volume.

Table 5.1: Adjusted R-squares of General Linear Models explaining body surface area to mass ratio of Common bulbuls across environmental conditions in West Africa. Full model includes latitude, altitude and one bioclimatic variable as predictors. Change in adjusted R - squares is the difference that results after dropping the variable in a column heading from the full model with all three variables. Models where bioclimatic variable made significant contribution to explaining body surface area to mass ratio are indicated in bold.

	R^2 of univariate model				**Δ R^2 after dropping variable from multivariate model**		
	Climatic Variable	**Latitude**	**Altitude**	**Full model**	**- Climatic variable**	**- Latitude**	**- Altitude**
Annual Mean Temperature	-0.01	0.48	-0.05	0.47	-0.01	-0.15	0
Mean Diurnal Range (Mean of monthly (max temp – min temp))	**0.5**	0.48	-0.05	**0.48**	**-0.02**	**0.03**	**0.01**
Isothermality	**0.63**	0.48	-0.05	**0.6**	**-0.14**	**0.02**	**0.01**
Temperature Seasonality (standard deviation *100)	**0.5**	0.48	-0.05	**0.46**	**0**	**0.02**	**0.03**
Max Temperature of warmest month	**0.33**	0.48	-0.05	**0.52**	**-0.06**	**0.03**	**-0.03**
Min Temperature of coldest month	**0.25**	0.48	-0.05	**0.51**	**-0.05**	**0.02**	**-0.05**
Temperature annual range	**0.58**	0.48	-0.05	**0.54**	**-0.08**	**0.02**	**0.02**
Mean Temperature of Wettest Quarter	0.009	0.48	-0.05	0.44	0.02	-0.12	0.03
Mean Temperature of Driest Quarter	-0.03	0.48	-0.05	0.43	0.03	-0.22	0.03
Mean Temperature of Warmest Quarter	0.13	0.48	-0.05	0.52	-0.06	0.02	-0.04
Mean Temperature of Coldest Quarter	-0.05	0.48	-0.05	0.45	0.01	-0.55	0.02
Annual Precipitation	**0.55**	0.48	-0.05	**0.51**	**-0.05**	**0.02**	**0.03**
Precipitation of the Wettest Month	**0.22**	0.48	-0.05	**0.44**	**0.02**	**-0.23**	**0.03**
Precipitation of the Driest Month	**0.49**	0.48	-0.05	**0.51**	**-0.05**	**-0.05**	**0.02**
Precipitation Seasonality (Coefficient of Variation)	**0.57**	0.48	-0.05	**0.53**	**-0.07**	**0.02**	**0.02**
Precipitation of the Wettest Quarter	**0.26**	0.48	-0.05	**0.44**	**0.02**	**-0.19**	**0.03**
Precipitation of Driest Quarter	**0.56**	0.48	-0.05	**0.55**	**-0.09**	**-0.01**	**0.01**
Precipitation of Warmest Quarter	**0.51**	0.48	-0.05	**0.57**	**-0.11**	**-0.06**	**0.01**
Precipitation of Coldest Quarter	**0.37**	0.48	-0.05	**0.44**	**0.02**	**-0.07**	**0.03**

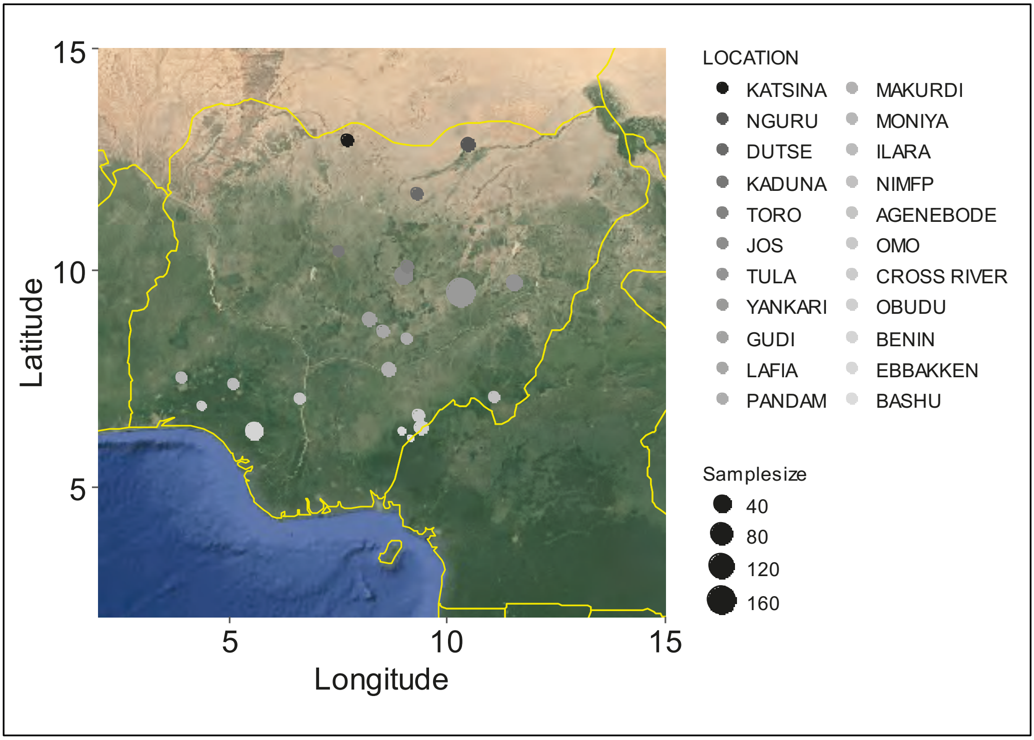

Figure 5.1: Map showing distribution of locations where body size measurements of Common bulbuls were obtained across West Africa. Sampling points are shaded darker with increasing latitude consistent with Figures 5.2, 5.3 and 5.4.

Statistical analyses

We built a General Linear Model (GLM) to predict average body surface area to mass ratio for each of the 22 capture locations where we trapped birds. We included pectoral muscle, subcutaneous fat scores and moult stage of birds in the GLM, but they did not improve model fit, hence they were dropped from the final model. Presence of brood patch (as a proxy for breeding status) explained 3% additional variation in body surface to mass ratio. But because only female birds carry brood patches, we could not control for breeding status when calculating predicted body surface area to mass ratio per location. Ignoring breeding status was unlikely to affect our conclusions because breeding in the Common Bulbul does not follow a consistent pattern along the environmental gradient (Unpublished data, see also Figure 5.2). The final model with which we estimated body surface area to mass ratio included only capture location as predictor variable ($r = 0.36$, $df = 516$, $p < 0.0001$). We then obtained predicted body surface area to mass ratio for each location using the predict function in R. Subsequently, we built a GLM to model predicted body surface area to mass ratio per location by latitude, altitude and one of 19 bioclimatic variables (Table 5.1). For each model, we sequentially dropped latitude and altitude to obtain R squares of resultant models (each including only a single local climatic variable).

We repeated same analyses (as we did with body surface area to mass ratio) using body mass and wing length on their own because both body mass and size may vary independently due to factors unrelated to thermoregulation, thus, confounding observations. Body mass should be higher in wetter and cooler environments where birds may breed earlier and thus, carry extra body reserves due to interrupted foraging (Macleod and Gosler 2006, Nwaogu et al. 2017). Also wing length should be shorter in more arid environments if net primary productivity determines overall body size (Hilderbrand et al. 1999; Madsen and Shine 2000), or relatively longer if Allen's rule is valid (Allen 1877). Compared to body surface area to mass ratio, variation in body mass and wing length alone were less well explained by bioclimatic variables (Table 5.S1 and 5.S2), thus it seems likely that there were no significant confounding effects of breeding or food availability on the estimated body surface to mass ratio.

To test the predictive power of bioclimatic variables independent of latitude, we compared the adjusted R squares of univariate models where bioclimatic variables predicted body surface area to mass ratio significantly (14 of 19, see Table 5.1) to multivariate models including latitude, altitude and a bioclimatic variable, using Wilcoxon matched pairs test. This was to confirm whether univariate models with a bioclimatic variable alone generally explained variation in body surface to mass ratio without including latitude and altitude in models. All analyses were carried out in R version 3.4.1 (R, Development Core Team 2006).

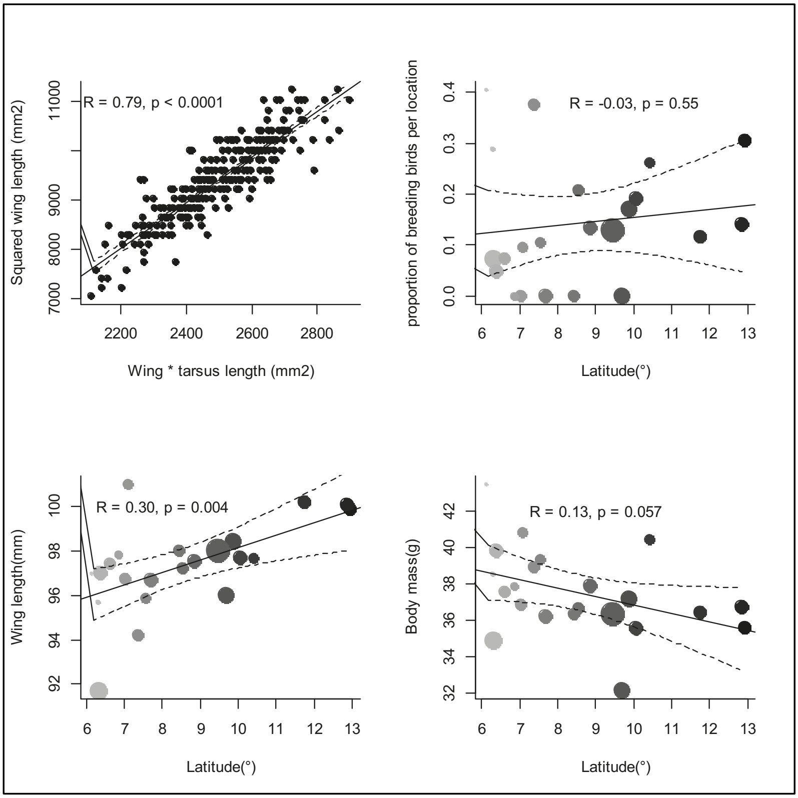

Figure 5.2: Top left: Correlation between squared wing length and product of wing and tarsus lengths of 308 Common bulbul *Pycnonotus barbatus* trapped in 15 of 22 study sites along the temperature gradient in Nigeria. **Top right**: No correlation between log proportion of breeding birds per location and latitude. **Bottom left**: Positive correlation between wing length and latitude suggests birds are significantly bigger in higher latitudes. **Bottom right**: No correlation between body mass and latitude – note that higher latitude locations are more likely to be arid (see Figure 5.1). Grey shading from light to dark is consistent with increasing latitude and point sizes indicate sample size (see Figure 5.1).

RESULTS

Body surface area to mass ratio of Common bulbuls across different environments was determined by local bioclimatic variables independent of latitude (Table 5.1, Figures 5.3 & 5.4). Birds had larger body surface area to mass ratio in hotter, arid and more seasonal environments compared to colder, wetter and less seasonal ones in Nigeria independent of latitude (Figures 5.1, 5.3 & 5.4). Body surface area to mass ratio was predicted significantly by 14 of the 19 bioclimatic variables (see Table 5.1 for significant variables). Mean annual temperature, and temperatures of the wettest, driest, warmest and coldest quarters did not explain much variation in body surface area to body mass ratio between locations (Table 5.1). Multivariate models including latitude and altitude as predictor variables were not better at explaining variation in body surface area to body mass ratio of Common bulbuls compared to univariate models of each of the 14 significant local bioclimatic variables alone ($V = 71.5$, $P = 0.12$, median = 0.51 *vs* 0.50, $n = 14$). Climatic variables alone explained between 0 – 63% of the variation in body surface area to mass ratio of Common bulbuls across location (Table 5.1). Latitude alone explained 48% while altitude alone explained 0% of the variation in body surface area to mass ratio of Common bulbuls across locations (Table 5.1). Nine out of 14 (64%) significant univariate models for bioclimatic variables had a higher R^2 than univariate models using latitude alone (Table 5.1). For the explorations of variation in wing length and body mass alone (Tables 5.S1 & 5.S2): bioclimatic variables explained 0 – 45% and 0 – 26% variation in wing length and body mass respectively. Latitude explained 30% and 13% variation in wing length and body mass respectively, while altitude explained 11% and 3% of wing length and body mass respectively.

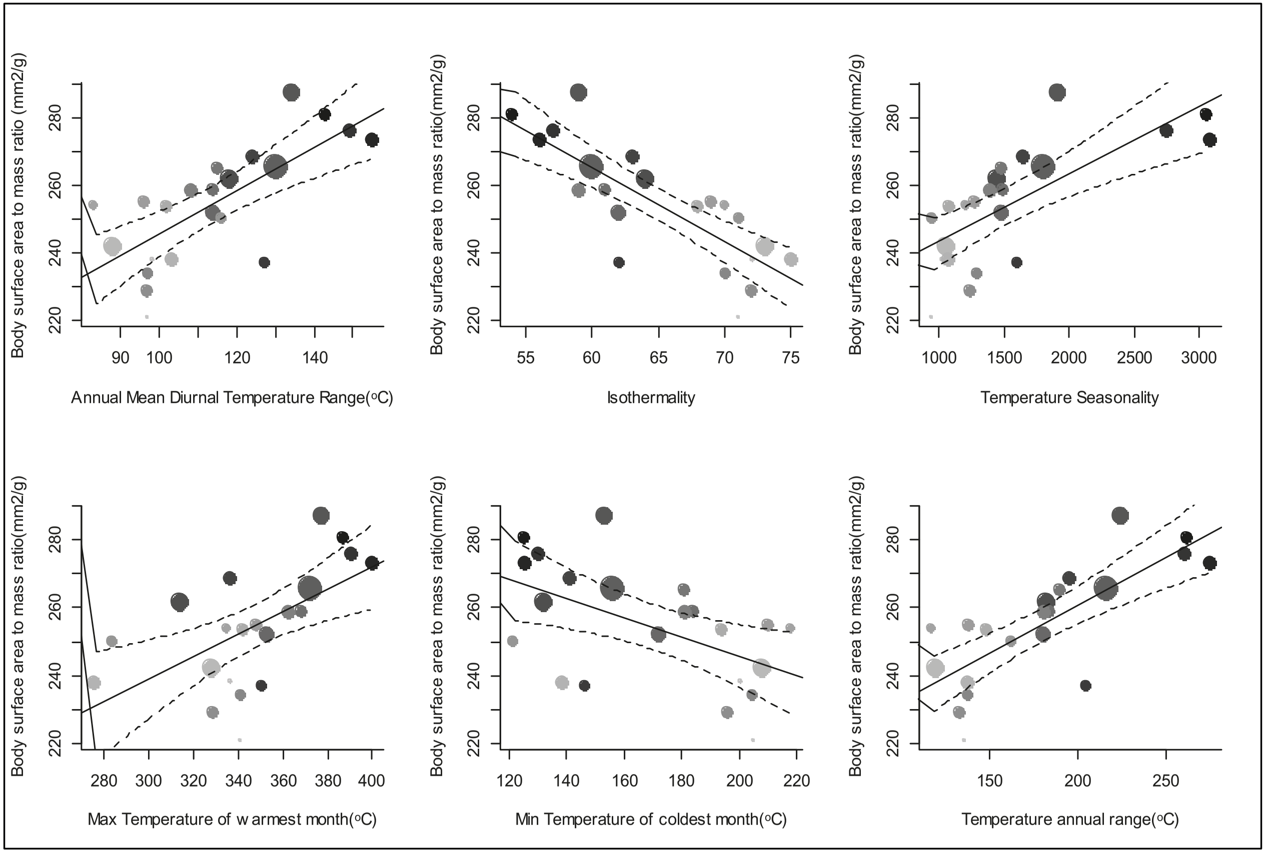

Figure 5.3: Correlations between body surface area to mass ratio of Common bulbuls *Pycnonotus barbatus* and six significant temperature variable predictors. Grey scale is ordered by increasing latitude to show independence of body surface to mass ratio and latitude. Grey shading from light to dark is consistent with increasing latitude and point sizes indicate sample size (see Figure 5.1).

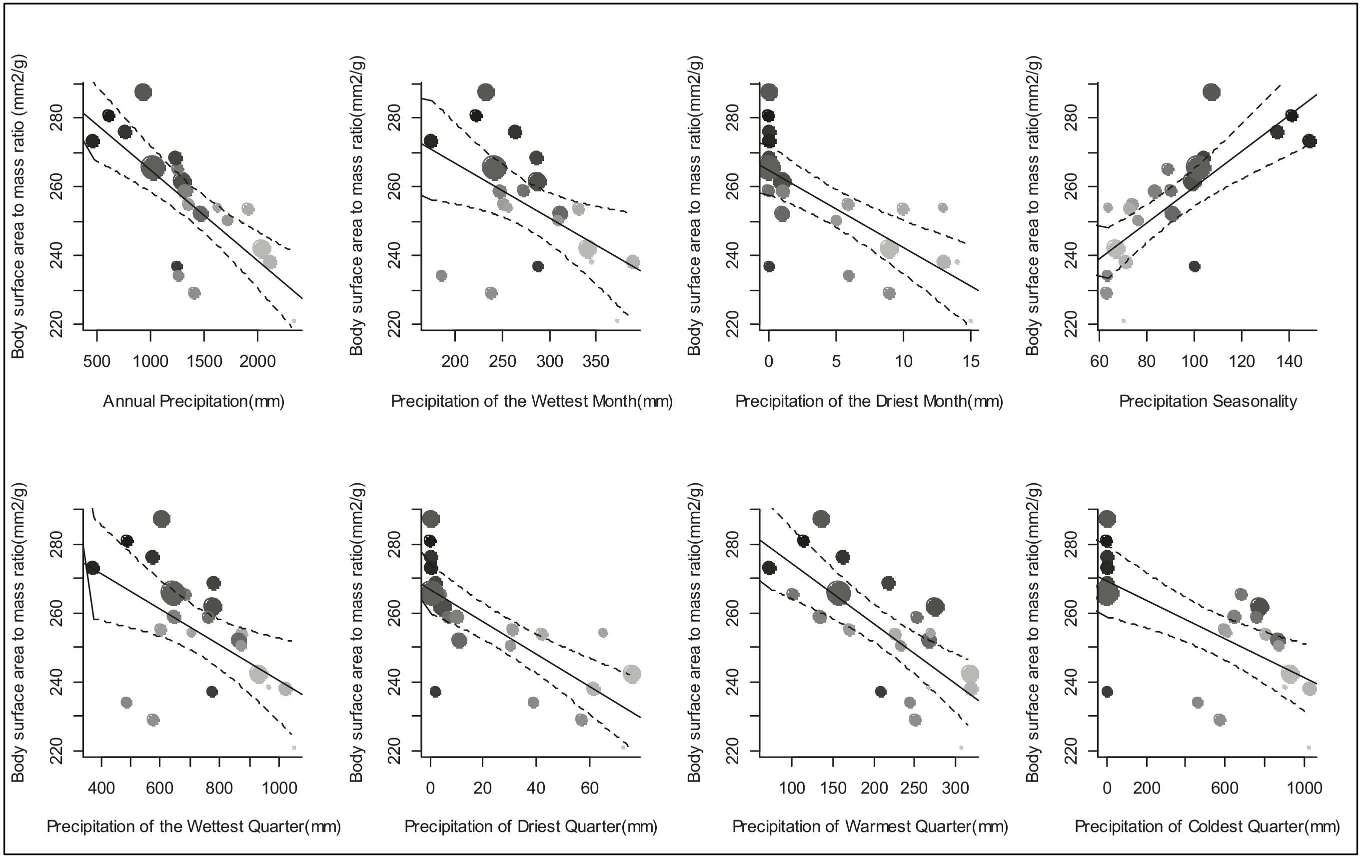

Figure 5.4: Correlations between body surface area to mass ratio of Common bulbuls *Pycnonotus barbatus* and eight significant precipitation variable predictors. Grey scale is ordered by increasing latitude to show independence of body surface to mass ratio and latitude. Grey shading from light to dark is consistent with increasing latitude and point sizes indicate sample size (see Figure 5.1).

DISCUSSION

Our results suggest that the link between relative body surface area and temperature variation along environmental gradient is valid and possibly related to thermoregulation in the Common Bulbul after taking local environmental conditions (Figure 5.3 & 5.4, Table 5.1), absolute body size variation (Figure 5.2, Table 5.S1 & 5.S2) and time of capture (Table 5.S3) into account. We discuss these results, that show that patterns of variation in body surface area to body mass ratio of a tropical songbird is consistent with Bergmann's rule independent of latitude.

Temperature varies globally on a latitudinal scale, but using latitude as a proxy for temperature variation may be misleading if local factors override global patterns (Meiri et al. 2007, Bourgault et al. 2010). Our data confirm that latitudinal differences do not often capture the combined effect of local factors on environmental conditions (Bourgault et al. 2010), because bioclimatic variables were relatively better at explaining body surface area to mass ratio compared to latitude (Table 5.1). In this case, body surface area to mass ratio also correlated with latitude, because temperature and aridity vary from north to south with timing and duration of rainfall in contrast with the global pattern of increasing temperature towards the pole, but consistent with decreasing local temperatures towards the Atlantic (Figure 5.1). This shows a strong association of body size variation and environmental temperature despite a reversal of the global latitudinal pattern.

Environmental temperature and aridity are closely linked (James 1970) and this may affect internal water balance (Tieleman and Williams 2000). Hence, the correlation between body surface area to body mass ratio and aridity in the Common bulbul is unlikely to be due to differences in net primary productivity as frequently suggested for other animals (Yom-Tov and Geffen 2006, Yom-Tov and Geffen 2011). Geographical variation in body mass has previously been reported for the Common bulbul (Crowe et al. 1981, Brittion 1972, Hanmer 1978): bulbuls tend to be heavier in localities with lower environmental temperature and high productivity. These results, although from body mass records uncorrected for size in four bulbul subspecies with different geographic range were interpreted as consistent with Bergmann's rule and the productivity hypothesis (Niles 1973). Our results do not negate these conclusions (Crowe et al. 1981), but they may have been arrived at by chance because the occurrence of heavier birds (rather than birds with larger body surface area to volume ratio) in cooler environment does not necessarily imply conformity to Bergmann's rule. Our raw body mass data also showed a negative non-significant trend with temperature (Figure 5.2), but wing length which is a comparatively better index of structural body size (Piersma and Davidson 1991) was significantly positively correlated with temperature (Figure 5.2). We suggest that correlations of body surface area to body mass ratio with both temperature and aridity suggests a link between thermoregulation and water balance in dry environments (James 1970, Hudson and Bernstein 1981). Common bulbuls had a smaller body surface area to mass ratio in more isothermal environments and larger body surface area to mass ratio in more seasonally arid environments (see negative correlations in Figure 5.3 & positive correlation in Figure 5.4, respectively), and this suggests that relative body surface area may be adapted to both the effect of environmental temperature and aridity on internal water balance (Williams and Tieleman 2005). We propose that beside several adjustments for thermoregulation (Tieleman and Williams 2000), birds may structurally adapt body size for non-evaporative heat loss so as to manage body temperature and reduce water loss in dry environments (Niles 1973).

Species with large distribution ranges or partial migratory status may show significant variation in body size and this may correlate strongly with latitude (Yom-Tov and Geffen 2006) even if unrelated to Bergmann's rule. We only confirm that the pattern observed in the locally resident Common bulbul conforms to Bergmann's rule after precluding any relationships between body mass and breeding with latitude and confirming a significant positive correlation of wing length and latitude (Figure 5.2). Therefore, we eliminate the possibility of misinterpreting an interrupted foraging response due to breeding (Nwaogu et al. 2017), starvation risk (Macleod and Gosler 2006) or migration (Hahn et al. 2015, Grilli et al. 2017) as conformity to Bergmann's rule. Unfortunately, both wing length and body mass have been used on their own to test Bergmann's rule (Watt et al. 2010) and this may lead to misleading interpretations. Bioclimatic variables explain 0 – 45% and 0 – 26% variation in wing length and body mass respectively (Tables 5.S1 & 5.S2), whereas they explain 0 – 63% variation in body surface area to mass ratio (Table 5.1). For birds we suggest that squared wing length by body mass appears a more informative proxy for relative body surface area than single measures of body size.

To conclude: although Bergmann (and several more recent authors) used latitude and single linear measurements of body size to test conformity to Bergmann's rule, its proposed mechanism (Salewski and Watt 2017) is independent of latitude and concerns body surface area to volume ratio which requires a combination of area and cubic measurement of body size. It is thus, likely that the validity of the mechanism proposed in Bergmann's rule (Mayr 1956, Watt et al. 2010, Salewski and Watt 2017) has not actually been tested empirically on living animals – yet this is crucial for assessing its validity and applicability to tests for, and predicting the effects of global warming. The problem associated with testing Bergmann's rule involves both data collection and utilisation, and our method may help with the latter given the wealth of available data from bird ringing schemes. Nonetheless, a combination of comparative morphometric analyses and translocation experiments may further test the validity of Bergmann's rule: by exposing different populations which show body size conformity to Bergmann's rule to controlled temperature conditions and measuring indices such as heat stress, metabolic rates and heat/water loss, thermoregulatory differences arising from relative differences in body surface area may be finally proved.

SUPPLEMENTARY INFORMATION

Table 5.S1: Adjusted R- squares of General Linear Models explaining wing length variation in Common bulbuls across environmental conditions in West Africa. Full model includes latitude, altitude and one bioclimatic variable as predictors. Change in adjusted R - squares is the difference that results after dropping the variable in a column heading from the full model with all three variables. Models where bioclimatic variable made significant contribution to explaining wing length are indicated in bold.

	R^2 of univariate model				**Δ R^2 after dropping variable from multivariate model**		
Climatic Variable	Climatic variable	Latitude	Altitude	Full model	-Climate variable	-Latitude	-Altitude
Annual Mean Temperature	0.008	0.3	0.11	0.39	0.01	-0.07	-0.02
Mean Diurnal Range (Mean of monthly (max temp - min temp))	**0.4**	**0.3**	**0.11**	**0.42**	**-0.02**	**0.03**	**-0.05**
Isothermality	**0.24**	**0.3**	**0.11**	**0.44**	**-0.04**	**0.03**	**-0.17**
Temperature Seasonality (standard deviation *100)	**0.24**	**0.3**	**0.11**	**0.39**	**0.01**	**0.03**	**-0.12**
Max Temperature of warmest month	-0.0006	0.3	0.11	0.44	-0.04	0.03	-0.12
Min Temperature of coldest month	**0.45**	**0.3**	**0.11**	**0.41**	**-0.01**	**0.03**	**0.03**
Temperature annual range	**0.36**	**0.3**	**0.11**	**0.44**	**-0.04**	**0.03**	**-0.11**
Mean Temperature of Wettest Quarter	-0.03	0.3	0.11	0.52	-0.12	0.02	-0.18
Mean Temperature of Driest Quarter	**0.2**	**0.3**	**0.11**	**0.37**	**0.03**	**-0.16**	**0.02**
Mean Temperature of Warmest Quarter	-0.04	0.3	0.11	0.39	0.01	0.02	-0.02
Mean Temperature of Coldest Quarter	0.14	0.3	0.11	0.37	0.03	-0.28	0.01
Annual Precipitation	**0.19**	**0.3**	**0.11**	**0.37**	**0.03**	**0**	**-0.1**
Precipitation of the Wettest Month	0.008	0.3	0.11	0.37	0.03	-0.14	-0.09
Precipitation of the Driest Month	**0.14**	**0.3**	**0.11**	**0.37**	**0.03**	**-0.12**	**-0.11**
Precipitation Seasonality (Coefficient of Variation)	**0.35**	**0.3**	**0.11**	**0.43**	**-0.03**	**0.02**	**-0.11**
Precipitation of the Wettest Quarter	0.03	0.3	0.11	0.37	0.03	-0.12	-0.09
Precipitation of Driest Quarter	**0.3**	**0.3**	**0.11**	**0.4**	**0**	**-0.01**	**-0.1**
Precipitation of Warmest Quarter	**0.18**	**0.3**	**0.11**	**0.45**	**-0.05**	**-0.01**	**-0.17**
Precipitation of Coldest Quarter	0.08	0.3	0.11	0.39	0.01	-0.16	-0.08

Table 5.S2: Adjusted R- squares of General Linear Models explaining body mass variation in Common bulbuls across environmental conditions in West Africa. Full model includes latitude, altitude and one bioclimatic variable as predictors. Change in adjusted R - squares is the difference that results after dropping the variable in a column heading from the full model with all three variables. Models where bioclimatic variable made significant contribution to explaining body mass are indicated in bold.

	R2 of univariate model				**Δ R2 after dropping variable from multivariate model**		
Climatic Variable	Climatic variable	Latitude	Altitude	Full model	-Climatic variable	Latitude	Altitude
Annual Mean Temperature	0.09	0.13	0.03	0.14	0.04	-0.03	0.05
Mean Diurnal Range (Mean of monthly (max temp - min temp))	0.09	0.13	0.03	0.14	0.04	0.04	-0.05
Isothermality	**0.26**	**0.13**	**0.03**	**0.21**	**-0.03**	**0.04**	**0.02**
Temperature Seasonality (standard deviation *100)	**0.16**	**0.13**	**0.03**	**0.14**	**0.04**	**0.04**	**-0.02**
Max Temperature of warmest month	**0.22**	**0.13**	**0.03**	**0.15**	**0.03**	**0.04**	**0.04**
Min Temperature of coldest month	-0.02	0.13	0.03	0.16	0.02	0.05	-0.02
Temperature annual range	**0.15**	**0.13**	**0.03**	**0.16**	**0.02**	**0.04**	**-0.05**
Mean Temperature of Wettest Quarter	0.07	0.13	0.03	0.17	0.01	-0.14	-0.02
Mean Temperature of Driest Quarter	-0.02	0.13	0.03	0.14	0.04	-0.03	0.03
Mean Temperature of Warmest Quarter	**0.19**	**0.13**	**0.03**	**0.17**	**0.01**	**0.05**	**0.04**
Mean Temperature of Coldest Quarter	0.02	0.13	0.03	0.16	0.02	-0.18	0.04
Annual Precipitation	**0.26**	**0.13**	**0.03**	**0.24**	**-0.06**	**0.03**	**0.01**
Precipitation of the Wettest Month	**0.15**	**0.13**	**0.03**	**0.15**	**0.03**	**-0.03**	**0**
Precipitation of the Driest Month	**0.26**	**0.13**	**0.03**	**0.27**	**-0.09**	**0.04**	**-0.05**
Precipitation Seasonality (Coefficient of Variation)	**0.15**	**0.13**	**0.03**	**0.16**	**0.02**	**0.04**	**-0.06**
Precipitation of the Wettest Quarter	**0.16**	**0.13**	**0.03**	**0.15**	**0.03**	**-0.01**	**0**
Precipitation of Driest Quarter	**0.19**	**0.13**	**0.03**	**0.22**	**-0.04**	**-0.07**	**0.04**
Precipitation of Warmest Quarter	**0.22**	**0.13**	**0.03**	**0.19**	**-0.01**	**0.01**	**0**
Precipitation of Coldest Quarter	**0.17**	**0.13**	**0.03**	**0.16**	**0.02**	**0.03**	**-0.03**

Table 5.S3: Summary statistics of general linear model predicting body surface area to mass ratio of common bulbuls *Pycnonotus barbatus* in 22 locations on a temperature and aridity gradient in Nigeria. Body surface area was calculated as the square of wing length. Time and date of capture were not significant predictors of body surface area to mass ratio and were not included in the final model used to estimate predicted body surface area to mass ratio for analyses reported in table 5.1, and figures 5.1 and 5.2.

Variable	Df	F	P	
Location	21	13.34	<0.001	***
Hour	1	0.91	0.34	
Date	1	1.98	0.16	

Chapter 6

Does inter-local variability in the timing of the wet season predicts the timing moult in a tropical passerine?

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Will Cresswell

Key words: Life history traits, spatio-temporal aridity, geography, breeding-moult overlap and West Africa.

Abstract

Rainfall is likely an important cue for species that live in seasonally arid environments, but because rainfall is often seasonal, it is difficult to distinguish its effect from other seasonal cues and co-varying life history traits. In central Nigeria Common Bulbuls *Pycnonotus barbatus* moult in the wet season, but this might be because other conflicting life history events like breeding are timed to occur at other periods, or just because moult is annual and occurs at the same time of the year in all locations regardless of environmental seasonality. To investigate the effect of local rainfall timing on moult, we examined variation in the extent of moult just before the wet season along a latitudinal gradient where the rains commence at different times. We predicted that moult should start earlier in sites where the rains commence earlier. We mist-netted 308 common bulbuls across 15 locations between latitude 6° N and 13° N within a three months period and modelled proportion of primary feather mass regrown as a function of precipitation of the driest quarter of the year which is a proxy for the timing of the wet season in each location. We found that moult advanced significantly with the timing of the wet season despite the absence of a similar pattern in the occurrence of breeding, suggesting that moulting is timed to local rainfall in each location rather than to the same period in the year across species range or to the timing of breeding. Thus, from one year of data along an environmental gradient, we show that the timing of moult may be organised to match suitable environmental conditions during the annual cycle of the Common Bulbul at each location independently of breeding. This pattern may apply to other species but will be less obvious if breeding is seasonal.

INTRODUCTION

Life history traits should be timed to coincide with suitable environmental conditions or at different times from other traits that may compete for common resources (Barta et al. 2006, McNamara and Houston 2008, Wingfield 2008, Visser et al. 2010, McNamara et al. 2011). Although the seasonality of many such traits are well documented, identifying the environmental cues that govern their occurrence are difficult because they are often correlated with multiple environmental variables, that are then themselves correlated with time (Dawson 2008). For example, in birds, breeding and moult are often timed to spring and summer conditions in temperate environments and to the wet season in most tropical environments (Baker. 1939, Hau 2001, Repenning and Fontana 2011) but it is unclear whether the timing of each life history event is related to independent cues, flexible to the timing of other events or is dominated by the need to time the most important annual life history event to a seasonal optimum.

Rainfall is an important determinant of seasonal environmental condition in the tropics (Poulin et al. 1992, Araujo et al. 2017), and is likely an important cue for species that live in seasonally arid environments (Chapman 1995, Lloyd 1999, Hau 2001, Houston 2012, Mares et al. 2017). However, rainfall is often seasonal, and so its occurrence correlates with seasonal life history traits. In central Nigeria, for example, we know that Common Bulbuls *Pycnonotus barbatus* specifically moult in the wet season despite weak breeding seasonality (Nwaogu et al. 2018a), but this may be because moult is unlikely to occur during breeding, or because moulting is controlled endogenously (Gwinner et al. 1995) or in response to variation in day length or other periodic cues like sunrise time (Goymann et al. 2012, Shaw 2017). Detailed analyses of the annual routine of the Common Bulbul at a single site in central Nigeria, suggests that timing of moult to the wet season may be important for optimising its annual cycle, since individuals are resident and capable of breeding year-round (Cox et al. 2013, Nwaogu et al. 2018a). However, we cannot conclude that rainfall exerts a direct effect on the timing of moult, because moult and rainfall are periodic, and vary little between years within a single location. However, because timing of the wet season varies over the geographic range of the Common Bulbul, we can test the effect of rainfall on the timing of moult using differences in the relative amount of feather material replaced by a moulting bird at a given time in its moult cycle (Summers 1976, Underhill and Joubert 1995). Such geographic variation in rainfall is similar to latitudinal temperature variation in temperate systems (Baker. 1939), which exerts predictable effects on the timing of life history events (Pimentel and Nilsson 2007, Bourgault et al. 2010).

The start and end of the wet season in Nigeria follows an annual predictable pattern from south to north. There is a single period of rainfall and one of drought each year, but the amount of rain and duration of the wet season varies latitudinally - the wet season is later and shorter going from south to north. The rains do not end abruptly, hence the commencement of the dry season and associated drying out of the environment is much more prolonged in more northerly locations. However, in all locations, the months before the onset of the rains are usually the driest, but more southerly locations where the wet season commence earlier remain relatively wet compared to more northern because they experience longer periods of precipitation leading into the quarter before the wet season. This creates an aridity gradient from the edge of the Sahara Desert in the north to the coast of the Atlantic Ocean in the south and this is consistent with the timing of the wet season (Nwaogu et al. 2018c).

Having established that moult is timed almost exclusively to the wet season, we test whether inter-local variability in the timing of the wet season specifically predicts the timing of moult in the Common Bulbul. We analyse the extent of primary moult across locations where onset and duration of rainfall vary over a four-month period. We predict that moult will be advanced in sites which experience earlier rains, modelled as precipitation of the driest quarter of the year per location. The amount of precipitation in the driest quarter of the year corelates negatively with the timing of the wet season. We modelled the proportion of primary feather mass grown by each bird as a function of the amount of precipitation in the driest quarter at capture locations ranging over 785 kilometres north to south.

METHODS

Study species

Common Bulbuls are resident across Nigeria (Elgood et al. 1973), they are capable of breeding year-round (Cox et al. 2013, Nwaogu et al. 2018) and typically long-lived (Stevens et al. 2013). Common Bulbuls are sexually monomorphic, usually weighing about 25 – 50g. They feed mainly on fruits and insects, whose availability vary with environmental conditions and phenology of plants. In central Nigeria, despite flexible breeding, wing moult at the population level lasts 138 ± 5 days – usually from 1st May (± 15 days) to 26th September (± 30 days) on average (Nwaogu et al. 2018a).

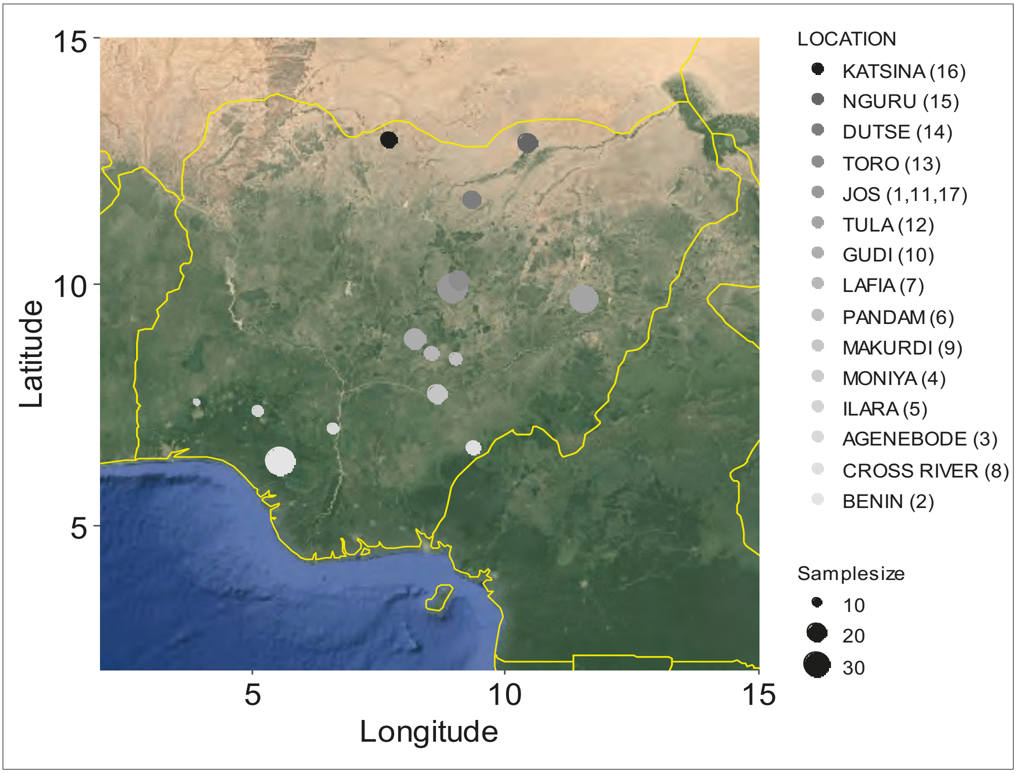

Figure 6.1: Map showing summary of moult records of Common Bulbul *Pycnonotus barbatus*, from 17th of January to 8th April 2017 across 15 locations along an aridity gradient in Nigeria where the wet season starts at different time in the year. Numbers in parenthesis against sampling locations indicate order in which sites where visited to assess moult.

Data collection

Within three months prior to the wet season, we travelled across Nigeria and mist-netted 308 Common Bulbuls across 15 locations between latitude 6 and 13° N (Figure 6.1). Mist netting was carried out between the 17th of January and 8th of April 2017. All locations were visited before the wet season. We sampled from the southernmost location (Benin) and advanced northward (but not necessarily always consistent with latitude increase; see Figure 6.1 for sampling order), apart from Jos which was sampled on three occasions. The pattern of sampling was aimed at avoiding a temporal bias towards southern locations, because we predicted that moult will commence later in more arid locations, and these were more likely in the north. Precipitation of the driest quarter and onset of the wet season correlate negatively with latitude, hence by sampling south to north we set sampling date on a negative trend with latitude, onset of the wet season and precipitation of the driest quarter, allowing us to interpret a positive correlation between the extent of moult and precipitation of the driest quarter as an effect of rainfall rather than sampling date within a given year. Note that this sampling order is likely to *weaken* the predicted positive correlation because based on a regular feather deposition rate (Summers 1976), moult will advance as we move towards more northerly locations.

For each bird, we assessed moult status by scoring each primary feather on an ordinal scale of 0 – 5: fully grown new feathers were scored 5 while un-moulted old feathers were scored 0, and feathers at different stages of growth were scored 1 – 4 depending on their size (Ginn & Melville, 1983). We also recorded wing length (± 1mm), brood patch score and body mass (± 0.1g, Ohaus Scout). We converted moult scores to proportion of feather material regrown at time of capture, using methods described by (Underhill and Zucchini 1988): each moulted feather was converted to feather mass using reference masses of individual fully grown primary feathers of the Common Bulbul obtained from Museum specimens at the A. P. Leventis Ornithological Research Institute in Nigeria. We used the function "ms2pfmg" provided with the package 'Moult' in R (Erni *et al*. 2013).

We extracted bioclimatic variables from http://www.worldclim.org/bioclim, using the GPS coordinates of locations where birds were caught with the aid of the 'maptools' and 'raster' packages in R. We obtained the precipitation of the driest quarter of the year in each location from the list of 19 variables provided from bioclim (see also Nwaogu et al. 2018).

Data analyses

We plotted the proportions of primary feather mass regrown against precipitation of the driest quarter of the year, latitude, sampling date and altitude at each location. This was to assess the effects of other environmental factors that may vary with latitude and altitude, and to prevent misinterpreting variation in sampling date or sample size as a spatial variation in the occurrence of moult. We also plotted proportions of breeding Common Bulbuls against precipitation of the driest quarter of the year, latitude, sampling date and altitude at each location, in order to confirm that breeding occurrence does not follow any latitudinal or sampling order. We used the proportion of breeding birds in each location instead of the breeding status of each bird because males do not incubate, so breeding status of male birds cannot be inferred from brood patch scores (Nwaogu et al. 2017). Accordingly, we did not consider sexes in the analyses because Common Bulbuls are sexually monomorphic. Finally, we plotted precipitation of the driest quarter of the year against latitude, sampling date and altitude at each location to verify consistency of sampling effect and precipitation pattern.

To test whether the correlation between the proportion of feather material replaced and the amount of precipitation in the driest quarter of the year is significant, we built a generalized linear mixed-effect model with a binomial error structure (and logit link function) predicting proportion of feather mass regrown as a function of precipitation of the driest quarter in each capture location. We included sampling location as random factor to account for the use of common site features. We used a binomial error structured generalized linear mixed-model so as to deal with boundedness, non-constant variance and non-normal errors that is characteristic of proportion data (Crawley 2012). With this approach, we model the proportion of feather mass grown as the probability of occurrence of moulted or non-moulted birds in each location – these categories bound the upper and lower limits of the distribution of feather mass grown in our data. We included altitude, sampling date and number of birds captured per location as possible confounding effects. Because precipitation of the driest quarter correlates strongly with latitude, we replaced precipitation of the driest quarter with latitude to test the effect of latitude on moult separately rather than include both in one model.

RESULTS

Moult advanced significantly before just before the wet season in environments with earlier rainfall (Figure 6.2A, Table 6.1), and this was independent of breeding, because the proportion of breeding birds per location was not correlated with the precipitation in the driest quarter of the year ($\chi^2_{1,\ 15}$=0.004 ± 0.01, P<0.8; model as Table 6.1 but using proportion of breeding as dependent variable; Figure 6.2E). Altitude, sampling dates and number of captured birds per location did not explain variation in proportion of primary feather mass regrown before the wet season (Table 6.1). Latitude did not significantly predict variation in proportion of primary feather mass regrown before the wet season ($\chi^2_{1,\ 15}$=- 0.12 ± 0.13, P<0.4; model as Table 6.1 but using latitude instead of precipitation of the driest quarter as an independent variable).

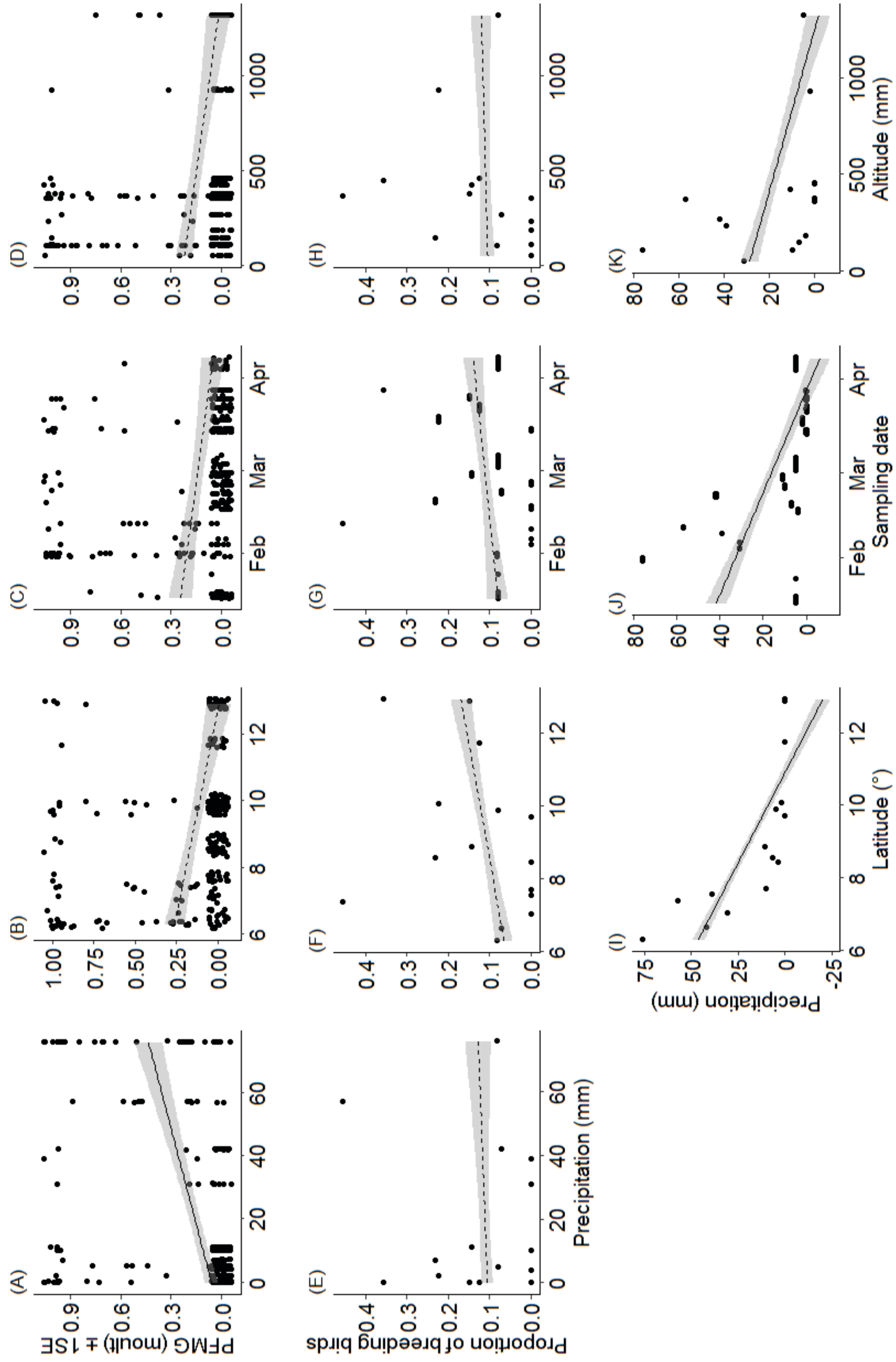
(A)
(B)
(C)
(D)
PFMG (moult) ± 1SE
0.9
0.6
0.3
0.0
1.00
0.75
0.50
0.25
0.00
(E)
(F)
(G)
(H)
Proportion of breeding birds
0.4
0.3
0.2
0.1
0.0
0
20
40
60
Precipitation (mm)
(I)
(J)
(K)
Precipitation (mm)
75
50
25
0
-25
6
8
10
12
Latitude (°)
80
60
40
20
0
Feb
Mar
Apr
Sampling date
500
1000
Altitude (mm)

Figure 6.2: First row: Relationship between proportions of primary feather mass regrown (PFMG) in Common Bulbuls *Pycnonotus barbatus* and (**A**) precipitation of the driest quarter of the year (**B**) latitude (**C**) sampling date and (**D**) altitude at locations along an aridity gradient in a seasonally arid West-African environment (See Figure 1). Second row: Relationship between proportion of breeding Common Bulbuls and (**E**) precipitation of the driest quarter of the year (**F**) latitude (**G**) sampling date and (**H**) altitude at locations along an aridity gradient in a seasonally arid West-African environment. Third row: Relationship between precipitation of the driest quarter of the year and (**I**) latitude (**J**) sampling date and (**K**) altitude at locations along an aridity gradient in a seasonally arid West-African environment. Non-significant correlations are indicated as broken lines. Moult records were obtained from 308 Common Bulbuls mist-netted in 15 locations around Nigeria (sample sizes per location are represented in Figure 1). Mist netting was carried between 17th January and 8th April 2017.

Table 6.1: Precipitation in the driest quarter of the year (before the onset of the main rains) predicts moult in Common Bulbuls *Pycnonotus barbatus*: output of generalized linear mixed model predicting proportion of feather material grown by Common Bulbuls in sampling locations along an aridity gradient in Nigeria (see Figure 6.1). Precipitation (precipitation of the driest quarter of the year) at each location was obtained from http://www.worldclim.org/bioclim. Moult records were obtained from Common Bulbuls mist-netted in 15 locations around Nigeria (sample sizes per location are represented in Figure 1). Sampling location was included as random factor in model (n=307 birds, location = 15, variance = 1.991e-16, SD = 1.411e-08). Mist netting was carried out between 17th January and 8th April 2017 (sampling order shown in Figure 6.1).

Variable	Estimate	Error	z	P	
Intercept	-2.22	0.41	-5.40	<0.01	***
Precipitation	0.02	0.01	3.00	<0.01	**
No. of captures	0.02	0.02	0.98	0.33	
Sampling date	0.00	0.00	-0.35	0.73	
Altitude	0.00	0.00	-1.65	0.10	.

DISCUSSION

By examining variation in the extent of primary moult along a latitudinal gradient where the wet season starts at different times in the year, we reveal that moult in Common Bulbuls is linked to local timing of rainfall. We discuss possible life history implications of this finding, which suggests that moulting in the Common Bulbul may be under a strong selection to match locally variable rainfall independently of the weak breeding seasonality shown for this species (Nwaogu et al. 2018a).

Locations where seasonal rainfall commence earlier show an advancement in moult shortly before the rains, and this is likely associated with earlier environmental productivity in locations with earlier rainfall. Such effect is similar to the influence of environmental productivity on north-south intra-African migration in some tropical species (Elgood et al. 1973; Nwaogu and Cresswell 2015). Rainfall increases environmental productivity in the tropics (Karr 1976; Silva et al. 2011), and is important for the acquisition of resources required to maintain life history events (Ward and Jones 1977, Poulin et al. 1992, Lloyd 1999, Mares et al. 2017). Advanced moult in less arid locations is unlikely a by-product of earlier breeding, because we found breeding bulbuls in all locations, despite variation in precipitation (Figures 6.2E - H) and was unlikely to be associated with other factors unrelated to rainfall because latitude did not explain variation in moult in place of precipitation.

Nonetheless, we encountered a few moulting birds in some locations where the wet season come later (Figure 6.2), and this may be due to local environmental productivity arising from variation in altitude or the presence of rivers which support riparian vegetation. The east-most sampling location – Tula (9° 8' N, 11° 27' E) (Figure 6.1), at proximity to a tributary of the Benue River was particularly striking in this regard for having 11.4% of 35 bulbuls trapped in main wing moult. This relatively high percentage of moulting birds is likely associated with higher local productivity along the river, because we observed bulbuls already exploiting ripe fruits of *Alchonia cordifolia* and *Syzigium guineense* that otherwise are usually unavailable before the rains in other locations of similar latitude (Nwaogu *Pers. obs*). Nonetheless, a location such as Markurdi (Figure 6.1) was also at proximity to the river Benue but no bulbuls were moulting at this site. Thus, moult may be associated with specific aspects of environmental productivity rather than just presence of water. Furthermore, traces of moulting in locations which experience the wet season later was likely because we sampled south to north (see Figure 6.1). These locations were visited within the last two weeks of sampling, and at this time (approximately 10 weeks after the start of sampling in the south), the wet season in these locations was 10 weeks closer than when we commenced sampling in the south. It is expected that moulting would have advanced 10 weeks further in more southerly locations at the time we sampled birds in the north. Thus, the significant positive correlation between moult and the onset of the wet season is likely indicative of a stronger correlation in nature than we observe from our data.

Although our data are preliminary because observations were in one year, they suggest local differences in the timing of the moult cycle (Helm and Gwinner 1999) in Common Bulbuls, but testing population level differences in the timing of the moult cycle will require simultaneous year-round sampling in multiple locations across the aridity gradient for more than one year. Consequently, our result may only be interpreted as a relationship between rainfall and moulting based on observed differences in the extent of primary moult among locations that vary in the onset of the wet season rather than timing of the population moult cycle (Newton and Rothery 2005). Our data, however, provide some evidence that the occurrence of moult is determined by local rainfall pattern, and is unlikely a consequence of the timing of breeding (Camacho 2013, Marmillot et al. 2016).

The correlation between precipitation and moult, suggest that the timing of moult rather than breeding may govern how the annual cycle of the Common Bulbul is organised to optimise seasonal aridity (Nwaogu et al. 2018a). Locally variable environmental signals often fine tune the timing of life history traits (Wingfield 2008), especially if their outcomes depend on environmental conditions (Harriman et al. 2017). Precise timing of moult may increase fitness by enhancing survival and future reproduction (Nilsson and Svensson 1996, Brinkhof et al. 2002). Thus a successful moult may be considered a life history alternative to current reproduction, and particularly so for tropical and south temperate birds that may depend strongly on future reproduction due to smaller clutch sizes and high nest predation (Martin 1996, Fontaine and Martin 2006). If timing of breeding is less dependent on the environment, because year-round conditions in the tropics meet minimal breeding requirements, then timing moult to more suitable annual conditions may optimise fitness (Ward 1969, Hinsley et al. 2003), and possibly also exert influence on the timing of other annual cycle stages. Nonetheless, the Common Bulbul does not necessarily represent the norm for tropical birds because tropical systems are highly diverse with respect to the timing of breeding and moult (Stouffer et al. 2013, Johnson et al. 2012), and this diversity may represent the fact that a wider range of solutions to life history challenges are possible in less constrained tropical environments. Such solutions may include moult-breeding overlaps which is common in tropical birds (Foster 1975, Marini and Durães 2001, Johnson et al. 2012, Stouffer et al. 2013, Repenning and Fontana 2011, Araujo et al. 2017). Our finding suggest that these overlaps may result from the absence of a causal link between breeding and moult coupled with interlocal variability in moult parameters due differences in environmental condition (Borras et al. 2004). Elucidating the mechanism governing the independent seasonal timing of moult in the Common Bulbul may contribute to our understanding of how the avian annual cycle is organized in relation to environmental conditions. In temperate systems, variation in the occurrence of moult is often considered a consequence of breeding or migration (Dietz et al. 2013, Tomotani et al. 2017), but our findings suggest that moult may be timed independently of breeding (Marmillot et al. 2016, Tomotani et al. 2017) and may require specific environmental conditions. Moult and breeding may require different resources - breeding may associate with short term food peaks that may vary throughout the year (Pimentel and Nilsson 2007, Ndithia et al. 2017b), while moult may depend on more stable and predictable food supplies, which may occur with more stable conditions in the wet season (Poulin et al. 1992, Araujo et al. 2017). Moreover, food limitation during breeding may be managed by brood reduction, brood desertion or increased parental effort (Ens et al. 1992), but the amount of feather material replaced annually is largely consistent and specific (Underhill and Joubert 1995). Moulting may be subject to environment suitability independently of breeding, but this may be less obvious in species with strong breeding seasonality. A key hypothesis, therefore, is that the seasonality of moult is maintained according to local environmental conditions independently of variation in breeding, hence breeding-moult overlap will correlate positively with weak breeding seasonality. This hypothesis is testable with the relatively large multi-species datasets of neotropical birds (Johnson et al. 2012b, Stouffer et al. 2013, Marini and Durães 2001, Repenning and Fontana 2011, Araujo et al. 2017)

Chapter 7

Geographical variation in baseline innate immune function does not follow a tropical environmental gradient of aridity

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Will Cresswell

B. Irene Tieleman

Keywords: spatial-temporal aridity, biogeography, West Africa, disease ecology, immune investment.

Abstract

Geographic variation in aridity determines environmental productivity patterns, including large-scale variability in pathogens, vectors and associated diseases, and disease risk is hypothesized to decrease with increasing aridity. If disease risk is matched by immune defense, then immune function should decrease with increasing aridity. We predicted a decrease in innate immune function from the cool-wet forest to the hot-dry Sahel zone of Nigeria. To test the prediction, we sampled blood and measured five innate immune indices, including haptoglobin, nitric oxide and ovotransferrin concentration, and haemagglutination and haemolysis titre from 286 Common Bulbuls *Pycnonotus barbatus* across 15 locations between 6 and 13°N. We sampled all birds within three months in the dry season, including resampling the first sampled location (Jos) at the end to test for temporal change in immune function. Immune indices did not decrease with increasing aridity, they were similar along the gradient, except nitric oxide concentration which unexpectedly increased with increasing aridity. Nonetheless, a significant proportion of the variations in haemagglutination and haemolysis titres were explained by differences among capture locations, suggesting that these immune indices are less variable within than among locations. Ovotransferrin concentration, haemagglutination and haemolysis titre were higher during the second sampling batch in Jos, contrary to expectations that immune indices will decrease later in the dry season. We conclude that in this tropical system, innate immune function does not decrease with increasing aridity and that temporal environmental conditions within a single location may affect immune function more strongly than spatial variation in aridity. In the dry season, Common Bulbuls are likely exposed to similar immune challenges or they are unconstrained by the availability of resources required for optimal immunity across locations. Our result underscore the need to quantify infectious agents and resource availability alongside immune indices and caution when predicting effects of climate variability on immune function or disease risk.

INTRODUCTION

Geographic variation in aridity determines environmental productivity patterns (Lieth 1975, Gaston 2000), including large-scale differences in infectious disease (Altizer et al. 2006), (Lisovski et al. 2017a). These patterns are largely driven by spatio-temporal variability in temperature and precipitation, and they underpin the mechanisms that together or independently shape interactions among infectious agents, vectors and hosts, including host contact rate, susceptibility, infectiousness and immunity (Hosseini et al. 2004, Lowen et al. 2007, Metcalf 2016). Consequently, innate immunity - the first line of defense against infection (Janeway et al. 2001), should vary with immune challenge (Horrocks et al. 2011b). Animal distributions should also be adapted to variation in disease risk (Piersma 1997, Mendes et al. 2005, O'Connor et al. 2018). Consequently, we might expect that under similar life history circumstances, immune investment should decrease along a gradient of decreasing environmental productivity (or increasing aridity) due to reduced immune challenge (Horrocks et al. 2011b) or limited resource availability for immune function (Lochmiller et al. 1993).

Both free living and parasitic species decrease with environmental productivity, and are expectedly more diverse in the tropics (Guernier et al. 2004, Nunn et al. 2005, Keesing et al. 2006, Salkeld et al. 2008). This high biological diversity in tropical environments is not only associated with high environmental productivity, but also with the diversity of environmental conditions within the tropics. But, despite the understanding that tropical environments are relatively disease prone and capable of imposing higher immune costs compared with temperate environments (Møller 1998, O'Connor et al. 2018), the relationship between environmental variability and immune function has rarely been investigated empirically within tropical systems (Ndithia et al. 2017a). Such investigations are important for understanding whether immune investment and infection risk determine habitat use in tropical systems and how the life histories of tropical animals may be affected by climate and land use changes (Lafferty 2009).

The environment, however, affects several factors which may affect immune function differently, so it is important to identify how specific environmental factors relate to immune variation in natural systems with clearly variable environmental patterns. Like disease risk, food availability, diet and life history patterns may also vary with environmental productivity in space and time, and these may also affect immune function (Sheldon and Verhulst 1996), (Klasing 1998). Populations may also be locally adapted or genetically varied across a species' range due to limited gene flow, and so populations may respond differently to variation in disease risk, immune challenge or resource constraints (Ricklefs and Wikelski 2002, Versteegh et al. 2014, Mangino et al. 2017). Such effects of environment versus genetic differences on life history traits, including immune function have been investigated over large spatial environmental gradients (Helm and Gwinner 1999, Nunn et al. 2005, Versteegh et al. 2014, Horrocks et al. 2012b, Horrocks et al. 2015) and across the annual cycle of temperate animals (Nelson et al. 2002, Hegemann et al. 2012b). Overall, results suggest that variation in some immune indices have a genetic background, while others are flexible or rigid to environment conditions (Versteegh et al. 2014).

The heterogeneity of environmental conditions in tropical systems allows for a sub-continental test of predicted global patterns of immune variation in accordance with the expected variation in disease risk (Altizer et al. 2006). For example, the gradient from the Sahara Desert in the north of West Africa to the coastal areas of the Atlantic Ocean in the south represents a gradient of decreasing environmental aridity and temperature (Figure 7.1). Within Nigeria, locations separated by less than 800 km may differ by over 1600 mm of rain annually. Nigeria experiences a single period of rainfall and one of drought each year, but the amount of rain and duration of the wet season varies latitudinally - the wet season is later and shorter going from south to north. In general, the onset of the wet season is usually rapid, and the effects of precipitation are quickly felt with the onset of the wet season, but the onset of the dry season and drying out of the environment is gradual. This feature creates a north-south spatial aridity gradient which is most pronounced at the end of the dry season, and a temporal aridity gradient from the onset of the dry season to its end at each location along the spatial gradient. This environmental gradient may drive spatio-temporal differences in food availability and infection risk and thus, may strongly influence life history traits, including immunity.

Some West African bird species are widespread residents across different locations, (Elgood et al. 1966, Elgood et al. 1973, Nwaogu and Cresswell 2015). Such species like the Common Bulbul *Pycnonotus barbatus* will experience different environmental conditions across their range. Common Bulbuls vary significantly in body size in relation to variation in temperature and aridity (Crowe et al. 1981, Nwaogu et al. 2018). They moult largely in the wet season, but breeding may be year-round (Nwaogu et al. 2018a). Across their range they feed on fruits, insects, nectar and seeds depending on availability (Milla et al. 2005, Okosodo et al. 2016). Such a species is therefore ideal for assessing the relationship between environmental aridity and immune function, assuming similar habitat use but variable infection risk and food availability across an environmental gradient.

Here, we test the hypothesis that immune function decreases with increasing aridity due to expected lower immune challenge and corresponding reduced immune investment due to limited resource availability in more arid environmental conditions. However, because site temperatures during sampling may affect immune function (Xu et al. 2017) independent of long-term environmental aridity, we also considered the effect of site temperature on immune indices. We sampled Common Bulbuls in the dry season, predicting that: (i) immune indices will decrease with increasing aridity from the cool-wet south to the hot-dry north of Nigeria, (ii) immune indices will become lower going further into the dry season within a single location mid-way along the environmental gradient.

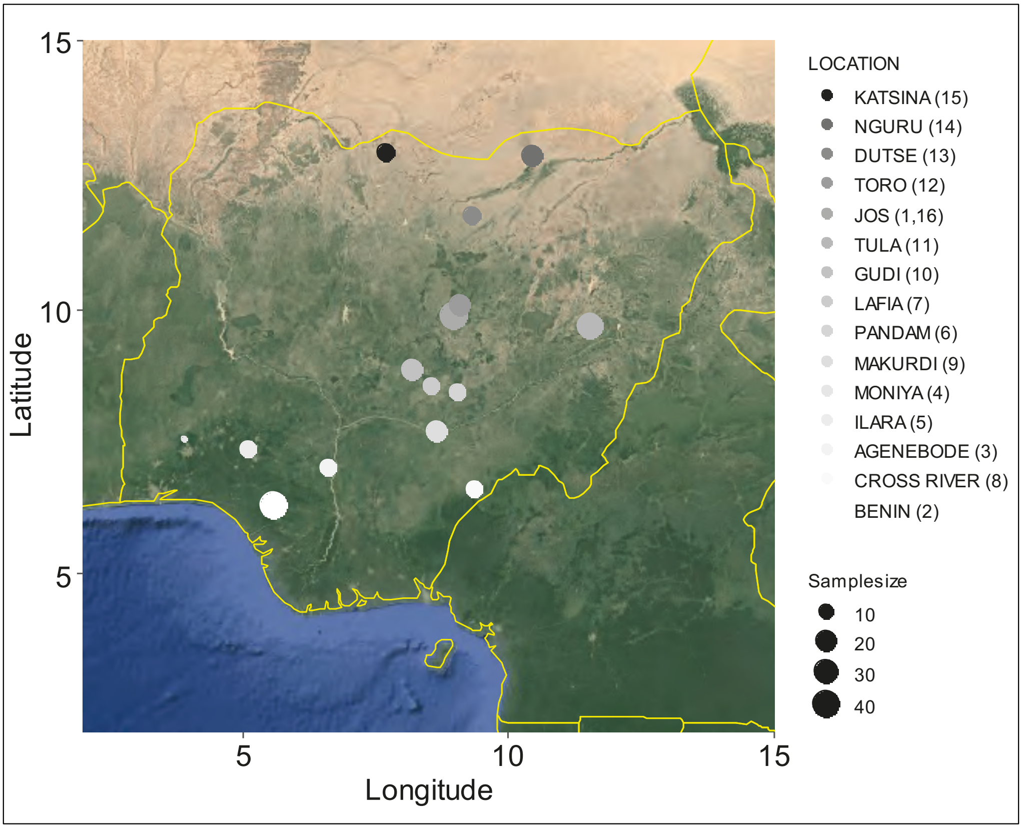

Figure 7.1: Map showing 15 locations along an aridity gradient in Nigeria where Common Bulbuls *Pycnonotus barbatus* were sampled within the end of the dry season between 17th of January and 8th of April 2017. Numbers in parenthesis against sampling location indicate order in which sites where visited for sampling

Table 7.1: Habitat description of 15 sampling locations along the environmental gradient north to south of Nigeria (Figure1) where Common Bulbuls where observed and sampled in the dry season between January 17 and April 8, 2017. Common Bulbuls were sampled at Jos at the start and end of the study.

LOCATION	N	Sampling Dates	Latitude (°N)	Longitude (°E)	Elevation (mm)	% Breeding	% Moulting	Habitat description	Diet
BENIN	39	Jan. 31 - Feb. 1	6.31	5.58	105.66	83.3	75.0	Forest clearing, cassava plantation, human settlement, fruit gardens	Insects from cassava farm, pawpaw fruits
CROSSRIVER	13	Feb. 21 - Feb. 22	6.62	9.35	264.01	7.1	7.1	Forest clearing, Riparian forest fragments, cassava plantation	Insects from cassava farm, Ficus fruits, *Alchonia cordifolia* fruits
AGENEBODE	11	Feb. 3 - Feb. 6	7.03	6.59	49	0.0	18.1	Farm settlement, cashew plantation, oil palm plantation, citrus plantation	Insects from cashew flowers, *Alchonia cordifolia* fruits, cashew fruits, oil palm fruits
ILARA	11	Feb. 10- Feb. 11	7.37	5.1	368.28	45.5	90.9	Cassava plantation, plantain plantation fruit gardens, oil palm plantation	Insects from cashew flowers, pawpaw fruits, oil palm fruits
MONIYA	2	Feb. 8 - Feb. 9	7.55	3.91	232.17	0.0	50.0	Forest clearing, fruit gardens	Insects from cassava farm, Ficus fruits
MAKURDI	20	Feb. 24- Feb. 25	7.71	8.66	107.46	0.0	0.0	Farm settlement, forest clearing, cashew plantation, cassava plantation	Insects from cashew flowers, pawpaw and Ficus fruits
PANDAM	12	Feb. 16-	8.44	9.04	183	0.0	0.0	Dry riverbank,	Ficus fruits

table continues

		Feb. 17						savannah woodland	
LAFIA	13	Feb. 18- Feb. 19	8.56	8.53	144.14	23.1	0.0	Human settlement, Neem plantation, riparian forest, fragments, rice farm	Insects from cashew and *Parkia biglobosa* flowers, neem fruits, ficus fruits
GUDI	21	Feb. 27-	8.86	8.2	422.59			Dense woody vegetation,	Ficus fruits
		Feb. 28				14.3	0.0	Inselbergs,	
								concrete water reservoir	
TULA	35	Mar. 14-	9.7	11.53	356.87			Mango plantation,	Insects, Mango fruits,
		Mar. 15				0.0	11.4	riparian forest fragments,	*Syzygium guineense* fruits,
								savannah woodland,	*Alchonia cordifolia* fruits
								inselbergs	ficus fruits
JOS	28	Jan. 17 -	9.88	8.98	1325			Riparian forest fragments,	Insects,
		Jan. 19				0.0	0.0	savannah woodland,	*Harungana madagascariensis* fruits
								inselbergs	*Rhus natalensis* fruits
JOSII	12	Apr. 4 -	9.88	8.98	1325			Riparian forest fragments,	Insects,
		Apr. 8				25.0	16.6	Savannah woodland,	*Rhus natalensis* fuits
								inselbergs	*Jasminum dichotomum* fruits
TORO	19	Mar. 17-	10.06	9.09	929.35			Riparian forest fragments,	Insects from cashew flowers,
		Mar. 19				22.2	5.5	vegetable farms	*Alchonia cordifolia* fruits
									ficus fruits.
DUTSE	16	Mar. 21-	11.73	9.33	457.94	12.5	6.3	Date plantation,	Insects from mango trees,
		Mar. 23						mango plantation	mango fruits
NGURU	20	Mar. 25- Mar. 26	12.86	10.45	375.13			Irrigated farmlands,	Insects from cashew flowers,
						15.0	10.0	neem plantation,	neem fruits

table continues

								human settlement	cashew fruits
KATSINA	14	Mar. 28- Mar. 30	12.92	7.72	447.53	35.7	0.0	Irrigated farmlands, neem plantation	Insect from onion farm, neem fruits

METHODS

Study species

Common Bulbuls are sexually monomorphic passerines which are 9 - 11 cm in body length and weigh 25 - 50g. In Table 7.1, we present detailed information on study populations and capture locations where we sampled Common Bulbuls between 17 January and 8 April 2017.

Bird sampling and environmental variables

We travelled between latitude 6 and 13° N in Nigeria and mist-netted 286 Common Bulbuls from 15 locations (Figure 7.1). We visited each location, including resampling of the first location visited (Jos) in the dry season. Apart from Jos, which was sampled at the start and end, we sampled from the southernmost location (Benin) and advanced northward. The sampling pattern (south to north) was designed to avoid sampling any location after the start of the rains since the rains commence earlier in the south, and because we predicted that immune function will decrease with increasing aridity. Sampling southern locations after the start of the rains will make any conclusions about the effect of environmental aridity on immune function invalid. By sampling south to north consistent with the onset of the rains, we were on average close to the start of the wet season in each location during our visit.

All birds were caught using mist-nets and sampled between 6:00 and 11:00 hours. We collected c.300 microliter of blood from each bird into heparinised micro capillary tubes after puncturing the brachial vein with a needle. On average, birds were bled 17.5 ± 8.6 minutes after capture. Samples were stored on ice in the field until processing to separate plasma from cellular fractions, then stored frozen at -20° C until immune assays were carried out.

We weighed (± 0.1g, Ohaus Scout), determined occurrence of moult and breeding from each bird before release in order to account for their possible effects on immune function. Breeding status could only be determined for female birds because males do not carry brood patches. Non-breeding females and all males were indistinguishable because Common Bulbuls are sexually monomorphic.

To assess the relationship between site temperature and immune function, we estimated the average local temperature at mid-day at each capture location during the sampling period using dry bulb temperature records from a hand-held thermometer. Since the entire sampling was carried out in the dry season there was no rainfall. To assess the relationship between environmental aridity and immune function, we extracted bioclimatic variables for each capture location (Table 7.1), including mean annual temperature, annual precipitation, temperature seasonality and precipitation seasonality from http://www.worldclim.org/bioclim, using the 'maptools' and 'raster' packages in R. We used the bioclimatic variables extracted to calculate De Martonne aridity index (Martonne 1926) as a measure of long term environmental aridity: De Martonne aridity index = annual precipitation (mm) / (mean annual temperature (°C) + 10). Locations with values less than 10 are classified as arid, while those with values greater than 40 are classified as humid.

Immune assays

We quantified plasma haptoglobin concentration using a functional colorimetric assay which quantifies the haeme-binding capacity of plasma. We followed instructions for the ‘manual method’ provided with a commercially available assay kit (Cat. No.: TP801; Tridelta Development Ltd, Maynooth, Co. Kildere, Ireland) (Matson et al. 2012).

We quantified nitric oxide concentration by a colorimetric assay described by (Sild and Hõrak 2009). The method estimates the concentration of nitrate and nitrite in plasma after reducing all nitrate to nitrite using copper-coated cadmium granules. A measurable colour development proportionate to the nitric oxide concentration follows reaction with Griess reagent, and its absorbance is measured by colorimetry.

We quantified ovotransferrin by estimating the maximum amount of iron required to saturate all ovotransferrin in a sample. We followed a three step process described by Horrocks et al. (2011): saturation of ovotransferrin with ferric iron under alkaline conditions, reduction of excess unbound iron by ascorbic acid, then dissociation of ovotransferrin-iron complex under acidic conditions, leading to a colour development whose absorbance is measured by colorimetry.

We quantified natural antibody-mediated haemagglutination and complement-mediated haemolysis titres of plasma samples against 1% rabbit red blood cells (Envigo RMS (UK) Ltd.) in phosphate buffered saline as described by Matson et al., (2005). Both haemagglutination and haemolysis titres were recorded as the number of serial dilution steps in which each function was still observable using an existing rubric (Matson et al. 2005).

Data analyses

First, we built general linear mixed-effect models to test the relationship between aridity index and local temperature, and haptoglobin, nitric oxide and ovotranferrin concentration, and haemagglutination and haemolysis titre. We included capture location as a random factor to account for differences between capture locations which may be unrelated to aridity or temperature and to account for the use of common site measures (aridity and temperature) to predict variation in the immune function in multiple birds within each location. We also included body mass and occurrence of moult as predictor variables because individual condition and moulting may affect immune function (Buehler et al. 2008). We did not include breeding status in our models because only females can be scored for breeding using brood patch occurrence. To test whether capture location contributed significantly to variation in immune function i.e. whether immune function differed among capture locations, we built general linear models with similar variables as included in the general linear mixed-effect models, but without the random factor, capture location. We compared model pairs using analysis of variance to determine whether the inclusion of capture location as random factor improved the explanatory power of the models. In addition, we calculated within and among location coefficient of variation for each immune index (Table 7.S1).

Secondly, to test whether immune indices were lower later in the dry season relative to the start of sampling, over the 12 weeks’ timeframe of the study, we built general linear models to compare the first and second sampling batch in Jos. We included sampling batch as a two-factor predictor variable, and body mass as a covariate to account for variation in individual condition.

All statistical analyses were performed in R 3.5.1 (R Development Core Team 2006).

RESULTS

Immune indices did not decrease with increasing aridity, unexpectedly, nitric oxide concentration increased with increasing aridity along the gradient and ovotransferrin concentration, haemagglutination and haemolysis titre increased later in the dry season in Jos. Nitric oxide concentration decreased with decreasing aridity, but ovotranferrin concentration, haptoglobin concentration, haemagglutination titer and haemolysis titre did not vary with aridity along the environmental gradient (Figure 7.2, Table 7.2). Haptoglobin concentration increased marginally insignificantly with local temperature along the environmental gradient during the sampling period, but ovotransferrin concentration, nitric oxide concentration, haemagglutination titre and haemolysis titre did not vary with local temperature during the sampling period (Figure 7.3, Table 7.2). Body mass explained variation in haemagglutination titre but not haptoglobin, nitric oxide and ovotransferrin concentration, and haemolysis titre (Table 7.2). Sampling location explained a significant proportion of the variation in haemagglutination titre (Va = 0.61 ± 0.78, P = 0.03) and haemolysis titre (Va = 0.01 ± 0.10, P = 0.04), but not in ovotransferrin concentration (Va = 0.91 ± 0.95, P = 0.08), nitric oxide concentration (Va = 0.07 ± 0.26, P = 0.85) and haptoglobin concentration (Va = 0.001 ± 0.02, P = 1). Individual bioclimatic did not correlate immune indices (see Figures 7.S1 - 5).

Ovotransferrin concentration and haemagglutination titre were significantly higher, while haemolysis titre was marginally insignificantly higher during the second sampling batch in Jos. Haptoglobin and nitric oxide concentration did not differ between sampling batches (Figure 7.4, Table 7.2).

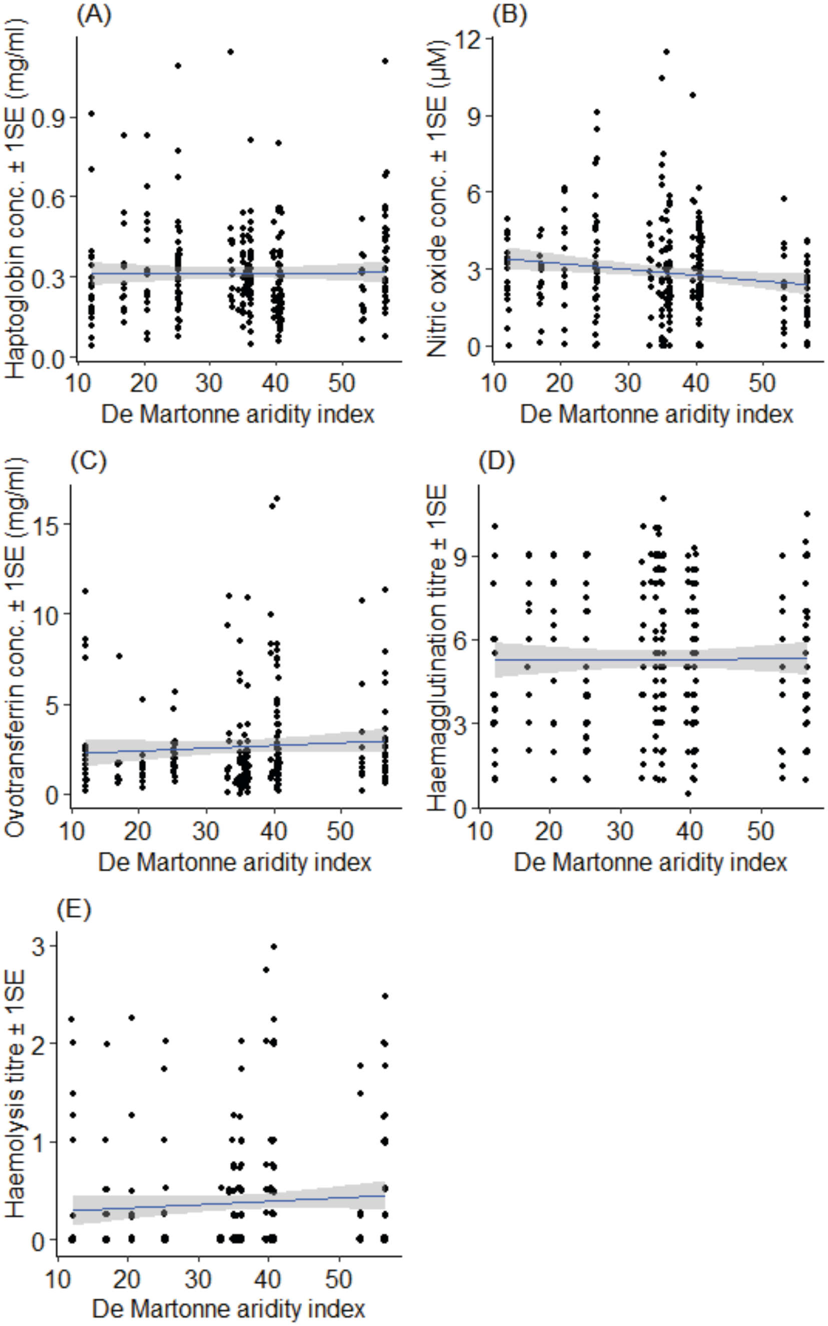

Figure 7.2: Relationship between (A) haptoglobin concentration (mg/ml), (B) nitric oxide concentration (µM), (C) ovotransferrin concentration (mg/ml), (D) haemagglutination titre and (E) haemolysis titre in Common Bulbuls and variation in aridity (De Martonne aridity index – high values indicates lower aridity i.e. increased humidity) along and environmental gradient in north to south of Nigeria.

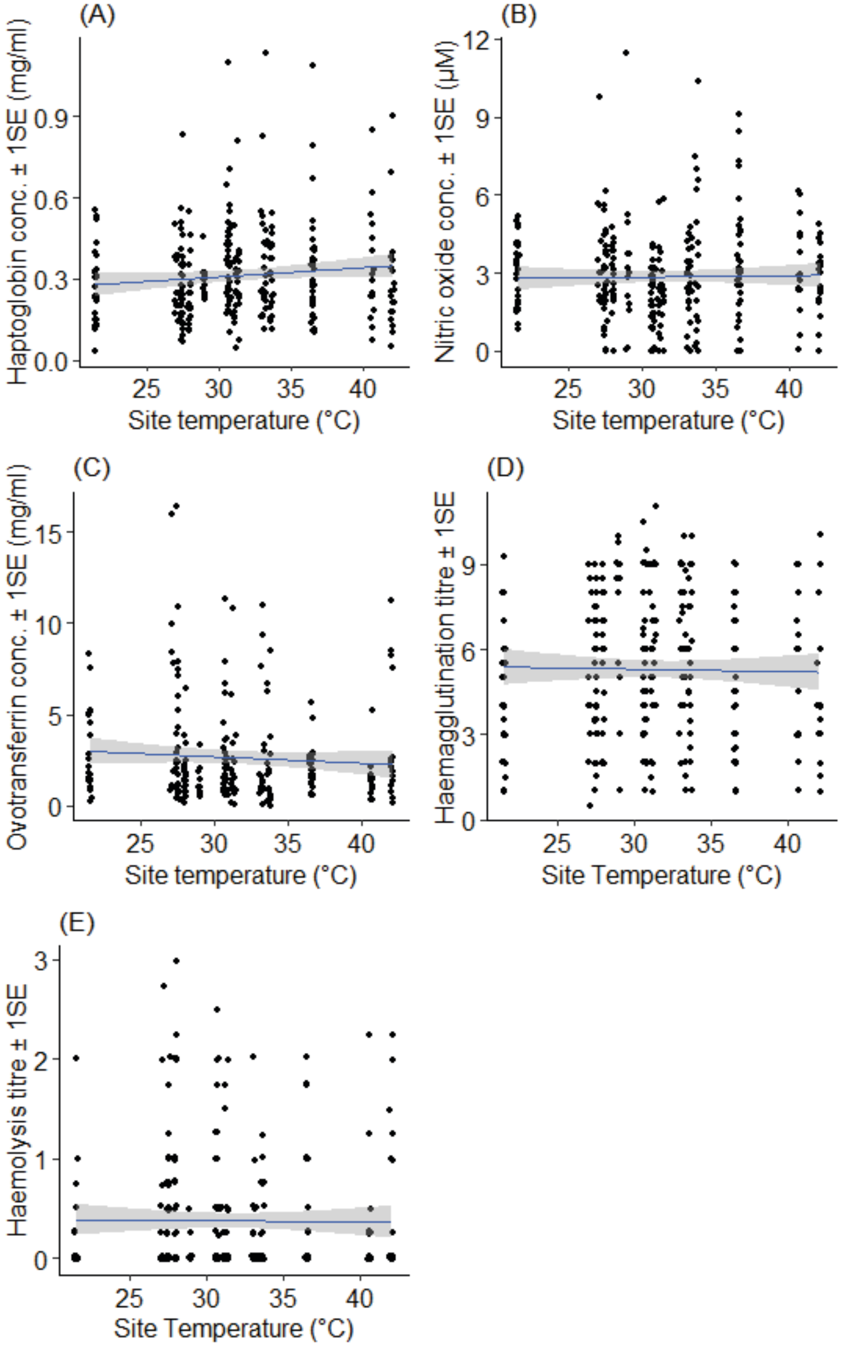

Figure 7.3: Relationship between (A) haptoglobin concentration (mg/ml), (B) nitric oxide concentration (μM), (C) ovotransferrin concentration (mg/ml), (D) haemagglutination titre and (E) haemolysis titre in Common Bulbuls and local temperature during sampling along and environmental gradient in north to south of Nigeria.

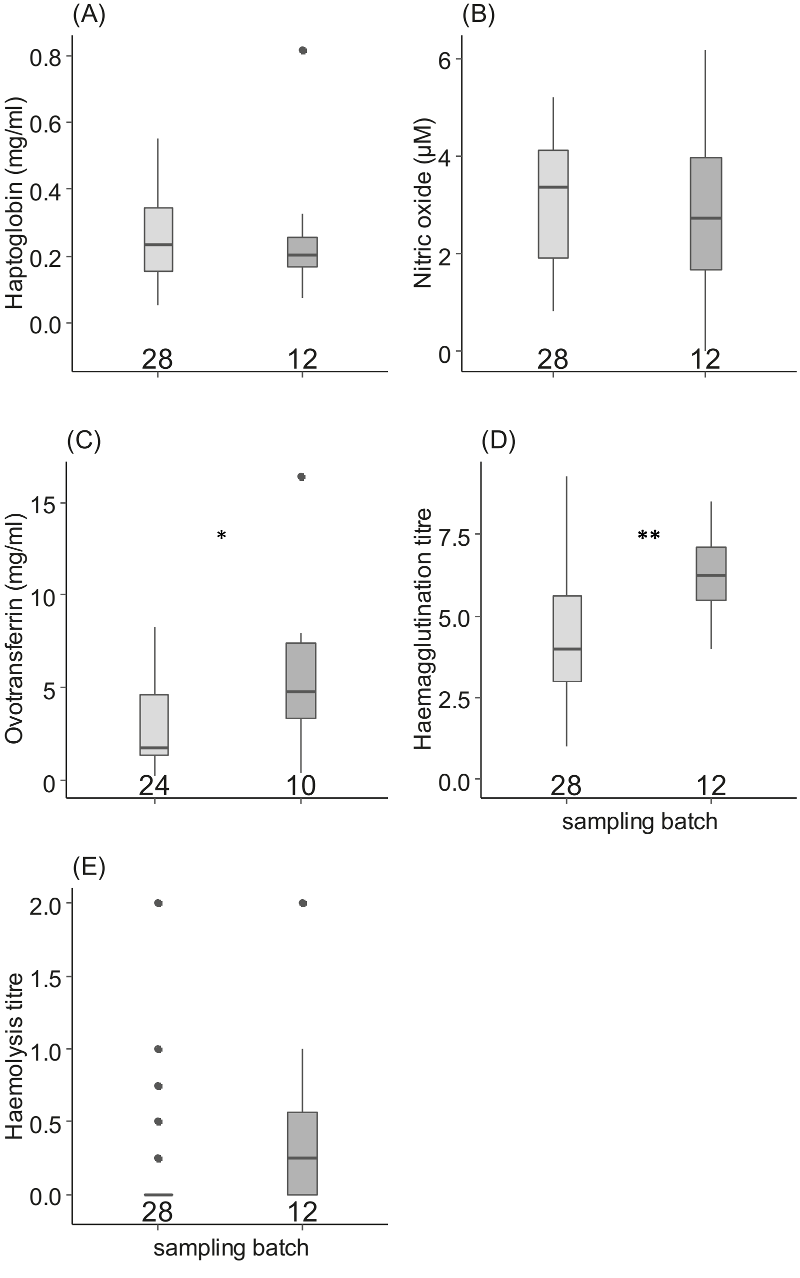

Figure 7.4: Difference in (A) haptoglobin concentration (mg/ml), (B) nitric oxide concentration (µM), (C) ovotransferrin concentration (mg/ml), (D) haemagglutination titre and (E) haemolysis between Common Bulbuls sampled in Jos (located about mid-way along an aridity gradient south to north of Nigeria (Figure 7.1)) at 12 weeks interval corresponding to the start and end of entire sampling period along the gradient. Box plots are arranged in sampling order with sample size indicated at the base of each plot.

Table 7.2: Summary of differences in immune indices of Common Bulbuls *Pycnonotus barbatus* sampled across 15 locations along an aridity gradient in Nigeria (Figure 7.1). Body mass and temperature during period of sampling were included in all models to test the effect of body condition and environmental condition during capture.

			Haptoglobin			**Nitric oxide**			**Ovotransferrin**			**Haemagglutination**			**Haemolysis**		
	Variable	**Df**	**Chisq**	**P**		**Chisq**	**P**		**Chisq**	**P**		**Chisq**	**P**		**Chisq**	**P**	
Gradient	**Aridity index**	1	0.97	0.33		**6.06**	**0.01**	*	0.09	0.76		0.00	0.99		0.25	0.62	
	Temperature	1	3.72	0.05	.	1.90	0.17		0.19	0.67		0.01	0.92		0.08	0.78	
	Body mass	1	0.07	0.80		0.43	0.51		0.09	0.77		**5.34**	**0.02**	*	1.20	0.27	
	Moulting	1	0.18	0.67		0.90	0.34		0.01	0.92		2.48	0.12		0.11	0.74	
	Variable	**Df**	**F**	**P**		**F**	**P**		**F**	**P**		**F**	**P**		**F**	**P**	
Jos	**Batch**	1	0.14	0.71		0.081	0.778		**5.01**	**0.03**	*	**7.99**	**0.01**	**	3.27	0.08	.
	Body mass	1	0.00	0.98		1.802	0.188		0.89	0.35		0.09	0.76		0.15	0.70	

DISCUSSION

We tested the hypothesis that immune function decreases with increasing aridity along an environmental gradient in Nigeria and found no evidence in support of this hypothesis. On the contrary, we observed that nitric oxide concentration increased with increasing aridity from south to north. Within the single site Jos, ovotransferrin concentration, haemagglutination and haemolysis titres increased 12 weeks later in the dry season, when environmental conditions were expected to be more arid. However, although immune indices were largely as variable within as among locations (Table 7.S1), variation in haemagglutination and haemolysis titres were partly explained by capture locations, independent of aridity. The lack of decrease in immune indices with increasing aridity was unexpected (Salkeld et al. 2008, Nunn et al. 2005, Horrocks et al. 2015), but it suggests that in this system, variation in innate immune function does not follow a simple environmental productivity pattern. We discuss the implications of this finding and present alternative perspectives on the observed pattern.

The hypothesis that immune function might be attenuated in arid environmental conditions is based on the assumption that disease risk decreases with increasing aridity (Horrocks et al. 2011, Horrocks et al. 2012b, Horrocks et al. 2015) and that immune investments are lower during periods of lower disease risk or limited resource availability (Hasselquist and Nilsson 2012). Apparently, this is not the case for this system. On the contrary, it appears that immune challenge is either similar along the gradient or that Common Bulbuls maintain a similar level of immune function in the dry season despite spatial variability in aridity at other times of the year. Moreover, variation in innate immune function may reflect infectious agents on animals rather than those in their environment (Horrocks et al. 2012a), and exposure to infection may depend strongly on habitat use and foraging habits (Johnson et al. 2009). So, regardless of the overall antigen abundance or diversity in the environment Common Bulbuls may encounter similar types of antigens due to similar habitat use, social interactions and foraging habits across their range, particularly when comparing populations at the end of the dry season (Table 7.1). Similar diets of fruit and insects in Common Bulbuls (Table 7.1) may also allow the maintenance of optimal immunity if immune challenge is similar along the gradient because innate immune function may be more affected by diet than food restriction or limitation (Klasing 1998, Cotter et al. 2011, Buehler et al. 2009b). Nonetheless, there is a need to quantify infectious agents in the environment and on animals alongside immune indices in studies of ecological immunology (Horrocks et al. 2012a), and this need is strongly highlighted by the unexpected lack of (or contra) correlation between immune indices and aridity in this study. Several studies have used immune indices or pathogen pressure alone to test hypotheses about immune function or disease risk (Piersma 1997, Møller 1998, Nunn 2002, Nunn et al. 2003, Blount et al. 2003, Mendes et al. 2005, Matson 2006, Spottiswoode 2008, Buehler et al. 2010). This may lead to misleading interpretations: every assumption of variation in disease risk or resource limitation due to variation in environmental conditions requires an empirical confirmation.

Temporal variation in environmental conditions within a single location or population may be a more important source of short-term variation in immune function than spatial variation in aridity, especially if environmental conditions along a gradient are suitable to support a minimum threshold of infectious agents or resources required to maintain immune responses. Three of the five immune indices we measured were higher 12 weeks after the first batch of sampling in Jos (mid-way along this aridity gradient). The reasons for these temporal differences are not obvious, but during the second batch of sampling in Jos, local temperatures were c. 6°C higher, and 25.0% and 16.6% of birds were breeding and moulting, respectively as compared to none during the first sampling (Table 7.1). However, although, the temporal differences in some immune indices were significant, they do not suggest an attenuation of immune function with increasing aridity (Horrocks et al. 2015) or occurrence of annual cycle stage (Martin et al. 2008b) but, they are consistent with findings from our year-round study on Common Bulbuls in the same location. In a two-year study, we unexpectedly found higher immune indices in the dry season compared to the wet season (Nwaogu et al. 2019). So, if immune indices are particularly high in the dry season, variation among locations may be minimal, because sampling was carried out at the end of the dry season in all locations, resulting in no correlation along the gradient. However, immune function may not be at maximum levels because there was still an increase between the first and second sampling batches in Jos. Overall, the similarity among locations and the high variability within locations (Table 7.S1) confirms that innate immunity is flexible to environmental conditions (Versteegh et al. 2014), but does not simply decrease with aridity.

Our result raises questions about the specific environmental factors that are responsible for influencing latitudinal variation in infection risk or immune function (Nunn et al. 2005), (Mendes et al. 2005). Except for nitric oxide which increased with increasing aridity and decreasing annual precipitation none of the other immune indices varied with aridity, local temperature or other selected bioclimatic variables, including mean annual temperature, annual precipitation, temperature seasonality and precipitation seasonality (Figure 7.S1 - 5). Studies involving larger latitudinal gradients show that mean annual temperature seem to explain variation in parasite load and immune indices better than precipitation (Salkeld et al. 2008, Horrocks et al. 2014). Results from studies that used similar immune indices to the ones used in this study are equivocal: (Horrocks et al. 2015) studied different species of adult Larks along an aridity gradient spanning desert, temperate and tropics and showed negative correlations between aridity and haemagglutination and haemolysis titres, and haptoglobin concentration, but no correlations between aridity and ovotransferrin concentration. However, using eggs from Lark species in different environments, they showed contrasting patterns for ovotransferrin and lysozyme concentration in egg albumin (Horrocks et al. 2014). Elsewhere in the tropics, (Ndithia et al. 2017a) recorded no differences between immune indices in chicks of Red-capped Larks *Calandrella cinerea* from three climatically distinct locations in Kenya, even though these chicks showed significant differences in growth patterns. Like Kenya, none of the locations from which we sampled Common Bulbuls qualifies to be described as ‘arid’ because none had a De Martonne aridity index (Martonne 1926) of less than 10 and all locations receive over 400 mm of rainfall annually despite strong seasonality in precipitation and temperature patterns. Therefore, since Common Bulbuls show a significant variation in body size (Nwaogu et al. 2018b) and in the extent of primary moult (Nwaogu et al. *in prep*) along the same environmental gradient, then immune function is perhaps, more flexible to local environmental conditions than body size and moult which seem more locally adapted. Alternatively, immune function requires greater environmental variability to show significant spatial variation (Horrocks et al. 2015, Horrocks et al. 2012b). The end of the dry season either minimised environmental variability or the environmental gradient in this system is not pronounced enough to generate variable innate immune phenotypes. A follow-up sampling during the wet season may yield a different outcome or elucidate the observed pattern because the rains may cause greater change to environmental conditions in arid than in humid locations, possibly resulting to greater immune challenge in arid environments at the onset of the rains.

In summary, we can reasonably conclude that in this tropical system, innate immune function does not follow a simple environmental productivity pattern, and this may apply to disease risk (Piersma 1997, Mendes et al. 2006, O'Connor et al. 2018). The observed pattern is unlikely to be a cancellation effect resulting from a decrease in immune indices further into the dry season because immune indices instead increased in Jos where sampling was repeated 10 weeks further into the dry season. This result underscores the importance of quantifying infectious agents and resource availability alongside immune indices in studies of ecological immunology where variation in infection risk and resource limitation form the underlying assumption for a hypothesis. More generally, caution should probably be exercised when predicting effects of climate variability on immune function and disease risk.

SUPPLEMENTARY INFORMATION

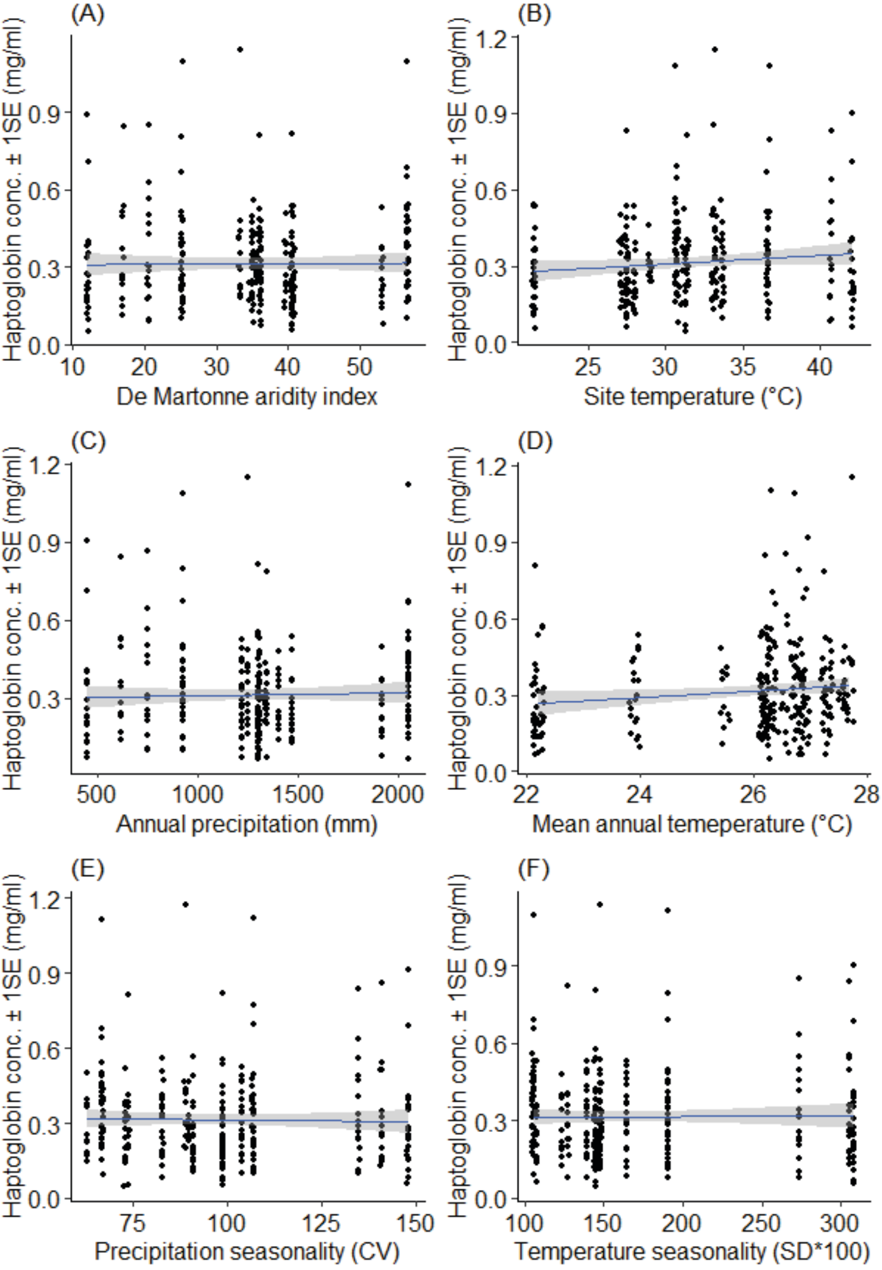

Figure 7.S1: Relationship between haptoglobin concentration (mg/ml) in Common Bulbuls sampled across 15 locations along an environmental in Nigeria and (A) De Martonne aridity index, (B) local temperature during sampling period, (C) annual precipitation (mm), (D) mean annual temperature, (E) precipitation seasonality (CV) and (F) temperature seasonality (SD*100).

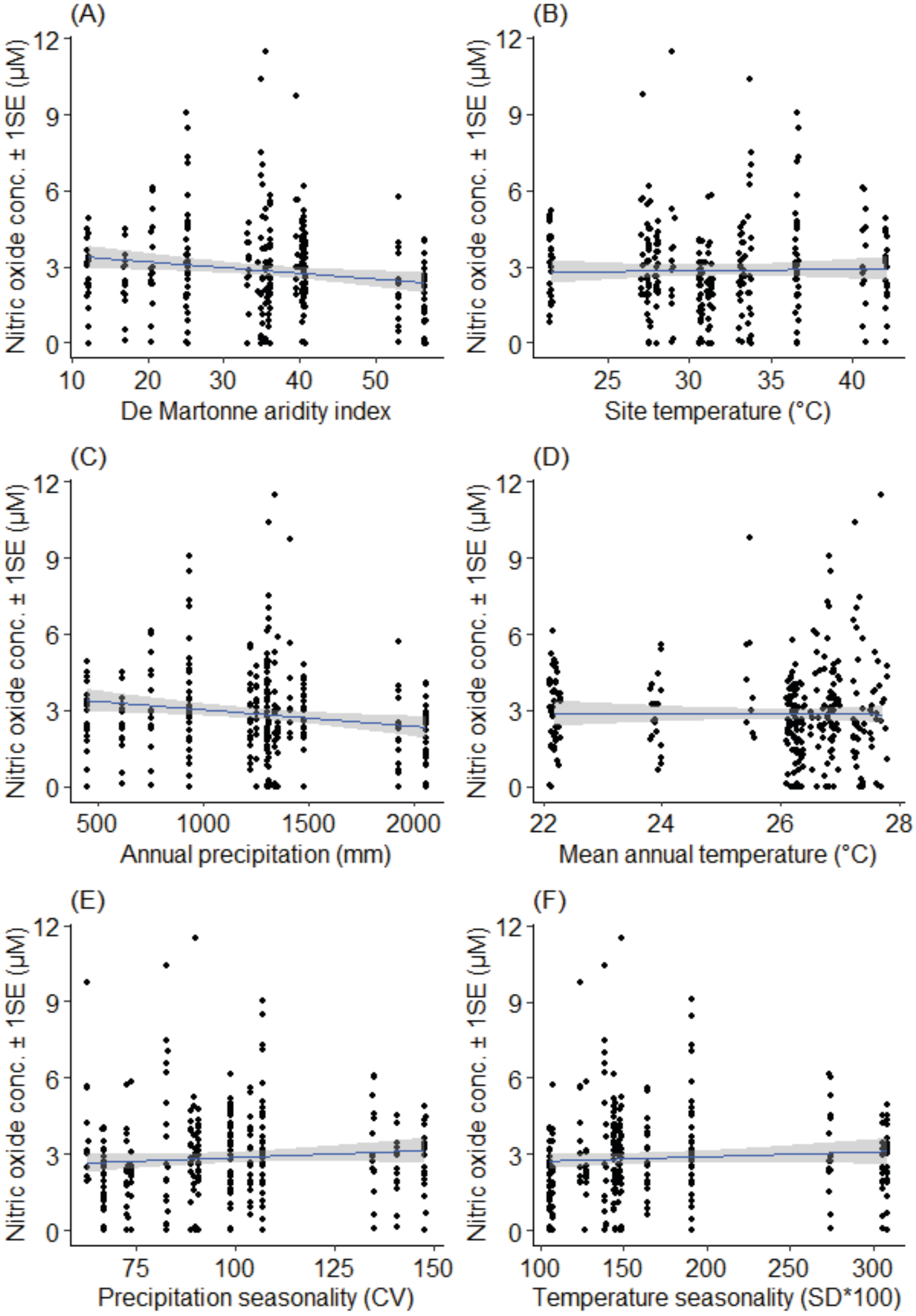

Figure 7.S2: Relationship between nitric oxide concentration (µM) in Common Bulbuls sampled across 15 locations along an environmental in Nigeria and (A) De Martonne aridity index, (B) local temperature during sampling period, (C) annual precipitation (mm), (D) mean annual temperature, (E) precipitation seasonality (CV) and (F) temperature seasonality (SD*100).

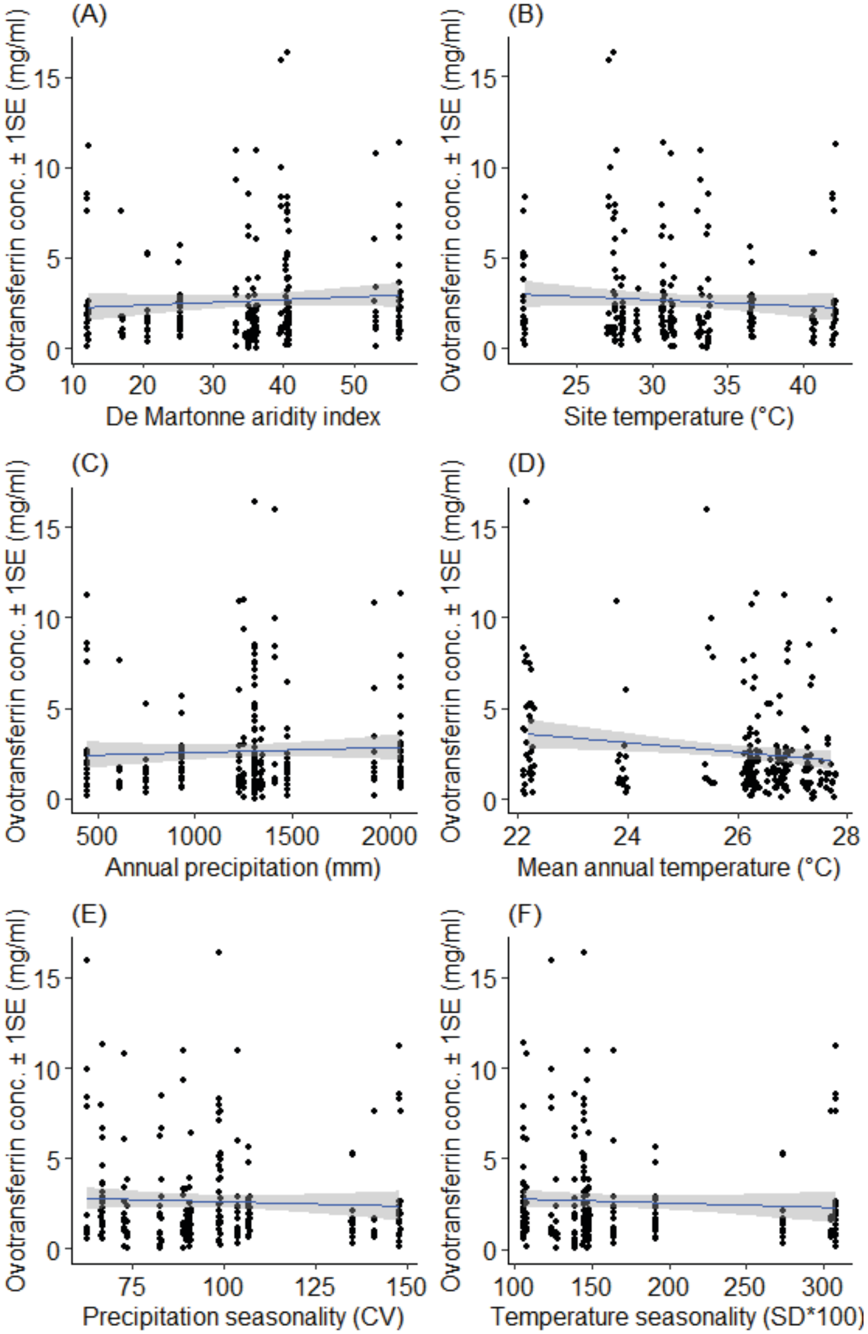

Figure 7.S3: Relationship between ovotransferrin concentration (mg/ml) in Common Bulbuls sampled across 15 locations along an environmental in Nigeria and (A) De Martonne aridity index, (B) local temperature during sampling period, (C) annual precipitation (mm), (D) mean annual temperature, (E) precipitation seasonality (CV) and (F) temperature seasonality (SD*100).

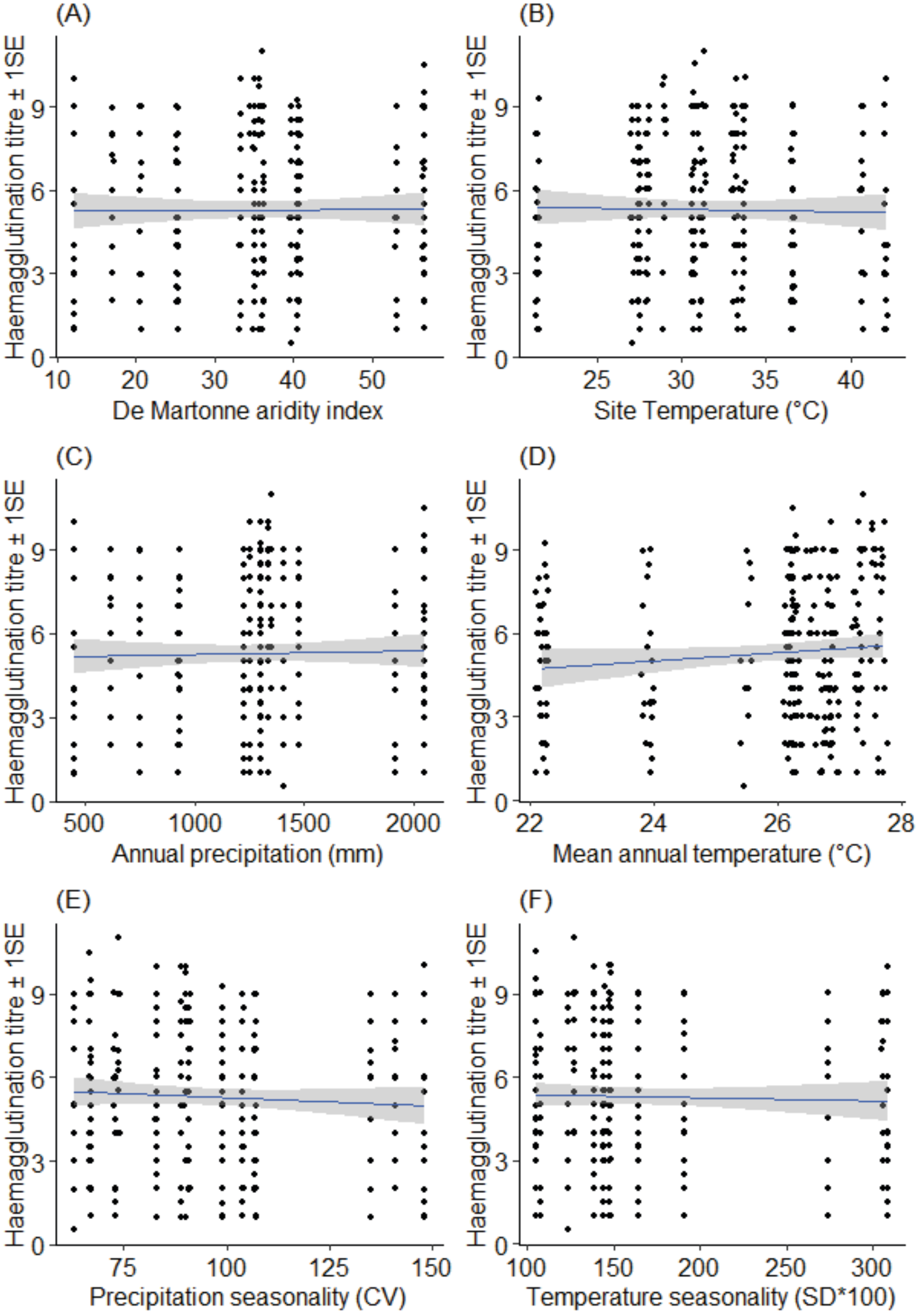

Figure 7.S4: Relationship between haemagglutination titre in Common Bulbuls sampled across 15 locations along an environmental in Nigeria and (A) De Martonne aridity index, (B) local temperature during sampling period, (C) annual precipitation (mm), (D) mean annual temperature, (E) precipitation seasonality (CV) and (F) temperature seasonality (SD*100).

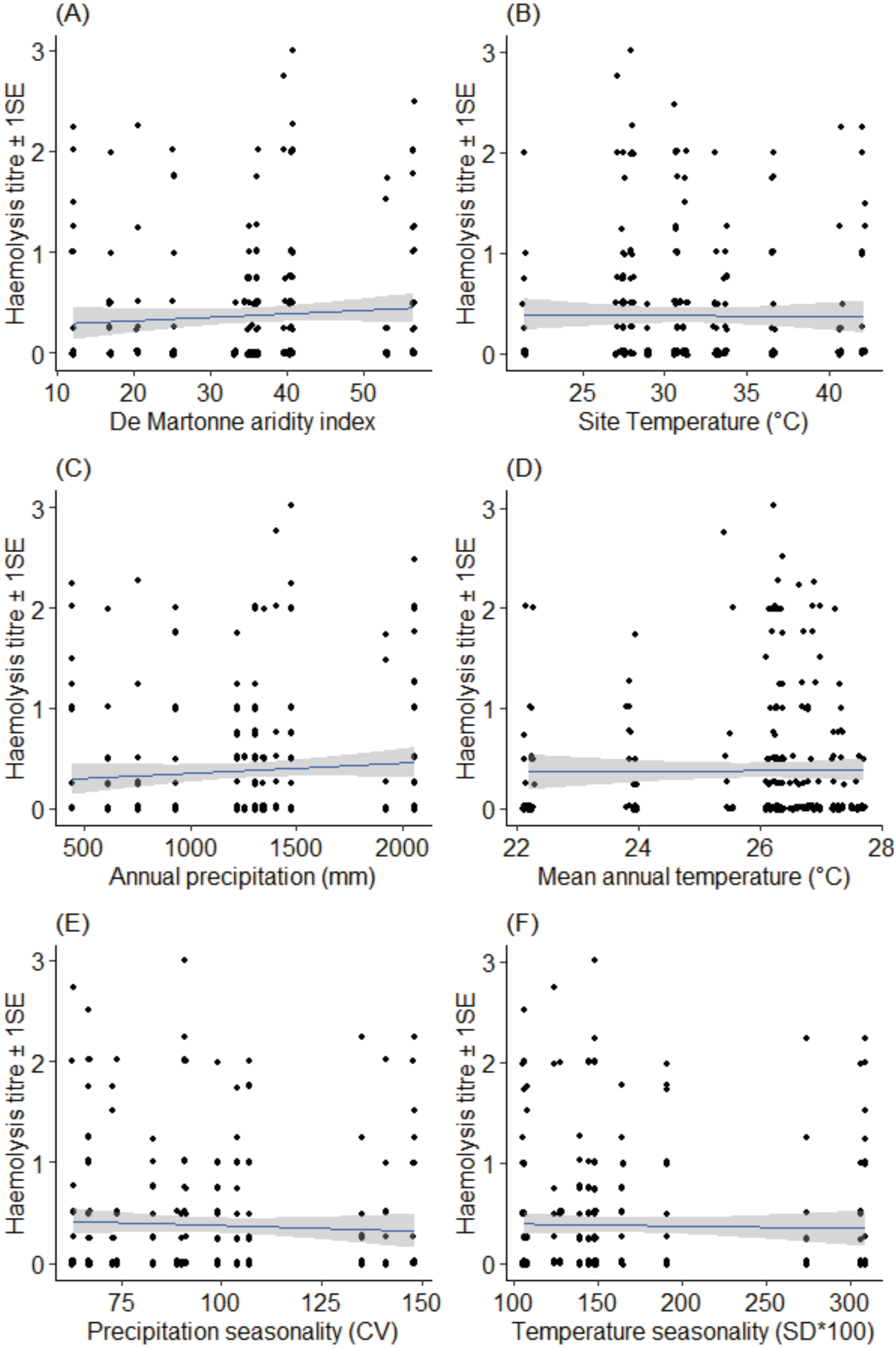

Figure 7.S5: Relationship between haemolysis titre in Common Bulbuls sampled across 15 locations along an environmental in Nigeria and (A) De Martonne aridity index, (B) local temperature during sampling period, (C) annual precipitation (mm), (D) mean annual temperature, (E) precipitation seasonality (CV) and (F) temperature seasonality (SD*100).

Table 7.S1: Variation immune indices of Common Bulbuls within and among 15 sampling locations along an environmental gradient of varying aridity north to south of Nigeria.

				Coefficient of variation		
Assay	**Variation**	**n**	**Mean**	**Mean**	**Max**	**Min**
Haptoglobin	Within-location	16 locations	0.31 (mg/ml)	0.51	0.77	0.18
	Among-location	285 samples		0.55		
Nitric oxide	Within-location	16 locations	2.85 (µM)	0.58	0.91	0.30
	Among-location	285 samples		0.64		
Ovotransferrin	Within-location	16 locations	2.63 (mg/ml)	0.87	1.17	0.24
	Among-location	234 samples		1.03		
Haemagglutination	Within-location	16 locations	5.29 (titre)	0.44	0.58	0.08
	Among-location	284 samples		0.48		
Haemolysis	Within-location	16 locations	0.38 (titre)	1.75	3.46	0.00
	Among-location	284 samples		1.69		

Table 7.S3: Summary test statistics for correlation between immune indices and De Martonne aridity index , local site temperature and selected individual bioclimatic variables - mean annual temperature, annual precipitation, temperature seasonality and precipitation seasonality (Figure 7.S1 - 5) all extracted from http://www.worldclim.org/bioclim.

		Haptoglobin			Nitric oxide			Ovotransferrin			Haemagglutination		Haemolysis	
Variable	**Df**	**Chisq**	**P**		**Chisq**	**P**		**Chisq**	**P**		**Chisq**	**P**	**Chisq**	**P**
Aridity index	1	0.05	0.83		3.31	0.07	.	0.68	0.41		0.00	0.97	0.32	0.57
Site temperature	1	2.31	0.13		0.00	0.95		0.74	0.39		0.08	0.77	0.06	0.81
Annual precipitation	1	0.00	0.97		3.70	0.05	.	0.26	0.61		0.02	0.89	0.30	0.58
Mean annual temperature	1	3.26	0.07	.	0.00	0.97		4.74	0.03	*	1.20	0.27	0.09	0.76
Precipitation seasonality	1	0.00	0.96		0.31	0.58		0.23	0.63		0.50	0.48	0.13	0.72
Temperature seasonality	1	0.14	0.71		0.24	0.63		0.41	0.52		0.13	0.72	0.02	0.89

Part IV

Effect of diet composition on body condition

Chapter 8

A fruit diet rather than invertebrate diet maintains a robust innate immune function in an omnivorous tropical songbird

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Keywords: Nutrient limitation, vegetarian diet, eco-immunology, immunomodulation, life-history trade-offs, path analyses and environmental change.

Abstract

Diet alteration may lead to nutrient limitations even in the absence of food limitation, and this may affect physiological functions, including immunity. Nutrient limitations may also affect the maintenance of body mass and key life history events that may affect immune function. Yet, variation in immune function is largely attributed to energetic trade-offs rather than specific nutrient constraints. We tested how diet composition affects innate immune function, body mass and moult separately and in combination with each other, and then used path analyses to generate hypotheses about their mechanistic connections. We performed a balanced parallel and crossover design experiment with omnivorous Common Bulbuls *Pycnonotus barbatus* in out-door aviaries in Nigeria. We fed 40 wild-caught bulbuls *ad libitum* on fruits or invertebrates for 24 weeks, switching half of each treatment between treatments after 12 weeks. We assessed innate immune indices (haptoglobin, nitric oxide and ovotransferrin concentrations, and haemagglutination and haemolysis titres), body mass and primary moult, fortnightly. We simplified immune indices into three principal components (PCs), but we explored mechanistic connections between diet, body mass and each immune index separately. Fruit fed bulbuls had higher body mass, earlier moult and showed higher values for two of the three immune PCs compared to invertebrate fed bulbuls. These effects were reversed when we switched bulbuls between treatments after 12 weeks. Exploration of correlations between immune function, body mass and moult, showed that an increase in immune function was associated with a decrease in body mass and delayed moult in invertebrate fed bulbuls, while fruit fed bulbuls maintained body mass independent of variation in immune function. Path analyses indicated that diet composition was most likely to affect body mass and immune indices directly and independently from each other. Only haptoglobin concentration was indirectly linked to diet composition via body mass. We demonstrated a causal effect of diet composition on body mass, innate immune function and moult: bulbuls were in better condition when fed on fruits than invertebrates, confirming that innate immunity is nutrient specific. Our results are unique because they show a reversible effect of diet composition on wild adult birds whose immune systems are presumably fully developed and adapted to wild conditions – demonstrating a short-term consequence of diet alteration on life history traits.

INTRODUCTION

Animals face nutrient limitations during their life or during the annual cycle, and such nutrient limitations may affect physiological and life history functions, including immunity (Klasing 2007), body mass maintenance (Krieger et al. 2006) and key life history events (Cotter et al. 2011), even in the absence of food limitation. Many annual omnivores are seasonal diet specialists, even showing changes in their digestive systems to accommodate diet shifts (Piersma et al. 1999, McWilliams and Karasov 2001). Such shifts in diet may be associated with seasonal limitations in nutrients required for optimal immune function. For example, protein-rich diets like insects may support leukocyte, antibody and acute phase protein synthesis (Mabuchi and Frankel 2016), but may be poor in antioxidants (Griffiths et al. 2016) which play immuno-modulatory and antioxidant functions (Klasing 2007, Isaksson 2015). Fruits and vegetables on the other hand are poor in proteins but rich in fibre, sugars, vitamins, flavonoids and carotenoids. Diet components also influence the gut microbiome (David et al. 2013, Pan and Yu 2014) and this may impact immune function. Nonetheless, it is difficult to predict which diet is best for immune function because different diets lack different nutrients (Cotter et al. 2011), and the immune system has different physiological pathways (Schmid-Hempel and Ebert 2003), but we may expect protein rich diets to be better because they contain essential amino acids that are not synthesised *de novo*.

How nutrient limitations modulate the relationship between immune function and other life history traits such as body mass maintenance and moult in birds is not well known (Pap et al. 2008, Pap et al. 2009 & Pap et al. 2011). Regulation of body mass and moult in birds are crucial aspects of self-maintenance, especially for long-lived species (Williams 1966) and like immune function, these are subject to nutrient availability (Murphy 1996, Murphy and Taruscio 1995). Dietary protein supply is crucial for the maintenance of body form and function because there is no storage form of protein in the body, and this implies that protein limitation may lead to breakdown of fat-free tissues (Krieger et al. 2006), including skeletal muscles and digestive organs (Piersma and Gill, 1998). Annual moult involves substantial tissue replacement and so moult may exploit tissue proteins if dietary supplies are limited (Podlaszczuk et al. 2017), especially because feather growth occurs during the post-absorptive state (Chen et al. 2015). However, although feather keratin requires high amounts of sulphur amino acids (methionine and cysteine) to synthesise (Murphy and King 1984), the ‘actual nutrient requirements’ of moult are unclear because moult may proceed with limited nutrients or under a wide variety of diets (Murphy and King 1992), albeit resulting in poor quality feathers or protraction (Murphy et al. 1988, Vágási et al. 2010).

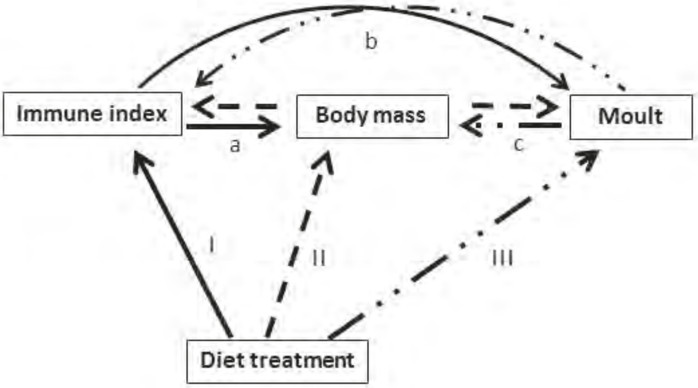

Figure 8.1: Diet composition may affect immune function, body mass and moult directly (I, II & III). Immune indices, body mass and moult may co-vary (a, b & c) if diet affects all or any of immune function, body mass or moult with resultant effect on others. Diet effect may follow alternative indirect pathways: **solid lines -** diet affects immune index and immune index affects body mass and/or moult, or **dashed lines -** diet affects body mass, body mass affects immune index and/or moult, or **dotdash lines** – diet affect moult and moult affects body mass and/or immune index. Note however, that the hypothetical indirect effect of diet which arise from a direct effect on moult can only be tested reasonably in the moulting season, and so was not considered.

Immune function, body mass and moult may co-vary if they exploit similar nutrients, especially if such nutrients are in short supply (a, b & c, Figure 8.1). Thus, exploring such covariations under different diet composition will aid the formulation of hypotheses that test how diet alteration and resultant nutrient limitations affect immune function, body mass and moult via potential trade-offs in natural systems (Sanz et al. 2004). Further complications to understanding variation in immune indices may be presented by our limited knowledge of the mechanistic connections between life history traits (Hegemann et al. 2013a). Diet alteration can affect several linked processes. For example, diet effects on immune function and moult may arise from its prior effect on body mass (dashed lines, Figure 8.1), or diet effects on body mass and moult may arise from its prior effect on immune function (solid lines, Figure 8.1). A prior effect of diet on moult may also affect body mass and possibly immune function (dotdashed lines, Figure 8.1), but this may be restricted to the moulting period in seasonally moulting birds. Poor nutrition, body mass loss and susceptibility to infection are intricately linked (Beldomenico et al. 2008, Schaible and Kaufmann 2007) and these may affect different immune indices differently, but, it still remains equivocal whether high immune indices are good or bad (Adamo 2004, Matson et al. 2006). Thus, by using path analyses to explore connections between immune indices and body mass, we can generate hypotheses on mechanisms linking immune indices and other life history traits. Path analyses generate hypotheses about causal connections based on correlative data sets (Shipley 2009). This knowledge is lacking, yet crucial for interpreting immune indices in light of individual fitness.

Omnivorous birds such as Common Bulbuls *Pycnonotus barbatus* are good models for testing the effect of diet composition on innate immune function, and more broadly, for understanding how specific immune indices associate with other life history traits during nutrient rather than food limitations. Some birds switch diets seasonally (Bairlein 1996, Jenni-Eiermann and Jenni 2003, Kissling et al. 2012, Marshall et al. 2016), but Common Bulbuls are year-round omnivores, feeding predominantly on fruits and invertebrates, and occasionally on nectar and seeds (Milla et al. 2005, Okosodo et al. 2016). By restricting Common Bulbuls to fruits or invertebrates only, we can explicitely identify their effects on the condition of Common Bulbuls.

Here we test how fruit and invertebrate diets affect innate immune function, body mass and extent of primary moult of wild-caught captive Common Bulbuls. First, we compared innate immune indices, body mass and extent of primary moult among and within Common Bulbuls fed on fruits or invertebrates, or switched between treatments after 12 weeks. We made comparisons before diet restriction, 12 weeks later on switching, and after a further 12 weeks after switching treatments. We predicted that: i) birds on protein-rich invertebrate diet will maintain higher body mass, earlier moult and higher innate immune indices. Secondly, we compared patterns of co-variation between immune function, body mass and extent of moult for fruit and invertebrate fed bulbuls. We expect that: (i) nutrient limitations due to diet manipulation will lead to negative correlations between immune response, body mass and extent of primary moult and this should be more pronounced in protein deprived fruit fed bulbuls. Finally, we used path analyses to generate hypothetical pathways to explain co-variations between immune indices and body mass. We explored whether diet effect on immune function and body mass is direct (I & II, Figure 1) or via two alternative indirect pathways: (i) diet effect on body mass is via diet effect on immune function (solid lines, Figure 8.1) or (ii) the effect of diet on immune function is via diet effect on body mass (dashed lines, Figure 8.1).

METHODS

Study species

Common bulbuls weigh 25 – 50g. They are sexually monomorphic, but males are slightly larger than females. They are resident in central Nigeria where they annually experience one wet and one dry season lasting about six months each. They breed year-round but moult almost entirely in the wet season (Nwaogu et al. 2018a). Common Bulbuls forage on different plants following availability in different seasons. Some of these plants are supported by gullies in riparian forest fragments which hold water in the dry season. This buffering of environmental conditions by riparian forest fragments and variable plant phenology supports year-round omnivory in wild Common Bulbuls (Nwaogu *pers. ob*s.).

Experimental set-up

We caught 40 adult Common Bulbuls using mist nets around the A. P. Leventis Ornithological Research Institute (APLORI) in Nigeria between 28 October to 7 November 2016 and housed them in groups of 10 birds in four adjacent out-door aviaries at APLORI. APLORI is located on the Jos Plateau in north central Nigeria (09°52'N, 08°58'E). The aviaries measured 3 x 1 x 2 m and consisted of a concrete floor, a metal frame, wire mesh and a thatched roof made from grass mats to provide shelter. Birds were fed fruits and invertebrates in captivity until the experiment started on the 2 December 2016. All birds were sampled for blood, assessed for moult and weighed to determine baseline body mass and innate immune function on 1 or 2 December, before diet restriction commenced on 2 December. During the experiment, birds in two aviaries were fed fruits (F), and the other two were fed invertebrates (I). We then sampled fortnightly. After 12 weeks of diet treatment, five birds from each aviary were switched between treatments, and the other five birds of each aviary remained on the same treatment (Figure 8.S1). The experiment continued for another 12 weeks. Thus, we grouped individuals as: II-invertebrate throughout, IF-invertebrate to fruit, FI-fruit to invertebrate and FF-fruit throughout.

Diet treatment

The invertebrate diet consisted of crushed air-dried grasshoppers, rehydrated crayfish and mealworms, and was supplemented with live insects caught in the reserve using sweep nets every other day. The fruit diet consisted of fruits available to Common Bulbuls in the wild at different parts of the year (Nwaogu *pers obs*). When available we provided bulbuls with ripe fruits of *Lantana camara, Phyllanthus muellerianus, Bridelia ferruginea, Harungana madagascariensis, Rhus natalensis, Jasminum dichotomum* and *Santaloides afzeli,* and supplemented these with chopped ripe papaya fruits daily. Both treatment groups were provisioned ad libitum.

Blood sampling, body mass and sexing

Birds were sampled between 6:00 and 10:00 hours daily in two consecutive days per sampling session. Two aviaries of alternate diet treatments were sampled per day, with sampling order rotating between sampling sessions. Birds were caught and sampled randomly per room and returned together after the last bird was sampled from a room. Birds were held in soft dark cloth bags after capture from aviary and after sampling to minimise stress. On average, each bird was bled within 6.04 ± 3.76 minutes of removal from the aviary, but because disturbance may commence from the first capture of the day, we also accounted for the time between the first capture and all subsequent captures each day. We collected c.300 microliter of blood from each bird into heparinised micro capillary tubes after puncturing the brachial vein with a needle. Samples were emptied into 1.5ml Eppendorf tubes and stored on ice until processing in the laboratory to separate plasma from cellular fractions. Separation was done within 4 hours of sampling. Plasma and blood cells were stored at -20° C until immune assays.

We weighed (± 0.1g, Ohaus Scout) each bird and assessed primary moult after blood sampling. Extent of primary moult was determined by scoring each primary feather on a scale of 0 – 5: newly full grown feathers were scored 5 while un-moulted old feathers were scored 0, and feathers at intermediate stages of growth were scored 1 – 4 (Ginn & Melville 1983). Scores were summed up to obtain the cumulative score of primary feathers per individual.

All birds were sexed using gel electrophoresis. We extracted DNA following methods by Richardson et al. (2001) and performed PCR with the P2/P8 primers (Griffiths et al. 1996).

Immune assays

(i) *Haptoglobin concentration*

Haptoglobin is a positive acute phase protein which circulates in low concentration but increases with inflammation (Jain et al. 2011), but see Hegemann et al. 2013b). Haptoglobin binds to and removes haem from circulation during infection, making haem unavailable to pathogens. We quantified plasma haptoglobin concentration using a functional colorimetric assay which quantifies the haem-binding capacity of plasma. We followed instructions for the 'manual method' provided with a commercially available assay kit (Cat. No.: TP801; Tridelta Development Ltd, Maynooth, Co. Kildere, Ireland) (Matson et al. 2012). We calculated within-assay variability (n=6 plates, maximum CV = 0.61, minimum CV = 0.42, mean CV = 0.51) and among-assay variability (n=450 samples, CV = 0.51) to verify consistency.

(ii) *Nitric oxide concentration*

Nitric oxide modulates inflammatory processes and participates in the direct killing of parasites and tumor cells (Sild and Hõrak 2009). We measured nitric oxide concentration by a colorimetric assay described by Sild and Hõrak (2009). The method estimates the concentration of nitrate and nitrite in plasma after reducing all nitrate to nitrite using copper-coated cadmium granules. A measurable colour development proportionate to nitric oxide concentration follows reaction with Griess reagent. We calculated within-assay variability (n=6 plates, maximum CV = 1.79, minimum CV = 1.16, mean CV = 1.49) and among-assay variability (n=443 samples, CV = 1.51) to verify consistency.

(iii) *Ovotransferrin concentration*

Ovotransferrin is a negative acute phase protein. Like haptoglobin, it binds to haem during infection, but its concentrations may decrease with increased inflammation due to temporarily high free hormones bound to ovotransferrin or the increased production of other acute phase proteins (Gruys et al. 2005, Jain et al. 2011, Giansanti et al. 2012). Ovotransferrin was quantified by estimating the maximum amount of iron required to saturate all ovotransferrin in a sample. We followed a three step process described by Horrocks et al. (2011): saturation of ovotransferrin with ferric iron under alkaline conditions, reduction of excess unbound iron by ascorbic acid, then dissociation of ovotransferrin-iron complex under acidic conditions, leading to a colour development whose absorbance is measured by colorimetry. We calculated within-assay variability (n=16 plates, maximum CV = 1.32, minimum CV = 0.30, mean CV = 0.63) and among-assay variability (n=427 samples, CV = 0.69) to verify consistency.

All colorimetric assays (i – iii above) were carried out using a Versamax plate reader (Molecular Devices Sunnyvale, California, US).

(iv) ***Haemagglutination/haemolysis titres***

We assessed natural antibody-mediated haemagglutination and complement-mediated haemolysis titres of plasma samples against 1% rabbit red blood cells (Envigo RMS (UK) Ltd.) in phosphate buffered saline as described by Matson et al. (2005). Both haemagglutination and haemolysis titres were recorded as the number of serial dilution steps in which each function was still observable. Haemagglutination and haemolysis titres were scored blind to individual and treatment using an existing rubric (Matson et al., 2005). We calculated within-assay variability (n=75 plates, haemagglutination: maximum CV = 0.27, minimum CV = 0.01, mean CV = 0.10; haemolysis: maximum CV = 2.45, minimum CV = 0.22, mean CV = 0.76) and among-assay variability (n=450 samples, haemagglutination: CV = 0.13; haemolysis: CV = 0.72) to ensure consistency.

Statistical analyses

Principal component analysis

We identified three principal components (PCs) with eigenvalues > 1 which cumulatively accounted for 66% of the total variation in immune indices (Table S2). Loading of the PC axes after varimax rotation revealed: PC1 (23.2%) – decreasing haptoglobin concentration and increasing haemolysis titre, PC2 (22.7%) – increasing ovotransferrin concentration and haemagglutination titre, and PC3 (20.0%) – increasing nitric oxide concentration (Table S2). 34% of total variation in immune indices was unexplained by the PCs, but we used the PCs instead of raw immune indices, because some simplification was achieved. Repeating analyses with the original measures give very similar results.

(i) ***Direct effect of diet composition on immune function, body mass and moult***

To test the effect of diet treatment on immune function, body mass and moult, we built a general linear mixed effect model each for the three principal components of immune indices, body mass and extent of primary moult. We included week, diet treatment and sex, and an interaction between week and diet treatment as main effects. For all principal components and body mass, we compared groups at week 0 and week 12 and between week 0 and 12 to test diet treatment effect, and then at and between week 12 and 24 to confirm effect after treatment switch. For moult we only compared extent of primary moult at week 24 relative to week 12 because there were no birds moulting before week 12. Moult in Common Bulbuls starts on the 1st of May on average (Nwaogu et al. 2018a) and this was after week 12.

For all models above, we included individual identity nested in aviary as random factor to account for individual variability and aviary effects. For models of the principal components of immune function and body mass, we also included the time lag between capture and sampling and that between the first capture of each day and the sampling of every bird to account for the effect of holding time on body mass and initial disturbance on immune indices.

(ii) *Diet effects on covariation between immune function, body mass and moult*

To compare covariation between body mass and innate immune function under fruit and invertebrate diets, we built three separate general linear mixed effect models, each with body mass as response to an interaction between diet treatment and a principal component of immune indices. We included week as main effect to account for the effect of temporal environmental factors (occurrence of rain and temperature variation (see Figure 8.S2)) on body mass and immune indices irrespective of diet. We also included the time lag between capture and sampling and that between the first capture of each day and the sampling of each bird in all models to account for the effect of holding time on body mass and initial disturbance on immune indices. We used all data from week 0 – 24, assigning diet treatment as the diet of an individual prior to sampling (I or F), regardless of whether the individual was switched between diets or not. However, we accounted for individual variability, diet switch and aviary effects by nesting individual identity in the four-level diet treatment (II, IF, FI and FF).

We tested for covariation between moult and immune indices at week 24 only, because Common Bulbuls were not moulting earlier. We built three separate general linear models with cumulative scores of primary feathers as response to an interaction between diet treatment and a principal component of immune indices. Similarly, we tested for covariation between moult and body mass at week 24. We built a general linear model with cumulative scores of primary feathers as response to an interaction between diet treatment and body mass. Again, we assigned diet treatment as the diet of an individual prior to sampling (I or F), regardless of whether individual was switched between diets or not.

(iii) Hypothetical pathways of diet effects on immune indices and body mass

To explore if diet composition affects immune function directly or indirectly through body mass, and – likewise - to explore if diet composition affects body mass directly or indirectly through immune function (Figure 8.1), we performed path analyses using the piecewiseSEM package in R (Lefcheck 2015). For each immune index, we built two structural equation models, each in turn comparing two linear models with each other: Model A compared the alternatives that the effect of diet on immune function was direct (Model I, Figure 8.1) or through body mass (Model a (dashed line), Figure 8.1). Model B compared the alternatives that the effect of diet on body mass was direct (Model II, Figure 8.1) or through immune function (Model a (solid line), Figure 8.1). We then compared the relative fit of the alternative structural equation models (Model A and Model B) using AIC scores. We included individual identity nested in week as random factor to account for individual and temporal variation.

RESULTS

(i) Diet composition affects body mass, moult and innate immune function

Innate immune function

Measures of innate immunity did not differ between the diet treatment groups at the start of the experiment (Figure 8.2), however, at week 12, fruit fed bulbuls had significantly higher PC1 (decreasing haptoglobin/increasing heamolysis) than invertebrate fed ones (Table 8.1 & 2). At week 24, treatment subsets did not differ significantly from each other (Table 8.2), however, the fruit fed subset that was switched to invertebrate diet decreased significantly in PC1 between weeks 12 and 24 ($F_{3,\,53} = 1.26$, $P = 0.02$) while the subset that remained on fruit diet did not ($F_{3,\,53} = 0.14$, $P = 0.75$). Accordingly, the invertebrate fed subset switched to fruit diet increased significantly in PC1 between weeks 12 and 24 ($F_{3,\,53} = -1.12$, $P < 0.01$) while the subset that remained on invertebrate diet did not ($F_{3,\,53} = -0.25$, $P = 0.60$).

PC2 (ovotransferrin/heamagglutination) was not different between treatment groups at the start of the experiment (Figure 8.2C) but was higher in fruit fed bulbuls compared to invertebrate fed ones at week 12 (Table 8.1 & 8.2). At week 24, treatment subsets did not differ significantly from each other (Table 8.2), however, the invertebrate fed subset that was switched to fruit diet increased significantly in PC2 between weeks 12 and 24 ($F_{3,\,53} = 1.01$, $P < 0.01$) while the other treatment subsets did not differ significantly between weeks 12 and 24 (all $P > 0.3$).

PC3 (nitric oxide) did not differ between diet treatments subsets at weeks 0, 12 or 24, however, the invertebrate fed subset switched to fruit diet increased significantly in PC3 between weeks

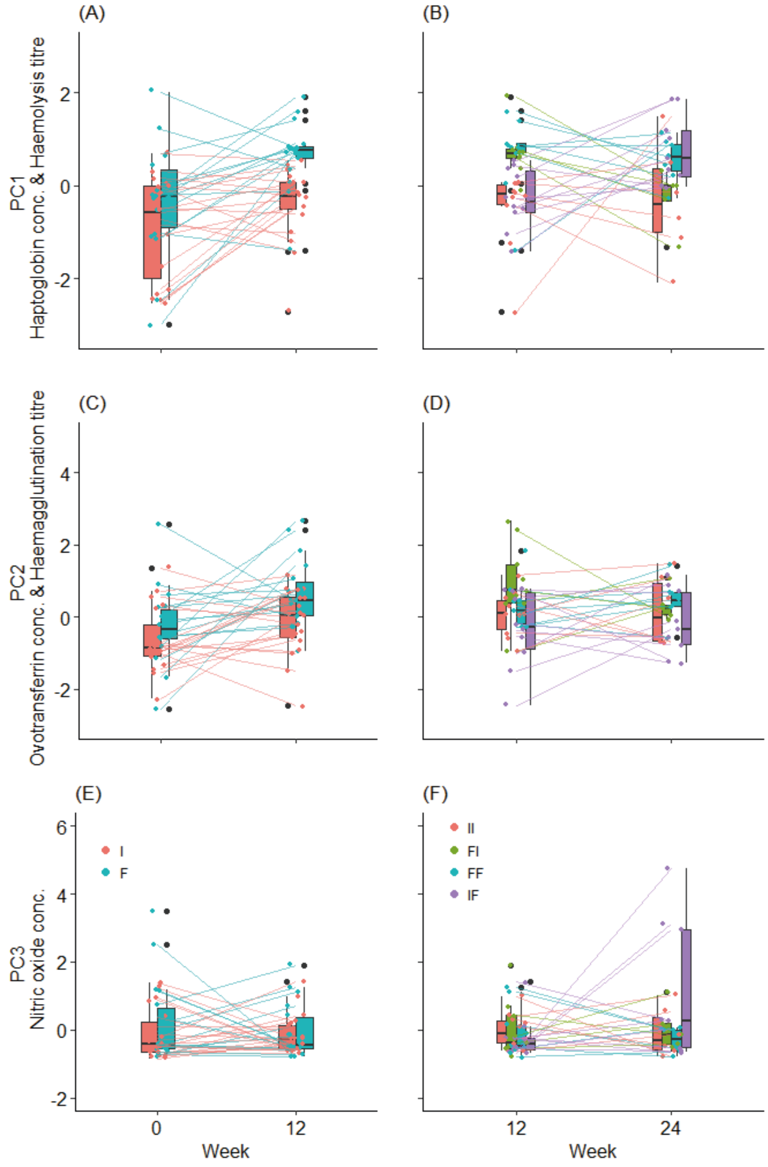

Figure 8.2: Effect of diet treatment on (A-B) PC1 (decreasing haptoglobin concentration and increasing haemolysis titre), (C-D) PC2 (increasing ovotransferrin concentration and haemagglutination titre) and (E-F) PC3 (increasing nitric oxide concentration) in the common bulbul. Bars show group medians while lines connect individuals. A subset of each treatment was switched to alternative diet treatment at week 12: FI-fruit to invertebrate, IF-invertebrate to fruit, FF- fruit throughout and II-invertebrate throughout. Black dots are outliers from box plots while coloured dots are individual points.

Table 8.1: Innate immune function differs significantly between fruit and invertebrate fed Common Bulbuls *Pycnonotus barbatus*. Differences in PC1 (decreasing haptoglobin concentration and increasing haemolysis titre), PC2 (increasing ovotransferrin concentration and haemagglutination titre) and PC3 (increasing nitric oxide concentration) between fruit and invertebrate fed Common Bulbuls between week 0 and 12, and week 12 and 24. A subset of each treatment was switched to alternative diet treatment at week 12, resulting in four treatment groups: FI-fruit to invertebrate, IF-invertebrate to fruit, FF- fruit throughout and II-invertebrate throughout.

			PC1			**PC2**			**PC3**		
			Haptoglobin and haemolysis			**Ovotransferrin and haemagglutination**			**Nitric oxide**		
	Factor	Df	Chisq	P		Chisq	P		Chisq	P	
Week 0 &12	**Sampling[1]**	1	0.12	0.72		**8.00**	**0.01**	**	0.27	0.60	
	Capture[2]	1	0.24	0.63		1.61	0.21		0.00	0.97	
	Sex	1	2.46	0.12		0.01	0.92		0.53	0.47	
	Diet (F or I)	1	**9.48**	**<0.01**	**	**14.82**	**<0.01**	***	1.96	0.16	
	Week	1	**16.88**	**<0.01**	***	**4.58**	**0.03**	*	0.89	0.35	
	Diet*Week	1	1.30	0.25		1.79	0.18		0.46	0.50	
Week 12 & 24	**Sampling**	1	0.08	0.78		**9.02**	**<0.01**	**	0.09	0.76	
	Capture	1	0.69	0.41		0.00	0.99		**7.08**	**<0.01**	**
	Sex	1	0.27	0.61		0.00	0.97		0.42	0.52	
	Diet (FF, FI, IF or II)	3	**11.25**	**<0.01**	*	**11.79**	**<0.01**	**	5.97	0.11	
	Week	1	0.67	0.41		2.71	0.10	.	**7.94**	**<0.01**	**
	Diet*Week	3	**12.85**	**<0.01**	**	3.87	0.28		3.68	0.30	

[1]**Sampling** - time lag between capture and sampling [2]**Capture** – time lag between the first capture of each day and the sampling of each bird

Table 8.2: Posthoc summary of pairwise differences (Table 3) in PC1 (decreasing haptoglobin concentration and increasing haemolysis titre), PC2 (increasing ovotransferrin concentration and haemagglutination titre) and PC3 (nitric oxide concentration) between fruit and invertebrate fed subsets of Common Bulbuls *Pycnonotus barbatus* within week 0, 12 and 24. A subset of each treatment was switched to alternative diet treatment at week 12, resulting in four treatment groups: FI-fruit to invertebrate, IF-invertebrate to fruit, FF- fruit throughout and II-invertebrate throughout.

	Difference			PC1 Haptoglobin and haemolysis					PC2 Ovotransferrin and Haemagglutination					PC3 Nitric oxide				
	Week	**Diet**	**Diet**	**Est.**	**Error**	**t**	**P**		**Est.**	**Error**	**t**	**P**		**Est.**	**Error**	**t**	**P**	
Week 0 & 12	0	I	F	-0.49	0.31	-1.59	0.12		-0.59	0.31	-1.90	0.06						
	12	I	F	-0.96	0.32	**-3.06**	**<0.01**	**	-1.18	0.32	**-3.69**	**<0.01**	***					
Week 24	24	II	FI	-0.07	0.50	-0.13	1.00		-0.25	0.47	-0.53	0.95		0.09	0.76	0.11	1.00	
	24	II	FF	-0.89	0.46	-1.92	0.23		-0.53	0.43	-1.23	0.61		-1.16	0.71	-1.64	0.35	
	24	II	IF	-1.10	0.44	-2.52	0.07	.	0.10	0.41	0.25	0.99		-1.74	0.68	-2.56	0.05	.
	24	FI	FF	-0.82	0.49	-1.69	0.34		-0.28	0.45	-0.62	0.92		-1.25	0.77	-1.62	0.37	
	24	FI	IF	-1.04	0.46	-2.24	0.13		0.35	0.43	0.82	0.85		-1.83	0.76	-2.42	0.07	.
	24	FF	IF	-0.21	0.42	-0.51	0.96		0.64	0.39	1.64	0.35		-0.58	0.62	-0.93	0.79	

12 and 24 ($F_{3, 53} = -2.08$, $P < 0.01$) while the other treatment subsets did not differ significantly between weeks 12 and 24 (all $P > 0.17$).

Body mass

Body mass before the experiment was similar for bulbuls later fed fruits and bulbuls later fed invertebrates (Figure 8.3A). However, at week 12, fruit fed bulbuls were significantly heavier than invertebrate fed ones (Table 8.3 & 8.4). Accordingly, at week 24, the subset that was switched from invertebrate to fruits at week 12 were significantly heavier than those that remained on invertebrate diet, while those that were switched from fruit to invertebrate diet became significantly lighter than those that remained on fruits (Figure 8.3B, Table 8.3 & 8.4). At week 24, treatment subsets differed significantly in body mass (Table 8.3). The subset switched from fruit to invertebrate diet decreased significantly in body mass between weeks 12 and 24 ($F_{3, 60} = 2.94$, $P < 0.01$) while the subset that remained on fruits did not ($F_{3, 60} = -0.04$, $P = 0.95$). The subset switched from invertebrates to fruits increased marginally in body mass between weeks 12 and 24 ($F_{3, 60} = -1.05$, $P = 0.07$) while the subset that remained on invertebrate diet decreased significantly ($F_{3, 60} = 2.05$, $P < 0.01$).

Extent of primary moult

Only bulbuls fed with fruits throughout the experiment and those switched from invertebrate to fruits at week 12 had commenced primary feather moult by week 24 (Figure 8.3E, Table 8.3 & 8.4). At week 24, the cumulative scores of moulted primary feathers was significantly higher compared with week 12 for the subset fed fruits throughout ($F_{3, 62} = -3.63$, $P = 0.03$) and the subset that was switched from invertebrate to fruits at week 12 ($F_{3, 62} = -6.80$, $P < 0.01$), but not for the subset fed invertebrates throughout ($F_{3, 62} = 0$, $P = 1$) and the subset that switched from fruits to invertebrates ($F_{3, 60} = -0.29$, $P = 0.86$).

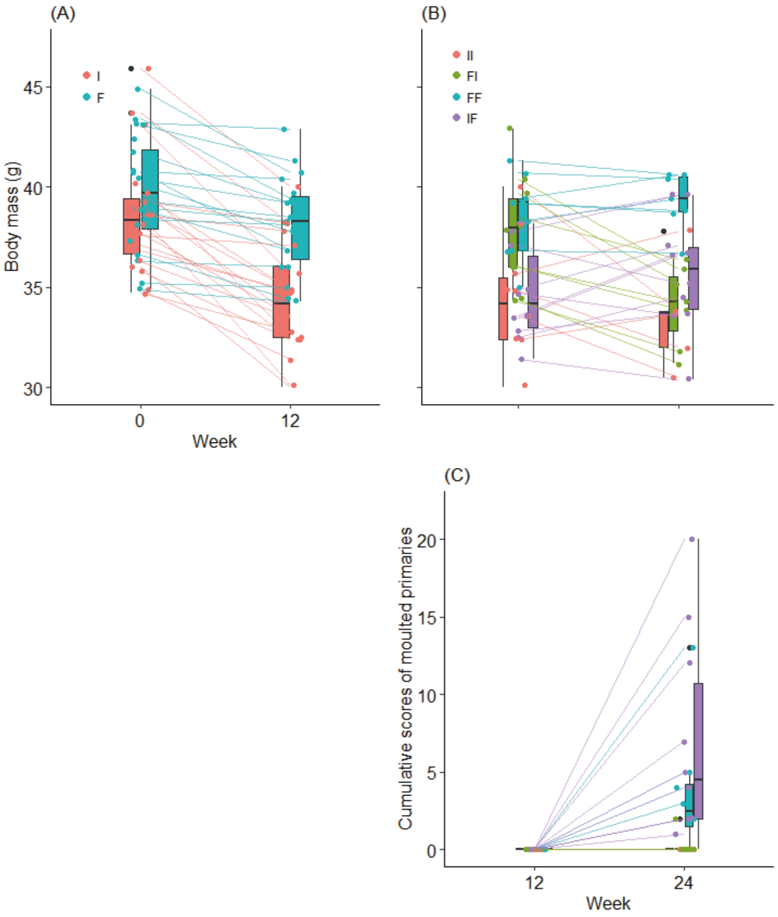

Figure 8.3: Effect of diet treatment on (A-B) body mass (g) and (C) extent of primary moult. Boxes show group median while lines connect individual birds before and after treatment. A subset of each diet treatment was switched to alternative treatment after 12 weeks. F-fruit, I-invertebrate, FI-fruit to invertebrate, IF-invertebrate to fruit, FF- fruit throughout and II-invertebrate throughout. Moult commenced only after the diet switch. Black dots are outliers from box plots while coloured dots are individual points.

Table 8.3: Differences in body mass and extent of primary moult between fruit and invertebrate fed Common Bulbuls *Pycnonotus barbatus* between week 0 and 12 and week 12 and 24. A subset of each treatment was switched to alternative diet treatment at week 12, resulting in four treatment groups: FI-fruit to invertebrate, IF-invertebrate to fruit, FF- fruit throughout and II-invertebrate throughout. Primary moult commenced after week 12 – all birds had new or old feathers before week 12.

			Body mass (g)			**Primary moult**		
	Factor	**Df**	**Chisq**	**P**		**Chisq**	**P**	
Week 0 & 12	**Sampling[1]**	1	0.03	0.87				
	Capture[2]	1	0.24	0.62				
	Sex	1	13.94	**<0.01**	***			
	Diet (F or I)	1	7.12	**0.01**	**			
	Week	1	85.08	**<0.01**	***			
	Diet*Week	1	14.63	**<0.01**	***			
Week 12 & 24	**Sampling[1]**		2.94	0.09	.			
	Capture[2]		0.28	0.59				
	Sex	1	9.20	**<0.01**	**	1.73	0.19	
	Diet (FF, FI, IF or II)	3	18.00	**<0.01**	***	11.64	**0.01**	**
	Week	1	4.69	**0.03**	*	17.75	**<0.01**	***
	Diet*Week	3	23.20	**<0.01**	***	16.52	**<0.01**	***

[1]**Sampling** - time lag between capture and sample [2]**Capture** – time lag between the first capture of each day and the sampling of each bird

Table 8.4: Posthoc summary of pairwise differences (Table 1) in body mass and extent of primary moult between fruit and invertebrate fed subsets of Common Bulbuls *Pycnonotus barbatus* within week 0, 12 and 24. A subset of each treatment was switched to alternative diet treatment at week 12, resulting in four treatment groups: FI-fruit to invertebrate, IF-invertebrate to fruit, FF- fruit throughout and II-invertebrate throughout. Primary moult commenced after week 12 – all birds had new or old feathers before week 12.

	Pairwise difference			**Body mass (g)**					**Primary feather moult**				
	Week	**Diet**	**Diet**	**Estimate**	**Error**	**t**	**P**		**Estimate**	**Error**	**t**	**P**	
Week 0 & 12	0	I	F	-0.76	0.81	-0.94	0.35						
	12	I	F	-3.20	0.81	**-3.96**	**<0.01**	***					
Week 24	24	II	FI	-1.57	1.19	-1.32	0.55		-0.29	1.63	-0.18	1.00	
	24	II	FF	-5.72	1.22	**-4.69**	**<0.01**	***	-3.63	1.58	-2.29	0.10	.
	24	II	IF	-3.60	1.12	**-3.20**	**0.01**	**	-6.80	1.51	**-4.50**	**<0.01**	***
	24	FI	FF	-4.15	1.19	**-3.47**	**<0.01**	**	-3.34	1.51	-2.21	0.12	
	24	FI	IF	-2.03	1.06	-1.91	0.22		-6.51	1.44	**-4.52**	**<0.01**	***
	24	FF	IF	2.12	1.16	1.82	0.26		-3.18	1.39	-2.29	0.10	

(i) Diet composition modulates covariation between body mass, moult and innate immune function

In invertebrate fed bulbuls body mass decreased significantly when PC1 or PC2 increased, while for fruit fed bulbuls we found no association between body mass and PC1 or PC2 (Figures 8.4 A & B, Table 8.5). Body mass and PC3 were not correlated in either diet treatment groups (Figure 8.4C, Table 8.5). Body mass differed significantly among weeks, and between males and females (Table 8.5). PC1, PC2 and PC3 also differed significantly between weeks irrespective of diet treatment, but not between sexes (Figure 8.S4, Table 8.S3).

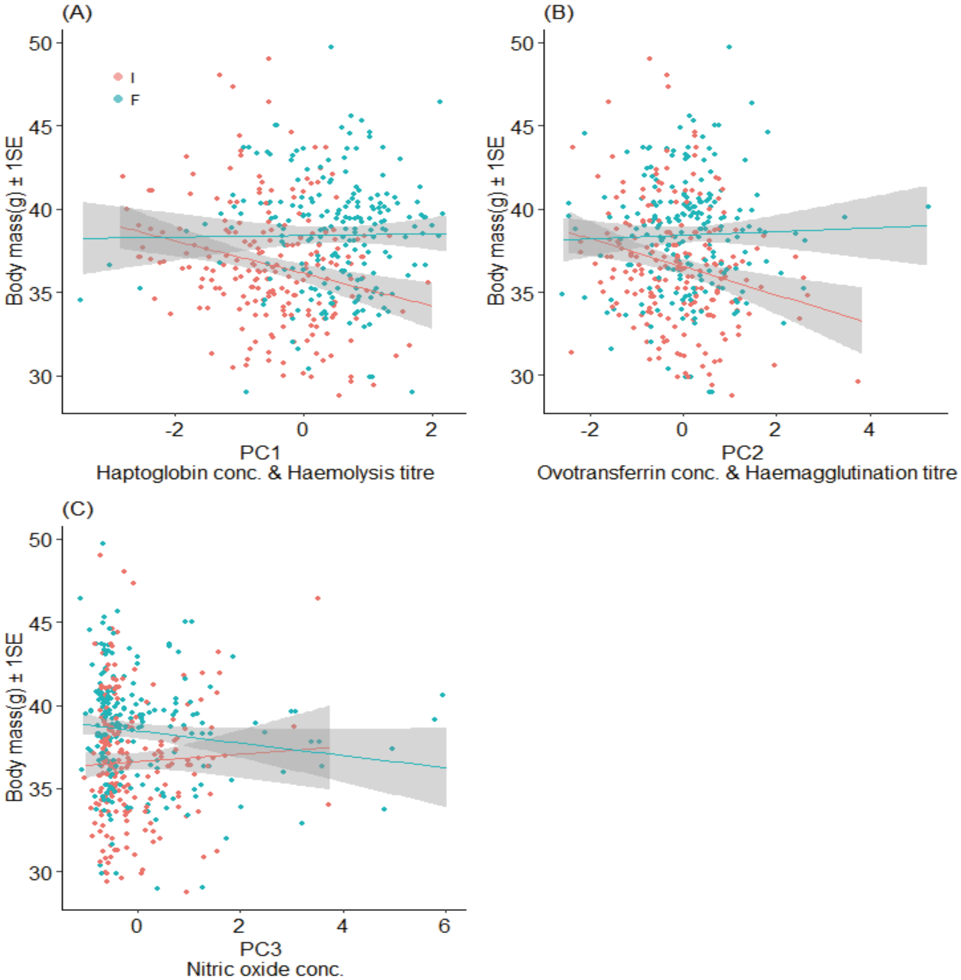

Figure 8.4: Correlation between body mass and (A) PC1 (decreasing haptoglobin concentration and increasing haemolysis titre), (B) PC2 (increasing ovotransferrin concentration and haemagglutination titre) and (C) PC3 (increasing nitric oxide concentration) in Common Bulbuls fed on fruit or invertebrates. Each individual was weighed and sampled before diet treatment and then subsequently, 2 weeks after restriction to invertebrate (I) or fruit (F) diet over a 24-week period.

Extent of primary moult was neither correlated with immune indices nor with body mass, but only fruit fed bulbuls had commenced moult of primary feathers at week 24 (Figure 8.4).

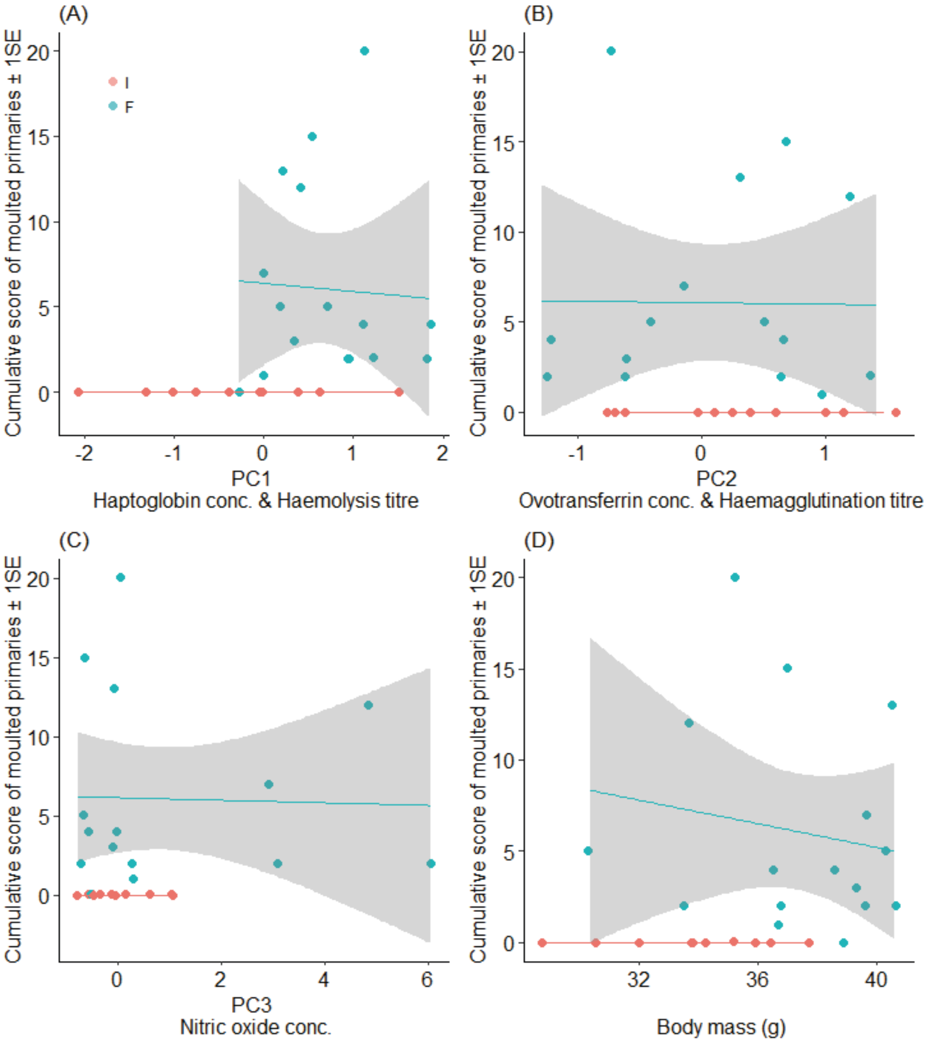

Figure 8.5: Correlation between cumulative scores of moulted primaries and (A) PC1 (decreasing haptoglobin concentration and increasing haemolysis titre), (B) PC2 (increasing ovotransferrin concentration and haemagglutination titre), (C) PC3 (increasing nitric oxide concentration) and (D) body mass (g) of Common Bulbuls fed on invertebrates(I) or fruits(F) for 24 weeks. Correlations for moult and immune indices obtained at week 24.

Table 8.5: Only invertebrate fed Common Bulbuls *Pycnonotus barbatus* decrease in body mass with increased immune function. Relationship between body mass and variation in PC1 (decreasing haptoglobin concentration and increasing haemolysis titre), PC2 (increasing ovotransferrin concentration and haemagglutination titre) and PC3 (increasing nitric oxide concentration) in Common Bulbuls fed on fruits or invertebrates. Each individual was sampled before diet treatment and then subsequently, fortnightly over a 24-week period. Week was included to account for temporal variation in environmental factors. Individual identity and diet switching history were included as random effects to control for individual variability and treatment switch effects respectively.

		PC1			**PC2**			**PC3**		
		Haptoglobin and haemolysis			**Ovotransferrin and haemagglutination**			**Nitric oxide**		
Factor	Df	Chisq	P		Chisq	P		Chisq	P	
Sampling[1]	1	**10.57**	**<0.01**	**	**7.62**	**<0.01**	**	**8.99**	**<0.01**	**
Capture[2]	1	**8.56**	**0.01**	**	**5.41**	**0.02**	*	**6.13**	**0.01**	*
Sex	1	**26.74**	**<0.01**	***	**25.29**	**<0.01**	***	**24.88**	**<0.01**	***
Immune function	1	**7.68**	**<0.01**	**	0.51	0.47		0.04	0.84	
Diet	1	**20.03**	**<0.01**	***	**12.84**	**<0.01**	***	**12.95**	**<0.01**	***
Week (0 - 24)	11	**292.92**	**<0.01**	***	**323.57**	**<0.01**	***	**315.06**	**<0.01**	***
Immune function*Diet	1	**5.56**	**0.02**	*	**7.04**	**<0.01**	**	0.38	0.54	

[1] **Sampling** - time lag between capture and sampling [2] **Capture** – time lag between the first capture of each day and the sampling of each bird

(ii) Diet composition more often affects body mass and immune indices directly

Diet treatment was more likely to affect immune function and body mass directly rather than indirectly via its effect on either immune function or body mass (Table 8.S4, Figure 8.5A- E). The only exception was haemagglutination titre (Figure 8.5D) which was not affected directly by diet treatment ($F_{1,\,436} = -0.05$, $P = 0.29$). We only found support for an indirect effect of diet treatment on the association between haptoglobin concentration and body mass (Figure 8.5A). Haptoglobin concentration was significantly lower for bulbuls on a fruit diet ($F_{1,\,434} = -0.49$, $P < 0.01$, Table 8.S4) and correlated negatively with body mass ($F_{1,\,434} = -0.13$, $P < 0.01$, Table 8.S4). The association between haptoglobin concentration and body mass was more likely to be an effect of haptoglobin on body mass rather than an effect of body mass on haptoglobin concentration because the pathway - diet treatment affects haptoglobin concentration and haptoglobin concentration affects body mass was better supported than the other alternative (Figure 8.5A).

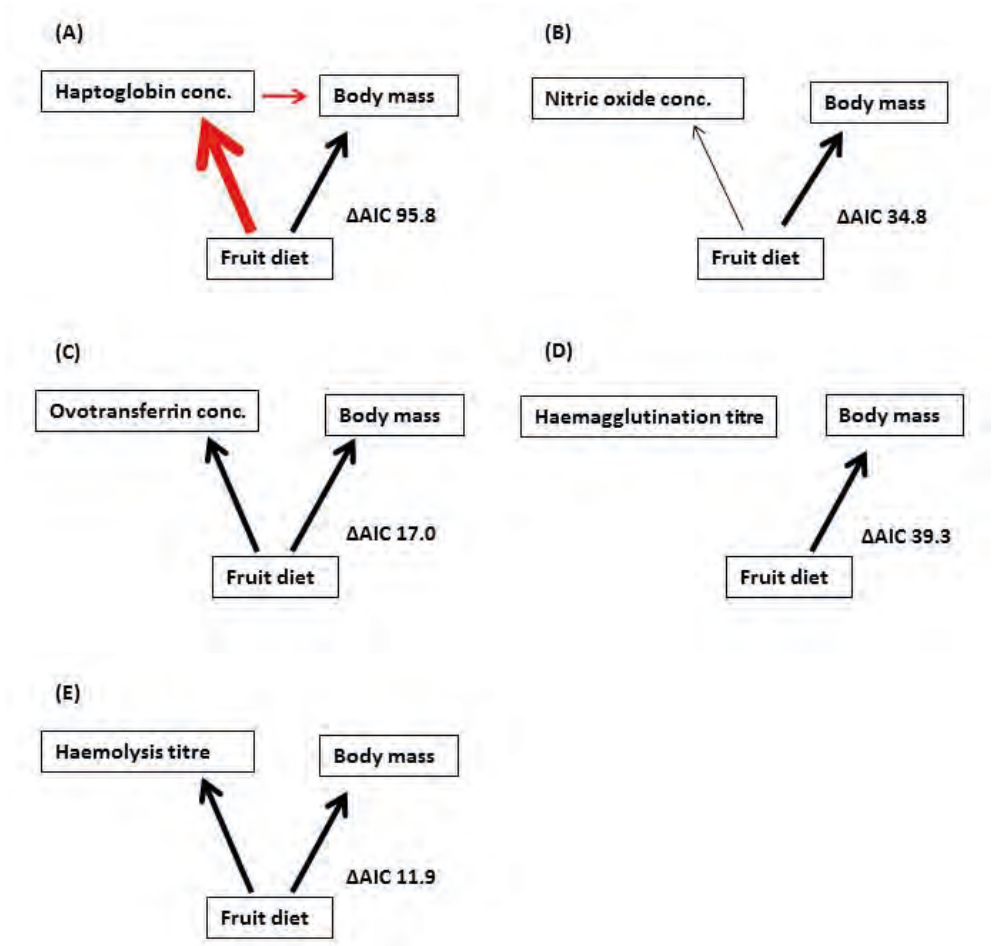

Figure 8.6: Rudimentary path diagrams showing path of diet treatment effect on body mass and immune indices in Common Bulbuls fed on fruit or invertebrates. Invertebrate diet was set as the model intercept. Only significant relationships are shown. Difference between the two alternative indirect pathways of diet effect tested are shown as ΔAIC (solid lines *vs* dashed lines, Figure 1). Arrows indicate directionality of causal relationships. Width of arrows indicate the strength of an effect (scaled based on standardised estimates from structural equation models – table S4). Red arrows indicate negative effects and black arrows positive effects. Overall, diet affects body mass and immune function directly for all immune indices except haemagglutination titre, but indirectly affects body mass through effect on haptoglobin concentration - body mass decreases with increased haptoglobin concentratio

DISCUSSION

Investigating how diet composition affects body mass, moult and immune fucntion, we found that Common Bulbuls fed on fruits maintained higher body mass, earlier moult and a more robust innate immune function relative to bulbuls fed on invertebrates. This was also reflected in the absence of body mass loss with higher immune response and lower haptoglobin concentrations in the fruit-fed birds. Invertebrate-fed birds on the other hand decreased body mass with higher immune response and had higher haptoglobin concentration. Exploration of the mechanistic connection between immune indices and body mass revealed that diet treatment was more likely to affect body mass and immune indices directly rather than via alternative indirect pathways. The only exceptions were haemagglutination titre which was not affected by diet treatment, and haptoglobin concentration which was associated with loss of body mass.

We found no indication that the fruit-fed bulbuls - that presumably suffered protein limitation – had impaired innate immune function, body mass or moult as we predicted (Lochmiller et al. 1993). On the contrary, bulbuls were in better condition when fed on fruits than invertebrates, and this was reversible. Apparently, essential amino acids from invertebrate diet are not the limiting nutrients for maintenance of immune function, body mass and moult in Common Bulbuls. The occurrence of lower body mass and absence of moult, coupled with lower indices of immune function combined, but higher haptoglobin concentrations specifically, in invertebrate-fed bulbuls indicates poorer health (Owen-Ashley and Wingfield 2006). Yet, the mechanism generating the opposite response in fruit-fed bulbuls is not clear. A striking difference between the two diets is the presence of carotenoids and sugars in fruits and their absence in invertebrates. Carotenoids and sugars have been proposed to function as antioxidants (Griffiths et al. 2016, Levin et al. 2017), and therefore, may have immune-modulatory and anti-inflammatory effects (Chew and Park 2004). Carotenoids especially, modulate a range of immune parameters, including stimulating T-cell and antibody production, and reducing oxidative damage during immune response (Chew and Park 2004). While the lower haptoglobin concentration which may be linked to the anti-inflammatory role of carotenoids (Park et al. 2010) in fruit-fed bulbuls, the exact links between carotenoids and the other immune indices we measured are not known. Carotenoids are often considered vital for immune function because their deficiency in diet is associated with dulling of carotenoid-based pigmentation during infection (Torres and Velando 2007, Baeta et al. 2008). However, compounds such as flavonoids, vitamins, iodine, fluorine and specific fatty acids (Demas et al. 2012), and the gut microbiome (Belkaid and Hand 2014) are also vital for immune function and may differ in composition between fruit and invertebrate-fed bulbuls.

We do not know whether the invertebrate-fed bulbuls became infected by new agents or became more susceptible to already harbored infections, but because their natural antibody and complement activities were lower (PC1 & PC2), they may be more prone to infection (Ochsenbein et al. 1999). Natural antibodies function as recognition molecules capable of opsonizing invading microbes and initiating the complement enzyme cascade, which ends in the destruction of the invading microbe (Reid et al. 1997, Belperron and Bockenstedt 2001).

Their activity forms a first line of defense and a useful link between innate and adaptive immunity (Ochsenbein and Zinkernagel 2000, Schmid-Hempel and Ebert 2003, Panda and Ding 2015). We propose that the observed direct effect of diet composition on immune indices, body mass and moult may reflect a cascade of events: fruit deprivation possibly impaired prophylactic components of innate immunity (such as natural antibody and compliment activities which clear early stages of infection), leading to increased inflammation, loss of body mass and ultimately delayed moult. Nonetheless, omnivory seems facultative rather than obligate because bulbuls survived temporarily on either diet, although prolonged fruit deprivation was clearly more detrimental than invertebrate deprivation.

The co-occurrence of decreased body mass with higher immune response and delayed moult was only present in invertebrate-fed bulbuls, not in fruit-fed bulbuls, and may indicate a diet-dependent trade-off between these traits. The contrasting pattern between treatments suggests that disease susceptibility increases when resources are insufficient to sustain optimal immunity (Nelson et al. 2002, Cornet et al. 2014). Previous experiments that used similar immune indices to our study, but restricting food availability rather than nutrients, found no evidence for downregulation of constitutive innate immune function (Buehler et al. 2009c, Schultz et al. 2017) in food restricted birds. Thus, our results confirm that the immune system requires specific nutrients not just energy (Cotter et al. 2011, Klasing 1998). We therefore hypothesize, that under low energy budgets, Common Bulbuls may still maintain aspects of innate immune function (Hegemann et al. 2012a) if required nutrients are sufficiently available. However, under specific nutrient limitation they may prioritise pathogen defence by trading-off body mass and moult to free resources for acute phase response, because disease presents an immediate risk to death (WHO 2018). Innate immunity is thus, a component of body condition that is associated with the maintenance of body mass and onset of annual moult - a key life history event for Common Bulbuls (Nwaogu et al. 2018a). However, although, invertebrate-fed bulbuls in poorer condition did not moult until the end of the experiment, the extent of moult in fruit-fed bulbuls was not correlated with body mass or immune function, suggesting that it is moult initiation and maintenance that is condition dependent not moult extent (Murphy and King 1992).

We found that immune indices and body mass were most likely affected directly by diet treatment. The only exceptions were haemagglutination titre which was not affected by diet treatment, and haptoglobin concentration which was negatively correlated to body mass. Note however, that diet composition affected haemagglutination titre during the first 12 weeks of the experiment (combined with ovotransferrin as PC1), thus the absence of a treatment effect in the full range of the experiment may be associated with increased variability in immune indices (Figure 8.S3) observed after the onset of the rains (Figure 8.S1). In the wild, innate immune function differs between the wet and dry season in Common Bulbuls (Nwaogu et al. 2019). The negative correlation between haptoglobin concentration and body mass may be due to a breakdown of muscle proteins to supply amino acids for hepatic acute phase protein synthesis (Jain et al. 2011, Londhe and Guttridge 2015). Curiously, ovotransferrin concentration was not associated with body mass variation and showed an opposite trend with haptoglobin concentration despite also being an acute phase protein. The reason for this might be that unlike haptoglobin, ovotransferrin is a negative acute phase protein which decreases with high inflammation because temporarily high free hormones may be bound to ovotransferrin during inflammation. Furthermore, haptoglobin may be produced at the expense of ovotransferrin (Gruys et al. 2005, Giansanti et al. 2012). The opposite trend between haptoglobin concentration and other immune indices suggest connections among immune indices, and this may result from cascading processes or trade-offs within the immune system. Innate immune indices may have adaptive features (Kvell et al. 2007) – one layer of immune defence may not be required until another is initiated, suppressed or surpassed (Ochsenbein et al. 1999, Panda and Ding 2015). Our results show unequivocally that high lytic capacity covaries with low haptoglobin concentration in healthier Common Bulbuls (PC1), demonstrating that depending on the immune index considered, immune function may vary positively, negatively, or show no association with body mass as proxy of general physical condition or other life history traits such as moult. Experimental studies testing trade-offs between immune function and other life history traits should aim at manipulating inflammatory response or nutrient availability because manipulations targeted at physical condition may be transferred to offspring (Tieleman et al. 2008) or only visible in the long term (Hegemann et al. 2013a). Disparities between studies that manipulated inflammatory response may have arisen from differences in resource constraints (Williams et al. 1999, Råberg et al. 2000), behavioural adjustments (Ardia 2005b) or individual quality (Ardia 2005a). Nonetheless, the extent of variation within the immune system is far from being fully understood.

Our results confirm that innate immunity is nutrient specific (Klasing 1998, Klasing 2007), but this study is unique because we show a reversible effect of diet composition on wild adult birds whose immune systems are presumably fully developed and adapted to wild conditions – demonstrating a short term consequence of diet alteration on life history traits. A crucial next step is decomposing dietary components to identify specific functions, and possibly the role of the gut microbiome. We propose that seasonal diet composition plays an important role in maintaining seasonal variation in immune function (Durand and Morel 2008), allowing animals to combat seasonal immune challenges. Therefore, if life history events coincide with sufficient availability of required nutrients, trade-offs between immune function and life history events (Sheldon and Verhulst 1996, Hasselquist and Nilsson 2012) can be avoided. This principle may explain the strict seasonal timing of life history events in seasonal environments and the blurring of seasonal patterns in life history events in environments with mild resource seasonality (Merrill et al. 2015).

SUPPLEMENTARY INFORMATION

Study species and environmental conditions in study site

We study Common Bulbuls *Pycnonotus barbatus* in the Amurum Forest Reserve (09°52'N, 08°58'E) at the A.P. Leventis Ornithological Research Institute on the Jos Plateau in north central Nigeria. The reserve is a heterogeneous woodland habitat with inselbergs and interspersed riparian forests, surrounded by farmlands and human settlements. It experiences a single wet and dry season annually. The wet season usually lasts from April to October. Minimum and maximum daily temperatures vary in a bimodal fashion due to increased cloud cover in the wet season and the occurrence of cold dry north-easterly trade winds during the periods between November and February.

Common bulbuls are 25 – 50g sexually monomorphic resident passerines. In central Nigeria, they breed year-round but with small breeding peaks at the end of the dry season/start of the wet season and the end of the wet season (Nwaogu et al. 2018a). Moult is seasonal and takes place in the wet season for most individuals. Males moult about 21 days earlier than females, but on average moult starts on the 1st of May annually at the population level (Nwaogu et al. 2018a).

Common Bulbuls feed largely on fruits and insects, although nestlings are predominantly fed insects and then fruits later. Adult birds also feed occasionally on nectar or seeds. Generally, fruits and insects are available year-round, because some plants flower and fruit during the dry season, attracting large numbers of insects and birds. A few gullies in riparian forest fragments retain water even in the dry season and provide drinking water for animals and nourishment for plants. Therefore, a combination of environmental heterogeneity, plant phenology and distribution, ensure year-round omnivory in the Common Bulbul, although with some seasonal variability in abundance of different food items.

Experimental set up

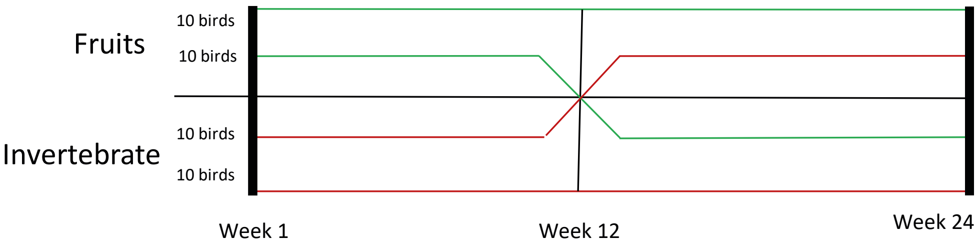

Figure 8.S1: Experimental set-up – 10 of 20 Common Bulbuls in each diet treatment were switch to alternative treatments 12 weeks after diet manipulation, while the other 10 of each treatment were maintained on the same treatment until week 24. Birds were held in four aviaries, two aviaries were supplied fruits while the other two were supplied invertebrates,

hence, five birds from each aviary was switched to alternative diet treatment while the other 5 was maintained on the same diet.

Table 8.S1: Sample sizes maintained between weeks 0 and 24 of experimental diet manipulation on captive wild caught Common Bulbuls *Pycnonotus barbatus*. The overall reduction in sample size was due to recorded mortalities during the experiment and one bird escaping from captivity.

Week		0	2	4	6	8	10	12	14	16	20	22	24
	Female												
Invertebrate		9	9	9	9	9	9	9	7	7	7	7	5
Fruit		6	6	6	6	6	6	6	8	7	6	6	6
	Male												
Invertebrate		11	11	11	11	11	11	11	12	12	11	10	8
Fruit		14	13	13	13	14	13	13	11	11	11	11	11

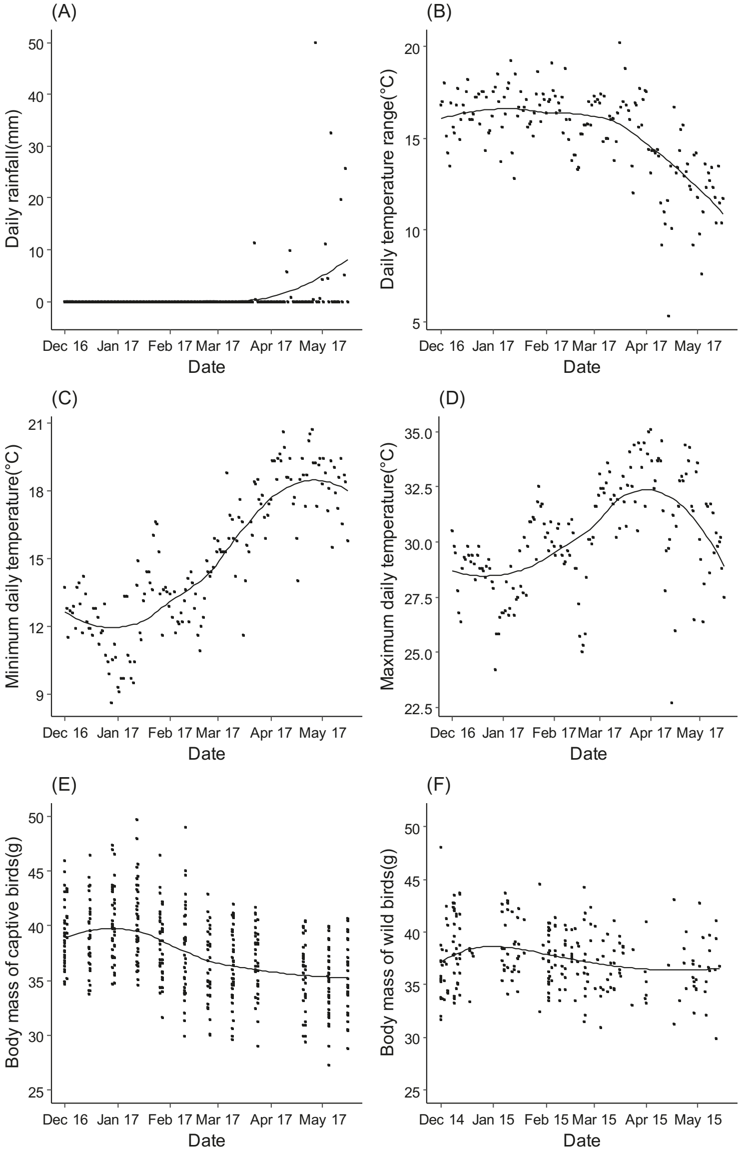

Figure 8.S2: Natural variation in environmental condition and body mass over the time course of the diet experiment: Daily rainfall (mm) (A), temperature range (°C) (B), minimum temperature (°C) (C), maximum temperature (°C) (D) and body mass (g) of captive Common Bulbuls during diet experiment (E). body mass (g) of wild Common Bulbuls (F) from December 2014 to May 2015, corresponding to the period of diet experiment in 2016/2017. Weather data was obtained from the Nigerian Metrological Agency at the Jos Airport located 26km from the A. P. Leventis Ornithological research Institute.

Table 8.S2: Principal component axes loadings of five innate immune indices. PC1 represents decreasing haptoglobin concentration and increasing haemolysis titre, PC2 represents increasing ovotransferrin concentration and haemagglutination titre, while PC3 represents increasing nitric oxide concentration.

Original variables	**PC1**	**PC2**	**PC3**
Ovotransferrin concentration	0.188	**0.736**	
Nitric oxide concentration			**0.992**
Haptoglobin concentration	**-0.742**	0.168	
Haemagglutination titre	-0.151	**0.722**	
Haemolysis titre	**0.742**	0.206	

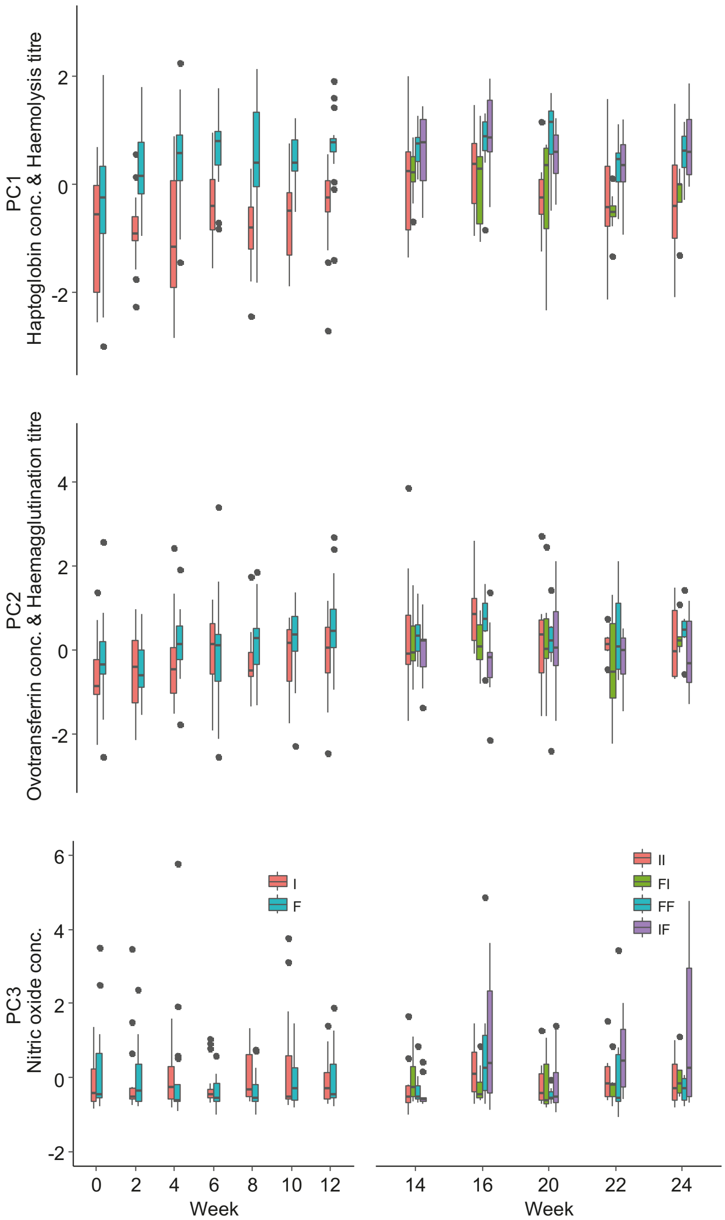

Figure 8.S3: Variation in innate immune function over 24 weeks of diet restriction to fruits or invertebrates in Common Bulbuls *Pycnonotus barbatus*: PC1 (decreasing haptoglobin and increasing haemolysis titre), PC2 (increasing ovotransferrin concentration and haemagglutination titre) and PC3 (increasing nitric oxide concentration). A subset of each treatment group was switched to alternative diet treatment after sampling at week 12. F-fruit, I-invertebrate, FI-fruit to invertebrate, IF-invertebrate to fruit, FF- fruit throughout and II-invertebrate throughout.

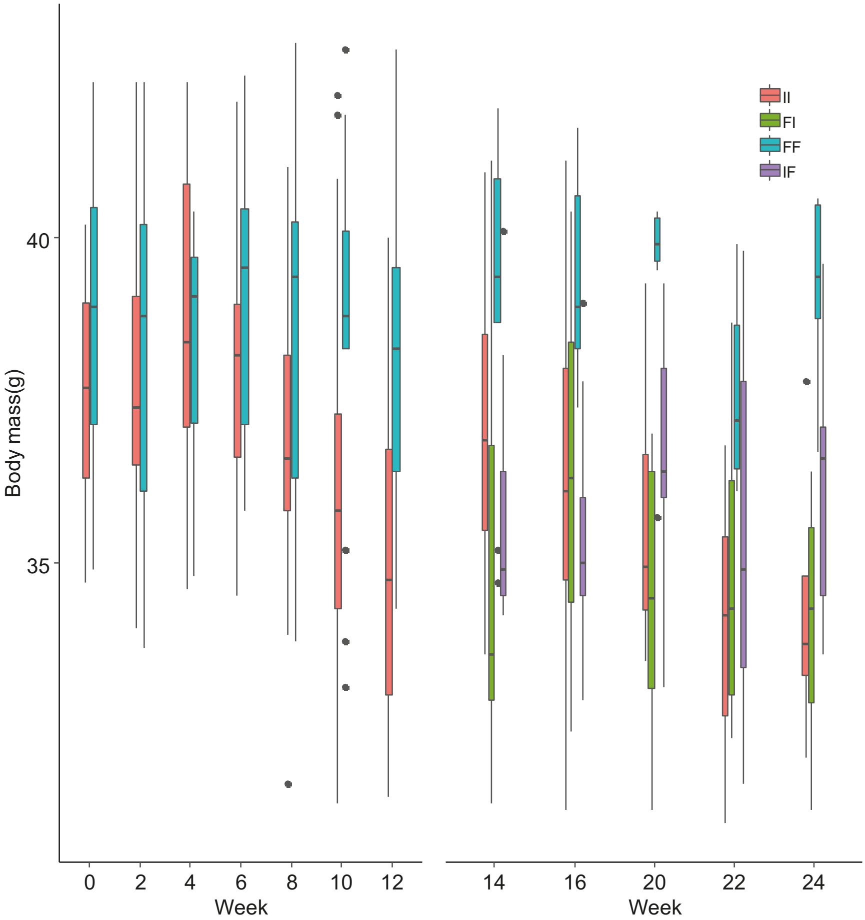

Figure 8.S4: Temporal variation in body mass (g) over 24 weeks of diet restriction to fruits or invertebrates in Common Bulbuls *Pycnonotus barbatus*. A subset of each diet treatment group was switched to alternative diet after sampling at week 12. F-fruit, I-invertebrate, FI-fruit to invertebrate, IF-invertebrate to fruit, FF- fruit throughout and II-invertebrate throughout.

Table 8.S3: Innate immune function differs significantly between weeks in Common Bulbuls *Pycnonotus barbatus* irrespective of diet treatment. Individual identity and diet switch history were included as random factors to control for individual variability and treatment effects, respectively.

PC1 - Haptoglobin and Lysis

Factor	**Df**	**Chisq**	**P**	
Sampling[1]	1	1.50	0.22	
Capture[2]	1	4.84	**0.03**	*
Diet (I or F)	1	98.14	**<0.01**	***
Week	11	52.75	**<0.01**	***
Body mass	1	2.87	0.09	.
Sex	1	2.08	0.15	
Diet*Week	11	13.15	0.28	

PC2 - Ovotransferin and Agglutination

Factor	Df	Chisq	P	
Sampling[1]	1	**3.91**	**0.05**	*
Capture[2]	1	0.37	0.54	
Diet (I or F)	1	2.49	0.11	
Week	11	**22.45**	**0.02**	*
Body mass	1	0.09	0.76	
Sex	1	0.23	0.63	
Diet*Week	11	13.02	0.29	

PC3 - Nitric oxide

Factor	Df	Chisq	P	
Sampling[1]	1	0.25	0.61	
Capture[2]	1	2.18	0.14	
Diet (I or F)	1	**5.71**	**0.02**	*
Week	11	**23.78**	**0.01**	*
Body mass	1	0.54	0.46	
Sex	1	0.02	0.88	
Diet*Week	11	17.09	0.11	

[1] **Sampling** - time lag between capture and sampling [2] **Capture** – time lag between the first capture of each day and the sampling of each bird

Table 8.S4: Summarised output from structural equation models testing alternative path of diet treatment effect on body mass and immune function in Common Bulbuls fed on fruits or invertebrates. Models and AICs highlighted bold indicate selected structural models explaining the most likely paths of diet treatment effect on body mass and/or specific immune indices (see Figure 1). Haptoglobin concentration is the only immune index that is significantly associated with body mass variation, otherwise diet treatment affects immune indices and body mass directly, except for haemagglutination titre which not affected by diet treatment.

	HP							
S-Model	AIC	L-Model	Response	Predictor	DF	Std Est.	P	
		1	Hp	DietF	434		0.00	***
A	150.0		Mass	DietF	435	0.26	0.00	***
		2	Hp	Mass	435	-0.13	0.01	*
		1	Mass	Diet	434		0.00	***
B	54.2		Hp	DietF	436	-0.49	0.00	***
		2	Mass	Hp	435	-0.13	0.00	**
	NOX							
		1	Nox2	DietF	425		0.04	*
A	26.6		Mass	DietF	426	0.25	0.00	***
		2	Nox2	Mass	426	-0.03	0.52	
		1	Mass	DietT	425		0.00	***
B	61.4		Nox2	DietF	426	0.09	0.07	
		2	Mass	Nox2	426	-0.01	0.74	
	OVO							
		1	Ovo	DietT	406		0.00	***
A	42.5		Mass	DietF	407	0.26	0.00	***
		2	Ovo	Mass	407	0.01	0.90	
		1	Mass	DietT	407		0.00	***
B	59.5		Ovo	DietF	407	0.20	0.00	***
		2	Mass	Ovo	5	0.00	0.98	
	AGG							
		1	Agg	DietT	434		0.16	
A	23.7		Mass	DietTF	435	0.26	0.00	***
		2	Agg	Mass	435	-0.06	0.25	
		1	Mass	DietF	434		0.00	***
B	63.0		Agg	DietF	436	0.05	0.29	
		2	Mass	Agg	435	-0.03	0.52	
	Lysis							
		1	Lys	DietF	434		0.00	***
A	50.4		Mass	DietF	435	0.26	0.00	***
		2	Lys	Mass	435	0.04	0.47	
		1	Mass	DietF	434		0.00	***
B	62.3		Lys	DietF	436	0.23	0.00	***
		2	Mass	Lys	435	0.04	0.41	

Chapter 9

General discussion: How variation in life history traits arises from environmental seasonality in a tropical environment – a synthesis

Nwaogu chima Josiah

My co-authors and I investigated how seasonal environmental conditions relate to the occurrence of breeding and moult in the Common Bulbul in order to further investigate how the separate and interactive effects of seasonal environmental conditions, breeding, moult and diet explain variation in innate immune function in the same species. In the preceding chapters, we showed that the weak breeding seasonality of Common Bulbuls arises from flexible between and within year individual breeding patterns and strict seasonal timing of moult to the wet season at the population level. We also showed that only relatively heavier females bred in the dry season, suggesting that although breeding is possible year-round in our study system, foraging conditions are less favourable in the dry compared to the wet season. On investigating seasonal variation in innate immune function, we found that Common Bulbuls varied innate immune function seasonally, but this variation was better explained by the occurrence of wet and dry seasons rather than breeding and/or moult, but unexpectedly immune indices were higher in the dry season compared to the wet season. Considering variation in life history traits along an environmental aridity gradient in space, we found that Common bulbuls had larger body surface area to body mass ratio in hotter and more arid environments, consistent with Bergmann's rule. We also observed that moult commenced earlier in locations with earlier rainfall consistent with our suggestion that moult is timed to the wet season rather than periodic. However, we did not find any suggestion that immune function varies with aridity along the same spatial environmental gradient along which body size and moult varied with aridity. On manipulating diet in wild-caught captive reared Common Bulbuls, we found a direct and reversible effect of diet treatment on immune function and body mass, and a subsequent effect on moult - fruit-fed Common Bulbuls maintained a more robust innate immune function, higher body mass and earlier moult compared to invertebrate-fed ones.

In this last chapter, I will discuss how insights gained from studying the Common Bulbul in its natural environment over time and space and under an experimental condition has improved our understanding of variation in innate immune function as a life history trait that interacts with the environment and other life history traits to determine fitness. I will highlight how the use of multiple immune indices can help us with interpreting what immune indices may indicate within the context of body condition and resource allocation trade-offs. Finally, I will discuss the promising potential of this decoupled system for understanding different aspects of immune function and life history evolution and point to specific investigations needed to further understand the patterns observed in our data.

How environmental conditions explain breeding, moult and breeding body mass variation

In Chapter 2 (Nwaogu et al. 2018a) we confirmed the weak breeding seasonality of Common Bulbuls and showed that this occurred at the individual level despite seasonally arid environmental conditions, but we found that the timing of moult remained seasonal and almost entirely within the wet season at the population level. This finding implies that moult is not causally linked to the timing of breeding, an idea which contrasts with the general notion that breeding is timed to seasonal food abundance and then in turn drives the timing of moult: a notion which prevails in temperate systems (Lack 1968, Perrins 1970, Verhulst and Nilsson

2008). We suggested that in Common Bulbuls moult is timed to match seasonal rainfall, and this suggestion is supported by our finding along the spatial gradient reported in Chapter 6. Along the aridity gradient, locations experiencing earlier rainfall had Common Bulbuls already advanced in moult compared to those with later rainfall. However, although, moult is timed to the wet season (when foraging conditions are more favourable) and breeding occurs in both seasons, I do not suggest that moult is more resource costly than breeding; rather I argue that a precise annual timing of moult may provide greater fitness benefit than when timed relative to breeding and this may apply to other species (Marmillot et al. 2016, Tomotani et al. 2017).

It is not completely clear whether the mechanism generating a seasonal moult rather than breeding reflects a strategy which prioritizes self-maintenance over reproduction due to low annual reproductive output (Williams 1966) or differences in response to environmental conditions by breeding and moult. In any case, we concluded that there is a stronger seasonal constraint on moult than breeding, and in Chapter 3 (Nwaogu et al. 2017) we showed that breeding females can adjust their body mass to accommodate the extra cost of breeding during the relatively more impoverished dry season - confirming that breeding is indeed flexible. Body reserves maintain vital functions during foraging unpredictability, a process possibly linked to the action of the adipocyte-derived hormone leptin which regulates physiological processes, including immune function during lean periods (Halaas et al. 1995, Lord et al. 2001, Demas et al. 2003).

I suggest that since breeding can be successful year-round, seasonal breeding probably offers little fitness advantage, so life history traits related to self-maintenance, such as moult and immune function may be controlled more strongly in relation to environmental seasonality. This suggestion is partly supported by our finding in Chapter 4, where we showed that variation in innate immune indices were better explained by variation in environmental conditions than the occurrence of annual cycle stages. Therefore, I propose that because birds are exclusively iteroparous (Bennett and Owens 2002), enhancing annual survival through self-maintenance may be a route to achieving higher life time fecundity (Schaffer 1974), especially if annual reproductive output is low. Common Bulbuls seem to prioritise their survival (Ghalambor and Martin 2001) by maintaining a regular moult schedule and investing in immune function, but they also optimise the value of successfully fledged chicks by extended post-fledging care. Thus, adult survival may influence the organisation of the annual cycle in Common Bulbuls (Bennett and Owens 2002), but its perceived low importance in small birds (Lack 1968) may be due to the low survival variability among temperate passerines. I will argue that tropical birds harbour enough survival variability (Karr et al. 1990, Mcgregor et al. 2007, Stevens et al. 2013), to allow selection to act strongly on self-maintenance traits in some species. This may inform whether we expect a self-maintenance trait like immune function to vary over the annual cycle according to maintenance cost or the survival benefit it offers animals (McNamara and Houston 2008).

Moult appears a highly conserved process in birds (Murphy and King 1992), and its observed independence from breeding in the Common Bulbul supports evidence presented for differential shifts among annual cycle stages due to climate change (Tomotani et al. 2017). The independent timing of moult from breeding may also explain the prevalence of breeding-moult overlap in less seasonal breeding tropical birds (Foster 1975, Johnson et al. 2012b, Stouffer et al. 2013) and its rarity in temperate birds (Camacho 2013). If moult is causally linked to breeding, and breeding seasonality is weak, then individuals should moult at variable periods in the year – but this may not be the case. Thus, the moult cycle may be an important 'clock' for timing the annual cycle of tropical birds that breed at any time of the year since it seasonality is relatively conserved. However, there is still need to investigate what constraints actually govern moult seasonality, because previous studies report that the nutrients deposited in feathers are relatively low compared to the energy expended during moult (Murphy and King 1992, Murphy 1996). For example, although, we showed in Chapter 8 that Common Bulbuls on relatively poorer invertebrate diet did not moult, the fruit-fed Common Bulbuls that moulted did not vary in the extent of moult according to their body condition. Hence, despite the fact that moult depends on nutrition and may be subject to foraging conditions in the wild (Ward 1969), other aspects of body condition or life history consideration maybe important for the timing of moult.

Occurrence and determinants of seasonal variation in immune function

Seasonal variation in innate immunity is hypothesised to vary according to the temporal patterns in disease risk (Horrocks et al. 2012a) or life history trade-offs that may arise from specific annual cycle stages (Martin et al. 2008a), but where annual cycle stages co-vary with seasonal environmental conditions, the effects of both factors cannot be separated. In Chapter 4, we investigated the separate and interactive explanatory power of seasonal environmental variation (i.e. occurrence of rainfall) and annual cycle stage on seasonal variation in immune function in Common Bulbuls. We found that seasonal differences in immune function were more often explained by seasonal environmental conditions than by the occurrence of annual cycle stages. But contrary to expectations, immune indices were higher in the dry season compared to the wet season. Although, this finding supports the overriding role of the environment in explaining variation in immune function (Horrocks et al. 2011b), it opposes the idea that environmental productivity or aridity is a simple proxy for disease risk (Horrocks et al. 2015). The latter point is further supported by our results in Chapter 7, where we found no correlation between environmental aridity and immune function along the spatial aridity gradient in Nigeria.

There is hardly any objection to the idea that there is seasonal variation in immune function (Nelson and Demas 1996, Nelson et al. 2002, Nelson 2004), however, the mechanism generating such seasonal variation is unresolved (Martin et al. 2008a, Buehler et al. 2008, Buehler et al. 2009, Hegemann et al. 2012). We show that in a system where annual cycle stages are seasonally decoupled, there is no evidence that immune function is suppressed during periods of limited food availability or during the occurrence of breeding or moult. However, it remains unclear whether higher immune indices in the dry season represent higher disease risk for Common Bulbuls as reported for free-range poultry birds (Miguel et al. 2013). In temperate systems, in which immune function is higher in winter (Nelson 2004), such immune elevations are explained by three factors: (i) an upregulation of immune function to counteract immunosuppressive stress effects of harsh environmental conditions (Sinclair and Lochmiller 2000), (ii) increased resource availability for immune function due to the absence of breeding and moult (Greenman et al. 2005), and (iii) higher occurrence of contact-transmitted infections (Nelson 2004). The first two explanations align with the notion that immune function is costly and governed by trade-offs (Sheldon and Verhulst 1996), while the third suggests a more beneficial importance of the immune system which anticipates or responds to infection (Horrocks et al. 2011b). Thus, if we liken dry season conditions in our system to the relatively impoverished temperate winter conditions, then our results favour the third explanation which suggests a response to higher disease risk. However, the first two explanations are generally favoured (Martin et al. 2008a) in temperate systems even though they seem opposed to each other, because it is not intuitive that immune function should be upregulated in winter under resource constraint if it is ‘costly’. A major shortcoming of explaining seasonal variation in immune function solely on the basis of resource allocation trade-offs when only few factors have been measured is that seasonal environmental factors and annual cycle stages covary and animals may still maintain seasonal patterns in the absence of major perturbations in environmental conditions which affect immune function even when other environmental factors change. For example, (Buehler et al. 2008) showed that captive Red Knots *Calidris canutus* maintained seasonal patterns of immune function despite temperature manipulation. In another study, they showed no evidence for melatonin immunoenhancement of immune function in the same species (Buehler et al. 2009a). Hegemann et al. (2012) on the other hand, working with Skylarks *Alauda arvensis* in the wild showed that seasonal patterns in immune function are not consistent between years, and this is presumably because the specific environmental conditions that affect immune function changed between years despite consistent environmental seasonality.

Clearly, much more information is required to interpret variation in immune function on the basis of resource allocation trade-offs between immune function and other competing functions (Norris and Evans 2000). Currently, it is not clear how much of a particular resource (energy or nutrients) is required to maintain immune function (Derting and Compton 2003). Moreover, several arms of the immune system may be activated in response to a single immune challenge (Hegemann et al. 2013b) and several other energy demanding processes (e.g breeding, moult and migration) may be functional at the same time. The effect of increased energy expenditure may then not necessarily be visible in the trait under consideration. For example, (Martin et al. 2003) proposed that the energy expended during immune activity in the House Sparrows *Passer domesticus* can produce half an egg, yet, (Williams et al. 1999) reports no evidence of reduced reproductive output after immunizing European Starlings *Sturnus vulgaris*. Hegemann et al. (2012a) also showed that Skylarks seasonally modulate energy budgets but maintain energetically costly inflammatory responses throughout their annual cycle, suggesting that animals may save resources (or energy) on other demanding processes to maintain aspects of immune function if needed. In Chapter 8, we demonstrated clearly using experimental diet manipulation that depending on the immune index considered, immune indices may correlate positively, negatively, or even show no relationship with other life history traits. For example, high haemolysis titre covaried with low haptoglobin concentration, higher body mass and earlier onset of primary moult in healthier Common Bulbuls. Thus, it is probably more appropriate to also consider the fitness benefits of an immune response in terms of future survival and reproduction (Ardia et al. 2003, Møller and Saino 2004), than to simple interpret a negative correlation between one immune index and one seasonal life history event as evidence of a seasonal resource allocation trade-off. However, I do not suggest that there are no trade-offs between immune function and other life history traits because studies have shown convincingly that activation of the immune system has fitness costs (Råberg et al. 2000, Moret and Schmid-Hempel 2000, Ardia 2005a). Rather, I propose that the effect of any trade-off may depend on environmental condition, individual quality (Ardia 2005a, Ardia 2005b) and possibly the risk of death to infection (Nelson et al. 2002), and this may not always lead to a negative correlation depending on the immune indices considered.

Differences between multiple immune indices in different annual cycle stages may help us better understand trade-offs between immune function and other life history traits in a slightly different way. In Chapter 4, we showed the co-occurrence of high haemolysis and low haptoglobin concentration in breeding females and the opposite trend in non-breeding ones. If we interpret higher haemolysis titre as enhanced capacity to destroy pathogens (Ochsenbein et al. 1999), and lower haptoglobin concentration to reflect lower infection (Owen-Ashley and Wingfield 2006), then it could mean that breeding females are more immunocompetent and/or less challenged compared to non-breeding ones, or that challenged females omit breeding. Therefore, higher haptoglobin concentration and lower haemolysis titre in non-breeding females may indicate a trade-off between breeding versus immune function (increased inflammatory response) (Råberg et al. 2000). This idea is further supported by Chapter 4 where we showed that delayed moult and lower body mass are associated with high haptoglobin concentration and low haemolysis titre. I propose that immunocompetence is crucial for maintaining the regular occurrence of annual cycle events but immunocompetence is likely a composite of several indices of immune function (Norris and Evans 2000). Although it is well documented that annual cycle stages like breeding and moult are costly (Perrins 1970, Murphy 1996), it is not apparent that their occurrence constitutes ‘stress’. Moreover, except where unpredictable events like harsh cold winters or drought severely deplete resources, the difference in resource availability between the breeding and the non-breeding period is hypothesized to balance the resource deficit between self-maintenance only and self-maintenance plus breeding (Ashmole 1963b). In my opinion, self-maintenance also includes optimal immune function, because pathogen defence is a crucial component of body condition. Chapter 2 and 3 confirm that environmental conditions do not limit breeding in the Common Bulbuls, so seasonal variation in immune function is unlikely due to direct resource allocation trade-offs between immune function and breeding. We only find a likelihood of resource allocation trade-off in Chapter 8 where fruit-deprivation led to high haptoglobin concentration, lower body mass and delayed moult. However, since Common Bulbuls are unlikely to be fruit or invertebrate deprived in the wild (Box B), such a trade-off can be avoided and therefore unlikely. I conclude that seasonal variation in immune function in the Common Bulbul is most likely in response to and/or anticipation of increased infection in the dry season. This could be confirmed by quantifying seasonal infection dynamics or disease risk over the same time scale within which I have measured immune function.

What drives variation in immune function along a spatial environmental gradient?

Geographical variation in environmental conditions drive patterns of life history variation and provide a useful way of verifying whether temporal patterns in life history events are a result of environmental conditions or simply periodic. In Chapter 5, we presented evidence for local body size adaptation along a spatial aridity gradient in Nigeria by showing that Common bulbuls had larger body surface area to body mass ratio in hotter and more arid environments consistent with Bergmann's rule (Salewski and Watt 2017). A possible interpretation is that Common Bulbuls are locally resident and presumably experience little or no gene flow between locations along the gradient. Local residency is further supported by results in Chapter 6, where we showed local differences in the extent of primary moult consistent with variation in rainfall. But unexpectedly, results in Chapter 7 showed that immune function did not vary along the aridity gradient, a finding in contrast with the hypothesis that innate immune function decreases with increasing aridity – a proposed proxy for disease risk (Horrocks et al. 2015). Although, the finding on immune function is contrary to prediction, it supports arguments in Chapter 4 that variation in immune function does not follow simple environmental productivity patterns. However, because Common Bulbul populations appear locally distinct along the gradient, then immune function is likely a more flexible life history trait than body size and timing of moult across environmental conditions. Nonetheless, the factors driving similar immune function along the aridity gradient are not obvious because we expected disease risk to decrease with increasing aridity and the aridity gradient to be more pronounced at the end of the dry season after different durations of drought among locations. However, the pattern is also not explainable by differences in the timing life history events of Common Bulbuls along the environmental gradient. Therefore, I conclude that any assumption of variation in disease risk or pathogen abundance along an environmental productivity gradient requires testing before drawing predictions on immune variation based on infection risk.

Innate immune function is nutrient specific

In Chapter 8 we confirmed that immune function depends on specific nutrients not just energy (Klasing 1998, Demas et al. 2012), but interestingly, we revealed that essential amino acids from protein-rich diets are apparently not the main limiting nutrients for innate immune function (Lochmiller et al. 1993) because fruit-fed Common Bulbuls were healthier overall compared to invertebrate-fed ones. The reversible diet effects on immune function, body mass and moult highlight the potential importance of the environment in influencing immune variation through diet determination. In addition, Chapter 8 demonstrated the diet specificity of immune indices and the life history implications of their variation. Previous studies focused on the energetics of immune function (Lord et al. 2001, Demas et al. 2012) and thus, were more likely to restrict food access rather than specific nutrients (French et al. 2007, Buehler et al.

2009b, Schultz et al. 2017, Xu et al. 2017). Although such studies improved our understanding of how resource limitation affects immune function, results remained equivocal, and this is partly because the nutrient needs (or allocation formula) during immune responses are largely unknown, so limitations cannot be determined by overall food restriction. This problem is exemplified in observational or experimental studies within systems where although environmental conditions are impoverished, animals still have enough resources to maintain vital functions (Hegemann et al. 2012a) – a problem that may further explain the lack of immune variation along the environmental gradient as reported in Chapter 7. Through diet alteration (and thus, nutrient limitation) we show unequivocally that low natural antibody and complement activities, low ovotransferrin and nitric oxide concentration, and high haptoglobin concentration are associated with overall poor body condition in Common Bulbuls that are deprived of nutrients obtainable from fruits. Therefore if Common Bulbuls were resource limited in the wild due to variation in environmental conditions along the aridity gradient we would expect to pick it up.

Results from Chapter 8 are unique because they reveal the sensitivity of immune function to short term diet alteration even in adult wild birds whose immune system is presumably well developed and adapted to wild conditions. Our knowledge of diet effects on immune function in birds is largely limited to studies on poultry and captive pet birds (Klasing 1998, Klasing 2007). Our observations of these rapid diet effects on wild Common Bulbuls imply consequences of environmental change for both resident and migratory birds that depend on seasonally available food items (Bairlein 1996, Jenni-Eiermann et al. 2011, Marshall et al. 2016, Guglielmo et al. 2017). Of all proxies of environmental variation considered in this thesis, diet had the strongest effect on all immune indices measured, thus, seasonal diet composition may play an important role in maintaining immune function and occurrence of other life history traits over the annual cycle. The role of seasonal diet variability in influencing seasonal variation in immune function of vertebrates has been largely ignored (Martin et al. 2008a) even in temperate systems where environmental seasonality is very pronounced.

Conclusion

Taken together, results from this thesis support a stronger role of the environment in driving variation in immune function (Horrocks et al. 2011b) than the possible effects of resource allocation trade-offs between competing annual cycle stages and immune function (Sheldon and Verhulst 1996). However, patterns of variation in immune indices reveal that variation in innate immune function does not follow simple environmental productivity patterns, as reported for disease risk and pathogen prevalence (Altizer et al. 2006, Nunn et al. 2005, Hawley and Dana 2011). Consequently, a quantification of host dependent and independent infectious agents is crucial to further our understanding of the variation observed in immune function in this system. In addition to environmental disease risk or antigen abundance, another factor shaping immune phenotypes of mammals and birds is the gut microbiome (McFall-Ngai et al. 2013) and ecologists now realize the possible impact and function of the gut microbiome in the life of wild animals (Koch and Schmid-Hempel 2012, Belkaid and Hand 2014). Studies that have manipulated the microbiome show significant effects on immunity (van de Crommenacker et al. 2010, Montalban-Arques et al. 2015). An analysis of the gut microbiome and their functional significance will specifically further our understanding of the interesting results in Chapter 8, where fruit-fed Common Bulbuls maintained immune function, body mass and moult despite protein deprivation, suggesting that proteins may be made available via alternative sources if needed. The activities of the gut microbiota is strongly linked to *de novo* synthesis of essential amino acid that are important for physiological functions including immunity (Lin et al. 2017). Experimental studies on the link between diet, the gut microbiome and immune function have rarely been carried out, especially in tropical environments where the linkage between food availability and occurrence of life history events such as breeding can be decoupled from environmental seasonality within a single species like the Common Bulbul.

Overall, our findings suggest that the long-standing paradigm which emphasizes the costly nature of immune function limits our appreciation of its survival benefit to animals in the wild and largely ignores the ultimate role of the immune system which is enhancing survival (Møller and Saino 2004). Pathogen defense is likely prioritized over several other functions when the risk of death is eminent, and disease presents an immediate risk to death (WHO 2018). By integrating natural history and investigation of variation in innate immune function in the Common Bulbul within the context of a tropical environment in a single study, this thesis provides evidence that an immune function connects different aspects of life history (Figure 9.1). This is because we showed that birds maintaining a robust immunity were in better body condition and are most likely to breed and moult. This idea is actually consistent with (Hamilton and Zuk 1982) hypothesis that only disease free animals can invest optimally in other traits like signalling to attract mates and the act of reproduction itself. I provide some insights into the benefit of immune function in the wild and reaffirm the importance of interpreting variation in immune function according to the functional relevance of the immune indices we measure (Adamo 2004). These insights gained by expanding studies of ecological immunology to tropical systems and studying variation in immune function within the context of a species' environment will help the future refinement of hypotheses in ecological immunology. Indeed, the historical bias towards studies on captive and/or temperate seasonally breeding animals may have led to misconceptions about resource allocation trade-offs being the most important factor governing seasonal variation in immune function in natural systems. Trade-offs are definitely an important source of variation in immune function like other physiological traits, but their validity as explanation for seasonal variation in immune function seems better rooted within an environmental context (Tieleman 2018a), and tropical systems provide great opportunities for further exploration of the composite influence of multiple factors on immune function within an environment.

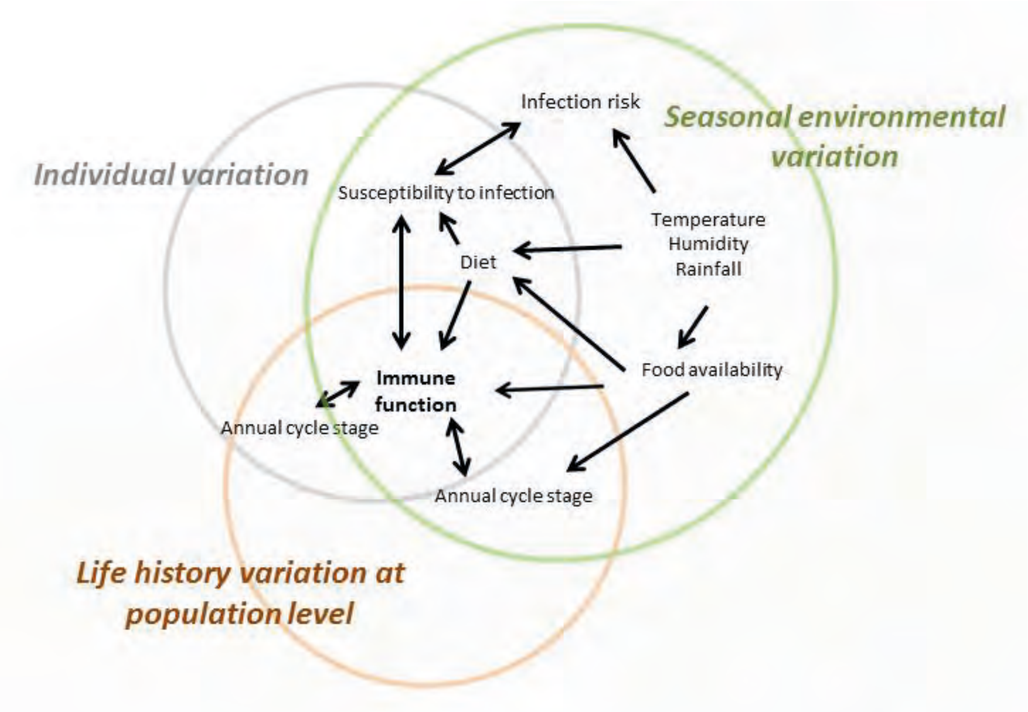

Figure 9.1. Conceptual model of how variation in immune function may arise via interactions between the seasonal environmental variation, life history variation at the population level and individual variation in the Common Bulbul. Key points: 1. Variation in environmental conditions alters the food availability and infection risk. Food availability may influence occurrence of annual cycle stages at the population level and diet decisions of individual birds. 2. Variation in environmental infection risk, individual variation and variation in diet may influence susceptibility to infection. 3. Variation in diet may affect the immune function directly or indirectly through effect on other aspects of individual condition that may affect infection susceptibility. 4. Occurrence of annual cycle stages may lead to variation in immune function at both the population and individual level due to resource allocation trade-offs or decisions due to variation in individual condition.

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NEDERLANDSE SAMENVATTING

Het doorgronden van de evolutie van de levensloop d.w.z. hoe organismen hun beschikbare energie en nutriënten verdelen tussen overleven en reproduceren, is een fundamenteel probleem. Het merendeel van onze kennis hierover komt voort uit onderzoek in gematigde klimaten, terwijl het leven echter in de tropen is geëvolueerd. Hoewel er groeiende aandacht is voor de biologische diversiteit en het unieke karakter van levensvormen in tropische systemen, is er nog weinig bekend over de verschillende selectiedrukken en evolutionaire aanpassingen waardoor diverse levensloopstrategieën zich hebben ontwikkeld. Dit proefschrift is bedoelt om bij te dragen aan het herstellen van deze scheve balans en is daarom gericht op het beter begrijpen van hoe seizoensgebonden omgevingsfactoren en dieetvariatie in de tropen leidt tot variatie in kenmerken van de levensloop.

Voor sommige tropische vogels is het broeden, op populatieniveau, niet sterk seizoensgebonden, ondanks dat er voorspelbare seizoensoptima voor omgevingsomstandigheden bestaan. Het blijft daarom onduidelijk of individuen zijn aangepast om te broeden op specifieke tijden durende een jaar, of juist flexibel zijn als gevolg van variabele omgevingsomstandigheden. In het tweede hoofdstuk (eerste data-hoofdstuk) van dit proefschrift testte ik of de broedactiviteit van de door het hele jaar broedende grauwe buulbuul *Pycnonotus barbatus*, is ontstaan als gevolg van individuele variabiliteit in broedperiode. Ik analyseerde de broedactiviteit en ruikenmerken van vogels gevangen gedurende een periode van twee jaar, en vond dat de broedgegevens van individuen net zo variabel kunnen zijn als in de populatie. Het voorkomen van broeden kon niet worden verklaard aan de hand van de hoeveelheid neerslag of temperatuur, hoewel vogels tijdens de rui zelden broedden. In tegenstelling tot broeden was de rui wel sterk seizoensgebonden maar was niet gecorreleerd aan neerslag of temperatuur. Ik stel daarom voor dat, gezien omgevingsomstandigheden het hele jaar door broeden in deze soort mogelijk maken en omdat voortplantingssucces onderhevig is aan een hoog predatierisico, er waarschijnlijk een zwakke selectie is voor individuen om het broeden samen te laten vallen met pieken in variabele omgevingsomstandigheden. In tegenstelling daarvan is het waarschijnlijk dat rui, een jaarlijks voorkomend fenomeen, onder sterke selectie staat om gelijktijdig plaats te vinden met de pieken in variabele omgevingsomstandigheden, waarbij een verhoogde overlevingskans het voortplantingssucces bevordert.

Het hele jaar door broeden kan het voortplantingssucces bevorderen, maar het individu moet in staat zijn zich aan te passen aan een variërende mate van voedselbeschikbaarheid tussen de seizoenen. Aannemelijk is dat vogels mogelijk lichaamsreserves opslaan als er een verhonger risico ontstaat vanwege beperkte beschikbare foerageertijd. Als het foerageren echter onbeperkt is, zou het lichaamsgewicht laag moeten blijven om het predatierisico te beperken dat het verkrijgen en meedragen van lichaamsreserves met zich meebrengt. In mijn tweede data-hoofdstuk testte ik aan de hand van 15 jaar aan gegevens over lichaamsmassa's van grauwe buulbuul vrouwtjes of lichaamsgewichtsvariatie kan worden verklaard door beperkte foerageertijd tijdens het broeden,. Ik vond dat de broedfase het lichaamsgewicht van vrouwtjes voorspelde: het lichaamsgewicht piekte abrupt tijdens de incubatietijd en daalde tijdens de broedzorg. Broedende vrouwtjes waren zwaarder in het droge seizoen dan in het natte seizoen. In het droge seizoen was het waarschijnlijker dat zwaardere vogels aan het broeden of incuberen waren, wat suggereert dat verhoogde lichaamsreserves nodig kunnen zijn als buffer voor de beperkte foerageertijd of verarmde foerageeromstandigheden, die het meest uitgesproken kunnen zijn tijdens respectievelijk de incubatie en in het droge seizoen.

Naast het vinden van voedsel moeten dieren zichzelf beschermen tegen infecties die per seizoen kunnen verschillen. De hoeveelheid beschikbare middelen voor verdediging kan echter afhankelijk zijn van hoeveel al is verbruikt in andere processen. Seizoensvariatie in immuniteit kan dus ofwel worden toegeschreven aan veranderingen in omgevingsomstandigheden als aan compromissen die optreden wanneer middelen worden toegewezen aan andere processen. Het loskoppelen van deze factoren is lastig in het wild. In mijn derde data-hoofdstuk heb ik, door gebruik te maken van niet-seizoensgebonden broeden en ruien in de grauwe buulbuul, het effect van seizoensgebonden omgevingscondities effectief losgekoppeld van dat van het jaarlijkse cyclusstadium, en getest hoe beide factoren de immuunfunctie beïnvloeden. Ik voorspelde dat indien de kans op infecties toeneemt met regenval het immuunsysteem sterker zou zijn in het natte seizoen, maar indien broeden of rui beperkingen oplegt aan vogels, dat dan de immuun parameters lager zijn tijdens het broeden of ruien. Door het kwantificeren van vijf aangeboren immuun parameters gedurende twee jaar vond ik dat seizoen en niet stadia van de jaarcyclus de variatie in alle immuun parameters verklaarde, behalve één: NO_x, waarvan de concentratie verschilde tussen de jaarlijkse cyclusstadia maar niet tussen seizoenen. Tegen mijn verwachting in waren de meeste immuun parameters hoger in het droge seizoen en tijdens het broeden, terwijl ik verwacht dat regen de infectiekans verhoogde en het broeden immuunfunctie verminderde. Ondanks dat, kon ik concluderen dat omgevingsfactoren de aangeboren immuniteit rechtstreeks beïnvloeden in plaats van via compromissen in het toewijzen van middelen tussen verschillende levensloopprocessen.

Zoals voorspeld door de regel van Bergmann kan lichaamsgrootte variëren als reactie op omgevingsomstandigheden. Deze regel is een biofysische generalisatie dat endotherme dieren de neiging hebben groter te zijn in koudere klimaten. In mijn vierde data-hoofdstuk testte ik of de lichaamsgrootte van grauwe buulbuul varieert in overeenstemming met de regel van Bergmann. Ik mat vogels op 22 locaties tussen 6 en 13°N en schatte vervolgens de gemiddelde verhouding tussen lichaamsoppervlak en -gewicht per locatie. In overeenstemming met de regel van Bergmann ontdekte ik dat vogels een grotere verhouding tussen lichaamsoppervlak en -gewicht hadden in warmere omgevingen, onafhankelijk van de breedtegraad en hoogte (ten opzichte van zeeniveau).

Neerslag is een belangrijke bepalende factor voor de omgevingstoestand in de tropen, maar omdat regenval vaak seizoensgebonden is, is het moeilijk om het effect ervan te onderscheiden van dat van andere seizoensgebonden factoren en co-variërende levensloopkenmerken. Bijvoorbeeld, grauwe buulbuuls ruien in het natte seizoen in centraal Nigeria, maar het is niet bekend of de rui op hetzelfde tijdstip op alle locaties plaatsvindt of op een tijdstip dat samenvalt met de lokale regenval. In het vijfde data-hoofdstuk van dit proefschrift modelleerde ik de mate van rui als een functie van de timing van neerslag op 15 locaties tussen 6° N en 13° N. Ik vond dat vogels eerder ruiden op plekken waar het natte seizoen eerder begon, wat suggereert dat de rui in grauwe buulbuuls is gekoppeld aan timing van de regenval.

Variatie in regenval en temperatuur zou ook het infectierisico kunnen bepalen langs een gradiënt van omgevingscondities. Zo voorspelde ik dat als het infectierisico afneemt met toenemende droogte, maar gepaard gaat met immuun functie, de aangeboren immuunfunctie zou moeten afnemen met toenemende droogte van het koud-natte bos tot de hete-droge Sahel-zone in Nigeria. Ik testte deze voorspelling in het zesde data-hoofdstuk van dit proefschrift door het meten van aangeboren immuun parameters in grauwe buulbuuls op 15 locaties tussen 6° N en 13° N. Tegen mijn verwachting in daalden de immuun parameters niet met toenemende droogte, maar in plaats daarvan waren ze op verschillende locaties vergelijkbaar, wat suggereert dat in dit systeem de aangeboren immuunfunctie niet varieert met verschillende mate van droogte. Dit resultaat onderstreept de behoefte aan voorzichtigheid bij het voorspellen van de effecten van klimaatvariabiliteit op ziekterisico.

Dieet is een cruciaal onderdeel van de omgeving en wordt sterk beïnvloed door omgevingsveranderingen, met soms grote gevolgen. In mijn laatste data-hoofdstuk, testte ik hoe de samenstelling van het dieet van invloed is op de aangeboren immuunfunctie, lichaamsgewicht en rui. Gedurende 24 weken voedde ik 40 in het wild gevangen grauwe buulbuuls *ad libitum* vruchten of ongewervelde dieren in buitenvolières in Nigeria. Ik beoordeelde aangeboren immuunindexen, lichaamsgewicht en primaire rui tweewekelijks en vond dat fruit-gevoede buulbuuls een robuustere aangeboren immuunfunctie, hoger lichaamsgewicht en eerdere handpenrui hadden in vergelijking met vogels gevoed met ongewervelde dieren. Deze resultaten zijn uniek omdat ze een effect aantonen van een dieetaanpassing bij wilde vogels - wat korte termijn gevolgen van omgevingsverandering aantoont.

Door klassieke natuurstudie en fysiologie te integreren in de context van de directe omgeving waarin een soort leeft, biedt dit proefschrift nieuw inzicht in hoe variatie in broed- en ruipatronen en immuunfunctie verschillende aspecten van de levensloop van een tropische vogel met elkaar verbindt. Mijn bevindingen in dit proefschrift suggereren met name dat het al lang bestaande paradigma dat de kostbare aard van immuunfunctie benadrukt, het belang van het overlevingsvoordeel ondermijnt en grotendeels voorbijgaat aan de uiteindelijke rol van het immuunsysteem, overleving. De voor- en nadelen van de immuunfunctie kunnen alleen worden bepaald als rekening wordt gehouden met de omgevingsomstandigheden én levensloop waaronder variaties optreden.

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Chima Nwaogu, Cape Town, 5th May 2019.

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Before Chima's PhD under the joint supervision of Irene Tieleman and Will Cresswell at the University of Groningen, the Netherlands and St. Andrews, UK respectively, he studied for a bachelor's degree in Zoology at the University of Jos Nigeria and a master's degree in Conservation Biology at the A.P. Leventis Ornithological Research Institute (APLORI) also at the University of Jos. For his master's thesis, he used bird ringing data collected at the APLORI to investigate trends in body mass of birds over a period of habitat management between 2001 and 2011 under the supervision of Yahkat Barshep and Alexa M. Lord. After his master's, he participated in a molecular ecology and evolution course at the Molecular Ecology and Evolution Lab. at Lund University in Sweden as visiting student. He returned to APLORI afterwards as a research associate until 2014 when he started his PhD. As a research associate at APLORI, Chima was involved with Constant Effort Site (CES) bird ringing and assisting students and interns with aspects of field ornithology and statistics. He was also involved with monitoring a Rosy Bee-eater breeding colony on the river Niger - the only known extant breeding colony of the species north of the equator. In April 2019 he started a postdoctoral fellowship at the Fitzpatrick Institute of African Ornithology in Cape Town, South Africa in collaboration with Arjun Amar, where he investigates morph related differences in breeding performance and physiological responses of Black Sparrow-hawks in Cape Town.

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