Reproduction, growth and immune function: novel insights in equatorial tropical birds

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by

Henry Kamau Ndithia

born on 31 December 1971
in NAIVASHA, Kenia

Supervisor
Prof. B.I. Tieleman

Co-supervisor
Dr. M. Muchai

Assessment Committee
Prof. B. Helm
Prof. M.E. Visser
Prof. L. Lens

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Three proximal equatorial tropical environments, increased aridity

Chapter 1

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General introduction and scope of the thesis

Henry K. Ndithia

Introduction

The goal of this section is to introduce the main topics covered in this thesis providing an overview of the expectations of each thesis chapter. The section provides a comparison between temperate and arctic zones, where several studies related to our topics have been carried out, on the one hand and the equatorial tropical environment where many scientific information gaps still exist.

1. Reproduction, growth and immune function in temperate and arctic zones

Temperate and arctic zones have predictable seasonal changes in environmental conditions and day-length is the main driver of the timing of reproduction in birds (Demas and Nelson 1998, Wingfield et al. 1997, Versteegh et al. 2014, Colwell 1974). Birds use day-length to time reproduction (Lambrechts et al. 1996, Nager and van Noordwijk 1995, van Noordwijk et al. 1995) while temperature and food availability serve as supplementary cues to fine tune the timing of reproduction and nestling growth to local environmental conditions (Hau 2001, Lambrechts et al. 1996, Van Noordwijk et al. 1995). This regularity in the timing of reproduction and nestling growth and the predictability in seasonal changes in day-length potentially results in synchronized spring breeding within and among species living at the same location (Nager and van Noordwijk 1995, Lambrechts et al. 1996). Avian growth rate and development is associated with the pace-of-life and nestlings in species and populations from temperate and arctic zones have comparatively faster growth rates compared to those at lower latitudes (Ricklefs 1976, McCarty 2001, Ricklefs and Wikelski 2002, Wikelski et al. 2003, Wiersma et al.. 2007). Raising of young in these zones is restricted to the spring season.

Like growth rates, immune function has been hypothesized to vary with the pace-of-life in birds, with reduced investment in the immune function associated with a faster pace-of-life (Ricklefs and Wikelski 2002, Martin et al. 2004, Tieleman et al. 2005). Physiological processes, e.g., reproduction, growth and immune function among others, require resources that are often limited and that come at a fitness cost. Due to the cost associated with the maintenance and use of an immune system, trade-offs have been shown to exist in temperate and arctic zone birds between immune defense and other life-history activities such as reproduction, growth and development, migration, thermoregulation (Sheldon and Verhulst 1996, Lochmiller and Deerenberg 2000, Norris and Evans 2000). In the case of reproduction and immune function, several studies involving increase in reproductive effort in birds have demonstrated reduced immune response when an immune system responds (Moreno et al 1999, Ilmonen et al 2000, Nordling et al 1998, Schmid-Hempel 2003). Birds also cushion increased demands on reproduction by allocating resources from immunity to reproduction in order to avoid reduction in reproductive output (Sheldon and Verhulst 1996). In addition, reduced reproductive effort has been shown to lower infection rates (Ots and Horak 1996) while prevalence and intensity of parasitic infection increase in animals during reproduction (Doreenberg et al 1997). Resource allocation from immunity to reproduction could increase the susceptibility to infection of individuals with increased reproductive investment (Sheldon and Verhulst 1996) leading to reduced fitness. Conversely, an increased investment in immune

function could lead to a correlated unavoidable loss in a fitness component such as reproduction (Stearns 1992).

Just as reproduction in temperate and arctic zone birds is sensitive to environmental variation (Nelson and Demas1996, Marra and Holberton 1998, Shepherd and Shek 1998, Ruiz et al. 2002, Tieleman et al. 2005), immune function of birds in these zones varies seasonally (Martin et al. 2008), partly due to trade-off between reproduction and immune function (Martin et al. 2008, Buehler et al. 2008, Pap et al. 2010a, Hegemann et al. 2012, Hegemann et al. 2012, Horrocks et al. 2012, Pap et al. 2010b). However, seasonal environmental changes such as temperature, disease risk and food availability have been linked to annual variation in immune function (Nelson et al. 2002). Seasonal changes in disease risk and strengthening of the immune defense in anticipation of disease, based on changes in environmental cues, have also been shown to be a cause for seasonal variation in the immune function in birds (Nelson et al 2002). Reproductive activity and seasonal variation in immune function in the temperate and arctic zone system both being influenced by environmental factors makes it difficult to decouple the effects of reproduction from those of environmental factors on the variation in immune functions.

2. Variation in life history traits: avian reproduction and nestling growth in the context of environmental conditions

a) Variation in environmental conditions in equatorial tropics and avian reproduction

In contrast to the temperate and arctic zones, environmental conditions in equatorial tropical environments are frequently unpredictable and highly variable (Grant and Boag 1980, Boag and Grant 1984, Wrege and Emlen 1991, Conway et al. 2005). Consequently, a diversity of breeding systems and rates of growth exists in this region. In relatively seasonal tropical environments, tropical birds can use tropical photoperiod on a long-term basis and environmental conditions on a short-term basis to time reproduction (Hau et al. 2000). In seasonal tropical environments with predictable changes in wet and dry seasons, birds also breed seasonally and use environmental conditions (rain and food) to time reproduction in much the same way temperate birds do (Wikelski et al. 2000, Hau et al. 2001). Conversely, some tropical birds living in unpredictable climate, breed whenever it rains (Hau 2001), with no physiological preparation for a breeding season, indicating plasticity which allows them to respond to short-term cues and flexibility in their regulation of reproduction.

The high spatiotemporal variability in rainfall and food resources in proximal equatorial tropical environments (Young 1994a, Grant and Boag 1980, Boag and Grant 1984, Wrege and Emlen 1991, Scheuerlein and Gwinner 2002, Stutchbury and Morton 2001, Conway et al. 2005) results in high variability in the timing of reproduction and in nestling growth among and within environments. In such circumstances, birds have to time their reproduction based on short-term and more irregular factors, e.g., rainfall and food resources (Stutchbury and Morton 2001, Dittami and Gwinner 1985), resulting to asynchronous reproduction (Moore et al. 2005). Reproduction in equatorial tropical birds has become more adaptable to their highly unpredictable habitats with large variability in environmental conditions depending on a variety of factors that influence their breeding including rain (Dittami and Gwinner 1985, Dittami 1986, Chapman 1995), food supply (Dittami and Gwinner 1985, Chapman 1995, Komdeur

1996, Hau et al. 2000, Scheuerlein and Gwinner 2002, Moore et al. 2005) and ambient temperature (Foster 1974, Tye 1991). Others tend to have more flexible breeding schedules breeding opportunistically (Boag and Grant 1984, Young 1994b), yet, in some equatorial tropical locations, birds may breed year-round (Ndithia per obs.). However, there have not been studies carried out to determine factors that may influence the timing of reproduction in opportunistically breeding birds and those that breed all year round. In addition, no studies have investigated several potential factors that may influence the timing of breeding in such species over time (several annual patterns) and comparing over space (different environments with different climatic conditions). It is the goal of this thesis to fill these gaps.

b) Variation in avian growth rates among and within equatorial tropical environments

Variation in avian growth rate and development occurs in equatorial tropical environments with faster growth rate in species and populations at high altitude (Khanna 2005, Scott 2011), in less arid environments (Tieleman et al. 2004, Tieleman 2005) and in early-hatched nestlings (Van Noordwijk 1995, Gebhardt-Henrich and Van Noordwijk 1991, Christians 2002). In addition, patterns of variation in nestling growth and development are hypothesized to reflect adaptation to specific environmental conditions (Starck and Ricklefs 1998, Demas and Nelson 2012). The spatiotemporal variation in climatic conditions associated with proximal equatorial tropical environments provide an opportunity to investigate how nestling growth rates have evolved in response to different within equatorial tropical climates (locations) and to different environmental conditions within the year. Nestling growth is hypothesized to decrease along aridity gradient with cool and wet environments, that are thought to provide more food resources, promoting faster nestling growth while more arid environments, thought to be devoid of food resources, fostering slower growth (Ricklefs 1976, Tieleman 2004). However, investment in growth rate in cool and wet environments is likely to be compromised by the cost associated with thermoregulation (Krijgsveld et al. 2003). In addition, differences in environmental conditions during the year within an environment with unpredictable and inconsistent patterns of rainfall and food resources is likely to promote variation in nestling growth rates (McCarty 2001) with nestlings raised during wet periods, presumably with food abundance, exhibiting faster growth rate compared to those raised during dry food deficient periods (Emlen and Wrege 1991). Studies that compare growth rates of nestlings in different environments with distinct climatic conditions and in nestlings hatched during different times of the year in unpredictable and highly variable equatorial tropics have not been done. This thesis seeks to fill that gap by providing insights into how variation in environmental conditions shape variation in nestling growth rates.

3. Ecological immunology

The immune system is critical in the protection of any organism from disease-causing agents and works to increase fitness in animals. The immune system is highly complex and is as costly as it is beneficial to animals. Development, maintenance and use of an immune system is costly in terms of energy, e.g. nutrients required for the maintenance of lymphoid tissue, turnover of leukocytes, and time (Schmid-Hempel and Ebert 2003). This cost is paid whether

an individual is attacked or not (Kraaijeveld et al 2001). Animals also incur immunopathological costs due to the damage to the body's own healthy cells as a result of an immune response. Increased formation of detrimental waste products causes damage to tissue, including the immune system as a result of high metabolic rate due to increased wear and tear of the body (Råberg et al. 1998). It is also costly to mount an immune response during infection or challenge (Råberg et al 1998, Schmid-Hempel 2003, Kraaijeveld et al 2001). These factors result in differences in immune responses depending on the magnitude of disease threat and resource availability based on the extent of investment in a defense mechanism by an individual (Sheldon and Verhulst 1996, Kraaijeveld et al 2001), all of which are regulated by ecological factors. The central goal of ecological immunology is to understand and explain immunological variation among and within species, typically in free-living animals, and uses immune measures to test ecological and evolutionary hypothesis (Sheldon and Verhulst 1996).

The innate immune system: a synopsis

All animals have natural enemies in the form of parasites and pathogens; a defense mechanism is therefore critical to fight these foreign bodies. Foreign bodies can either be non-biological or biological (antigens). Non-biological foreign substances are less harmful and are dealt with by the liver and kidneys (Clark 2008). Antigens are microbes found in the system of animals that live and reproduce in hosts and produce biologically-active molecules that are released into the body of the host (Clark 2008). The vertebrate immune system is composed of innate and adaptive immune systems.

Innate immune system

The innate immune system has evolved together with an organism over evolutionary times; its proteins are encoded in genes in the DNA passed from generation to generation in a species. Innate immune system responds to infection through two interconnected mechanisms.

a) Microbial pattern recognition (MPR) is based on the fact that microbes have certain structural features that they cannot change without losing their ability to survive and function and which become prime target for a chemically-based innate immune defense (Clark 2008). MPR have genetically encoded proteins that are able to recognize and bind to those unique, unalterable foreign microbial structural patterns called pathogen-associated molecular patterns (PAMPs); the innate immune system uses pattern recognition receptors (PRR) to interact with PAMPs in a similar way antibodies interact with antigens (Clark 2008).
b) Inflammation involves changes in blood flow, release of cytokines and altered white blood cell flow (Clark 2008). When white blood cells encounter microbes, the cells become activated, release pro-inflammatory cytokines and kick off an inflammatory reaction which involve redness and heat (caused by blood rushing to affected site), swelling and pain (caused by accumulation of lymph as a result of pressure on local nerves) (Clark 2008).

Complement and macrophages are components of the innate immune system. Complement helps antibodies kill microbes. Antibodies do not kill antigens, but only bind them causing agglutination (Clark 2008). When two antibody molecules bind close enough together on the same cell, their protruding antigen combining site (Fc tails) serve as an anchoring site for an assembly of a complement complex at that spot on the cell; the complement complex form pore structures, punch a hole in the cell membrane of microbe and allow water to enter the cell, killing it through osmotic rupture (Clark 2008). Macrophages are huge microbicidal cells which kill microbes by engulfing (Clark 2008). Macrophages have pattern recognition receptors (PRR) and therefore are PAMP-recognizing, and kill bacteria, funguses and protozoan parasites through phagocytosis (Clark 2008). To improve their efficiency, macrophages use their Fc receptors and work together with antibodies - antibodies tag many microbial cells through its antigen-combining sites, macrophages kill them through phagocytosis in their lysosomes, therefore killing hundreds of times more efficiently (Clark 2008).

Adaptive immune function

The adaptive (also acquired) immune function, which mainly consists of B and T cells, uses 'memory' pool which acts as a record of the antigens the immune system has encountered in the past, and is thus able to compete genetically with the rapidly reproducing microbes (Clark 2008). Adaptive immune system focuses and intensifies the innate immune mechanisms and therefore provides synergistic interaction with the innate immune function (Clark 2008, Ochsenbein and Zinkernagel 2000, Matson et al. 2005). Innate and adaptive immunity are equal 'partners' and work together in a coordinated response mounted against microbial infection. However, we only focus on the innate immune function in this thesis because it is the only defense system present to protect organisms against pathogen and parasite exposure before the acquired immune response develops (Matson et al. 2005, Starck and Ricklefs 1998, Mauck et al. 2005, Pihlaja et al. 2006, Stambaugh et al. 2011, Schmid-Hempel 2003, Clark 2008).

4. Variation in immune function

Inter-and-intra-tropical variation in immune function

In the face of the large variation in environmental conditions in equatorial tropical environments, the ontogeny of the immune function may vary widely among conspecifics living in different environments with distinct climate and within populations of conspecifics over time. These variations reflect adaption to specific environmental conditions (Starck and Ricklefs 1998, Demas and Nelson 2012). Hypothesized to be related to the pace-of-life (Tella et al. 2002,Lee et al. 2006, Lee et al. 2008) innate immune function of nestlings is expected to match the parasitic and pathogen pressure exerted by different environments and at different periods of the year through the maternally-derived antibodies (Starck and Ricklefs 1998,Mauck et al. 2005,Stambaugh et al. 2011). Besides, nestlings raised under favourable environmental conditions, e.g., wet conditions with presumably more abundant food resources, are expected to have better innate immune function through better diet and by parent females depositing

high concentrations of maternal antibodies and defences compared to those raised under resource-strenuous conditions.

Unlike in the temperate and arctic zones, equatorial tropical environments that are in close proximity to each other are characterized by large inter-and-intra-annual variations in rainfall and food supply (Dittami and Gwinner 1985, Conway et al. 2005). Immune responses can be sensitive to environmental variation (Nelson and Demas1996, Marra and Holberton 1998, Shepherd and Shek 1998, Ruiz et al. 2002, Tieleman et al. 2005), which in equatorial tropical environments can vary within an environment, leading to variation in the occurrence, distribution, abundance and diversity of pathogens and parasites. This variation in environmental conditions and disease risk within an environment may result in sympatric species occupying different ecological niches within the same environment and/or having different reproductive strategies, leading to differences in immune responses. The co-occurrence of two study species in such a set up provides an opportunity (unique to this region) for interspecific comparison of reproduction-induced variation in immune function.

Additionally, just like disease risk, immune function may also vary with the climatic conditions of the environment of the host (Horrocks 2015, Horrocks 2012b, Zamora-Vilchis et al. 2012, Sehgal et al. 2011, Rubenstein et al 2008), and weather conditions of their environment during the year, resulting in differences in immune responses of conspecific species depending on disease threat in their geographical location (Ardia 2007). Conspecifics may also vary in their immune responses based on their sex due to differences in the roles males and females play during reproduction (Emerson and Hess 1996, Sossinka 1980, Hau et al. 2004, Møller et al. 2003). In Red-capped Larks, only females build nests and incubate but both sexes feed nestlings, with expected immunosuppression in females compared to males. In addition, immune responses of males may be selectively suppressed due to activation of reproductive hormones (Nunn et al. 2009). Immune responses may also vary due to fundamental differences in male and female life-histories, for instance body size differences (upregulated in males) (Nunn et al. 2009, Hasselquist 2007, Zuk 1996). Yet, factors influencing variation in immune function in equatorial tropical birds have not been studied. Some equatorial tropical bird species breed year round but it is unknown whether immune function of such year-round breeding equatorial species varies with reproduction and how that happens. In our lark species reproduction occurs year-round, while their environment(s) are characterized by varying environmental conditions across the year among and within environments. These larks are potentially opportunistic breeders whose timing of reproduction is potentially not related to biotic and abiotic environmental factors. Equatorial tropical system provides the unique opportunity to investigate the effects of breeding behaviour on immunity, while excluding effects of the environment. By confirming that environmental conditions do not differ between periods at which we sampled breeding and non-breeding birds, we are sure that any differences in immune function between breeding and non-breeding birds do not result from environmental variation.

5. The study system and the lark family

Birds have been used for a variety of ecological studies and can be used to assess what the drivers of patterns in ecosystems are (Rahbeck 1997, Sanders et al. 2003) because they are well-studied, are taxonomically stable, are easily surveyed and are widely-distributed across many habitats. The larks family *Alaudidae* has closely related species that occur in a broad range of climatic and environmental conditions (Alström et al. 2013, Zimmermann et al. 1999). This makes the family an ideal choice as model to investigate spatiotemporal variations in physiology and life-history (Alström et al. 2013). The larks family has been used to investigate interspecific variation in immune function along climate gradient as proxy for environmental disease risk (Horrocks et al. 2015, Horrocks et al. 2012, Williams and Tieleman 2005) and to provide an ecological perspective on microbes and immune defense in eggs (Grizard et al. 2015). These evaluations using the lark family have provided a perfect study system for comparative inter-and-intra-specific variation in immune indices, reproductive strategies and growth patterns particularly in an equatorial tropical region with high intra-and-inter-tropical variation in environmental conditions.

Our study system uses the Red-capped *Calandrella cinerea* and the Rufous-naped Larks *Mirafra africana*, two sedentary species living and breeding simultaneously in different environments with differing climatic conditions in Kenya: cool and wet South Kinangop; warm and wet North Kinangop and warm and dry Kedong. South Kinangop receives on average 939 ±132.7 (SD) mm of rain per year, and experiences variation in monthly mean T_{min} between 3.0 and 8.2ºC, and monthly mean T_{max} between 21.2 – 30.0ºC. North Kinangop receives on average 584 ± 62.6 (SD) mm of rain per year, and experiences variation in monthly mean T_{min} between 3.0 and 13.7ºC, and monthly mean T_{max} between 22.1 and 30.5ºC, while Kedong receives on average 419 ± 96.8 (SD) mm of rain per year, and experiences variation in monthly mean T_{min} between 6.2 – 15.7ºC, and monthly mean T_{max} between 25.3 – 34.9ºC (for details of climatic conditions, see Ndithia et al. (2017a). Variation in climatic conditions in these environments help to comparatively examine environmentally induced adaptations of reproduction and physiology, and is important in understanding how environmental conditions of different locations shape the reproductive strategies of different populations of the same species. In addition, as a result of the variation of environmental conditions of an environment during the year, this set up allows for the evaluation of across-species within-location variation in costs and benefits of different reproductive strategies and immune response to understand why species have certain reproductive strategies/immune response compared to others.

This comparative study benefits from the fact that the two lark species have similar ecological traits therefore avoiding the complications that arise from different evolutionary histories. Both species are ground-nesters, build open-cuped nests, breed year-round, have similar clutch size and incubation patterns, consume similar diet and have similar foraging behaviour. Despite both species inhabiting open grasslands, they use different micro-habitats occasioned by variation in weather conditions within an environment, and with potentially different disease risk and immune responses. However, up to now, factors that influence variation in immune function among sympatric species during the year and factors that influence variation in immune function within a single species living in different environments differing in climatic conditions are unknown in an equatorial tropical region. Overall, based on

existing knowledge on their behaviour, physiology, life history and environmental conditions, these two lark species and the associated spatiotemporal differences in climatic conditions of their environments provide a perfect and unique model for investigating and understanding the ecology and evolution of immune function with respect to reproduction and environmental conditions in an equatorial tropical region.

6. General objectives and thesis outline

Factors that influence the timing of reproduction, variation in growth and immune function among and within environments in equatorial tropical birds are largely unknown. This is the general objective of this thesis. The unpredictability and high variability of environmental conditions in equatorial tropical environments results in different factors playing the role of influencing reproduction in birds among and within environments in this region. To understand environmental conditions of equatorial tropics in order to evaluate how they may influence the ecology and physiology of birds living in this region, we started in **Chapter 2** by quantifying and examining (to understand the patterns) intra-annual variation in rainfall, average minimum (T_{min}) and average maximum (T_{max}) temperatures and invertebrate abundance and occurrence and intensity of breeding in Red-capped Larks within each of our three climatically distinct environments. We had expected a bimodal pattern of rainfall which subsequently could influence pattern of food availability and breeding in larks. We consequently related breeding in each environment with these biotic and abiotic factors to determine which among them influenced breeding. Essentially, we investigated the hypotheses that in equatorial tropical environments, birds time their breeding to match periods of high rainfall and temperature (Hau 2001, Tieleman and Williams 2005, Foster 1974, Skutch 1950, Tye 1991) and/or peak periods of food supply (Tieleman and Williams 2005, Young 1994b, Boag and Grant 1984, Poulin et al. 1992, Skutch 1950) using larks as a model. Subsequently among these environments, we compared these biotic and abiotic factors and the occurrence and intensity of breeding to determine their differences, and whether reproduction in Red-capped Larks is influenced by the same factors in different environments, in which case it would point to an evolutionary link of reproduction to environmental conditions.

Variation in environmental conditions is hypothesized to cause variation in patterns of growth and development and variation in the ontogeny of immune function among and within populations (Starck and Ricklefs 1998, Demas and Nelson 2012). We exploited the spatiotemporal variation in climatic conditions in our three climatically-distinct study environments to understand variation in life-history strategies among and within tropical locations which formed the basis of our study in **Chapter 3** of this thesis. We compared growth and immune function in lark nestlings from South Kinangop, North Kinangop and Kedong and studied growth and immune function as a function of year-round variation in breeding intensity and rain within Kedong. We proposed that differences of our three study locations in orography and in altitude, and the fluctuating and inconsistent patterns of rainfall and food availability within and between years in Kedong (see **chapter 2** of this thesis) may have consequences on growth. In addition, we hypothesized that the large environmental variation among equatorial environments is likely to cause variation in immune function in nestling birds, variations that may reflect the local pathogen pressures exerted by different environments through the

maternally derived antibodies (Sheldon and Verhulst 1996, Norris and Evans 2000). Again, breeding under favorable environmental conditions during the year allows females to access better diet and are thus able to deposit higher concentrations of maternal antibodies and defenses. Females are therefore able to give rise to nestlings with better immune function compared to those bred during periods with poor environmental conditions. We investigated how nestling growth rates and variation in immune function have evolved in response to differences in environmental conditions among locations and during different periods of the year respectively, questions that are still unexplored in equatorial tropical environments.

Reproduction is reported in multiple studies to trade-off with immune function; they are both costly physiological processes that compete for allocation of the often limited resources (Sheldon and Verhulst 1996, Lochmiller and Deerenberg 2000, Norris and Evans 2000). At the same time, immune function can be sensitive to environmental variations (Nelson and Demas1996, Marra and Holberton 1998, Shepherd and Shek 1998, Ruiz et al. 2002, Tieleman et al. 2005). However, our study species breed year-round as opposed to temperate and arctic zone birds which breed only in the spring hence the effect of reproduction on immune function can also be confounded by the effects of environmental conditions. In **Chapter 4,** we exploited this unique system of equatorial tropical environments by examining the role of reproduction in the variation of immune function and mass while controlling for the effects of environmental conditions in two sympatric species in North Kinangop, the Red-capped *Calandrella cinerea* and the Rufous-naped Larks *Mirafra africana*. Variation in environmental condition is likely to result in different ecological niches within a location (with potentially different pathogen pressure), leading to sympatric species occupying these different niches or exhibiting different reproductive strategies. We investigated whether our two species differed in their immune responses and mass during breeding and non-breeding periods, and whether males and females of each of these species differed in their immune response as a result of the different roles they play during reproduction (Emerson and Hess 1996, Sossinka 1980, Hau et al. 2004, Møller et al. 2003) or due to fundamental differences in male and female life-histories (Nunn et al. 2009, Hasselquist 2007, Zuk 1996). We expected non-breeding birds to generally have more robust immune function compared to breeding ones (Nelson and Demas 1996, Bentley et al. 1998, Martin et al. 2008). We checked if environmental conditions differed between periods at which we sampled breeding and non-breeding birds. We also did not expect environmental variables to differ between breeding and non-breeding birds in any of the two species. This study of the influence of reproduction on immune function while excluding the effects of environmental conditions is novel in equatorial tropics.

Variation in immune function has been attributed to life history trade-offs and to variation in environmental conditions (Sheldon and Verhulst 1996, Tieleman 2018), the latter of which may reflect pathogen pressure in an environment. In **Chapter 5** of this thesis, we investigated the role of reproduction in the variation of immune function by exploiting a unique study system of three populations of year-round breeding Red-capped Larks living and breeding simultaneously in environments with large intra-and inter-annual variations and unpredictable temporal patterns of rainfall and food availability. We investigated if immune function and body mass differed among chick-feeding and non-breeding (males and females), and incubation (females only) from three climatically distinct environments that are generally permissive of year-round breeding (Ndithia et al. 2017a). Based on resource trade-offs, within

each location, we expected non-breeding birds to generally have more robust immune function (Bentley et al. 1998, Nelson and Demas 1996, Martin et al. 2008) and higher body mass (Moreno 1989) compared to breeding ones. Based on the antigen exposure hypothesis which predicts reduced microbial abundance in arid environments (Horrocks et al. 2012), we expected immune function to decrease along a gradient of aridity from South Kinangop to North Kinangop and Kedong. We checked if environmental conditions differed between periods at which we sampled breeding and non-breeding birds, therefore excluding the potential role of environmental condition in influencing variation in immune function. We expected that environmental conditions would not differ according to breeding stages and hence could be excluded as confounding factors in explaining any reproduction-associated variation in immune function. Again, like in **Chapter 4** above, we explored the role of sex in the variation of immune function and expected that during breeding, females would have depressed immune function due to their higher reproductive effort (nest building, incubating and chick-feeding as opposed to males' chick-feeding only).

Chapter 6 synthesizes the findings of each individual chapter in this thesis putting the findings in the context of expected and/or predicted results and compare/contrast with what is known from literature.

Chapter 2

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Year-round breeding equatorial Larks from three climatically-distinct populations do not use rainfall, temperature or invertebrate biomass to time reproduction

Henry K. Ndithia,
Kevin D. Matson,
Maaike A. Versteegh,
Muchane Muchai,
B. Irene Tieleman

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Abstract

Timing of reproduction in birds is important for reproductive success and is known to depend on environmental cues such as day length and food availability. However, in equatorial regions, where day length is nearly constant, other factors such as rainfall and temperature are thought to determine timing of reproduction. Rainfall can vary at small spatial and temporal scales, providing a highly fluctuating and unpredictable environmental cue. In this study we investigated the extent to which spatio-temporal variation in environmental conditions can explain the timing of breeding of Red-capped Lark, *Calandrella cinerea*, a species that is capable of reproducing during every month of the year in our equatorial east African study locations. For 39 months in three climatically-distinct locations, we monitored nesting activities, sampled ground and flying invertebrates, and quantified rainfall, maximum (T_{max}) and minimum (T_{min}) temperatures. Among locations we found that lower rainfall and higher temperatures did not coincide with lower invertebrate biomasses and decreased nesting activities, as predicted. Within locations, we found that rainfall, T_{max}, and T_{min} varied unpredictably among months and years. The only consistent annually recurring observations in all locations were that January and February had low rainfall, high T_{max}, and low T_{min}. Ground and flying invertebrate biomasses varied unpredictably among months and years, but invertebrates were captured in all months in all locations. Red-capped Larks bred in all calendar months overall but not in every month in every year in every location. Using model selection, we found no clear support for any relationship between the environmental variables and breeding in any of the three locations. Contrary to popular understanding, this study suggests that rainfall and invertebrate biomass as proxy for food do not influence breeding in equatorial Larks. Instead, we propose that factors such as nest predation, female protein reserves, and competition are more important in environments where weather and food meet minimum requirements for breeding during most of the year.

Introduction

Ecological and environmental factors, such as food availability and weather, shape reproductive decisions in many bird species. These factors can act alone or in combination, and they may fluctuate in predictable or unpredictable ways within and between years. For birds living at mid-latitude locations in the temperate zone, predictable seasonal changes in day length and other environmental conditions function as reproductive cues. These birds can potentially time reproduction with regularity (Colwell 1974, Hau et al. 2000, Wikeski et al. 2000, Hau 2001). Increasing photoperiod, a characteristic of temperate zone spring, triggers reproduction in birds via neuroendocrine mechanisms (Lambrechts et al. 1996, Hau 2001, Gwinner 2003). Food availability and temperature serve as supplementary cues to fine-tune the timing of reproduction to local environmental conditions (Nager and van Noordwijk 1995, van Noordwijk et al. 1995, Lambrechts et al. 1996, Hau 2001). The net result is synchronized spring breeding within and among species living at the same location (Nager and van Noordwijk 1995, Lambrechts et al. 1996).

In contrast, birds living in equatorial locations experience little predictable intra-annual variation in day length (Moreau 1949, Young 1994), but instead experience large, and frequently unpredictable, variation in rainfall and food availability (Grant and Boag 1980, Boag and Grant 1984, Wrege and Emlen 1991, Young 1994, Scheuerlein and Gwinner 2002, Stutchbury and Morton 2001, Conway et al. 2005, Moore et al. 2005, Ndithia et al. 2007). By living in environments without predictable seasonal cues, equatorial birds are thought to time reproduction based on shorter-term and more irregular factors, such as rainfall and food availability (Dittami and Gwinner 1985, Stutchbury and Morton 2001). Additionally, these birds tend to have more flexible breeding schedules and may breed opportunistically (Boag and Grant 1984, Young 1994). For example, initiation of breeding with the onset of rain (Dittami and Gwinner 1985, Dittami 1986, Chapman 1995) greatly promotes nesting success in low-latitude birds (Lloyd et al. 2001, Lepage and Lloyd 2004, Monadjem and Bamford 2009). Likewise in some equatorial birds, timing of reproduction coincides with peaks in food supply (Dittami and Gwinner 1985, Chapman 1995, Komdeur 1996, Hau et al. 2000, Scheuerlein and Gwinner 2002, Moore et al. 2005). These observations that in unpredictable equatorial environments birds preferentially breed at times of the year with higher rainfall and food, match with the general pattern that environments that are more arid have lower primary productivity and select for reduced reproductive effort (Tieleman et al. 2003a , Tieleman et al. 2004). Other factors, such as wind and mist, both of which effectively lower ambient temperature, may also be important (Foster 1974, Tye 1991).

One possible consequence of commencing breeding in response to unpredictable localized conditions is that a single species living in distinct environments might show variation in breeding patterns on a small geographical scale (Moore et al. 2005). Exploiting such small-scale variation in environmental aridity within the tropics, we intensively investigated year-round breeding in three equatorial populations of Red-capped Larks *Calandrella cinerea* (Gmelin 1789) in Kenya for 39 consecutive months. These resident populations, despite their close geographic proximity, experience different patterns of temperature and rainfall, with climates ranging from warm and dry to cool and wet, and representing an expected gradient of increasing primary productivity (Peñuelas et al. 2007). Thus, the study system allows for a

comparative, intraspecific analysis of environmentally induced spatio-temporal variation in reproduction. Rarely have studies assessed year-round breeding activities of equatorial species and related breeding to biotic and abiotic characteristics of climatically-distinct locations.

The overall objective of our study was to compare and understand breeding in Red-capped Larks in relation to spatio-temporal variation in weather conditions and food resources. Specifically, we 1) compared spatial variation in rainfall, temperature, invertebrate biomass and breeding across our three study locations, 2) described within-location year-round patterns of rainfall, temperature and invertebrate biomass and how these variables co-vary with breeding and, 3) determined which, if any, biotic and abiotic factors are related to occurrence and intensity of breeding by Red-capped Larks in each location. We predicted that the drier and warmer the location, or the drier and warmer the time of the year, the lower the productivity of invertebrates and the lower the intensity of breeding by Larks.

Materials and methods

Study system

Red-capped Larks are small grassland birds that are widely distributed across Africa. They prefer habitats dominated by short grasses or almost bare ground, including fallow and cultivated agricultural fields. Red-capped Larks feed mostly on invertebrates (including beetles, wasps, caterpillars, butterflies and moths, earthworms, and grasshoppers) and occasionally on grass seeds (pers. obs.). Pairs build ground-level open-cup nests that are placed next to a scrub or grass tuft. They typically lay two eggs per clutch (mean 1.89 ± 0.33 (SD) eggs, n = 279, range 1-3 eggs; pers. obs.). During breeding, birds defend the area around the nest but neighboring nests can be as close as 10 m; outside breeding they occur in flocks (pers. obs.). Before our study, nothing had been documented about timing, number of breeding attempts and other breeding parameters at the individual or population level.

From January 2011 to March 2014, we worked simultaneously in multiple plots in South Kinangop, North Kinangop and Kedong (see Table 1 for details per plot), three locations in central Kenya with distinct climates. Distances between locations are 19 km (South Kinangop - North Kinangop), 29 km (South Kinangop - Kedong) and 34 km (North Kinangop – Kedong). Accessible plots within locations were chosen based on observations of Red-capped Larks made by local bird watchers and by us (H.K.N., B.I.T.). We set up a weather station (Alecto WS-3500, Den Bosch, Netherlands) at each location (Table 1) to measure daily rainfall (mm) and minimum (T_{min}) and maximum (T_{max}) temperatures ($^{\circ}$C). Using these measurements from three full calendar years (March 2011 – February 2014), we calculated annual and monthly rainfall, and annual and monthly T_{min} and T_{max}.

Table 1. Coordinates, altitude (m above sea level (ASL)), surface area (km^2) and distance to weather station (km) for each plot in our three study locations South Kinangop, North Kinangop and Kedong.

Location	Plot name (altitude, m ASL)	Plot surface area (km^2)	Distance to weather station (km)
South Kinangop	Kenyatta road (2679); 0^{0}49'23"S, 36^{0}34'39"E	0.3	18.9
	Sasumwa (2508); 0^{0}45'03"S, 36^{0}39'22"E	0.2	9.4
	Seminis (2556); 0^{0}42'30"S, 36^{0}36'30"E	1.2	5.2
North Kinangop	Joshua (2451); 0^{0}36'00"S, 36^{0}28'27"E	0.2	3.8
	Mbae (2425); 0^{0}36'54"S, 36^{0}30'48"E	0.35	2.5
	Ndarashaini (2412); 0^{0}34'33"S, 36^{0}29'41"E	0.3	1.8
Kedong	A (2064); 0^{0}53'07"S, 36^{0}24'32"E	0.5	7.3
	B (2075); 0^{0}52'45"S, 36^{0}23'29"E	0.4	10.4
	C (2076); 0^{0}53'37"S, 36^{0}23'54"E	0.9	9.6
	D (2075); 0^{0}53'44"S, 36^{0}24'32"E	0.9	6.8

South and North Kinangop lie on a plateau of montane grassland along the Aberdare mountain ranges. Study plots in South and North Kinangop flood periodically during rains and standing water remains after rains have stopped (pers. obs. 2010 - 2014). In South Kinangop, birds bred only in Seminis, despite initially observing them also in the other two plots (Table 1). Flooding made Seminis unavailable for breeding from April – December 2012 and April 2013. Flooding in North Kinangop affected nests located in parts of Joshua and Ndarashaini in October 2011 and October 2012; these plots also received heavy rainfall in April 2013 that affected nesting activities. Kedong, a privately owned ranch in the Rift Valley in Naivasha, consists of large grassland patches that did not flood (pers. obs. 2010 – 2014).

The study species involved is not and endangered or protected species. The National Museums of Kenya approved this research and owners of the land gave permission to conduct the study on their respective sites.

Estimating invertebrate availability

To assess invertebrate biomass as proxy for food availability in each location, we used pitfall traps and sweep nets to sample ground and flying invertebrates respectively once per month (Ganihar 1997). We assessed within-location variation in invertebrate biomass using data from plots within a location. For pitfall traps, we used plastic cups with a 26 cm circumference that contained ≈100 ml of 5% formaldehyde solution and that we buried so that rims of the cups were at ground level. We placed five pitfall traps in each plot and left them in place for five days each month. We placed the traps 70 m apart along a 280m-long transect in all plots except one plot in South Kinangop (Seminis), where instead we equally spaced 10 pitfall traps along a 630m-long transect. For sweep-netting (net diameter 0.4m), we established permanent 50m long transects in each plot, subjectively selected as representative for the plot. Per location, one

field assistant collected invertebrates between 9:00 – 10:00 am. If it rained during this hour, we postponed sweep-netting to the same hour on a day without rain. All field assistants were trained to sample in the same manner. We standardized the analyses of pitfall and sweep net sampling data per location (see statistical analysis section below). To calculate annual average and monthly average biomasses, we used two complete calendar years (24 months, March 2011-February 2012 and March 2013-February 2014), excluding the year in which flooding caused multi-month gaps in the data (March 2012-February 2013). Our resulting 24-month data sets had three missing values for ground invertebrates (October 2013, North Kinangop; October and December 2011, Kedong) and three missing values for flying invertebrates (October 2011, October 2013, North Kinangop; February 2014, South Kinangop). For calculations of annual and monthly averages, we substituted each missing value with the average value of the preceding and subsequent months.

We preserved collected specimens in 70% alcohol, later identifying and sorting them based on morphology (Picker and Griffiths 2004). For biomass estimation, we classified invertebrates into 10 categories based on size and shape: ants; bees and wasps; beetles and bugs; butterflies and moths; caterpillars, caddisflies, and stoneflies; diplura, millipede, centipede, and earthworms; flies; grasshoppers, crickets, and mantis; spiders, ticks, and mites; and the rest (woodlice, cicadas, cockroaches and earwigs).

We estimated biomass of each of our invertebrate categories as a proxy for food availability. To do this, we first used a subsample of 2198 invertebrate specimens, representing all invertebrate categories from all locations, to develop a category-specific calibration curve relating dry mass as a function of length and width (Ganihar 1997, Benke et al. 1999, Gruner 2003). For every individual in the subset, we measured length (anterior-most part of the head to the tip of abdomen) and width (the widest point of abdomen) using vernier calipers, dried them in an oven for 48 hours at 65°C (Sample et al. 1993, Benke et al. 1999, Gruner 2003), and measured dry mass on an analytical balance (model KERN ACS 220-4N, KERN and Sohn of Belingen, Germany). We used a log-transformed power model to describe the length-width-mass relationship; the power model has been shown to give the highest adjusted r^2 compared to length-mass and length-area relationships [Sample et al. 1993, Benke et al. 1999, Gruner 2003): biomass = a + b log(length) + c log (width), where a, b and c are coefficients of the model from each of the invertebrate categories whose biomass we estimated.

We used calibration curves per invertebrate category to predict body mass from length and width (for details on the adjusted r^2 and the range of length and width, see Appendix 1). Overall, we collected, measured, dried, and applied the biomass estimation protocol to 23,628 specimens from pitfall traps and 3260 captured by sweep-netting (including calibration subset).

Lark reproduction

To determine the year-round breeding activities of Larks, we spent on average 134 person-hours per month searching for nests in the three locations combined (Table 2 provides a breakdown for effort per location).

Table 2. Search effort (in days (days had a minimum of 2 searching hours) and hours per month) for nests of Red-capped Larks *Calandrella cinerea* in our three study locations South Kinangop, North Kinangop and Kedong, from January 2011 to March 2014.

	Search effort (days/month)		Search effort (hours/month)	
Location	Average + SD	Range	Average + SD	Range
South Kinangop	6.6 ± 2.94	1 - 13	43.9 ± 24.24	3 - 130
North Kinangop	8.6 ± 2.20	3 - 13	40.3 ± 17.06	7 - 87
Kedong	14.1 ± 5.30	7 - 24	49.8 ± 35.95	17 - 193

Our nest search strategy included observing breeding behavior (e.g., transport of nest materials or food, breeding-related alarm calls, nervous parental behavior around nest sites) and routinely walking plots to flush parents incubating eggs or brooding young. We quantified nest-searching effort as person-hours, i.e., number of hours searching for nests multiplied by the number of persons searching. For each month we calculated a "nest index" (i.e., level of breeding) value, which we defined as the total number of nests found in a month per 10 person-hours of effort. To calculate annual average and monthly average nest indices we used the two complete calendar years (24 months) of March 2011-February 2012 and March 2013-February 2014, excluding the year in which flooding caused multi-month gaps in the data (March 2012-February 2013). Our resulting 24-month data set had one missing value (April 2013, South Kinangop), for which we substituted the average of March and May 2013.

Statistical analyses

For all analyses, we tested and confirmed that the dependent variable and the final models observed the assumptions of normality and homoscedasticity of variance through graphical and statistical methods. We tested for among-location differences in rainfall, T_{min} and T_{max}, ground and flying invertebrates, and nest index (continuous variable) using mixed models (R-package lme) with location as fixed factor and month as random factor. To compare invertebrate biomasses among plots and locations, we standardized ground and flying invertebrate sampling by expressing biomass per five pitfall traps and one sweep net session per plot or location per month. For among-location comparisons, we log-transformed ground and flying invertebrate data because they were not normality distributed. We found no significant among-plot differences in ground invertebrate biomasses within South Kinangop ($F_{2, 47} = 0.89$, $P = 0.42$) or in Kedong ($X^2 = 3.98$, P=0.26). For North Kinangop, among-plot differences in ground invertebrate biomasses were significant ($X^2 = 6.49$, P=0.04), although post-hoc tests showed no significant differences among-plots. There were no significant among-plot differences in flying invertebrate biomasses for any of the three locations (all $X^2 < 5.74$, $P > 0.06$). Therefore, we used the mean monthly biomass per location to test for among-location differences.

We investigated if and how environmental conditions in the month before breeding ("prior") and in the month of breeding ("current") were associated with the occurrence and intensity of breeding. We calculated pairwise correlation coefficients between the environmental factors per location (supplementary material 2), to identify potential

collinearity. We used model selection based on the Akaike Information Criterion corrected for sample size (AICc) because this allows for exploration of multiple models simultaneously (Burnham and Anderson 2002). To investigate what determines occurrence of breeding, we transformed the continuous variable nest index into the new variable "occurrence of breeding" (binomial: presence/absence). We then used generalized linear mixed models with a binomial distribution to construct for each location a "full" model with the new dependent variable "occurrence of breeding". These full models included ten explanatory variables (i.e., prior and current rain, T_{min}, $T_{max,}$ ground invertebrate biomass, and flying invertebrate biomass) and four two-way interactions, i.e., prior and current rain and corresponding ground invertebrate biomass and prior and current rain and corresponding flying invertebrate biomass. We compared all the possible models and ranked them in order of their AICc, such that the lowest values were considered to have more statistical power (Burnham and Anderson 2002). The model with the highest weight and the lowest AICc value was considered the most parsimonious, although all models within 2 AICc of the best model were included in further analysis (Grueber et al. 2011). We explored the relative contribution of the various environmental parameters to breeding by applying model averaging and standardization based on all models with ΔAICc values < 2 (the "best model-set"), compared with the top model (Grueber et al. 2011). Although AICc values of models in the best model-set without month as random effect were higher than models with random effect, we added month as a random effect to the models to correct for potential seasonal effects. We analyzed all data using R statistical software (version 3.0.3) (R core Team).

In the second part of our analysis we investigated how environmental conditions in the month before breeding and in the month of breeding were associated with the intensity of breeding. For this, we analyzed only the months in which breeding occurred (i.e. nest index > 0). We used linear models with a Gaussian distribution and constructed a "full" model with continuous variable "nest index", for each location. We used the same explanatory variables and statistical approach as in the analysis above. Because month never improved the models in the analysis of occurrence of breeding (see above), and in order to maximize power for the tests of the effects of environmental factors we did not include month as a random effect in these models. In addition, because of low sample size in South Kinangop (n = 7 months), we performed the analyses of intensity of breeding only for North Kinangop and Kedong.

Additional analyses, in which we explored the effects of different time windows and time lags of the environmental variables (up to six months preceding breeding) on breeding occurrence and intensity, did not result in qualitatively different results (see supplementary material 1).

Results

Spatial differences in environmental factors, invertebrates and breeding of Larks

Mean annual and monthly rainfall were highest in South Kinangop, intermediate in North Kinangop and lowest in Kedong (Table 3; Fig. 1A, 2A, 3A). South Kinangop received on average 123% more rain than Kedong, while North Kinangop received 40% more rain than Kedong. T_{min} and T_{max} were lowest in South Kinangop (annual average T_{min} = 5.5°C, annual average T_{max} = 24.7°C), intermediate in North Kinangop (annual average T_{min} = 9.1°C, annual average T_{max} = 25.4°C) and highest in Kedong (annual average T_{min} = 10.5°C, annual average T_{max} = 28.6°C) (Table 3; Fig. 1B, 2B, 3B).

Table 3. Annual (n = 3 years) and monthly (n = 36 months) rainfall (average ± SD, and range), and monthly average minimum and average maximum temperatures (n = 36 months, average ± SD, and range) as measured by our weather stations in South Kinangop, North Kinangop and Kedong, during March 2011 to February 2014.

Location	Annual rain (mm)	Monthly rainfall (mm)		Monthly average minimum (^{0}C)		Monthly average minimum (^{0}C)	
	Mean ± SD	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
South Kinangop	939 ± 132.7	78 ± 69.7^a	0 - 309	5.5 ± 1.06^a	3.0 ± 8.2	24.7 ± 2.09^a	21.2 - 30.0
North Kinangop	584 ± 62.6	49 ± 35.3^b	0 - 55	9.1 ± 2.42^b	3.0 ± 13.7	25.4 ± 2.27^a	22.1 - 30.5
Kedong	419 ± 96.8	35 ± 39.2^b	0 - 153	10.5 ± 1.92^c	6.2 ± 15.7	28.6 ± 2.44^b	25.3 - 34.9

Superscripts indicate subsets of significant differences ($P<0.05$) among locations in post-hoc tests, after mixed-model analyses. Note: This table contains data for three complete calendar years, but data used for analyses of breeding (Figs 1, 2 and 3) comprise the entire study period of January 2011 to March 2014.

Biomasses of ground invertebrates (log pitfall, mg) did not differ significantly among locations, but biomasses of flying invertebrates (log sweepnet, mg) were highest in Kedong, intermediate in South Kinangop, and lowest in North Kinangop (Table 4). Flying invertebrate biomasses were on average 42% lower in North Kinangop than in Kedong and 27% lower in South Kinangop than in Kedong; flying invertebrate biomasses did not differ significantly between South and North Kinangop (Table 4). Despite differences in climate and invertebrate biomass, Red-capped Larks bred in all three locations (Table 5). In the period January 2011 – March 2014, we found 74 nests in South Kinangop, 63 nests in North Kinangop and 153 nests in Kedong (Table 5). Calculating nest index corrected for search effort, we found the highest numbers in Kedong, followed by North Kinangop (63% lower than Kedong) and South Kinangop (84% lower than in Kedong).

Year-round variation in environmental conditions, invertebrates and breeding of Larks

In all three locations, rainfall occurred in all calendar months of the year, but the amount of rainfall in any given month was highly variable and unpredictable among years (Fig. 1A, 2A, 3A). The only consistent annually recurring observation was that January and February were dry in all three locations in all four years (with the exception of Kedong in 2014). Outside of this annually recurring dry season, there was no month without rain in North Kinangop and only one month was without rain in South Kinangop (March 2014). However, Kedong received no rain at all during six months in 2013 (June-November), in contrast to 2011 and 2012 when this location received rain every month. Average monthly T_{min} and average monthly T_{max} varied unpredictably throughout the year in all locations and years, but generally, T_{min} were lowest and T_{max} were highest in January and February each year (Fig 1B; 2B; 3B). Average monthly T_{max} varied between 21.2°C and 30.0°C in South Kinangop, between 22.1°C and 30.5°C in North Kinangop, and between 25.3°C and 34.9°C in Kedong (Table 3). Likewise, average monthly T_{min} varied between 3.0°C and 8.2°C in South Kinangop, between 3.0°C and 13.7°C in North Kinangop, and between 6.2°C and 15.7°C in Kedong (Table 3).

Ground and flying invertebrates were present in all months in all locations, but biomasses varied among months and among years in an unpredictable manner (Fig 1C, 2C, 3C). Overall, we observed Red-capped Larks breeding in all calendar months, but they did not breed in every month in every year in any of the three locations (Fig 1A, 2A, 3A). In South Kinangop, we found nests in the calendar months January-April and June-August, and in 10 out of 30 months total (33%). In North Kinangop, we found nests in all calendar months except June and July, and in 21 out of 39 months total (54%). Finally, in Kedong, we found nests in all calendar months and in 20 out of 39 months total (51%). In all locations, year-to-year variation in nest index was present, with highest nest indices in 2012 (Fig 1A, 2A, 3A).

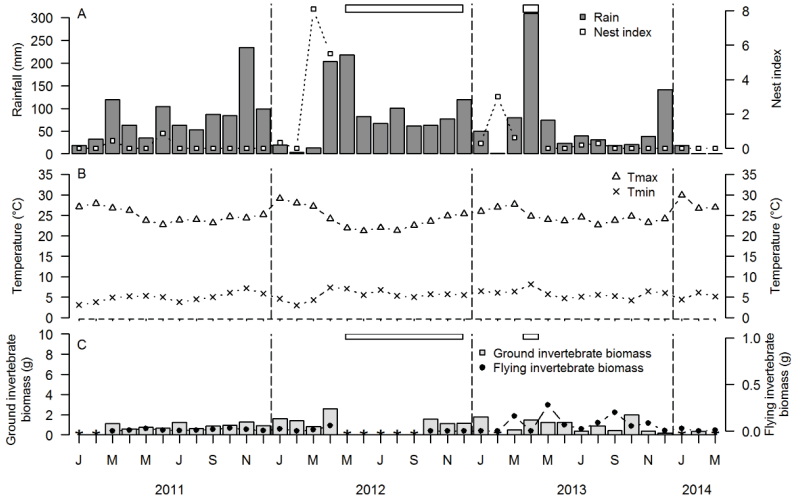

Fig 1. Temporal variation during January 2011-March 2014 of A. rainfall (mm) and nest index (number of nests/10 search h) of Red-capped Larks *Calandrella cinerea*, B. average monthly maximum (Tmax) and minimum (Tmin) temperature (^{0}C), C. biomass (g dry weight) of ground-dwelling and flying invertebrates in South Kinangop. Horizontal open rectangles represent periods of flooding (i.e. standing water in the study location). Data gaps other than from flooding in ground and flying invertebrates represent missing data due to e.g., vandalism (see Methods).

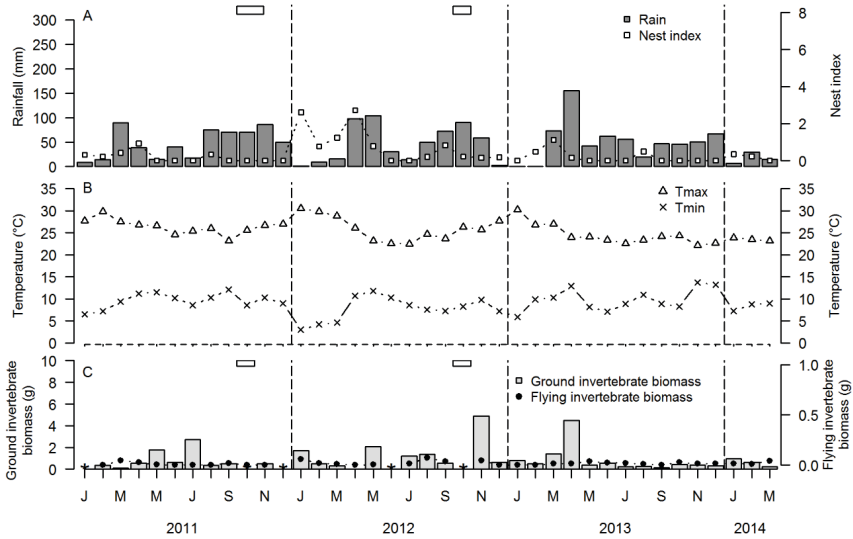

Fig 2. Temporal variation during January 2011-March 2014 of A. rainfall (mm) and nest index (number of nests/10 search h) of Red-capped Larks *Calandrella cinerea*, B. average monthly

maximum (Tmax) and minimum (Tmin) temperature (ÊC), C. biomass (g dry weight) of ground-dwelling and flying invertebrates in North Kinangop. Horizontal open rectangles represent periods of flooding (i.e. standing water in the study location). Data gaps other than from flooding in ground and flying invertebrates represent missing data due to e.g., vandalism (see Methods).

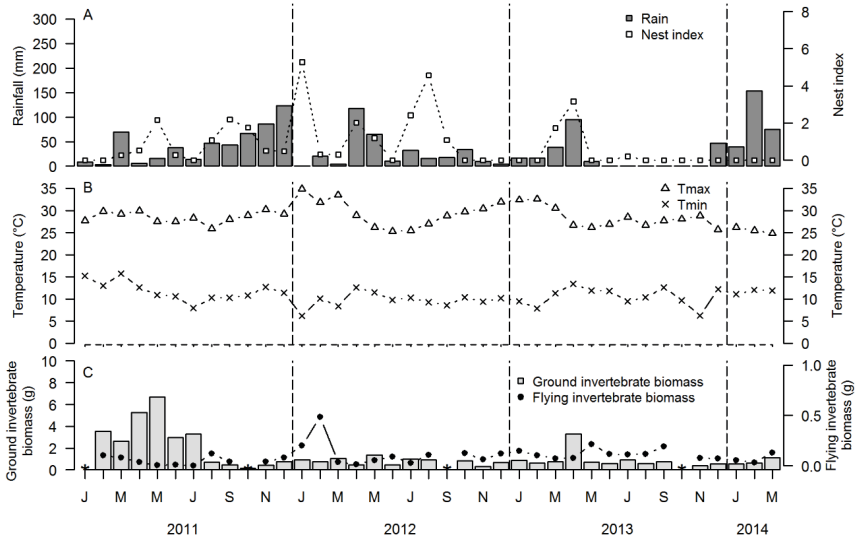

Fig 3. Temporal variation during January 2011-March 2014 of A. rainfall (mm) and nest index (number of nests/10 search h) of Red-capped Larks *Calandrella cinerea*, B. average monthly maximum (Tmax) and minimum (Tmin) temperature (^{0}C), C. biomass (g dry weight) of ground-dwelling and flying invertebrates in Kedong. Data gaps in ground and flying invertebrates represent missing data due to e.g., vandalism (see Methods).

Table 4. Annual and monthly biomass of ground invertebrates (log(mg dry mass/5 pitfalls)) and flying invertebrates (log(mg dry mass/sweep-net session)) in South Kinangop, North Kinangop and Kedong. Annual values (average ± SD) were based on two calendar years (March 2011-February 2012 and March 2013-February 2014). Data for the third year (March 2012-February 2013) were excluded because flooding caused incomplete data sets for South and North Kinangop (see Methods). Likewise, monthly values (average ± SD, range) were based on 24 months (March 2011- February 2012 and March 2013-February 2014).

Location	Annual biomass ground invertebrates log(pitfall)	Monthly biomass ground invertebrates log(pitfall)		Annual biomass flying invertebrates log (sweep-net session)	Monthly biomass flying invertebrates log(sweep-net session)	
	Mean ± SD	Mean ± SD	Range	Mean ± SD	Mean ± SD	Range
South Kinangop	34.6 ± 1.74	2.88 ± 0.27^{a}	2.26 - 3.30	15.7 ± 3.92	1.31 ± 0.65^{a}	0.00 - 2.45
North Kinangop	33.2 ± 0.96	2.77 ± 0.38^{a}	2.06 - 3.65	12.4 ± 2.12	1.04 ± 0.56^{a}	0.00 - 1.80
Kedong	35.6 ± 1.88	2.97 ± 0.41^{a}	2.10 - 3.83	21.5 ± 3.21	1.79 ± 0.55^{b}	0.00 - 2.69

Superscripts indicate subsets of significant differences ($P<0.05$) among locations in post-hoc tests, after mixed-model analyses.

Table 5. Total number of nests found, percentage successful nests, and annual and monthly nest index (number of nests/10 h search effort) in South Kinangop, North Kinangop and Kedong. Total number of nests found is based on the period January 2011 ±March 2014. Nests failed due to a variety of reasons, including predation, abandonment, flooding and human destruction. Average nest indices are based on two complete calendar years (March 2011 - February 2012, and March 2013 ±February 2014; 24 months) and excluded the year in which flooding occurred (see Methods). Superscripts indicate subsets of significant differences ($P<0.05$) among locations in post-hoc tests, after mixed model analyses.

Location	**Number of nest found (% successful)**	**Annual nest index (number of nests/10h of search effort)**	**Monthly nest index (number of nests/10h of search effort)**	
		Mean ± SD (n = 2 years)	Mean ± SD (n = 24 months)	Range
South Kinangop	74 (12%)	1.5 ± 0.16	0.13 ± 0.23a	0.00±0.86
North Kinangop	63 (26%)	3.7 ± 1.91	0.31 ± 0.59ab	0.00±2.61
Kedong	153 (18%)	10.0 ± 6.89	0.83 ± 1.30b	0.00±5.28

Associations between nest index, environmental factors and invertebrate biomass

The abiotic (rainfall, T_{min} and T_{max}) and biotic (ground and flying invertebrate biomass) factors, in the month before or the month of breeding, explained little variation in occurrence or intensity of breeding of Larks, in any of the three locations (Table 6). For each location, and for both sets of analyses, no single best model emerged. For the analysis of occurrence of breeding there were subsets of six (South Kinangop), 13 (North Kinangop), and four (Kedong) models with $\Delta AICc < 2$ (Table 6A). These models contained zero to two of the ten abiotic and biotic factors included in the analysis; each of the subsets contained four to seven of the ten factors (Table 6A). The explained variation (D^2) of models was consistently low, and for South Kinangop and North Kinangop, the model sets included the intercept-only "null" model (i.e., no environmental factors; Table 6A).

Using model averaging and standardization to explore the relative contribution of the environmental parameters to breeding, we could not identify one or more significant environmental factors that explained breeding in Larks (Table 7A; Figure 4 A- D).

For the analysis of intensity of breeding there were subsets of six (North Kinangop) and three models (Kedong) with $AIC_c < 2$ (Table 6B). These models contained zero to two of the ten abiotic and biotic factors included in the analysis; each of the subsets contained two to seven of the ten factors (Table 6B). The explained variation (R^2) of models was consistently low, and for North Kinangop, the model sets included the intercept-only "null" model (i.e., no environmental factors; Table 6B). Using model averaging and standardization to explore the relative contribution of the environmental parameters to breeding, we could only identify one significant environmental factor that explained breeding in Larks (i.e. T_{max} in North Kinangop; Table 7B).

Discussion

This study showed that year-round breeding activities of Red-capped Larks in three climatically-distinct equatorial populations were not associated with rainfall, temperature and invertebrate biomass, across and within locations. Across locations that represent a gradient of rainfall and temperature, we found no support for the prediction that drier and warmer locations had lower invertebrate biomass and less breeding activity of Larks. In line with these results, within each location we also found no evidence that breeding was timed to co-occur with rainfall, higher or lower temperatures or invertebrate biomass. Instead, we observed highly unpredictable and irregular variation in environmental variables, invertebrate biomass and breeding of Larks, among months and among years. Red-capped Larks bred in all calendar

Table 6. Model selection results of (A) occurrence of breeding of Red-capped Larks in South Kinangop, North Kinangop and Kedong, and (B) intensity of breeding in North Kinangop and Kedong as a function of biotic and abiotic factors in the month prior to the breeding observation and the month of the breeding observation (see Methods for details). Parameters, degrees of freedom, AICc, ΔAICc, weights and the explained variation (D2 in (A) and R2 in (B)) of the top-model and models with a ΔAICc <2.

A	Model ranking	Parameters	DF	AICc	ΔAICc	Weight	D^2
South Kinangop (n = 22 months)	1	Intercept	2	32.1	-	0.296	0.00
	2	Intercept +Tmin[prior]	3	33.1	0.98	0.181	0.08
	3	Intercept +Flying invertebrates[prior]	3	33.6	1.43	0.145	0.06
	4	Intercept +Tmax[prior]	3	33.6	1.47	0.142	0.06
	5	Intercept +Tmax[current]	3	33.8	1.71	0.126	0.04
	6	Intercept + Ground invertebrates[current]	3	34.1	1.96	0.111	0.03
North Kinangop (n = 27 months	1	Intercept +Tmax[current]	3	39.9	-		
	2	Intercept +Tmax[prior]	3	40.0	0.07	0.119	0.15
	3	Intercept +Tmin[prior]	3	40.2	0.26	0.115	0.15
	4	Intercept +Tmax[current]+Flying invertebrates[prior]	4	40.2	0.31	0.105	0.15
	5	Intercept	2	40.2	0.34	0.101	0.23
	6	Intercept +Tmax[prior]+Flying invertebrates[prior]	4	40.3	0.42	0.096	0.23
	7	Intercept + Tmax[current] +Flying invertebrates[current]	4	41.4	1.45	0.058	0.20
	8	Intercept +Tmin[prior]+ Ground invertebrates[current]	4	41.4	1.45	0.058	0.19
	9	Intercept +Ground invertebrates[prior]	3	41.4	1.5	0.057	0.11
	10	Intercept +Tmax[current]+Tmin[prior]	4	41.5	1.58	0.053	0.19
	11	Intercept + Flying invertebrates[prior]	3	41.8	1.91	0.046	0.09

	12	Intercept +Ground invertebrates[current]	3	41.9	1.98	0.045	0.09
	13	Intercept +Tmax[prior]+Tmin[prior]	4	41.9	1.98	0.045	0.18
Kedong (n = 31 months)		Intercept + Ground invertebrates[current]	3	43.6	-	0.372	0.17
		Intercept + Tmax[prior]+Ground invertebrates[current]	4	44.1	0.47	0.295	0.23
		Intercept + Tmax[current]+ Ground invertebrates[current]	4	45.2	1.55	0.172	0.20
		Intercept +Tmin[prior]+ Ground invertebrates[current]	4	45.3	1.68	0.161	0.20
B	Model ranking	Parameters	DF	AICc	ΔAICc	Weight	D^2
North Kinangop (n = 14 months)	1	Intercept +Tmax[current]+Ground invertebrates[prior]	4	14.1	-	0.270	0.35
	2	Intercept +Tmax[current]	3	14.9	0.73	0.188	0.16
	3	Intercept	2	15.1	1.00	0.164	-
	4	Intercept +Tmax[prior]	3	15.4	1.25	0.145	0.13
	5	Intercept +Tmin[prior]+Ground invertebrates[prior]	4	15.7	1.59	0.122	0.27
	6	Intercept +Ground invertebrates[prior]	3	15.9	1.77	0.112	0.10
Kedong (n = 17 months)	1	Intercept	2	66.7	-	0.332	-
	2	Intercept +Tmin[current]	3	66.9	0.16	0.307	0.10
	3	Intercept + Rainfall[prior]	3	67.6	0.83	0.220	0.06
	4	Intercept +Tmax[prior]	3	68.4	1.71	0.141	0.01

months overall, but they did not breed in every month in every year in every location. Our findings raise the question of which factors do trigger equatorial Red-capped Larks to initiate breeding. They suggest that environmental conditions vary at a small spatio-temporal scale and often provide minimum requirements for breeding, at least for some individuals in each population.

Despite the small geographical distances (19-34 km) separating the three locations, our weather data allowed us to quantitatively confirm the distinct spatial patterns in rainfall and temperatures that we expected among locations due to orography (Table 1). However, against our predictions, the warmest and driest location, Kedong, had the highest flying invertebrate biomasses and the highest Lark nest index. In contrast, the cool and wet climatic extreme, South Kinangop, had intermediate ground and flying invertebrate biomasses and the lowest Lark nest index. Overall, the general global trend of lower primary productivity in more arid environments (Pérez-Sáchez et al. 2013, Peñuelas et al. 2003, Tieleman et al. 2004) is not reflected in our invertebrate and Lark breeding data.

Other factors that, in combination with rainfall and temperature, may affect Lark ecology and that differ among locations include soil type, land use, and the occurrence of climatic excesses. South Kinangop's climate and fertile soil allow for intensive crop cultivation, but the combination of heavy rains and poor soil drainage also make the area prone to flooding. Seminis, a study site in South Kinangop with communally grazed land, experiences

Table 7. Model averaging and standardization results of occurrence of breeding of Red-capped Larks in South Kinangop, North Kinangop and Kedong, as a function of biotic and abiotic factors in the month prior to the breeding observation and the month of the breeding observation (see Methods for details). Standardized estimates (± SE, 95% confidence intervals) for biotic and abiotic factors of the average model per location are based on the model subsets in Table 6.

A	Parameter	Estimate	S.E.	95% CI
South Kinangop	Intercept	-0.81	0.50	-1.80±0.18
	Tmax[current]	0.12	0.12	-0.11±0.35
	Tmax[prior]	0.15	0.15	-0.14±0.44
	Tmin[prior]	-0.25	0.21	-0.67±0.16
	Ground invertebrates[current]	0.10	0.11	-0.13±0.32
	Flying invertebrates[prior]	-0.16	0.15	-0.45±0.13
North Kinangop	Intercept	-0.01	0.36	-0.73±0.70
	Tmax[current]	0.41	0.25	-0.09±0.90
	Tmax[prior]	0.33	0.23	-0.12±0.78
	Tmin[prior]	-0.26	0.19	-0.62±0.10
	Ground invertebrates[current]	0.07	0.07	-0.06±0.21
	Flying invertebrates[current]	0.04	0.04	-0.03±0.12
	Flying invertebrates[prior]	0.28	0.24	-0.19±0.76
Kedong	Intercept	0.33	0.43	-0.51±1.18
	Tmax[current]	0.02	0.02	-0.02±0.06
	Tmax[prior]	0.08	0.05	-0.03±0.18
	Tmin[prior]	-0.14	0.15	-0.44±0.15
	Ground invertebrates[current]	2.37	1.19	0.04±4.71

B				
North Kinangop	Intercept	0.553	0.09	0.38±0.72
	Tmax[current]	0.168	0.08	0.02±0.32
	Tmax[prior]	0.046	0.03	-0.01±0.10
	Tmin[prior]	-0.042	0.02	0.08±0.00
	Ground invertebrates[prior]	-0.168	0.09	-0.34±0.00
Kedong	Intercept	1.656	0.36	0.94±2.37
	Rainfall[prior]	0.234	0.16	-0.09±0.56
	Tmax[prior]	-0.117	0.11	-0.33±0.09
	Tmin[current]	-0.37	0.22	-0.81±0.07

multiple months of standing water in some years, which might negatively affect productivity and diversity of plants and invertebrates (McMullen and Lytle 2012). In contrast, Kedong's grasslands are extensively grazed by diverse wild and domestic herbivores, a process that might increase primary productivity and favor invertebrate and Lark reproduction (Veen et al. 2010, Van Klink et al. 2015, Tieleman et al. 2004, Tieleman and Williams 2005).

All three locations showed a lack of predictable intra-annual patterns in rainfall, temperature and invertebrate biomasses. Lark breeding was most unpredictable in Kedong where we observed breeding in 51% of the months but in all 12 calendar months, and most predictable in South Kinangop, where breeding occurred in 33% of the months restricted to seven calendar months. In addition, variation among years contributed to the unpredictability in all locations. Although we did not identify relationships among rainfall and breeding during the 39 months of our study, we did observe that breeding activities were affected by multi-month rainfall excesses: during a six-month drought in Kedong breeding was almost (but not fully) absent, while extended flooding prevented birds from breeding in South Kinangop and destroyed nests in both South and North Kinangop. These observations are in line with other studies in highly variable and unpredictable environments that also show that flooding from heavy rainfall disrupts breeding by destroying nests, by directly killing eggs and young [Foster 1991, Poiani 2006), and by reducing food availability or foraging efficiency (Foster 1974, Tye 1991, Young 1994, McMullen and Lytle 2012). Moreover, drought can result in food and foraging limitations, and birds may avoid breeding during dry seasons (Boag and Grant 1984, Dittami 1986, Poulin 2006, Young 1994, Tieleman and Williams 2002, Tieleman and Williams 2003b, Lepage and Lloyd 2004).

The results of our model selection analysis, which did not strongly identify any of the environmental factors as relevant for Larks reproduction, have two potential explanations. Either the spatial scale at which we sampled rainfall, temperature, and invertebrates was not the scale that Red-capped Larks used to time breeding, or Red-capped Larks used other factors to initiate breeding. We sampled rainfall and temperature with one weather station per location, placed centrally among the multiple plots within a location but as a result also at varying distance to each plot (Table 1). Likewise, we sampled invertebrates at only one transect in each plot. One might

wonder if the small-scale spatio-temporal variation in environmental and ecological factors that we discovered calls for measuring rainfall, temperature and invertebrates at the level of Lark territories. While the addition of territory-level data might be interesting, we do not believe that sampling scale underlies our population-level findings about Lark breeding. Moving around in small flocks when not breeding, Red-capped Larks do not appear to stay within their breeding territory year-round (pers. obs. based on color-ringed individuals), a behavior that contrasts with some other Lark species (e.g., Hoopoe Larks, *Alaemon alaudipes*, in the Arabian Desert (Tieleman and Williams 2002).

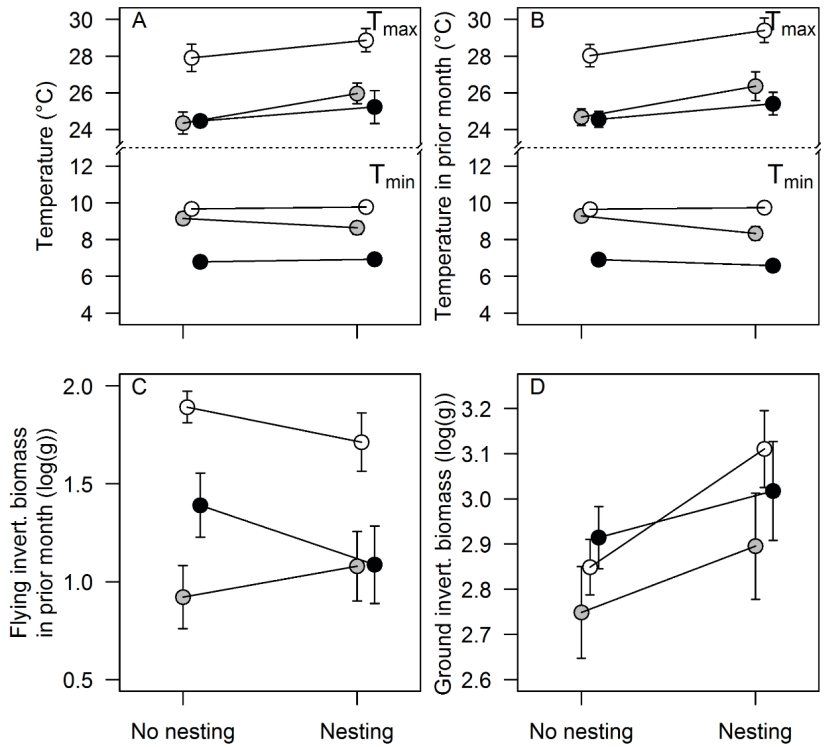

Fig 4. Average monthly (± SE) values during non-breeding and breeding months, for the biotic and abiotic factors that were selected in the model selection analysis (Table 6, Table 7) for South Kinangop (black symbols), North Kinangop (grey symbols), and Kedong (white symbols). A. average minimum (T_{min}) and average maximum (T_{max}) temperature (^{0}C) in current month, B. average minimum (T_{min}) and average maximum (T_{max}) temperature (^{0}C) in prior month, C. flying

invertebrate biomass (log(g dry weight)) in prior month. D. ground invertebrates biomass (log(g dry weight)) in current month.

We therefore propose that Red-capped Larks use other factors to time reproduction, with prime candidates being nest predation (Martin 1995, Ghalambor and Martin 2002, Decker et al. 2012, Praus et al. 2014, Toyama et al. 2015), female protein reserves (Ward 1969, Fogden 1972, Jones and Ward 1976, Fogden and Fogden 1979), or social factors. Although a detailed study remains to be done, nest predation in our study sites is generally high, with only 53 nests that fledged from the total of 290 nests found in different stages of the nesting cycle (pers. obs.). In the face of such intense nest predation Red-capped Larks may breed opportunistically (Grant and Boag 1980, Zann et al 1996, Grant et al. 2000, Tieleman and Williams 2002, Tieleman and Williams 2005). If environmental conditions are always permissive, birds may breed whenever they have resupplied their reserves after a failed nest attempt (Scott et al. 1987). This would also be in line with earlier studies on equatorial passerines with opportunistic breeding schedules, in which the authors suggested that protein reserves of individual females may determine whether and when they breed, leading to asynchronous year-round breeding activities at the population level (Ward 1969, Fogden 1972, Jones and Ward 1976, Fogden and Fogden 1979, Zann et al. 1996). Social factors can affect breeding decisions in multiple ways. For example, some individuals may time their breeding to benefit from peak food availability (Dittami and Gwinner 1985, Komdeur 1996, Hau et al. 2000, Scheuerlein and Gwinner 2002), while others may avoid competition for food or nesting space by conspecifics or other species by choosing "unpopular" times between food peaks (Minot 1981). In addition, timing of breeding may be influenced by predation-avoidance strategies (i.e., a pair avoids being the only one breeding at a particular time due to the high predation risk associated with that position (Sanson et al. 2009, Fontaine and Martin 2006) and prolonged nestling dependence on parents (Langen 2000). A better understanding of these possible mechanisms will come from studies at the individual level that complement our population level findings.

Supporting information

S1 Appendix.

Number of samples (n) and range of length (minimum L in mm, maximum L in mm) and width (minimum W in mm, maximum W in mm) of invertebrates used in generating calibration curves, and statistics per curve (coefficients a, b, c; adjusted r^2, degrees of freedom, F and P-values), per invertebrate category we used to predict body mass from length and width. Calibration curves were fit with the formula

Log(biomass in mg dry weight) = log(*a*) + *b* log(length in mm) + *c* log (width in mm),

where *a*, *b* and *c* are coefficients of the model (see Methods).

S1 File. Outcome of the investigation of the effect of average maximum temperature, average minimum temperature, rainfall, ground and flying invertebrates on nest index at time windows and time lags up until 6 month prior to the current month in Red-capped Larks living in three climatically-distinct Kenya locations, South Kinangop, North Kinangop and Kedong during March 2011 - February 2014. Please note the inclusion of Table A and Figure A in S2 File.

S1 Table. Correlation coefficients (below the diagonal) and P-values (above the diagonal) of pairwise correlations among the three weather variables and two invertebrate variables in three Kenyan locations, South Kinangop, North Kinangop and Kedong during March 2011
- February 2014.

S2 Table. Coefficients of variation of monthly (n = 36 months) rainfall, average minimum temperature and average maximum temperatures as measured by our weather stations in South Kinangop, North Kinangop and Kedong, during March 2011 - February 2014. F-tests of equal variances indicated that there were no significant differences among locations $P<0.05$; indicated by superscripts).

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Chapter 3

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Geographical and temporal variation in environmental conditions affects nestling growth but not immune function in a year- round breeding equatorial lark

Henry K. Ndithia,
Samuel N. Bakari,
Kevin D. Matson,
Muchane Muchai,
B. Irene Tieleman

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Abstract

Variation in growth and immune function within and among populations is often associated with specific environmental conditions. We compared growth and immune function in nestlings of year-round breeding equatorial Red-capped Larks *Calandrella cinerea* from South Kinangop, North Kinangop and Kedong (Kenya), three locations that are geographically close but climatically distinct. In addition, we studied growth and immune function of lark nestlings as a function of year-round variation in breeding intensity and rain within one location. We monitored mass, wing, and tarsus at hatching (day1) and at 4, 7, and 10 days post-hatch, and we quantified four indices of immune function (haptoglobin, agglutination, lysis and nitric oxide) using blood samples collected on day 10. Nestling body mass and size at hatching, which presumably reflect the resources that females allocated to their eggs, were lowest in the most arid location, Kedong. Contrary to our predictions, nestlings in Kedong grew faster than nestlings in the two other cooler and wetter locations of South and North Kinangop. During periods of peak reproduction within Kedong, nestlings were heavier at hatching, but they did not grow faster over the first 10days post-hatch. In contrast, rainfall, which did not relate to timing of breeding, had no effect on hatching mass, but more rain did coincide with faster growth post-hatch. Finally, we found no significant differences in nestling immune function, neither among locations nor with the year-round variation within Kedong. Based on these results, we hypothesize that female body condition determines nestling mass and size at hatching, but other independent environmental conditions subsequently shape nestling growth. Overall, our results suggest that environmental conditions related to food availability for nestlings are relatively unimportant to the timing of breeding in equatorial regions, while these same conditions do have consequences for nestling size and growth.

Introduction

Patterns of growth and development, and ontogeny of immune function vary widely among avian species and populations, variation that is hypothesized to reflect adaptation to specific environmental conditions (Starck and Ricklefs 1998, Demas and Nelson 2012). Growth rates are associated with pace-of-life, with faster growth rates associated with species and populations that live at high latitudes (Ricklefs 1976, McCarty 2001, Ricklefs and Wikelski 2002, Wikelski et al. 2003, Wiersma et al. 2007) at high altitudes (Khanna and Yadav 2005, Scott 2011), and in less arid environments (Tieleman and Williams 2004, Tieleman 2005). For a given pace-of-life, i.e. within populations and within seasons, early-hatched broods have been shown to grow faster than late-hatched broods due to changes in food abundance and quality of diet (Van Noordwijk et al. 1995, Gebhardt-Henrich and Van Noordwijk 1991, Christians 2002). Like growth rates, immune function has also been hypothesized to vary with pace-of-life in birds, with reduced investment in the immune system associated with a faster pace-of-life (Ricklefs and Wikelski 2002, Martin et al. 2004, Tieleman et al. 2005). However, several studies show that environmental conditions can be more important determinants of immune function than pace-of-life (Møller et al. 2006, Horrocks et al. 2012, Versteegh et al. 2012, Versteegh et al. 2014, Horrocks et al. 2015). In addition, within a given pace-of-life, immune function is not fixed, but changes seasonally in adult birds (Nelson and Demas 1996, Horrocks et al. 2013, Hegemann et al. 2012, 20] and nestlings (Dubiec and Cichoń 2001, Christe et al. 2001, Dubiec and Cichoń 2005).

Within equatorial regions, which on the global scale are associated with comparatively slow avian growth rates (Ricklefs 1976), spatial and temporal variation in climatic conditions still exist. This spatio-temporal variation in climatic condition provides a strong opportunity to understand variation in life-history strategies among tropical locations and species. For example, orography and altitudinal differences can lead to large variation in rainfall and temperature over small geographic distances, and rainfall patterns are often unpredictable within regions (Boag and Grant 1984, Wrege and Emlen 1991, Ndithia et al. 2017a). This within-region variation raises questions about how nestling growth rates have evolved in response to different tropical climates, questions that have generally not been investigated. In general, cool and wet locations are thought to provide more food, promoting faster nestling growth; more arid locations are thought to be food deficient, favoring slower growth (Ricklefs 1976, Tieleman and Williams 2004). In contrast however, investment in growth rate for nestlings in cool and wet locations is expected to compete with requirements for thermoregulation, possibly reducing growth rate in such locations compared to drier ones (Krijgsveld et al. 2003). Studies including costs of thermoregulation could identify the relative importance of these factors. Differences in environmental conditions within and between years in a location with fluctuating and inconsistent patterns of rainfall and food availability are likely to promote variation in nestling growth rates (McCarty 2001). Likewise, nestlings raised in food abundant wet seasons grow faster than those raised in food deficient dry

seasons (Emlen et al. 1991), the latter of which result in hatching asynchrony commonly recorded among tropical artricial birds (Ricklefs 1976, Ricklefs 1997, Royle et al. 1999, Ricklefs 2002).

Immune defences of organisms living in a particular environment are expected to match pathogen pressure experienced in that environment (Horrocks et al 2011). Although high parasite pressures associated with tropical regions might also result in tropical birds having relatively robust immune systems compared to their temperate counterparts, environmental variation within equatorial regions should select for intra-tropical variation in immune function in adult and nestling birds. Immune function in young birds may be particularly revealing about the parasite pressures exerted by different locations within a region. Since the time for possible parasite exposure and for subsequent acquired immune responses is short, innate immune function and maternally derived antibodies are proposed to be most important for young birds (Starck and Ricklefs 1998, Mauck et al. 2005, Pihlaja et al. 2006, Stambaugh et al. 2011). Innate immune function is hypothesized to relate to pace-of-life (Tella et al. 2002, Lee et al. 2006, Lee et al. 2008), and maternally derived antibodies reflect maternal exposure to the local parasite pressures (Gasparini et al. 2001, Lemke et al. 2003). Assuming that immune function is traded off against reproduction (Sheldon and Verhulst 1996, Norris and Evans 2000) , one would expect that breeding under favorable environmental conditions allows females to allocate more resources to nestlings and improve their immune systems indirectly (e.g., via more diverse diet) or directly (e.g., through depositing higher concentrations of maternal antibodies and defenses) .

In order to better understand the role of intra-tropical variation on patterns of avian growth and development, and on immune function, we exploited the spatial and temporal variation in climate found in equatorial Kenya. This is an understudied component of the pace-of-life debate and one to which this study can strongly contribute. Here, locations that are in close proximity to one another have distinct rainfall and temperature patterns, and within locations, seasonal patterns of rainfall can be unpredictable (Ndithia et al. 2017a). Despite these differences, our study species, the Red-capped Larks *Calandrella cinerea*, occurs across locations. This provides the opportunity for intraspecific comparisons of environmentally-induced variation in nestling growth and immune function. We have previously found that Red-capped Larks breed year-round, particularly in Kedong, one of our study locations, and that nesting activities fluctuate throughout the year without direct associations with rainfall, temperature or invertebrate abundance (Ndithia et al. 2017a). Assuming that a high nesting intensity indicates a favorable set of environmental conditions, this system allows for the study of nestling growth and immune function in relation to temporal variation in environmental conditions that are favorable for females to breed or to rear nestlings.

Our overall objective was to investigate variation in growth and immune function in Red-capped Lark nestlings in relation to intra-tropical variation in environmental conditions. Specifically, we compared nestling growth rates and immunological indices among three climatically-distinct locations. We predicted that nestlings raised in cooler and wetter locations, with expected generally higher food availability, would display faster growth and higher investments in immune indices. In Kedong, we also examined consequences of hatching at

different times of the year on growth rates and on immunological indices. In particular, we compared times of the year with more and less nesting activity and with more and less rain. We expected nestlings hatched during times of the year when more larks bred to grow faster and to have more robust immune defences assuming that a high nesting intensity indicates a favorable set of environmental conditions for breeding or rearing nestlings. Because we previously found timing of breeding to be unrelated to rain and food availability (Ndithia et al. 2017a), we predicted that rainfall would not affect the allocation of resources to nestling growth and immunity.

Methods

Study species

The Red-capped Lark is a widespread grassland species occurring in large parts of Africa. In Kenya, the species' distribution ranges from dry and warm lowlands about 1200 m above sea level (a.s.l.) to cool and wet montane grasslands 2600 m a.s.l. (Zimmerman et al. 1999). Red-capped Larks build open-cup nests on the ground often next to a scrub or grass tuft, and lay clutches of two eggs; only two of the 290 nests we found had a clutch of three eggs. They feed on a variety of invertebrates and occasionally on grass seeds. Color ring re-sightings suggest that at least part of our study populations is resident to our study locations year round (pers. obs. H.K.N, S.N.B.).

Study areas and environmental conditions

We conducted our study from January 2011 to March 2014 at three locations in central Kenya: South Kinangop ($0^0$42'30''S, $36^0$36'31''E, 2556 m a.s.l.), North Kinangop ($0^0$36'55''S, $36^0$30'48''E, 2428 m a.s.l.), and Kedong ($0^0$53'37''S, $36^0$23'54''E, 2077 m a.s.l.). In the presented order, the locations experience increasing temperature and decreasing precipitation (Table 1). South and North Kinangop are high altitude montane grasslands that lie along the Aberdare ranges. During and after heavy rains, South Kinangop can experience flooding for some but not all months of the year (normal breeding continues in the absence of flooding) and North Kinangop partial flooding (affecting a few nests), causing damage to nest and death to nestlings [30]. Kedong, a privately owned and extensively grazed ranch in the Rift Valley, consists of large grassland patches that never flood. Direct maximum distances between these

Table 1. Annual (n = 3 years) and monthly (n = 36 months) rainfall (average ± SD, and range), and monthly minimum and maximum temperatures (n= 36 months, average ± SD, and range) as measured by our weather stations in South Kinangop, North Kinangop and Kedong, during March 2011 – February 2014 (from Ndithia et al. 2017).

Location	**Annual rainfall (mm)**	**Monthly rainfall (mm)**		**Monthly minimum temperature (^{0}C)**		**Monthly maximum temperature (^{0}C)**	
	Mean ± SD	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
South Kinangop	939 ± 132.7	78 ± 69.7	0 – 309	5.5 ± 1.06	3.0 – 8.2	24.7 ± 2.09	21.2 – 30.0
North Kinangop	584 ± 62.6	49 ± 35.3	0 – 155	9.1 ± 2.42	3.0 – 13.7	25.4 ± 2.27	22.1 – 30.5
Kedong	419 ± 96.8	35 ± 39.2	0 – 153	10.5 ± 1.92	6.2 – 15.7	28.6 ± 2.44	25.3 – 34.9

Table 2. Clutch sizes, sample sizes of nests and nestlings for growth measurements, and sample sizes of nests and nestlings for immune function of Red- capped Larks *Calandrella cinerea* in South Kinangop, North Kinangop and Kedong, three Kenyan locations with a gradient in climatic conditions, during January 2011 to March 2014. Nestlings normally fledged at day 10 or within one or few days thereafter.

	Clutch size		**Number of nests for growth (number of nestlings)**				**Number of nests for immune indices (number of nestlings)**
Location	Mean ± sd	Range (number of nests)	Day 1	Day 4	Day 7	Day 10	Day 10
South Kinangop	1.8 ±0.39	1-2 (62)	13* (19)	11 (17)	12 (18)	9 (14)	9 (14)
North Kinangop	1.9±0.33	1-2 (49)	8 (14)	10 (18)	8 (14)	10 (17)	9 (15)
Kedong	1.9±0.35	1-3 (133)	23 (44)	19 (36)	15 (29)	11[#] (19[#])	12 (18)

*number of nests for mass=12

[#]number of nests for wing=10; number of nestlings for wing=18

locations are 19 km (South Kinangop - North Kinangop), 29 km (South Kinangop - Kedong) and 34 km (North Kinangop – Kedong). Although we cannot fully exclude the possibility of exchange among locations, we never observed any movements between locations based on the total of 344 color-ringed birds; we observed our birds to be relatively resident. In addition, the three locations are not connected by grassland corridors but in contrast, are separated by natural barriers including escarpment and forest patches that make movement between locations less likely. Within each location, we worked in multiple plots, including Seminis in South Kinangop, Joshua, Mbae and Ndarashaini in North Kinangop and four grassland patches in Kedong. To obtain monthly rainfall in Kedong, we used a weather station (Alecto WS-3500, Den Bosch, Netherlands) that measured daily rainfall (mm).

Fieldwork: nest search, nestling growth, and nesting index

Searching year round over the entire study period, we found a total of 290 nests: 74 in South Kinangop, 63 in North Kinangop, and 153 in Kedong (for distributions over time, see (Ndithia et al. 2017a). Because of high nest loss through predation, flooding, and other causes, sample sizes of nests with nestlings varied by location and with nestling age (see Table 2 for details). We aimed to find nests at the construction or egg stage, and we monitored nests daily or every other day to determine with certainty hatching dates and nestling order. We made extra effort around hatching date to visit nests to establish hatching order. In cases when we did not distinguish first from second-hatched nestling because both hatched before we could make the distinction, we scored 1.5 for both unknown nestlings. At hatching, we clipped the tip of the claw of the hind toe of the first-hatched nestling to distinguish first- and second-hatched nestlings, which generally hatched a few hours apart. For 19 nests that we found already with nestlings, we estimated age of nestlings based on morphological characteristics, including presence of downy feathers and openness of the eyes.

We measured body mass, wing length, and tarsus length of nestlings at days 1 (hatching), 4, 7 and 10. We measured body mass using a 50g Pesola (accuracy, 0.1g), measured wing length on a flattened and straightened wing using a 150mm ruler specially designed for measuring birds (accuracy, 0.5mm) and measured tarsus length from the knee to the base of the last complete scale before the toes diverge using a Vernier calipers (accuracy, 0.1mm) (Spencer 1984, Svensson 1992). Three field assistants and H.N. worked in all three locations and took these measurements on birds. In addition, at the beginning of the project, H.K.N. trained the three assistants to harmonize the measuring skills and avoid observer bias. Nestlings normally fledged between day 10 and 12 of age. On day 7, we trapped both parents using a cage trap at the nest to record morphological parameters.

Red-capped Larks breed year-round but the number of nests varies from month to month (Ndithia et al. 2017a). To quantify the month-to-month variation in nesting intensity at the population level in Kedong, we calculated a “nest index”: the total number of nests found in a month per 10 hours of nest searching effort (Ndithia et al. 2017a). From January 2011 to March 2014, our mean monthly search effort in Kedong was 14.1 ± 5.30 days (SD, n = 39, range = 7-24 days) or 49.8 ± 35.95 hours (SD, n = 39, range = 17-193 hours).

Nestling immune function

Using heparinized capillary tubes, we collected blood samples in the field from the brachial wing vein of 47, 10-day-old nestlings (n = 30 nests) in the three locations combined (see Table 2 for breakdown per location). Blood samples were kept on ice and centrifuged at the end of each fieldwork day. The plasma fraction was then frozen for future analyses of haptoglobin, natural antibodies and complement, and nitric oxide.

Haptoglobin, an acute phase protein, increases in concentration in blood in response to acute infection, inflammation, or trauma (Quaye 2008, Matson et al. 2012). We determined the concentration (mg/ml) of haptoglobin (or more specifically, haptoglobin-like functional equivalents) using an assay that measures the haem-binding capacity of plasma (TP801; Tridelta Development limited, Maynooth, Ireland) following the instructions provided by the manufacturer and with the 5 minute incubation step at 30°C (for details, see Matson et al. (2012). Each of the three assay plates, included an among-plate standard which we run in duplicate within each plate (Matson et al. 2012) (mean within-plate coefficient of variation (CV) = 2.4%; mean among-plate CV=2.7%).

Natural antibodies and complement are constitutive components of the innate immune system (Matson et al. 2005). We quantified natural antibody-induced agglutination and complement-induced lysis of rabbit red blood cells (Envigo, Belton, UK) following the protocol of (Matson et al. 2005). We scored lysis and agglutination titers from randomized images of assay results. All scoring of lysis and agglutination (HLHA) were done blind to sample and plate identity, and all HLHA samples were scored at least twice by the same person. If the first two scores were < 1 titer apart, we used the mean value in statistical analyses. If the difference between the first two scores was > 1, we re-scored the sample a third time and used the median in analyses. We assigned half scores when samples showed a lysis or agglutination result that was intermediate between two titers. We calculated among-plate and within-plate variation for agglutination (mean among-plate CV=9.7%; mean within-plate CV=7.7%) and for lysis (mean among-plate CV=18.6%; mean within-plate CV=9.8%)

Nitric oxide is a multifunctional signaling molecule that can provide information about an individual's condition, and whose functions include the modulation of inflammatory processes and the destruction of parasites, virus-infected cells, and tumor cells [53] . We determined nitric oxide production (mmol/ml) through the reduction of nitrate to nitrite following the assay of (Sild and Hõrak 2009). We used the Griess reaction assay kit from Promega and recorded absorbance at 542 nm.

Statistical analyses

We first checked, per location, for differences in mass, wing and tarsus between nestlings whose ages we knew and those whose age we estimated. Over-or-under estimation of age of nestlings may lead to incorrect data of nestling mass, wing and tarsus. We did not find significant differences between these groups (all t values < 2.14, all P values > 0.07) and therefore pooled them in further analyses. We described growth in mass, wing length and tarsus length using logistic

growth curves (Ricklefs 1979, Tieleman 2005) for each location that we fitted by the R-package "car (Fox and Weisberg 2011)." To compare among locations and ages, we calculated residual values relative to a single overall curve for all locations combined. We fitted this overall curve using average values per age for each location, to account for sample size differences among locations. We expressed residuals as percent deviation from this curve. In further analyses, we used these residuals in linear mixed-effects models ("lme" in the R-package "nlme"; (Pinheiro et al. 2012).

To compare nestling mass, wing length, and tarsus length across locations, we used models with location, age, hatching order and the interaction between location and age as explanatory variables, and with individual nested within nest as random factors. These random factors accounted for the lack of independence between nest mates due to shared genetic background and parental care (Sofaer et al. 2013) and repeated measurements on individual nestlings. We used residuals of absolute nestling size (i.e., absolute mass, wing length, or tarsus length) and residuals of relative nestling size (i.e., % of adult mass, wing length, or adult tarsus length) in this comparison. To determine nestling size relative to size at maturity (i.e., % of average adult size), we first calculated sex- and population-specific mean values of mass, wing length, and tarsus length since we did not know the sex of nestlings. Then we averaged the male and female values to approximate generalized adult values (Appendix 1). When the interaction between location and age was significant, we ran models per age (day 1, 4, 7, 10) to discover at which age(s) the location effect was significant. We subsequently tested for differences among locations using Tukey post-hoc tests.

For the within-Kedong analyses of nestling mass, wing length, and tarsus length in relation to nest index and total monthly rainfall, we calculated residuals relative to the logistic curve for Kedong only. Our models contained these residuals as dependent variables; nest index (or rainfall), age, hatching order, and the interaction between nest index and age as explanatory variables; and nest as random factors. We repeated all analyses of mass using instead mass divided by tarsus (an index for body condition); results from the two analyses were similar, so we only report results from the first mass analyses.

For analyses of haptoglobin, we log-transformed data, because the residuals of the final model were not normally distributed. For the among location comparison, we first tested and found that sample redness at 450 nm did not affect haptoglobin ($F_{1, 14} = 1.13$, $P = 0.31$) but sample age did ($F_{1, 14} = 6.36$, $P = 0.02$) (Matson et al. 2012). We then constructed a model that included log haptoglobin as the dependent variable; location, hatching order, sample age, and the interaction between location and hatching order as explanatory variables; and nest as a random factor. For the within Kedong analysis, we established that sample redness ($F_{1, 5} = 2.89$, $P = 0.15$) and sample age ($F_{1, 5} = 2.80$, $P = 0.16$) did not significantly affect haptoglobin concentration. Models then included explanatory variables hatching order, and either nest index or monthly rainfall, and the interactions. Again, nest was included as a random factor.

In comparisons of agglutination (log-transformed to obtain normality) and nitric oxide across locations, we found no effect of plasma sample age (agglutination $F_{1, 17} = 0.06$, $P = 0.81$;

nitric oxide $F_{1, 9} = 0.11$, $P = 0.75$). We therefore included location, hatching order, their interaction as explanatory variables, and we included nest as a random factor. For within Kedong analyses, plasma sample age did not affect agglutination ($F_{1, 6} = 0.02$, $P = 0.89$) or nitric oxide ($F_{1, 3} = 0.39$, $P = 0.58$) also. We therefore constructed models with nest index or monthly rainfall, hatching order, and the interaction as explanatory variables and nest as a random factor.

Additionally among the three locations, we explored effects of mass and tarsus at day 10 (sample sizes did not allow including growth, and measurement at days 1, 4 and 7) on the three immune measures, because of possible trade-offs between growth and immune measures. We did not find any significant effects of location or mass/tarsus ($F_{2, 25} < 1.98$, $P>0.16$); we do not report these results.

For all analyses, we tested and confirmed assumptions about normality and homoscedasticity of variance through graphical and statistical methods. We simplified models using backward elimination by excluding one-by-one the most insignificant terms ($\alpha = 0.05$) until we arrived at a final model. We used R statistical software version 3.0.3; (R Core Team) in all our analyses.

Results

Nestling growth but not immune function varies among three climatically distinct locations

Growth curves for mass and wing differed among locations in a similar fashion with nestlings in Kedong starting at the lowest mass and shortest wing, but having the highest growth constant K for mass and wing, and nestlings in South Kinangop starting at the highest mass and longest wing but having the lowest K for both variables (Fig. 1, Table 3). Comparing residuals among locations for both mass and wing length, we found a significant interaction between location and age, and no significant effect of hatching order (Table 4). Subsequent analyses per age for mass revealed that at hatching (day 1), the location effect was significant ($F_{2, 40} =15.59$, $P < 0.001$) and nestling body mass (in g) was 34% higher in South Kinangop than in Kedong ($z = 4.98$, $P < 0.001$) and 47% higher in North Kinangop than in Kedong ($z = 3.85$, $P < 0.001$); nestling body masses in South and North Kinangop did not significantly differ from each other ($z = 0.46$, $P = 0.89$). Among-location differences in residual mass on days 4 ($F_{2, 37} = 2.02$, $P = 0.15$), day 7 ($F_{2, 32} = 0.21$, $P = 0.82$) and day 10 ($F_{2, 27} = 0.91$, $P = 0.42$) were not significant.

Subsequent analyses per age for wing length revealed that wing lengths differed significantly among locations on day 7 ($F_{2, 32} = 6.23$, $P = 0.01$) but not on days 1 ($F_{2, 41} = 1.25$, $P = 0.30$), 4 ($F_{2, 37} = 0.25$, $P = 0.78$) and 10 ($F_{2, 26} = 0.17$, $P = 0.85$) (Fig 1). Wing lengths at day 7 were significantly shorter in South Kinangop than in Kedong ($z = 3.52$, $P = 0.001$), but not significantly different between South and North Kinangop ($z = 1.51$, $P = 0.29$) or between North Kinangop and Kedong ($z = 1.56$, $P = 0.26$). Analyses of relative nestling body mass and relative wing length (% of adult mass and wing length, see Appendix 1 for adult masses and wing length) provided qualitatively similar results (not shown).

Tarsus growth curves differed among locations with nestlings in Kedong starting with intermediate tarsus lengths and having the highest K, whereas nestlings in South Kinangop had the shortest tarsi and the lowest K (Fig. 1, Table 3). In the model comparing residuals for tarsus lengths among locations, the interaction between location and age was significant and hatching order was insignificant (Table 4). Analyses per age showed that tarsus lengths differed

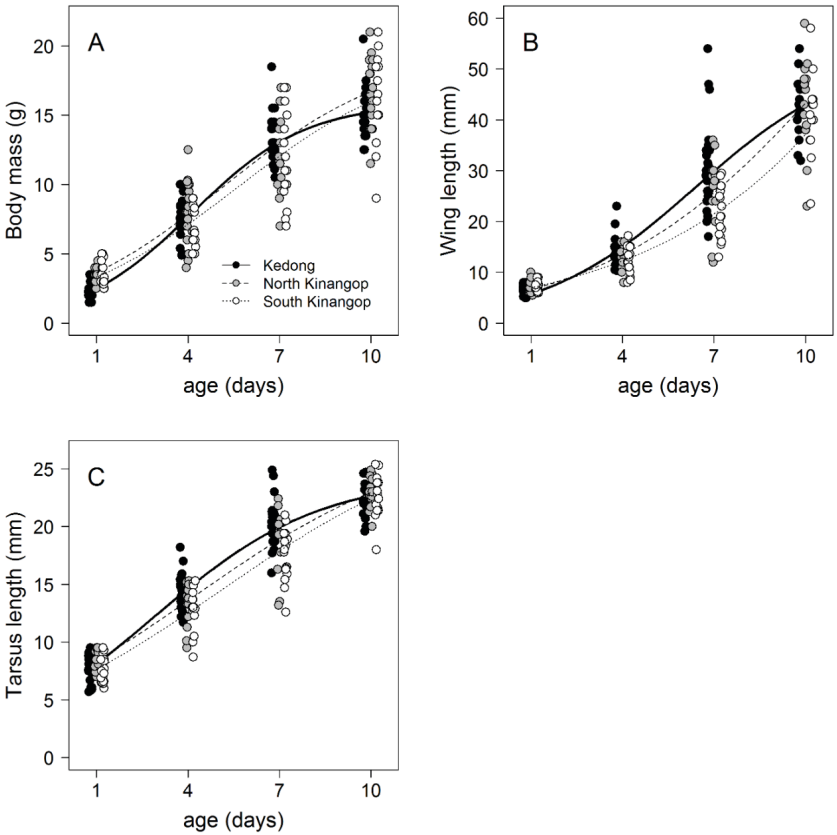

Fig. 1. Mass (g, A), wing length (mm, B) and tarsus length (mm, C) of Red-capped Lark *Calandrella cinerea* nestlings as a function of age in South Kinangop (cool and wet), North Kinangop (warm and wet) and Kedong (warm and dry), three Kenyan populations with distinct climates. Data for the three locations are plotted apart to increase visibility rather than that the age at measurements differed between locations significantly among locations on days 1 ($F_{2, 41} = 3.82$, $P = 0.03$) and 7 ($F_{2, 32} = 6.58$, $P = 0.004$), but not on day 10 ($F_{2, 27} = 0.45$, $P = 0.64$) (Fig. 1). On day 4, the difference was marginally insignificant for absolute tarsus length ($F_{2, 37} = 2.64$, $P = 0.08$) and significant for relative tarsus length ($F_{2, 37} = 3.89$, $P = 0.03$). Results for relative and absolute

tarsus length for other ages were qualitatively similar (not shown). Pairwise comparisons among locations showed that tarsus length in South Kinangop was shorter than in Kedong (day 1, $z = 2.40$, $P = 0.04$; day 7, $z = 3.63$, $P < 0.001$), tarsus length in South Kinangop was shorter than in North Kinangop only on day 1 ($z = 2.40$, $P = 0.04$), while tarsi did not significantly differ between North Kinangop and Kedong on day 1 or 7 (day 1, $z = 0.60$, $P = 0.82$; day 7, $z = 1.49$, $P = 0.29$)

Table 3. Logistic growth curve variables for growing nestlings of Red-capped Larks in South Kinangop, North Kinangop and Kedong, in addition to the overall curve based on the averages of the three locations. The logistic function is $W(t) = A / (1 + \exp(-K (t - t_i))$, where $W(t)$ is the weight at age t, A is the asymptote of the growth curve, K is the growth rate constant, and t_i is the inflexion point or age at maximal growth rate. $K_{restriced}$ is the growth constant when restricting the data set to individuals with repeated measures on days 1, 4, 7 and 10 (Kedong n = 10 nestlings, North Kinangop n = 3 nestlings, South Kinangop n = 10 nestlings). Values in parentheses represent 1 SE. For K, the 95% confidence intervals are given. Hatching day is defined as day 1.

	A (S.E.)	t_i (S.E.)	K (S.E.)	95% C.I.	$K_{restricted}$ (S.E.)	95% C.I.
Body mass (g)						
South Kinangop	19.5 (2.66)	5.6 (1.00)	0.34 (0.065)	0.21 - 0.47	0.43 (0.108)	0.21 - 0.65
North Kinangop	19.7 (2.16)	5.2 (0.82)	0.36 (0.068)	0.21 - 0.49	0.55 (0.115)	0.29 - 0.80
Kedong	15.9 (0.38)	4.1 (0.15)	0.54 (0.031)	0.48 - 0.60	0.57 (0.034)	0.51 - 0.64
Overall	17.5 (0.59)	4.7 (0.23)	0.43 (0.029)	0.38 - 0.49		
Wing length (mm)						
South Kinangop	558.9 (3882.94)	23.7 (6.88)	0.19 (0.057)	0.07 - 0.30	0.21 (0.083)	0.04 - 0.37
North Kinangop	101.4 (65.63)	11.1 (4.49)	0.26 (0.065)	0.13 - 0.39	0.37 (0.087)	0.17 - 0.56
Kedong	53.2 (4.17)	6.4 (0.50)	0.39 (0.037)	0.32 - 0.46	0.39 (0.074)	0.24 - 0.54
Overall	72.4 (10.84)	8.8 (1.05)	0.28 (0.060)	0.14 - 0.41		
Tarsus length (mm)						
South Kinangop	29.0 (3.02)	5.1 (0.97)	0.25 (0.034)	0.18 - 0.31	0.30 (0.043)	0.21 - 0.38

North Kinangop	28.0 (2.32)	4.2 (0.75)	0.26 (0.038)	0.18 - 0.33	0.34 (0.052)	0.22 - 0.45
Kedong	24.3 (0.60)	2.9 (0.18)	0.37 (0.022)	0.32 - 0.41	0.36 (0.033)	0.29 - 0.42
Overall	25.5 (0.71)	3.5 (0.22)	0.30 (0.038)	0.21- 0.38		

Table 4. Results of linear mixed-effect models examining the relationship of residuals for mass, wing and tarsus lengths as a function of location, age and chick hatching order for nestlings of Red-capped Larks *Calandrella cinerea* from South Kinangop, North Kinangop and Kedong

Mass

	d.f.	F	P
Body mass			
Location*Age	6, 143	10.54	<0.001
Location	2, 57	19.51	<0.001
Age	3, 143	17.97	<0.001
Hatching order	1, 142	0.20	0.66
Wing			
Location*Age	6, 142	9.24	<0.001
Location	2, 58	1.91	0.16
Age	3, 142	11.54	<0.001
Hatching order	1, 141	1.70	0.19
Tarsus			
Location*Age	6, 143	3.62	0.002
Location	2, 58	7.33	0.001
Age	3, 143	2.26	0.08
Hatching order	1, 142	0.11	0.74
NI 1	13	2.31	0.15
Day 8			
NI 1	9	0.33	0.58

Growth constants restricted to complete individual records

To further explore possible causes of the differences in growth constant K among locations, we restricted the data sets in each location to individual nestlings for which we had complete sets of repeated measurements (i.e., days 1, 4, 7, and 10; Kedong n = 10, North Kinangop n = 3, South Kinangop n = 10). With this approach we excluded nestlings that disappeared from the data set as a result of starvation, nest predation or flooding. Because we had observed nestlings in poor condition especially in South and North Kinangop, we hypothesized that selective disappearance

of this subset of nestlings might have affected the difference in K-values among locations. Indeed, restricting the analyses to healthy nestlings that successfully grew and fledged, yielded increased K values in North Kinangop and South Kinangop, confirming our observations that nestlings died of poor condition in these locations, but these values remained lower than in Kedong (Table 3.Note: based on 95% confidence intervals, differences were not significant).

Immune function

Log haptoglobin (mg/ml) were highest in Kedong, intermediate in North Kinangop and lowest in South Kinangop (Fig. 2A), a non-significant location effect ($F_{2, 25} = 2.84$, $P = 0.08$). Log agglutination titre did not differ significantly among the three locations (Fig. 2B), ($F_{2, 26} = 0.12$, $P = 0.88$).Lysis titre was zero for 45 out of the 47 nestlings and lysis titre was one for two 10-day old nestlings, one individual each from South and North Kinangop. We therefore did not test for among-location differences in lysis. There was no location effect among the three locations in nitric oxide (mmol/ml) (Fig. 2C), ($F_{2, 23} = 0.55$, $P = 0.59$). Hatching order did not affect log haptoglobin ($F_{1, 14} = 0.12$, $P = 0.73$), log agglutination ($F_{1, 17} = 0.01$, $P = 0.93$) or nitric oxide ($F_{1, 10} = 1.30$, $P = 0.28$).

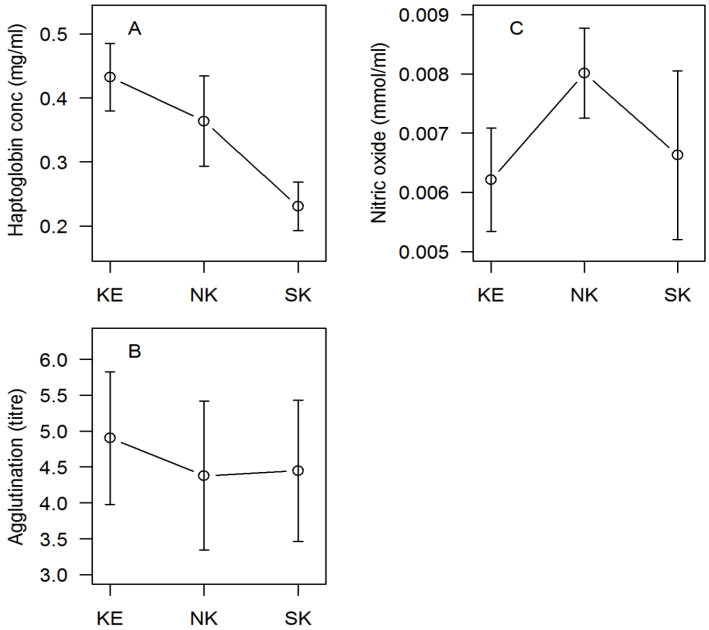

Figure 2. Haptoglobin concentration (mg/ml ±SE, A) , agglutination (titre±SE, B) and nitric oxide concentration (mmol/ml±SE, C) of 10-day old nestlings of Red-capped Larks *Calandrella cinerea* in South Kinangop (SK, cool and wet), North Kinangop (NK, warm and wet) and Kedong (KE, warm and dry), three Kenyan locations differing in climatic conditions.

Nestling growth but not immune function changes with population breeding intensity and rainfall in a year-round breeder in Kedong

Analysing residual mass as a function of the nest index of the month of hatch, we found a significant interaction between nest index and age, and no significant effect of hatching order (Fig. 3, Table 5A). Analyses per age showed that at hatching nestling body mass was higher when nest index was higher (day 1: $F_{1,21} = 8.80$, $P = 0.01$), but at days 4, 7, and 10 we observed no significant relation with nest index (all $F < 2.3$, all $P > 0.15$; Fig. 3). Analyses of residuals for mass with monthly rainfall also revealed a significant interaction between rainfall and age. When analysed per age, the analysis revealed a significant difference on day 7 ($F_{1,13} = 5.78$, $P = 0.03$, Table 5B): with more rain, 7-day-old nestlings were heavier (Fig. 3). At other ages, mass did not correlate with rainfall (all $F < 0.76$, all $P > 0.39$; Fig. 3). At day 10, the range of rainfall values is limited,

prohibiting robust evaluation at this age. Wing and tarsus lengths were unrelated to nest index or rainfall in Kedong at any age (Fig. 3, Table 5). Wing and tarsus lengths were also unrelated to hatching order (Table 5).

Table 5. Results of linear mixed-effect models examining the relationship of residuals for mass (in g) wing and tarsus lengths (in mm) in relation to A) nest index, age and chick hatching order and B) monthly rainfall (mm), age and chick hatching order, in Red-capped Larks C*alandrella cinerea* nestlings in Kedong.

A.

	d.f.	F	P
Mass			
Nest index*Age	3, 66	3.42	0.02
Nest index	1, 28	13.05	0.001
Age	3, 66	2.39	0.08
Hatching order	1, 65	0.14	0.71
Wing			
Nest index*Age	3, 64	0.87	0.46
Nest index	1, 28	1.30	0.26
Age	3, 67	35.71	<0.001
Hatching order	1, 26	0.58	0.45
Tarsus			
Nest index*Age	3, 65	1.81	0.15
Nest index	1, 28	0.30	0.59
Age	3, 68	2.25	0.09
Hatching order	1, 26	0.31	0.58

B.

	d.f.	F	P
Body Mass			
Rainfall*Age	3,65	3.21	0.03
Rainfall	1,28	2.07	0.16
Age	3,65	1.41	0.25
Hatching order	1,26	0.14	0.71
Wing			
Rainfall*Age	3,64	2.01	0.12
Rainfall	1,28	0.41	0.53
Age	3,67	35.42	<0.001
Hatching order	1,26	0.55	0.47
Tarsus			
Rainfall*Age	3,65	2.07	0.11
Rainfall	1,28	1.42	0.24
Age	3,68	2.13	0.10
Hatching order	1,26	0.28	0.60

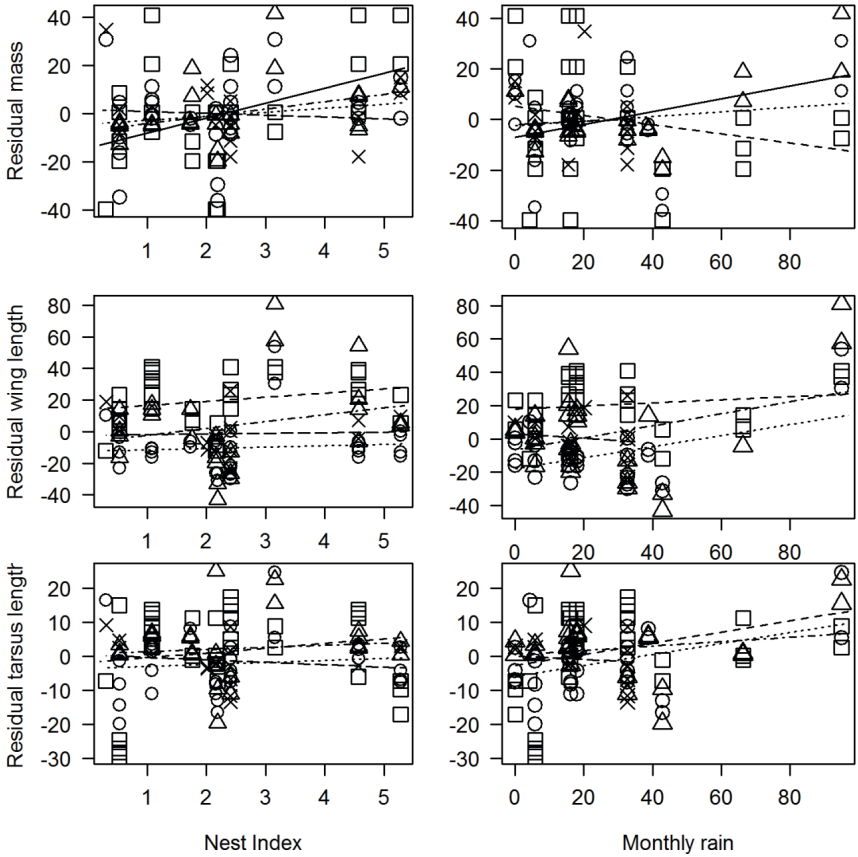

Figure 3: Mass, wing and tarsus lengths (residuals in % of the logistic growth curve) of four age-classes of nestlings of Red-capped Larks *Calandrella cinerea* as a function of nest index (number of nests/10 search hours) and monthly rainfall (mm) in Kedong (warm and dry), Kenya. Residual mass showed a significant relationship with nest index for nestlings at hatching (day 1) and with monthly rainfall for nestlings at day 7; both of these lines are represented by open squares and continuous line and open triangles and continuous line respectively. Legend for the non-significant relationship: open square and dotted line represent nestlings aged day 1, open circles and dotted line day 4, open triangles and dot-dashed line day 7, crosses and line with long dashes day 10

Immune function

The three immune indices were unrelated to nest index or rainfall in Kedong (Fig. 4): log haptoglobin (nest index $F_{1,10} = 1.31$, $P = 0.28$; rainfall $F_{1,10} = 0.14$, $P = 0.71$), log agglutination (nest index $F_{1,9} = 1.37$, $P = 0.27$, rainfall $F_{1,9} = 0.11$, $P = 0.75$) and nitric oxide ($F_{1,10} = 2.85$, $P = 0.12$, rainfall $F_{1,10} = 0.79$, $P = 0.40$). Similarly, hatching order was unrelated to log haptoglobin ($F_{1,6} = 0.08$, $P = 0.78$), log agglutination ($F_{1,6} = 0.01$, $P = 0.94$) or nitric oxide ($F_{1,3} = 0.17$, $P = 0.70$).

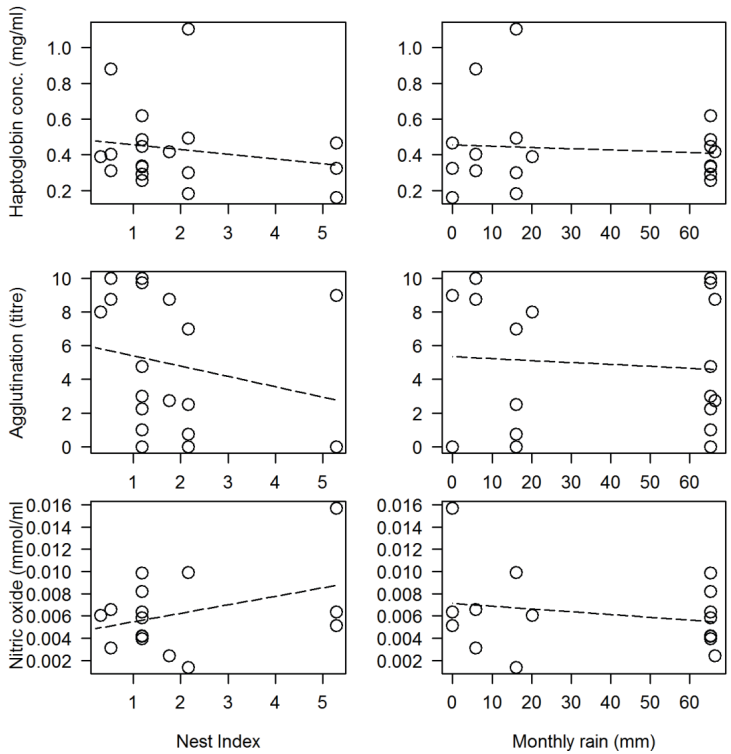

Figure 4. Haptoglobin (mg/ml), agglutination (titre) and nitric oxide (mmol/ml) of 10-day old nestlings of Red-capped Larks *Calandrella cinerea* as a function of nest index and monthly rainfall in Kedong (warm and dry), Kenya.Dashed lines means that the relationship is non-significant.

Discussion

Red-capped Lark nestlings from three climatically-distinct, but geographically close tropical environments differed in growth in ways that suggest independently acting influences of female body condition and food availability for nestlings. Nestling body mass and size at hatching, which presumably reflect the resources that females allocated to their eggs (Arnold 1992, Houston and Donnan 1995, Nager et al. 1997, Christians 2002), were lowest in the most arid location, Kedong. However, contrary to our predictions, nestlings in this location grew faster than in the other two cooler and wetter locations of South and North Kinangop. Consistent with these among-location findings, the differences in growth of nestlings at different times of the year within Kedong, although partially in contrast with our predictions, also pointed to independent effects of female body condition and food availability for nestling growth. At times of the year when more individuals in the Kedong population bred, suggesting conditions for breeding for females were favorable, nestlings had indeed higher body mass at hatching, but they did not grow better in the days thereafter. Where we previously had found that rainfall did not affect the timing of breeding (Ndithia et al. 2017a), we now discovered that rainfall also had no effect on nestling mass at hatching, but that more rain did coincide with faster growth post-hatch. Finally, and unexpectedly, neither the among-location comparison, nor the within-location analysis in Kedong revealed any significant differences in nestling immune function. The results on the variation in nestling growth rate demonstrate the strong roles of female body condition and that of food availability in defining the pace-of-life and variation in life-history strategies within-tropical environments.

Slow growth rates in the tropics have been attributed to the poor food quality and low food availability (Ricklefs 1969), and our within-tropics study also implicates a role of food in explaining differences in growth. Whereas increasing aridity has been associated with slower growth (Tieleman and Williams 2004), growth of Red-capped Larks in our study was actually fastest in the most arid location (Kedong). We propose that the local ecology of the three locations in the current study may not represent a typical aridity gradient with a decrease in primary productivity, and an associated decrease in invertebrates. In a related study, (Ndithia et al. 2017a) – unexpectedly – found that flying invertebrate biomasses were highest in the most arid location, Kedong, and lowest in the cooler and wetter locations, South and North Kinangop. The large amounts of precipitation in South Kinangop, and to a lesser extent in North Kinangop, led to frequent flooding, which we hypothesized negatively impacted the food quality and quantity for nestling larks, with negative consequences for growth (Ricklefs 1976, Boag 1987, Martin 1987). When we fitted growth curves based on data for nestlings that we monitored from hatching to fledging, instead of including all nestlings irrespective of their fate, the growth constants K, increased in South and North Kinangop, but not in Kedong. We interpret this result as additional evidence of food limitation for nestlings in South and North Kinangop, but not in Kedong. Inclusion of nestlings that may have starved to death before getting to fledging age in South and North Kinangop pulled down the K value for these locations. The increased growth constant when restricting analyses to nestlings that fledged, adds to the likelihood that food is a more important

factor in determining nestling growth rate (and immune function) than thermoregulation in this study system.

The within-Kedong findings that nestlings hatched at a higher mass when more larks were breeding, but grew better in times with more rain (that do not coincide with higher numbers of breeding females) raise the question why females did not preferentially breed at times that were best for nestling growth. Numerous studies, mostly of nest-box breeding birds in temperate zones, have shown that females time breeding such that nestlings benefit optimally from the food peak that is common in temperate zone springs (Lack 1950, Perrins 1970, Martin 1987, Visser et al. 1998) . Our results suggest that the timing of breeding by Red-capped Larks is affected by other factors than food, for example nest predation, protein reserves of individual females or social factors (Ndithia et al. 2017a). However, the success of these birds' breeding attempts is at least partly determined by environmental factors, such as rain, that typically correlate with food availability. A lesser role for food may also be concluded from the small and typically constant clutch size of two eggs that fits with a bet-hedging strategy in a high-risk environment (Slagsvold 1984).

A comparison among growth constants (K) of larks from other regions in the world (Tieleman and Williams 2004) indicates that our within-tropical variation in growth of Red-capped Larks spans a range similar to, but lower than the range found among a variety of lark species from different environments. K varied from 0.34 to 0.54 in Red-capped Larks within the tropics (Table 3), and from 0.41 to 0.62 along a gradient from desert to temperate environments (Tieleman and Williams 2004). In general, neotropical passerines have been shown to grow 23% slower than temperate birds (Ricklefs 1976), but within-tropics variation has not received much attention. In contrast with many tropical species (Ricklefs 1997, Ricklefs 1976), but in line with other lark species (Tieleman and Williams 2004), Red-capped Larks did not show hatching asynchrony: hatching order did not affect nestling size, growth or immune function.

The three measured immune indices, haptoglobin, agglutination, and nitric oxide, showed more variation within than among locations. Furthermore, while varying substantially among individual nestlings, the indices did not significantly co-vary with nest index or rain within Kedong. The reliance on measurements at a single time point (i.e., day 10) can complicate comparisons among locations and through time, since the immune systems of nestlings, including components measured in the current study, may develop at different rates across individuals or populations e.g., (Matson et al. 2014). Immunity of nestlings may partly reflect the immunological status of their parents (Hasselquist and Nilsson 2009, Pihlaja et al. 2006, Stambaugh et al. 2011). The fact that only two nestlings had lysis (45 out of 47 nestlings had a value of zero) also supports the idea that the nestling immune system is not yet fully developed, a finding consistent with other studies (Stambaugh et al. 2011, Hegemann et al. 2013). When comparing haptoglobin in 10-day old tropical Red-capped Lark nestlings (Fig. 2) with 8-day old temperate Skylarks (0.28 mg/ml, (Hegemann et al. 2013), haptoglobin values for the latter are closest to the tropical South Kinangop population, which were about twice as low as Kedong and North Kinangop (although the difference was statistically insignificant). Of the three tropical locations, South Kinangop's

relatively cool and wet conditions resemble most closely a temperate environment. Agglutination titres of the Skylark nestlings (2.45) are lower than in all three Red-capped Lark populations (Fig. 2). Combined with the observation that agglutination of adults is higher in Skylarks than in Red-capped Larks (Horrocks et al. 2015), this finding suggests among-species differences in the ontogeny of their immune systems.

In conclusion, we found that nestlings of Red-capped Larks differed in size at hatching and growth rate among three climatically-distinct tropical locations and with year-round variation within location in Kedong. We propose that female quality and resource availability played independent roles in determining these findings. Whereas females in the resource-scarce cool and wet locations (Ndithia et al. 2017a) presumably allocated more resources to their eggs, giving rise to nestlings with larger body mass and size at hatching, parent birds in the more arid but resource-abundant Kedong (Ndithia et al. 2017a) committed more resources to feeding nestlings post-hatch, leading to higher growth rates. In addition, nestlings hatched during times of the year when more individuals were breeding in Kedong, presumably when conditions for breeding for females were favorable, had higher body mass at hatching. Parents in the different locations and at different times of the year in the same location, apply different life-history strategies (adjustment of female body condition and utilization of food availability) leading to differences in the pace-of-life. Innate immunity did not vary among locations and within Kedong it did not co-vary with nesting intensity or rain. Because we measured the immune system at one specific time point (day 10) only, it would be interesting to study the entire development trajectory of the nestling immune system and compare potential differences among and within populations, in relation to environmental conditions

Appendix 1. Mean (±SE), the pairwise locational differences, the mean of the means of males and females and sample sizes per location for adult Red-capped Lark *Calandrella cinerea* that we studied from January 2011 to March 2014. Different letters indicate significant within sex differences among locations (not between sexes within location) in the post-hoc tests.

Variable	Location	Mean ± SE, with pairwise locational differences (P<0.05)		Mean of males and females	Sample sizes per sex	
		Females	Males		Females	Males
Mass (g)	South Kinangop	25.9 ± 0.31^{a}	25.8 ± 0.54^{ab}	25.9	34	17
	North Kinangop	25.5 ± 0.25^{a}	25.7 ± 0.39^{a}	25.6	39	27
	Kedong	24.1 ± 0.18^{b}	24.4 ± 0.30^{b}	24.3	100	53
Wing length (mm)	South Kinangop	90.2 ± 0.46^{a}	94.8 ± 0.74^{a}	92.5	36	17
	North Kinangop	89.9 ± 0.46^{ab}	94.1 ± 0.60^{ab}	92.0	39	27
	Kedong	88.9 ± 0.20^{b}	92.8 ± 0.37^{b}	90.9	100	55
Tarsus (mm)	South Kinangop	25.3 ± 0.20^{ab}	25.9 ± 0.26^{a}	25.6	36	19
	North Kinangop	25.4 ± 0.20^{a}	25.4 ± 0.23^{ab}	25.4	39	26
	Kedong	24.9 ± 0.10^{b}	25.2 ± 0.13^{b}	25.1	100	55

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Chapter 4

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No downregulation of immune function during breeding in two year-round breeding bird species in an equatorial East African environment

Henry K. Ndithia,
Maaike A. Versteegh,
Muchane Muchai,
B. Irene Tieleman

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Abstract

Some equatorial environments exhibit substantial within-location variation in environmental conditions throughout the year and yet have year-round breeding birds. Breeding in birds in such systems are potentially unrelated to the variable environmental conditions. By confirming that environmental conditions do not differ between sampling periods of breeding and non-breeding birds, we become sure that any differences in immune function between breeding and non-breeding birds do not result from environmental variation, therefore allowing for exclusion of the confounding impact of variation in environmental conditions. This create a unique opportunity to test if immune function is downregulated during reproduction compared with non-breeding periods. We compared immune functions of sympatric male and female chick-feeding and non-breeding Red-capped *Calandrella cinerea* and Rufous-naped Larks *Mirafra africana* in equatorial East Africa. These closely-related species occupy different niches and have different breeding strategies in the same grassland. Red-capped Larks prefer areas with short grass almost bare ground and breed during low rainfall periods. Rufous-naped Larks prefer areas with tall grass with scattered shrubs and breed during high rainfall. We measured immune indices nitric oxide, haptoglobin, agglutination and lysis, and measured total monthly rain, monthly average minimum (T_{min}) and maximum (T_{max}) temperatures. Contrary to prediction, we found no downregulation of immune function during breeding: breeding birds had higher nitric oxide than non-breeding ones in both species, while the other three immune indices didn't differ between breeding phases. Red-capped Larks had higher nitric oxide than Rufous-naped Larks, which in turn had higher haptoglobin than Red-capped Larks. The environmental data confirmed that T_{max} was higher during breeding than during non-breeding for Red-capped Larks only, suggesting potential confounding effect of T_{max} on the comparison of immune function between breeding and non-breeding birds for Red-capped Larks. Overall, we conclude that in two year-round breeding equatorial larks, immune function is not downregulated during breeding.

Introduction

Hypothesized as costly, immune function has been proposed to be compromised during demanding life cycle events, most particularly during reproduction when animals allocate resources to production and care of offspring (Bonneaud et al. 2003a, Ardia 2005a, Greenman et al. 2005, O'Neal and Ketterman 2012). If life cycle events such as reproduction or migration demand resources that could otherwise be invested in immune function, the result will be seasonal variation in immune function. Trade-offs between immune function and reproduction have been proposed to be especially manifest in short-and-fast lived species that have evolved a life-history strategy which favours reproduction over self-maintenance. In contrast, long-and-slow lived species are hypothesized to maintain functions that increase survivorship, even under challenging conditions (e.g. reproduction, incremental weather) (Vindervogel et al. 1985, Hughes et al. 1989, Allander and Sundberg 1997, Christe et al. 2000). Although direct evidence from experimental studies for a trade-off between immunity and reproduction is mixed (see Tieleman 2018 for a review), seasonal variation in immune function has been reported in multiple studies of temperate and arctic zone birds (Martin et al. 2008, Buehler et al. 2008, Pap et al. 2010a, Pap et al. 2010b, Hegemann et al. 2012, Hegemann et al. 2012, Horrocks et al. 2012). During non-breeding, immune function has been shown to be elevated as individuals are free from reproductive activities that can be energetically and physiologically immunosuppressive (Lee 2006, Martin et al. 2008, Pap et al. 2010a, Pap et al. 2010b).

Because in temperate and arctic zones reproduction is restricted to the spring season, seasonal variation in immune function in these regions could also be explained by seasonally changing environmental conditions. Physiological changes in birds from temperate and arctic zones are mainly driven by day-length (e.g., Gwinner 2003, Versteegh et al. 2014), which also plays a major role in determining seasonal changes in environmental factors such as temperature, food availability and pathogen pressure that may also have more direct consequences on immune function of birds. Although in some tropical environments there are examples of tropical bird species, e.g., stonechats *Saxicola torquatus axillaris* and Spotted antbirds *Hylophylax naevioides naevioides*, that use small changes in sunrise and sunset times to regulate annual cylce activities (Goymann et al 2012, Dittami and Gwinner 1985, Hau et al. 1998, Hau 2001), some equatorial tropical environments have been referred to as aseasonal (environmental variation occurring in any month of the year) or have seasonality orchestrated by rainy and dry seasons instead of day-length and temperature (Conway et al. 2005, Ndithia et al. 2017a) and many bird species breed opportunistically and asynchronously. Large variation among and within tropical regions makes general characterization of environmental conditions at equatorial latitudes difficult. Yet, with generally low variability in day-length and with occurrence of substantial within-location variation in environmental conditions, many equatorial tropical bird species breed year round. If and how immune function of such year-round breeding equatorial species varies with reproduction, independent of environmental conditions, is not known.

Immune responses can be sensitive to environmental variation (Nelson and Demas1996, Marra and Holberton 1998, Shepherd and Shek 1998, Ruiz et al. 2002, Tieleman et al. 2005), and

sympatric species can differ in their immune responses, for example if they occupy different ecological niches or have different reproductive strategies. In addition, immune function of the same bird species may differ between sexes due to differences in the roles males and females play during reproduction (Sossinka 1980, Emerson and Hess 1996, Møller et al. 2003, Hau et al. 2004) or due to fundamental differences in male and female life-histories (Zuk 1996, Hasselquist 2007, Nunn et al. 2009). Studying males and females of different bird species under the same tropical environmental conditions creates the opportunity for a broader perspective of life history trade-offs in tropical birds (Stutchbury and Morton 2008). To our knowledge, no study has yet evaluated the effects of reproduction on immune function, while excluding those of environmental conditions.

Additionally, immune function has been hypothesized to vary with the pace-of-life in birds. Temperate and arctic bird species exhibit reduced investment in the immune function and increased investment in reproduction (Ricklefs and Wikelski 2002, Martin et al. 2004, Tieleman et al. 2005). Conversely, equatorial tropical birds optimize survival (investment in immune defense) over reproduction (Martin et al. 2006, Cox et al. 2010, Previtali et al. 2012) through small clutch sizes. Longer-and-slower lived species are known to manifest a strategy that favors the maintenance of functions that increase survivorship, such as immune capacity, even under challenging conditions such as reproduction (Ardia 2005, Lee 2006, Lee et al. 2008, Tella et al. 2002).To explore if immune function is downregulated during reproduction, while excluding the potential confounding effects of environmental conditions, we exploited a unique study system of year-round breeding by two sympatric tropical bird species, Red-capped Larks *Calandrella cinerea* and Rufous-naped Larks *Mirafra africana*, in North Kinangop, Kenya. Our equatorial study location is characterized by large and unpredictable intra-and-inter-annual variations in rainfall (Ndithia et al. 2017a). The co-occurrence of the two study species, and their occupation of different niches within the same grassland environment, provides an opportunity for interspecific comparison of reproduction-induced variation in immune function. Our previous study on Red-capped Larks in three Kenyan locations including North Kinangop revealed that, at the population level, nesting activities in this species fluctuate year-round and are unrelated to rainfall, temperature or invertebrate abundance (Ndithia et al. 2017a). Experiencing the same unpredictable intra-and-inter-annual variations in rainfall, Rufous-naped Larks also exhibit year-round breeding although often not synchronously with Red-capped Larks (H.K.N. pers. obs.). We therefore presumed that breeding in Rufous-naped Larks was also unrelated to rainfall, temperature or invertebrate abundance. Since environmental conditions did not influence reproductive decisions (Ndithia et al. 2017a), this novel study system enables investigating associations between reproductive activities and immune function.

We asked how immune function of males and females of Red-capped and Rufous-naped Larks differed between breeding (chick-feeding) and non-breeding birds living in the same equatorial environment that is generally permissive of year-round breeding, and where timing of breeding is not governed by day length, rainfall, temperature or resource availability (Ndithia et al. 2017a). We compared immune functions of these two species that live in the same open

grasslands, yet occupy different niches within these grasslands and exhibit different reproductive strategies (see methods for further details). Then, to confirm that any differences in immune function between breeding and non-breeding in the two species do not result from environmental variation, we tested if rainfall, average minimum (T_{min}) and average maximum (T_{max}) temperatures differed between breeding and non-breeding. We expected non-breeding birds to generally have increased investment in immune function and breeding (chick-feeding) ones to have depressed immune function due to expected trade-off between these two physiological processes (Nelson and Demas 1996, Bentley et al. 1998, Martin et al. 2008). Because we previously did not find any relationship between rain, T_{min} or T_{max} and nesting activity at the population level in North Kinangop (Ndithia et al. 2017a), we did not expect these environmental variables to differ between breeding and non-breeding birds in any of the two species.

Methods

Study species and study area

Red-capped and Rufous-naped Larks are sympatric bird species with wide distributions ranging from savannas with altitudes of 1200 m above sea level (ASL) to highland grasslands 2600 m ASL (Zimmermann 1999). Red-capped Lark is a small (mean mass, 25.6 ± 1.54 (SD), n = 66) gregarious bird of short grass to bare ground. Rufous-naped Lark is a larger (mean mass, 46.6 ± 4.11 (SD), n = 14) territorial bird that prefers areas with tall grass and scattered shrubs. Both species feed on a variety of invertebrates and occasionally on grass seeds. The two species breed year-round but potentially with different timing of breeding (H.K.N. pers. obs.). They build open-cup nests on the ground, often next to a scrub or grass tuft. Both species have a clutch size of two (Red-capped Lark, mean, 2.0 ± 0.00 (SD), n = 59; Rufous-naped Lark, mean, 2.0 ± 0.00 (SD), n = 31). Incubation and nestling phase each lasts ca. 10-12 days in both Red-capped and Rufous-naped Larks (Ndithia pers. obs.). In both species, only females build nest and incubate but both sexes feed nestlings. Red-capped Larks occurs in large non-territorial flocks when not breeding, but in pairs defending the area around the nest during breeding periods. In contrast, Rufous-naped Larks defend territories in pairs during breeding and non-breeding periods (H.K.N. pers. obs.). Color ring re-sightings suggest that both species are sedentary to our study locations year-round.

We studied both Red-capped and Rufous-naped Larks in three plots in North Kinangop, including Joshua ($0^0$36'00"S, $36^0$28'27"E, 2451 m ASL), Mbae ($0^0$36'54"S, $36^0$30'48"E, 2425 m ASL) and Ndarashaini ($0^0$34'33"S, $36^0$29'41"E, 2412 m ASL). Local variation in soil type, hydrology and rainfall among and within these plots created distinct grassland micro-habitats which the two species occupied and utilized. Red-capped Larks preferred the drier parts of the grassland with very short grass, almost bare ground, and bred during low rainfall periods. On the other hand, Rufous-naped Lark preferred the wetter areas with tall grass and scattered shrubs and bred during high rainfall periods. These micro-habitats has the potential of harboring different pathogen pressure (microorganisms and parasites). We selected plots based on information from local bird watchers and ourselves about the occurrence of the lark species, and based on provision

of permission to access the areas. We worked year-round and simultaneously in these plots from January 2011 to March 2014.

North Kinangop receives on average 584 ± 62.6 (SD) mm of rain per year, and experiences variation in monthly mean T_{min} between 3.0 and 13.7ºC, and monthly mean T_{max} between 22.1 and 30.5ºC (for details of climatic conditions, see Ndithia et al. (2017a). Annual variation in sunrise and sunset times at our study location is less than 35 minutes (Gwinner and Scheuerlein 1999). Despite some tropical species, e.g., the African stonechat and the Spotted Antbird using small changes in sunrise and sunset times to regulate their annual cycle activities (e.g., reproduction, moult) (Goymann et al. 2012, Dittami and Gwinner 1985, Hau et al. 1998, Hau 2001), environmental variation in rainfall and temperature in our study location are independent of calendar month (they are non-seasonal and occur in month of the year) (Ndithia et al. 2017a), our study species breed year round, opportunistically and asynchronously, and breeding is unrelated to any of the possible proximate factors – rainfall, temperature or food supply (Ndithia et al. 2017a).

Field sampling and recording of environmental abiotic variables

We caught non-breeding adult males and females using mist nets and we used cage traps to catch adult males and females at the nest sites during chick feeding. For Red-capped Larks, we sampled five and 13 female non-breeding and chick-feeding birds respectively, and 10 males each for non-breeding and chick-feeding. Only one of these birds, a male, was sampled during both non-breeding and chick-feeding. For Rufous-naped Larks, we sampled four and five female non-breeding and chick-feeding birds respectively, and five and three male non-breeding and chick-feeding birds respectively. We sampled only one male and one female during both non-breeding and chick-feeding. Sampling of the two species partly co-occurred in the same calendar month, and partly occurred in different calendar months, depending on their breeding activities.

From each individual, we collected a blood sample for immune function analyses using heparinized capillary tubes after carefully puncturing the brachial vein on the wing. We put blood samples in eppendorf tubes, temporarily stored them in ice and centrifuged them at the end of each fieldwork day. We stored the plasma fraction in the freezer (-20^0C) for future analyses. To obtain total monthly rainfall (mm), monthly average minimum (T_{min}) and monthly average maximum (T_{max}) temperatures ($^{\circ}$C), we set up a weather station (Alecto WS-3500, Den Bosch, Netherlands) in a secure location placed centrally to the three field sites. Direct distances from weather station locations to field sites were as follows: 3.8 km to Joshua; 2.5 km to Mbae and 1.8 km to Ndarashaini (Ndithia et al. 2017a).

Immune assays

Haptoglobin (mg/ml) is an acute phase protein that scavenges haemoglobin in the event of haemolysis and increases several fold in the event of infection, injury or malignancy (Quaye 2008). We determined haptoglobin concentration using an assay that measures the haem-binding capacity of plasma (TP801; Tridelta Development limited, Maynooth, Ireland) following instructions

provided by the manufacturer and with incubation at 30^0C for 5 minutes following Matson et al. (2012). Each of the three assay plates, included an among-plate standard which we ran in duplicate within each plate (Matson et al. 2012) (mean within-plate coefficient of variation (CV) = 2.4%; mean among-plate CV=2.7%).

Nitric oxide (mmol/ml) is a multifunctional signalling molecule that, among others, modulates inflammatory processes and participates in destroying parasites, virus-infected cells and tumor cells, providing information about animal condition (Sild and Hõrak 2009). We determined nitric oxide production through the reduction of nitrate to nitrite by copper-coated cadmium granules, followed by color development with Griess reagent (Promega; Sild & Hõrak 2009) and absorbance measurement at 542 nm (Versamax, Molecular Devices Sunnyvale, California, US) (Sild and Hõrak 2009).

Complement (hemolysis) and natural antibodies (hemagglutination) are constitutively present in the innate immune system (Matson et al. 2005). We quantified complement lysis titres and natural antibody agglutination titres against red blood cells of rabbit (Envigo, Belton, UK) through serially diluting plasma samples according to the assay of Matson et al. (2005). Lysis indicates the interaction of complement and natural antibodies. Agglutination reflects the interaction between natural antibodies and antigens of rabbit red blood cells. We scored hemolysis and hemagglutination titres blind to sample and plate identity at least twice. We used the mean in the analyses if the first two scores were less than one titre apart. If the difference of the first two scores was more than one, we scored a third time and used the median in analyses (Matson et al. 2005). We assigned half scores to samples that showed intermediate lysis and agglutination. We calculated among-plate and within-plate variation for lysis (mean among-plate CV=18.6%; mean within-plate CV=9.8%), and for agglutination (mean among-plate CV=9.7%; mean within-plate CV=7.7%).

Statistical analyses

We used generalized linear models (glm) with normal (Gaussian) distribution for analyses of haptoglobin, nitric oxide and agglutination, and with binomial distribution for analysis of lysis. Although perhaps ideal, using a mixed-effects model with either bird ID or nest ID as random factor was precluded by the design of the data set: we sampled different individuals during breeding and non-breeding, while during breeding we mostly sampled males and females that attended different nests (all individuals for Rufous-naped Larks, and 40% of the individuals for Red-capped Larks). To test for differences in immune function and mass (g) between breeding and non-breeding in different sexes of Red-capped and Rufous-naped Larks, we constructed a model of each immune index (haptoglobin, nitric oxide, agglutination and lysis) as dependent variables and with explanatory variables breeding status, species, sex and all three-way and two-way interactions. We square-root transformed data of haptoglobin and log-transformed data of nitric oxide to obtain normality because the residuals of models for these two indices were not normally distributed.

The haptoglobin assay may be affected by plasma sample redness due to sample hemolysis (Matson et al. 2012). During the assay, we did a 450 nm pre-scan to enable us to statistically correct for plasma sample redness. In addition, plasma sample age (range in sample age: 82 – 1256 days) possibly affects immune assays involving haptoglobin, nitric oxide, agglutination and lysis. In cases where plasma sample redness significantly affected haptoglobin and where plasma sample age significantly affected any of the immune indices, we included them in the respective models as a covariate. Haptoglobin was affected by plasma sample age ($F_{1, 49}$ =9.78, P = 0.003) and plasma sample redness ($F_{1, 49}$ =10.49, P = 0.002), and plasma sample age affected nitric oxide ($F_{1, 46}$ = 5.88, P = 0.02), agglutination ($F_{1, 48}$ = 8.12, P = 0.01) and lysis (X^2 = 5.07, d.f. =1, P = 0.02). We tested the effect of breeding status on mass for both lark species using two separate models because Rufous-naped Larks are almost twice the mass of Red-capped Larks. Each model included mass as dependent variable and explanatory variables breeding status, sex and their interaction.

To check whether environmental conditions confound the effect(s) of breeding on immunity, we tested if environmental conditions (rain, T_{min} and T_{max}) differed during chick-feeding and non-breeding periods in males and females of the two species by matching the date (month) of the immune measurement of each individual bird with the corresponding total monthly rainfall, T_{min} and T_{max}. Using rain, T_{min} and T_{max} as dependent variables, we build models that included explanatory variables breeding status, species, sex, and all three-way and two-way interactions.

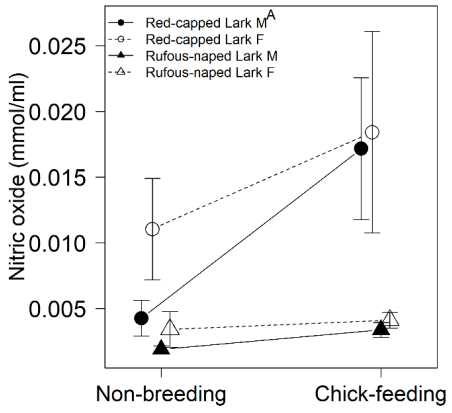
We used type III sum of squares in the anova summary of results to test main effects in the light of interaction terms as well as in the light of other main effects (Mangiafico 2015). Whenever an interaction was significant, we made a new variable consisting of all the separate variables in the interaction and did a Tukey's post hoc test on this new variable. For all analyses, we tested and confirmed that the residuals of the final models observed the assumptions of normality and homoscedasticity of variance through graphical and statistical methods. We simplified models using backward elimination by deleting the least significant terms from the model until we arrived at the final model and used $P < 0.05$ as selection criterion. The final model consisted of all the significant terms, any of the non-significant main effects of breeding status, species and sex and co-variates (sample age, sample redness) if any and applicable. We used R statistical software (version 3.0.3) (R Core Team 2014) in all our analyses.

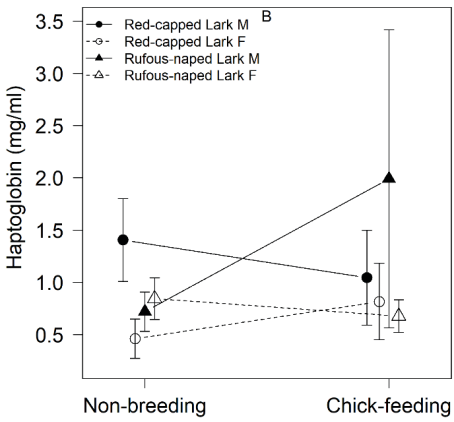
Results

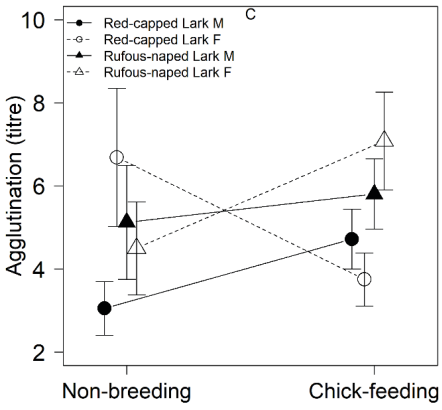
Immune function and body mass during breeding and non-breeding

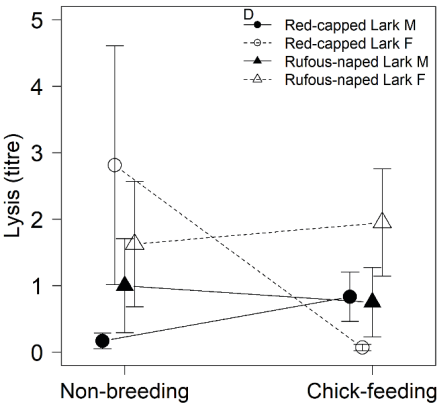
We found no uniform differences between chick-feeding and non-breeding larks for the four immune indices, but some indices did vary with breeding status and between species and sexes (Fig 1A-D, Table 1). We found significant effects of breeding status and species for nitric oxide, a significant effect of species on haptoglobin and a significant three-way interaction of breeding status *x* species *x* sex for agglutination (Fig 1A, 1B, 1C; Table 1). Chick-feeding birds had significantly higher nitric oxide than those that were not breeding in both species, while Red-capped Larks had significantly higher nitric oxide than Rufous-naped Larks (Fig 1A). Conversely,

Rufous-naped Larks had significantly higher haptoglobin than Red-capped Larks (Fig 1B). Post-hoc tests to further explore the significant three-way interaction breeding status *x* species *x* sex for agglutination only revealed that non-breeding females had higher agglutination than non-breeding males in Red-capped Lark (t = 3.39, P = 0.02, Fig 1C); all other pairwise comparisons were non-significant (all P > 0.18). We did not find an effect of breeding status on haptoglobin or effects of breeding status and species on lysis, while nitric oxide, haptoglobin and lysis did not differ significantly among sexes (Fig 1A, 1B, 1D, Table 1).). Body mass tended to be lower during chick-feeding than during non-breeding in Red-capped Larks males and females and in Rufous-naped Larks females, but not in Rufous-naped Larks males (Fig 1E, 1F).









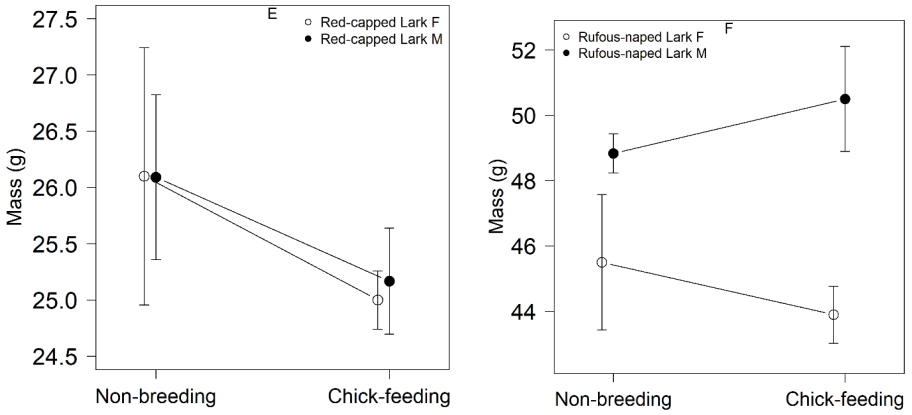


Figure 1. A. Nitric oxide (mean ± SE, mmol/ml), B. Haptoglobin (mean ± SE, mg/ml), C. agglutination (mean ± SE, titre), D. Lysis (mean ± SE, titre), E. and F. mass (g) in chick-feeding and non-breeding Red-capped Larks *Calandrella cinerea* and Rufous-naped Larks *Mirafra africana* in North Kinangop, Kenya. Samples sizes were as follows: Red-capped Lark females, non-breeding = 5, chick-feeding = 13, Red-capped Lark males, non-breeding = 10, chick-feeding = 10: Rufous-naped Lark females, non-breeding = 4, chick-feeding = 5, Rufous-naped Lark males, non-breeding = 5, chick-feeding = 3).

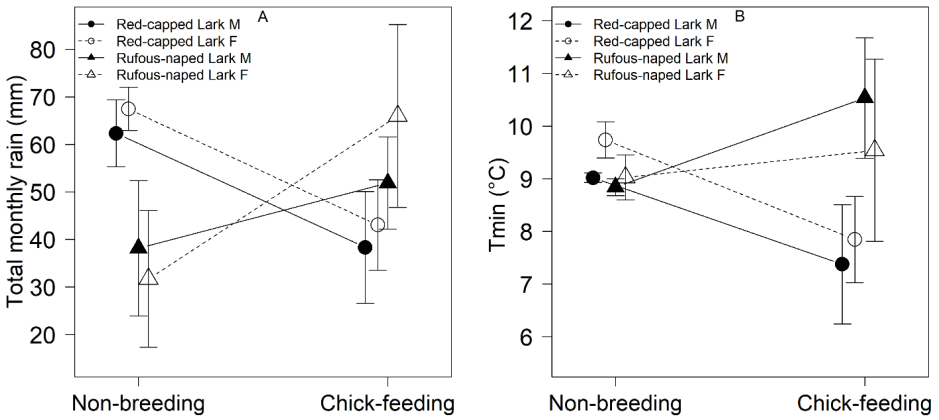
Breeding status had a marginally non-significant effect on body mass in Red-capped Larks, while sex had no effect (Fig 1E, Table 1). In contrast, body mass in Rufous-naped Larks did not significantly differ between breeding and non-breeding, but it did with sex where males were significantly heavier than females (Fig 1F, Table 1).

Table 1. Results of models examining variation in immune function between chick-feeding and non-breeding male and female Red-capped Larks *Calandrella cinerea* and Rufous-naped Larks *Mirafra africana* in North Kinangop, Kenya. Nitric oxide data was log-transformed and haptoglobin data was square root transformed to obtain normality. P values < 0.05 are indicated in bold.

Variable	Explanatory variable	DF	F	P
Nitric oxide (mmol/ml)	breeding status x species x sex	1,39	0.34	0.56
	species x sex	1, 40	0.15	0.70
	breeding status x species	1, 41	0.43	0.51
	breeding status x sex	1, 42	2.84	0.10
	sex	1, 43	1.34	0.25
	breeding status	1, 44	5.17	**0.03**
	species	1, 44	8.69	**0.005**
Haptoglobin (mg/ml)	breeding status x species x sex	1, 41	0.65	0.43
	breeding status x species	1, 42	0.46	0.50
	breeding status x sex	1, 43	0.62	0.44
	species x sex	1, 44	2.70	0.11
	breeding status	1, 45	1.82	0.18
	species	1, 45	4.85	**0.03**
	sex	1, 45	2.82	0.10
Agglutination (titre)	breeding status x species x sex	1,41	6.46	**0.01**
Lysis (titre)	breeding status x species x sex	1,41	1.34	0.25
	breeding status x species	1, 42	0.006	0.94
	species x sex	1, 43	0.37	0.54
	breeding status x sex	1, 44	2.82	0.09
	sex	1, 45	0.10	0.76
	species	1, 45	0.14	0.71
	breeding status	1, 45	0.25	0.62
Mass (g), Red-capped Lark	breeding status x sex	1, 35	0.01	0.93
	sex	1, 36	0.01	0.94
	breeding status	1, 36	3.37	0.07
Mass (g), Rufous-napped Lark	breeding status x sex	1, 11	1.25	0.29
	breeding status	1, 12	0.04	0.85
	sex	1, 12	11.65	**0.005**

Rainfall and temperature during breeding and non-breeding

Red-capped and Rufous-naped Larks appeared to experience differences in rainfall, T_{min} and T_{max} between chick-feeding and non-breeding, despite living in the same environment (Fig. 2). Remarkably, patterns were opposite in the two lark species: Red-capped Larks appeared to experience relatively low rainfall, low T_{min} and high T_{max} when they fed chicks compared to when they did not breed. In contrast, Rufous-naped Larks appeared to experience higher rainfall, higher T_{min} and lower T_{max} when they were feeding chicks than when not breeding (Fig 2). When statistically testing these patterns, the interaction breeding status *x* species was significant for rain and T_{max} but not for T_{min} which was marginally non-significant and for which the main effects were also not significant (Table 2). Subsequent post-hoc tests revealed that rainfall was not significantly lower during chick-feeding compared to when not breeding in both species (Red-capped Larks $t = 2.35$, $P = 0.08$; Rufous-naped Larks $t = 1.69$, $P = 0.29$). Although insignificant, rainfall tended to be higher when Red-capped Larks were not breeding than when Rufous-naped Larks were not breeding ($t = 2.31$, $P = 0.08$), while the difference in rainfall was non-significant when the two species were feeding chicks ($t = 1.57$, $P = 0.34$). Post hoc tests further showed that Red-capped Larks fed chicks at significantly higher T_{max} than when they were not breeding ($t = 4.50$, $P < 0.001$), but T_{max} did not differ between chick-feeding and non-breeding periods in Rufous-naped Larks ($t = 0.68$, $P = 0.88$). Red-capped Larks fed chicks at significantly higher T_{max} than Rufous-naped Larks ($t = 2.77$, $P = 0.03$), but T_{max} did not differ between the two species when they were not breeding ($t = 1.73$, $P = 0.27$).



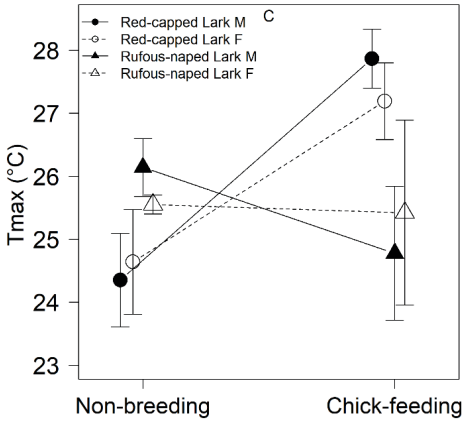


Figure 2. Relationships between (A) = rain, (B) = T_{min}, (C) = T_{max}) and the different breeding status of male and female Red-capped Lark *Calandrella cinerea* and Rufous-naped Lark *Mirafra africana* in North Kinangop, Kenya. Samples sizes were as follows: Red-capped Lark females, non-breeding = 5, chick-feeding = 13, Red-capped Lark males, non-breeding = 10, chick-feeding = 9: Rufous-naped Lark females, non-breeding = 4, chick-feeding = 5, Rufous-naped Lark males, non-breeding = 5, chick-feeding = 3).

Table 2. Results of models testing relationships between abiotic environmental factors (rain, mm) average minimum temperature ($T_{min,}$ ^{0}C) and average maximum temperature (T_{max} ^{0}C) and chick-feeding and non-breeding male and female Red-capped Larks *Calandrella cinerea* and Rufous-naped Larks *Mirafra africana* in North Kinangop, Kenya. Significant P values <0.05 are in bold.

Environmental variable	Explanatory variable	DF	F	P
Rain (mm)	breeding status *x* species *x* sex	1, 46	0.32	0.58
	species *x* sex	1, 47	0.0021	0.96
	breeding status *x* sex	1, 48	0.15	0.70
	sex	1, 49	0.26	0.61
	breeding status *x* species	1, 49	7.59	**0.01**
T_{min} (^{0}C)	breeding status *x* species *x* sex	1, 46	0.10	0.75
	breeding status *x* sex	1, 47	0.17	0.69
	species *x* sex	1, 48	0.42	0.52
	breeding status *x* species	1, 49	3.51	0.07
	breeding status	1, 50	1.43	0.23

	species	1, 50	1.83	0.18
	sex	1, 50	0.11	0.74
T_{max} (^{0}C)	breeding status *x* species *x* sex	1, 46	0.79	0.38
	species *x* sex	1, 47	0.04	0.85
	breeding status *x* sex	1, 48	0.06	0.81
	sex	1, 49	0.15	0.70
	breeding status *x* species	1, 49	10.06	**0.003**

Discussion

Studying four immune indices in two sympatric bird species, Red-capped and Rufous-naped Larks in equatorial East Africa, we found that haptoglobin, agglutination and lysis did not differ between breeding and non-breeding, but nitric oxide did, although contrary to prediction; chick-feeding birds had higher nitric oxide than those that were not breeding. Although sex did not affect any of the immune indices, there was high variation in all immune indices (except nitric oxide) and in body mass of Rufous-naped Larks between males and females, suggesting that immune function of different sexes responded either due to their different reproductive role, or due to differences in life-histories. It also depict the complexity of the immune function. Nitric oxide and haptoglobin differed between species with Red-capped Larks having higher nitric oxide than Rufous-naped Larks, which in turn had higher haptoglobin than Red-capped Larks, suggesting differences in life history adaptations of sympatric species facing variable and unpredictable environmental conditions. Non-breeding females had higher agglutination than non-breeding males in Red-capped Larks, the only immune index affected by sex. Body mass did not differ between breeding and non-breeding in any of the two species. Sex had an effect on body mass only in Rufous-naped Larks, with heavier males than females. The environmental data confirmed that rainfall and T_{min} did not differ between breeding and non-breeding birds for both species. This was also the case for T_{max} for Rufous-naped Larks, but for Red-capped Larks T_{max} was higher during chick-feeding than during non-breeding. Hence, for Red-capped Larks we cannot fully rule out a confounding effect of environmental conditions (i.e. T_{max}) on the comparison of immune function between breeding and non-breeding birds. Overall, we conclude that two tropical larks do not downregulate immune function during breeding. We propose that a productive future step would be to study if and how the highly variable environmental conditions shape variation in immune function in this system.

We had expected chick-feeding birds to have depressed immune function due to the expected trade-off between reproduction and immune function. On the contrary, our results indicated chick-feeding birds of both species to have higher nitric oxide than those that were not breeding, while the other immune indexes did not differ between breeding and non-breeding. This suggest that these species have the capacity to maintain both of these physiological processes simultaneously without adjustment of either. This is in line with other studies that show that

immune function vary with the pace-of-life (Martin et al. 2006, Cox et al. 2010, Previtali et al. 2012). We can conclude that the hypothesized immunosuppression due to the cost of reproduction is not generally applicable to all birds. Our findings could be in support of the more nuanced hypothesis that immunosuppressive costs of reproduction are more manifest in short-and-fast lived species than in long-and-slow lived birds. Although we have no data on the life expectancy of these lark species, tropical birds are generally thought to be longer-and-slower lived with well-developed immune defences (Martin et al. 2006, Lee et al. 2008). Hence it may not be surprising that these tropical larks follow a strategy that favours the maintenance of functions that increase survivorship such as immune capacity even under challenging conditions such as reproduction (Ardia 2005, Lee 2006, Lee et al. 2008, Tella et al. 2002). Several other studies have also demonstrated immunocompetence in equatorial tropical birds even during reproduction (Christe et al. 2000, Allander and Sundberg 1997, Vindervogel et al. 1985, Hughes et al. 1989). This strategy may in fact be less demanding because of the relatively small clutch size (both of these species have a clutch size of two, see methods) (see Deerenberg et al. 1997, Moreno et al. 1999, Hanssen et al. 2005) compared to temperate Skylarks *Alauda arvensis* (mean = 3.53 + 0.43, SE, range, 3-5, n=33) (Wilson et al. 1997, Delius 1965) and Woodlarks *Lullula arborea* (mean = 4.05+ 0.06, SE) (Wright et al. 2009). The elevation instead of downregulation of nitric oxide could mean that breeding individuals are more immunocompetent and/or less challenged than non-breeding ones. An alternative explanation is that challenged females omit breeding, automatically selecting only for birds with low nitric oxide in our sample. Both explanation would be in line with a long life expectancy.

In the case of the Red-capped Larks, the elevated nitric oxide during breeding coincided with higher T_{max}. This co-occurrence raises the possibility that high T_{max} provided a conducive environment for growth, development and reproduction of microorganisms and parasites (Sehgal et al. 2011, Zamora-Vilchis et al. 2012), and that birds responded to this with elevation of nitrogen oxide. Immune function is known to vary based on prevailing environmental conditions in tropical (Rubenstein et al 2008), desert (Horrocks et al. 2012) and temperate (Christe et al. 2001, Altizer et al. 2006, Hegemann et al. 2012) birds, and birds increase their immune function due to abundance of parasites and microbes in their environments (Christe et al. 2001, Møller et al. 2003, Horrocks et al. 2012).

Red-capped Larks had relatively high nitric oxide and low haptoglobin compared with Rufous-naped Larks while the two species live in the same environment. This either suggests within-location variability in exposure to disease and parasites, interspecific differences in susceptibility or interspecific differences in immune strategies to deal with the same problem. Although the two species generally take similar diets and have access to similar amounts of food supply, they occupy different niches within these grasslands (see methods for details of these differences). High variability in patterns of rainfall within North Kinangop tends to create distinct micro-habitats occupied by the two species with potentially different vulnerabilities. Microbial communities and parasites can vary in space even within a population (Bensch and Åkesson 2003, Knowles et al. 2010, Froeschke et al. 2010, Angel et al 2010). Other studies comparing birds living

in the same environment have also found that some species are more susceptible to disease than others (Perkins and Swayne 2003, Tumpey et al. 2004). In addition, the immune function of different species may respond differently to the same parasite or microbial infection in their environment (Blount et al. 2003, Matson et al. 2005, Pap et al 2010a, Pap et al 2010b), and immune function may also differ among species due to body size differences (Hasselquist 2007).

Females had higher agglutination than males in Red-capped Larks during non-breeding periods. Females frequently display more robust immune response than males owing to either different roles of sexes or to fundamental differences in male and female life-histories (Zuk 1996, Hasselquist 2007, Nunn et al. 2009). In our earlier study, we proposed that Red-capped Larks may be an opportunistic breeder, breeding whenever environmental conditions are permissive (Ndithia et al. 2017a). Opportunistically breeding males may invest in maintaining their reproductive capacity activated all year round in order to respond quickly to favourable reproductive conditions (Sossinka 1980, Emerson and Hess 1996, Hau et al. 2004), in which case the relatively high levels of reproductive hormones in males may suppress the immune function (Møller et al. 2003).

Our study system exemplifies the high variability in environmental conditions in equatorial tropics and how sympatric bird species living under these conditions utilize them differently to presumably maximize reproductive success and optimize their protection against diseases and parasites. Our study draws a sharp contrast in temporal variation in immune function between temperate and equatorial tropical birds, in light of the timing of life cycle events such as reproduction. It supports the proposition that equatorial tropical birds, exhibiting a slow pace-of-life strategy, optimize survival (investment in immune function) over reproduction (small clutch sizes). Further studies need to investigate the temporal patterns of pathogen and parasite pressures year-round, and to investigate the potential role of environmental conditions, particularly T_{max}, in the temporal variation of immune function. Further studies also need to investigate the different functions of the different components of immune function year-round and to relate this to the temporal patterns of pathogen and parasite pressure and year-round environmental conditions. An interesting further study topic would to be investigate variation in immune function on individuals during the year and during different breeding events.

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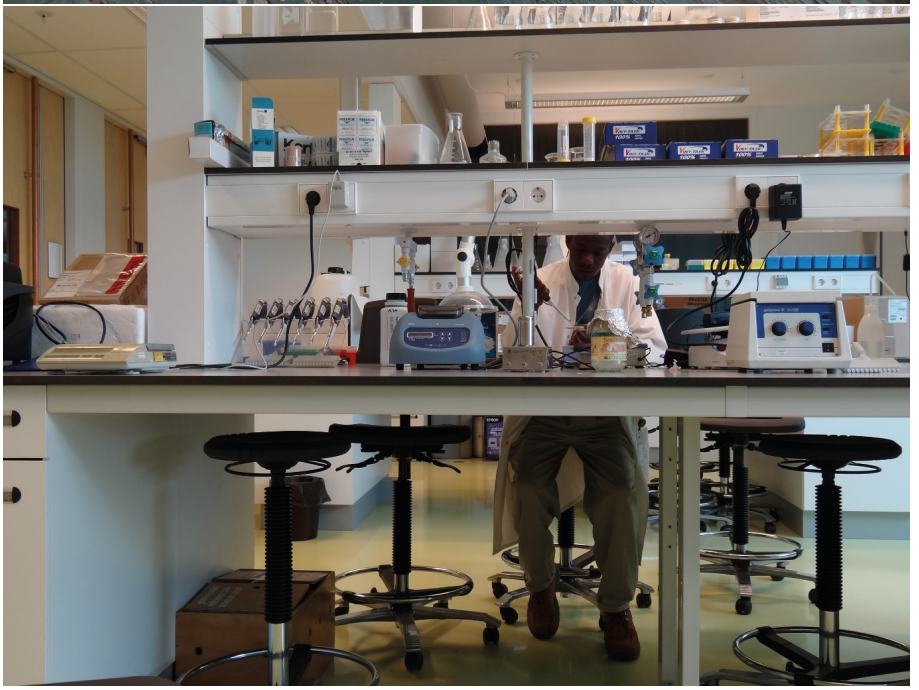

Chapter 5

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Environment, not reproduction explains variation in immune function in three year-round breeding equatorial lark populations

Henry K. Ndithia,
Kevin D. Matson,
Muchane Muchai,
B. Irene Tieleman

Unpublished manuscript

Abstract

Seasonal variation in immune function has been attributed to life history trade-offs, including downregulation during reproduction, and to variation in environmental condition. However, because life cycle stage and environmental conditions co-vary in the temperate and arctic zones where seasonal variation in immune function has been mostly studied, their separate contributions thus far have not been determined. We compared immune function and body mass of incubating (females only), chick-feeding (males and females), and non-breeding (males and females) Red-capped Larks *Calandrella cinerea* in three equatorial tropical environments, where birds breed year round. Working with birds in the relatively cool and wet South Kinangop, warm and wet North Kinangop and warm and dry Kedong, Kenya, we measured body mass and four immune indices: haptoglobin, nitric oxide, agglutination, and lysis. In order to confirm that variation in immune function between breeding and non-breeding was not confounded by environmental conditions, we tested if rainfall, average minimum temperature (T_{min}) and average maximum temperature (T_{max}) differed between breeding stages per location. We found higher concentrations of nitric oxide, hence upregulation instead of downregulation, during incubation in South Kinangop and during chick-feeding in North Kinangop compared to non-breeding birds in the same locations. Agglutination, haptoglobin, lysis, and body mass did not differ between breeding and non-breeding in any of the locations. During breeding, T_{min} was lower compared to non-breeding in all three locations, while T_{max} was higher during breeding compared to non-breeding periods in North Kinangop and Kedong. T_{max} was also higher when females were breeding compared to when they were not breeding in Kedong. Populations in the three climatically distinct locations differed in multiple immune indices. Thus, the immune indices we measured were seemingly more influenced by environmental conditions rather than by investment in reproduction. We propose that bird populations living in different environments develop immune strategies that are shaped by the prevailing environmental conditions through the environmental influence on disease risks.

Introduction

Seasonal variation in immune function has been attributed to life history trade-offs and to variation in environmental conditions (Sheldon and Verhulst 1996, Tieleman 2018). But these factors co-vary in temperate and arctic areas where seasonal variation in immune function has been studied. Thus, disentangling the effects of life history and environmental variation has presented a challenge to ecologists.

Certain events associated with an organism's life history, such as reproduction and migration, can be resource demanding (Martin et al. 2008, Piersma 1997). Consequently, these events may result in trade-offs with the immune system, a critical component of self-maintenance and survival (Buehler et al. 2008, Hegemann et al. 2012, Hegemann et al. 2012, Horrocks et al. 2012, Ilmonen et al. 2000, Martin et al. 2008). Seasonal variation in constitutive innate immune function in birds from temperate and arctic zones has been attributed to such trade-offs (Buehler et al. 2008, Hegemann et al. 2012a, Hegemann et al. 2012b, Horrocks et al. 2012a, Ilmonen et al. 2000, Martin et al. 2008). Yet other studies provide contrary evidence showing that immune function is maintained in the face of reproduction and other supposedly competing physiological processes, e.g. endocrinological changes (e.g. Møller et al., 2003, Alonso-Alvarez et al. 2007, Christe et al. 2000, Allander and Sundberg 1997, Vindevogel et al. 1985).

Immune function also varies with the abiotic conditions of an animal's environment (Horrocks 2015 - Oecologia, Horrocks 2012b, Lowen et al 2007, Tang 2009, Zamora-Vilchis et al. 2012, Sehgal et al. 2011, Rubenstein et al 2008), and a single species can mount different immune responses depending on geographical location (Ardia 2007). This type of immunological variation may reflect resource availability, "pathogen pressure", or some combination of the two. Pathogen pressure encompasses the abundance and diversity of parasites, pathogens and even commensal microorganisms in the environment (Horrocks et al. 2012b, Horrocks et al. 2011, Tschirren and Richner 2006, Møller et al. 2003, Christe et al. 2001, Sheldon & Verhulst 1996) and on the animal itself (Horrocks et al. 2012). High temperature is known to provide a conducive environment for growth, development, and reproduction of microorganisms and parasites (Zamora-Vilchis et al. 2012, Sehgal et al. 2011), and rain can correlate positively with microbial load (Atherholt et al 1998, Landesman et al. 2011). Parasites and microbial communities may vary in space (Knowles et al. 2010, Bensch and Åkesson 2003, Froeschke et al. 2010, Angel et al 2010) and time.

Seasonal or temporal variation in immune function in equatorial tropical birds is poorly studied in comparison to their temperate and arctic zone counterparts. Yet, many birds at or near the equator exhibit ecological characteristics, such as year-round breeding, that are useful for such investigations. Year-round breeding means reproduction is not tightly confounded with predictable intra-annual seasonal variation, as it is at mid-to-higher latitudes. In addition, some equatorial tropical regions are characterized by large variations in environmental conditions over short distances (Ndithia et al. 2017a), which can be exploited for studying environmental effects on immune function. Equatorial tropical bird species that are both widespread and year-round breeders allow for simultaneous comparisons of variation in immune function in breeding and non-

breeding individuals within and among populations. Thus, such a system is ideally suited for disentangling the effects of life history and environmental variation.

To understand the roles of reproduction and the environment in influencing immune function, we studied three populations of year-round breeding Red-capped Larks *Calandrella cinerea* living in three locations in equatorial Kenya (South Kinangop, North Kinangop and Kedong), which are geographically nearby one another but climatically distinct (Ndithia et al. 2017a). These three locations have distinct differences in average annual rainfall, average minimum temperature (T_{min}), and average maximum temperature (T_{max}), but they are also characterized by large intra- and inter-annual variations in quantity and timing of rainfall (Ndithia et al. 2017a). Our study species occurs and breeds in the three locations, providing an opportunity to study 1) reproduction-induced variation in immune function within each location, considering within-location variation in environmental conditions, and 2) intraspecific variation in immune function among climatically different locations.

We investigated if immune function and body mass differed among birds in three different reproductive states and from three climatically distinct environments that are generally permissive of year-round breeding (Ndithia et al. 2017a). The reproductive states included incubation (females only), chick-feeding (males and females), and non-breeding (males and females). We expected that environmental conditions (rain, T_{min}, T_{max}) would not differ according to breeding stages and hence could be excluded as confounding factors in explaining any reproduction-associated variation in immune function. Based on resource trade-offs, within each location, we expected non-breeding birds to generally have more robust immune function (Bentley et al. 1998, Nelson and Demas 1996, Martin et al. 2008) and higher body mass (Moreno 1989) compared to breeding ones. Based on the antigen exposure hypothesis which predicts reduced microbial abundance in arid environments (Horrocks et al. 2012), we expected immune function to decrease along a gradient of aridity from South Kinangop to North Kinangop and Kedong.

Methods

Study species

The Red-capped Larks is a small (mean mass, 25.6 ± 1.54 (SD), n = 66) gregarious bird in the family *Alaudidae*, occurring in grasslands with short grass to bare ground. Its distribution ranges from lowland savanna with altitude of 1200 m above sea level (ASL) to highland grasslands 2600 m ASL (Zimmermann et al. 1999). The species feeds on invertebrates including beetles, wasps, caterpillars, butterflies and moths, earthworms, grasshoppers, and occasionally on grass seeds (H.K.N pers. Obs.). Red-capped Larks breed year-round and build an open-cup nest on the ground, often next to a scrub or grass tuft. Each female lays two eggs (mean, 2.0 ± 0.00 (SD), n = 59), which she incubates on her own for 10-12 days; both parents feed the nestlings for about 10 days (H.K.N. N pers. Obs.). The species can breed in all calendar months, but it does not breed in every month in every year (Ndithia et al 2017a). Pairs of Red-capped Larks defend the area around the

nest during breeding periods, but the birds form large non-territorial flocks when not breeding. Color ring re-sightings suggest that the species is resident year-round in our study areas.

Study areas and environmental conditions

We conducted our study in three locations that are geographically close to each other but climatically distinct (Table 1, Ndithia et al. 2017a). We worked year-round and simultaneously in these locations from January 2011 to March 2014.

Table 1. Geographical and climatic characteristics of our three Kenyan study locations where we investigated the role of reproduction on the variation in immune function in Red-capped Lark *Calandrella cinerea*

Location	Character	Lat/Long	Elevation (m)	Average annual rain (mm ± SD)	Monthly mean T_{min} (range, ^{0}C)	Monthly mean T_{max} (range, ^{0}C)
S. Kinangop	cool and wet	0^{0}42′30″S, 36^{0}36′30″E	2556	939 ±132.7	3.0 – 8.2	21.2 – 30.0
N. Kinangop	Warm and wet	0^{0}36′55″S, 36^{0}30′48″E	2428	584 ± 62.6	3.0 – 13.7	22.1 – 30.5
Kedong	Warm and dry	0^{0}53′37″S, 36^{0}23′54″E	2077	419 ± 96.8	6.2 – 15.7	25.3 – 34.9

Field sampling and recording of environmental abiotic variables

In each location, we used mist nets to catch non-breeding adults of both sexes, and we used cage traps to catch females during incubation and both sexes during chick feeding (see Table 2 for details on sample sizes of breeding status, location and sex). From each individual, we collected a blood sample for immunological analyses from a needle puncture of the brachial vein using heparinized capillary tubes. We transferred these samples to micro centrifuge tubes, temporarily stored them on ice, and centrifuged them at the end of each fieldwork day. We stored the plasma fraction in the freezer for future analyses. We used a weather station (Alecto WS-3500, Den Bosch, Netherlands) in each location to obtain monthly total rainfall (mm), average monthly $T_{min,}$ and average monthly T_{max} ($^{\circ}$C).

Table 2. Sample sizes of males and females, and of females only for different breeding statuses and sex in Red-capped Larks *Calandrella cinerea* during the study of the role of reproduction in the variation of immune function versus that of the environment in South Kinangop, North Kinangop and Kedong in equatorial Kenya.

Parameter	breeding status	sex	South Kinangop	North Kinangop	Kedong
Immune function of males and females	non-breeding	f	4	5	22
		m	5	9	21
	chick-feeding	f	12	13	21
		m	11	10	15
Environmental variables of males and females	non-breeding	f	4	5	24
		m	5	10	22
	chick-feeding	f	11	13	20
		m	12	9	16
Immune function of females	non-breeding	f	4	5	22
	incubating	f	10	10	19
	chick-feeding	f	12	13	21
Environmental variables of females	non-breeding	f	4	5	24
	incubating	f	12	13	21
	chick-feeding	f	12	14	20

Immune assays

Haptoglobin is an acute phase protein that scavenges haemoglobin, which can be released into circulation by haemolysis or normal red blood cell turnover (Quaye, 2008) and which outside of erythrocytes is highly toxic (Alayash 2004). Concentration of haptoglobin in plasma increases intensely after an inflammatory stimulus, e.g., infection or injury (Quaye, 2008). We determined haptoglobin concentration using an assay that measures the haem-binding capacity of plasma (TP801; Tridelta Development limited, Maynooth, Ireland) following the manufacturer's instructions and at 30 ^{0}C during the 5 minute incubation (for more details see Matson et al. 2012). Each of the three assay plates included a standard that was run in duplicate in each plate (Matson et al. 2012). Mean within-plate coefficient of variation (CV) equalled 2.4%; mean among-plate CV equalled 2.7%.

Nitric oxide is a multifunctional signalling molecule that, among others roles, is important for modulating inflammatory processes and destroying parasites, virus-infected cells, and tumor cells. Therefore, the molecule provides information about variation in physiological condition, health state, and work load of an animal (Sild and Hõrak 2009). We determined nitric oxide (mmol/ml) production through the reduction of nitrate to nitrite by copper-coated cadmium

granules, followed by color development with Griess reagent (Promega; Sild and Hõrak 2009) and absorbance measurement at 542 nm (Versamax, Molecular Devices Sunnyvale, California, US).

Natural antibodies (hemagglutination) and complement (hemolysis) are constitutively present as part of the innate immune system, which provides a first line of defence against infectious agents (Matson et al. 2005). The production of natural antibodies does not require previous exposure to particular antigens. Instead, they bind to a range of antigens associated with foreign red blood cells, parasites, microorganisms, and toxins, and they can initiate the complement enzyme cascade that leads to cell lysis (Matson et al. 2005, Carroll, 1998, Belperron and Bockenstedt, 2001, Greenberg, 1985, Ochsenbein et al. 1999, Congdon, Farmer, Longenecker, and Breitenbach, 1969, Reid et al. 1997). We quantified lysis and agglutination titres against rabbit red blood cells (Envigo, Belton, UK) following the protocol of Matson et al. (2005). Agglutination reflects the interaction between natural antibodies in plasma and antigens of rabbit red blood cells. Lysis results from the interaction of complement and natural antibodies. We scored lysis and agglutination titres blind to sample and plate identity at least twice, assigning half scores to samples that showed intermediate result. We used the mean value in statistical analyses if the first two scores were less than one titre apart. If they were more than one titre apart, we scored a third time and used the median value (Matson et al. 2005). For lysis, mean among-plate CV equalled 18.6% and mean within-plate CV equalled 9.8%. For agglutination, mean among-plate CV equalled 9.7% and mean within-plate CV equalled 7.7%.

Statistical analyses

Because only females in this species incubate, we used separate analyses for data sets of males and females combined and for females only, with the corresponding tests to check for potential effects of the environmental conditions during breeding on immune function. To test if immune function and mass are determined by breeding status or environmental conditions in male and female Red-capped Larks from South Kinangop, North Kinangop and Kedong, we constructed generalized linear models (glm) with each immune index or mass as a dependent variable and with breeding status, location, sex and their two-way and three-way interactions as explanatory variables. We log-transformed haptoglobin and nitric oxide values to obtain normality. We used a normal (Gaussian) distribution for analyses of haptoglobin, nitric oxide, agglutination and body mass, and with a binomial distribution for the analysis of lysis.

The haptoglobin assay may be affected by plasma sample redness from hemolysis (Matson et al. 2012). Therefore, we pre-scanned samples at 450 nm to enable us to statistically correct for plasma sample redness. Additionally, plasma sample age (range in sample age: 81 – 1275 days) may affect quantification of the immune indices. Using one-way ANOVA, we found that log haptoglobin was affected by plasma sample redness at 450 nm ($F_{1, 127} = 8.49$, $P = 0.004$), and plasma sample age affected log haptoglobin ($F_{1, 127} = 12.54$, $P = 0.001$), agglutination ($F_{1, 139} = 10.76$, $P = 0.001$) and lysis ($X^2 = 38.96$, d.f. 1, $P < 0.001$) but not log nitric oxide ($F_{1, 130} = 1.10$, $P = 0.30$). In the case of a significant effect, we retained these methodological covariates in all relevant models.

To check whether or not environmental conditions confound the possible effects of breeding on immunity, we tested if total rain (mm), T_{min} (^{0}C), and T_{max} (^{0}C) differed between chick-feeding and non-breeding males and female birds in the three locations. We constructed models with each of these environmental conditions as dependent variables and with breeding status, location, sex, and their two-way and three-way interactions as explanatory variables. We matched the month of an immune measurement of an individual bird with the corresponding total monthly rainfall, T_{min}, and T_{max}.

To test for differences in immune function and mass during non-breeding, incubating and chick-feeding periods in females in the three locations, we built separate models for each immune index and mass as dependent variables and with explanatory variables breeding status, location, and their interaction. We log-transformed data of haptoglobin and data of nitric oxide to obtain normality. Tests did not reveal any significant effects of plasma sample age ($F_{1, 99} = 2.77$, $P = 0.10$) and plasma sample redness at 450 nm ($F_{1, 99} = 1.35$, $P = 0.25$) on haptoglobin, so both were excluded from the model with haptoglobin. Plasma sample age did not affect log nitric oxide ($F_{1, 105} = 0.99$, $P = 0.32$) but did affect agglutination ($F_{1, 98} = 6.11$, $P = 0.02$) and lysis ($X^2 = 21.48$, d.f. $= 1$, $P < 0.001$), so, like with the male and female data, it was retained in the models for the latter two.

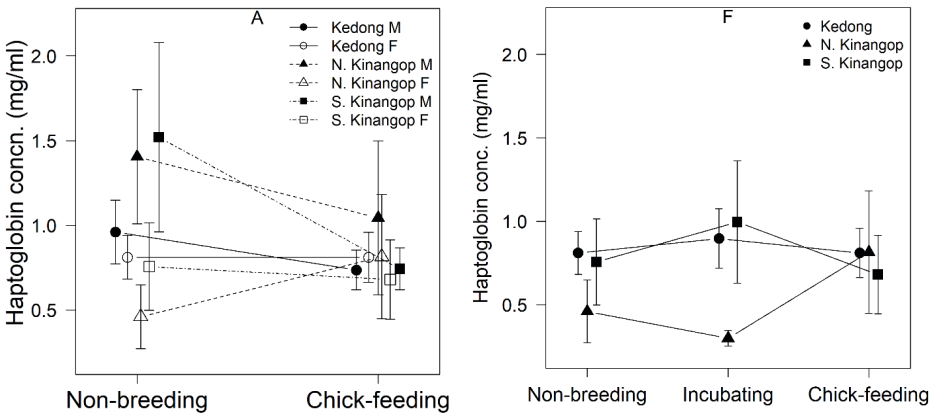
Like for the data set combining males and females (but excluding incubation), for the female only data set, we checked whether or not environmental conditions confound the possible effects of breeding on immunity. We tested if total monthly rain, T_{min}, and T_{max} differed among the non-breeding, incubating and chick-feeding periods in females in the three locations. We matched the month in which we sampled each immune index for an individual bird with the corresponding total monthly rainfall, T_{min}, and T_{max}. We built models with each of the different environmental conditions as dependent variable and with breeding status, location and their interaction as explanatory variables.

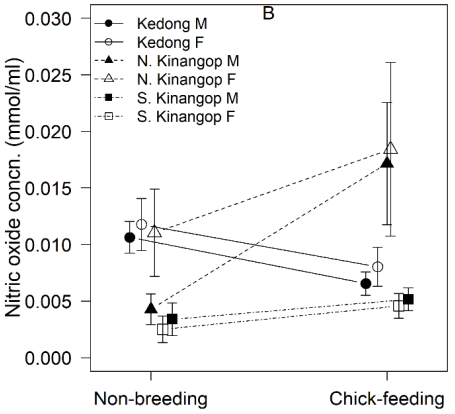
We simplified models using backward elimination by deleting the least significant terms from the model until we arrived at the final model and used $P < 0.05$ as selection criterion. We used type III sum of squares in the ANOVA summary of results to test main effects in the light of interaction terms as well as in the light of other main effects (Mangiafico 2015). The final model consisted of all the significant terms, plus breeding status, location, and sex (where applicable) regardless of significance and any applicable methodological co-variates. For all analyses, we tested and confirmed that the residuals of the final models observed the assumptions of normality and homoscedasticity of variance through graphical and statistical methods. Whenever an interaction was significant, we made a new variable consisting of all the separate variables in the significant interaction, conducted a Tukey's post hoc test on this new variable, and reported significant post hoc test results. We used R statistical software (version 3.0.3; R Development Core Team 2014) in all our analyses.

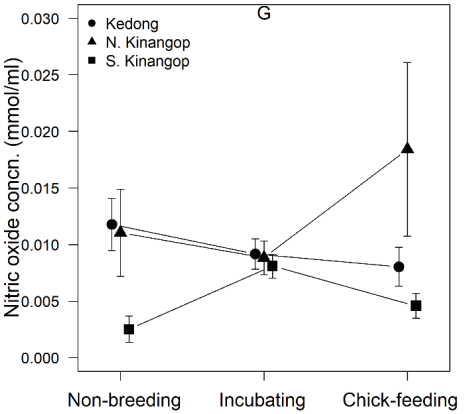
Results

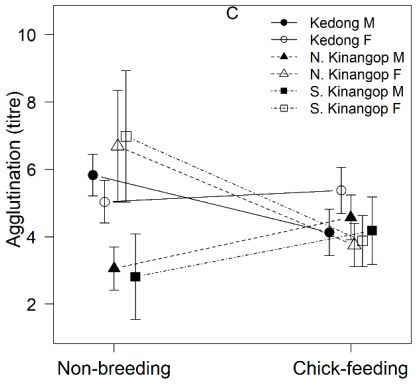
Immune function and body mass in chick-feeding and non-breeding Red-capped Larks from three locations

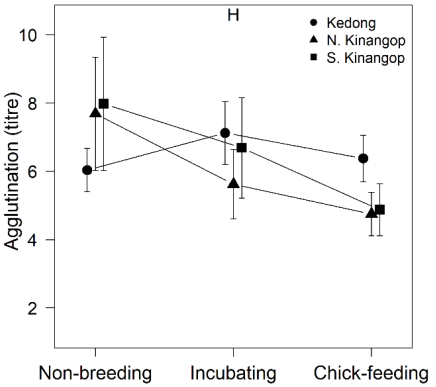
We found no consistent differences between chick-feeding and non-breeding individuals for haptoglobin, nitric oxide, agglutination or lysis, but sometimes locations and sexes did differ (Fig 1 A-D). Breeding status did not significantly affect haptoglobin in males and females in any of the locations, but we found a significant interaction of breeding status *x* location for nitric oxide and significant three-way interaction (breeding status *x* location *x* sex) for agglutination and lysis (Table 3a). Although there was a significant interaction of location *x* sex for haptoglobin (Fig 1 A, Table 3), post hoc tests revealed only borderline non-significant differences: males tended to have higher haptoglobin than females in North Kinangop ($t = 2.61$, $P = 0.07$), and among locations females in Kedong tended to have higher haptoglobin than females in North Kinangop ($t = 2.50$, $P = 0.09$). All other pairwise comparisons for haptoglobin were highly non-significant (all $t < 1.50$, all $P > 0.59$). With further exploration of the significant interaction of breeding status *x* location for nitric oxide, we found that values were higher during chick-feeding than during non-breeding within North Kinangop ($t = 3.39$, $P = 0.01$). Among locations during non-breeding, birds in Kedong had higher nitric oxide than those in South Kinangop ($t = 4.70$, $P < 0.001$) and North Kinangop ($t = 2.86$, $P = 0.04$). Among locations during chick-feeding, birds in North Kinangop had higher nitric oxide than those in South Kinangop ($t = 4.13$, $P < 0.001$) and Kedong ($t = 2.80$, $P = 0.04$). All other pairwise comparisons for nitric oxide were non-significant (all $t < 2.12$, all $P > 0.22$). Sex did not affect nitric oxide (Table 3). Similarly, we further explored the significant three-way interaction of breeding status *x* location *x* sex for both agglutination and lysis (Fig 1 C-D, Table 3).




B
Kedong M
Kedong F
N. Kinangop M
N. Kinangop F
S. Kinangop M
S. Kinangop F
Nitric oxide concn. (mmol/ml)
0.030
0.025
0.020
0.015
0.010
0.005
0.000
Non-breeding
Chick-feeding


G
Kedong
N. Kinangop
S. Kinangop
Nitric oxide concn. (mmol/ml)
0.030
0.025
0.020
0.015
0.010
0.005
0.000
Non-breeding
Incubating
Chick-feeding


C
Kedong M
Kedong F
N. Kinangop M
N. Kinangop F
S. Kinangop M
S. Kinangop F
Agglutination (titre)
10
8
6
4
2
Non-breeding
Chick-feeding


H
Kedong
N. Kinangop
S. Kinangop
Agglutination (titre)
10
8
6
4
2
Non-breeding
Incubating
Chick-feeding

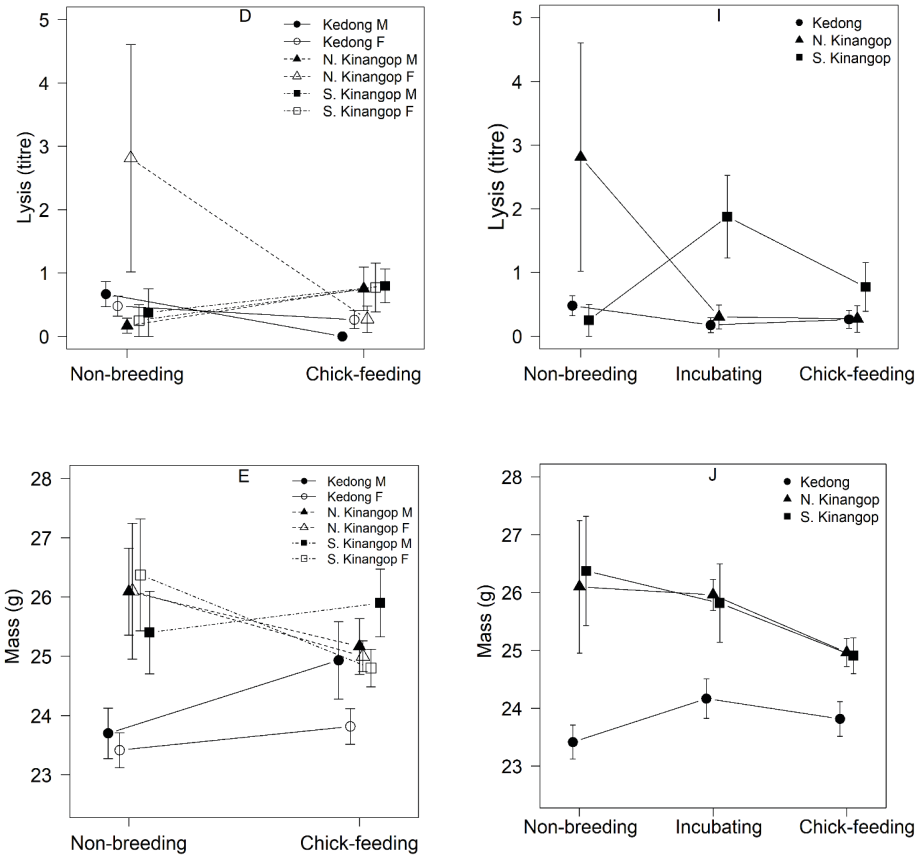


Figure 1. Haptoglobin (mean ± SE, mg/ml), nitric oxide (mean ± SE, mmol/ml), agglutination (mean ± SE, titre), lysis (mean ± SE, titre) and mass (g) in chick-feeding and non-breeding males and females (A-E), and in chick-feeding, incubating and non-breeding females (F-J) of our study species, Red-capped Larks *Calandrella cinerea*, in South Kinangop, North Kinangop and Kedong in equatorial Kenya.

Post-hoc tests revealed that agglutination was higher in non-breeding males in Kedong than in non-breeding males in North Kinangop ($t = 3.35$, $P = 0.02$), while lysis was higher in chick-feeding males in South Kinangop than in chick-feeding males in Kedong ($t = 3.51$, $P = 0.01$). All other pairwise comparisons involving agglutination and lysis were not significant (all $t < 2.79$, all $P > 0.11$).

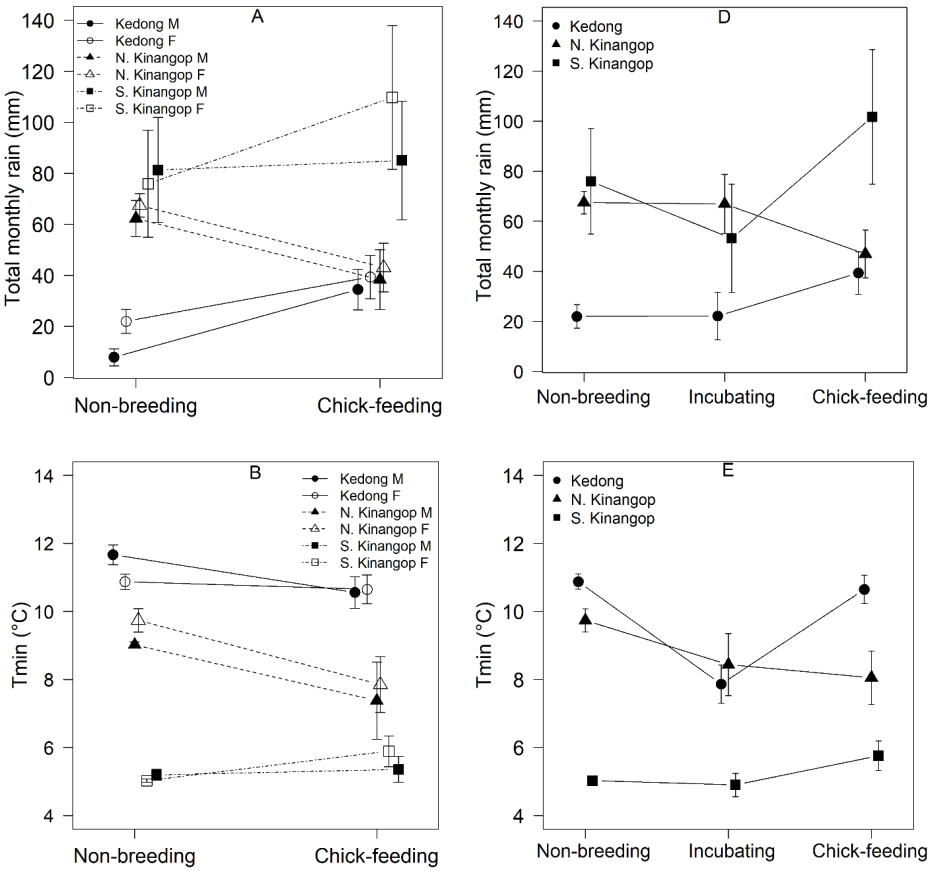
Body mass was lower during chick-feeding than during non-breeding in males and females in North Kinangop and in females in South Kinangop while males and females in Kedong and males in South Kinangop displayed the opposite pattern (Fig 1 E). The interaction of breeding status *x* location was significant, but there was no significant effect of sex on mass (Table 3). Post hoc tests on this significant interaction revealed that during non-breeding, larks weighed less in Kedong than in South Kinangop ($t = 4.22$, $P < 0.001$) and in North Kinangop ($t = 4.95$, $P < 0.001$). All other pair-wise comparisons were not significantly different (all $t < 2.07$, all $P > 0.24$).

Table 3. Results of models examining variation in immune function between chick-feeding and non-breeding male and female Red-capped Larks *Calandrella cinerea* in South Kinangop, North Kinangop and Kedong in equatorial Kenya. Data of haptoglobin and nitric oxide were log transformed to obtain normality. P values < 0.05 are indicated in bold.

Immune index	**Explanatory variable**	**DF**	**F**	**P**
Haptoglobin (mg/ml)	breeding status *x* location *x* sex	2, 115	1.61	0.20
	breeding status *x* location	2, 117	0.67	0.51
	breeding status *x* sex	1, 119	1.75	0.19
	location *x* sex	2, 120	3.14	**0.047**
	breeding status	1, 120	1.19	0.28
Nitric oxide (mmol/ml)	breeding status *x* location *x* sex	2, 120	1.79	0.17
	breeding status *x* sex	1, 122	0.37	0.54
	location *x* sex	2, 123	0.80	0.45
	sex	1, 125	0.12	0.73
	breeding status *x* location	2, 125	9.23	**< 0.001**
Agglutination (titre)	breeding status *x* location *x* sex	2, 128	6.71	**0.002**
Lysis (titre)	breeding status *x* location *x* sex	2, 130	11.86	**0.003**
Mass (mg)	breeding status *x* location *x* sex	2, 150	1.32	0.27
	location *x* sex	2, 152	1.10	0.34
	breeding status *x* sex	1, 154	1.78	0.18
	sex	1, 155	2.37	0.13
	breeding status *x* location	2, 155	4.00	**0.02**

Rainfall and temperature experienced by chick-feeding and non-breeding Red-capped Larks in three locations

Red-capped Larks experienced differences in rainfall, T_{min}, and T_{max} between non-breeding and chick-feeding, depending on the location in which they lived (Fig 2 A-C). Overall, rainfall showed a mixed pattern, T_{min} was lower, constant or higher during chick-feeding compared to non-breeding, and T_{max} was higher during chick-feeding than during non-breeding in all locations (Fig 2 A-C). The interaction of breeding status *x* location was significant for rainfall and T_{max} but was borderline non-significant for T_{min}, which instead differed according to both breeding status and location (Table 4).



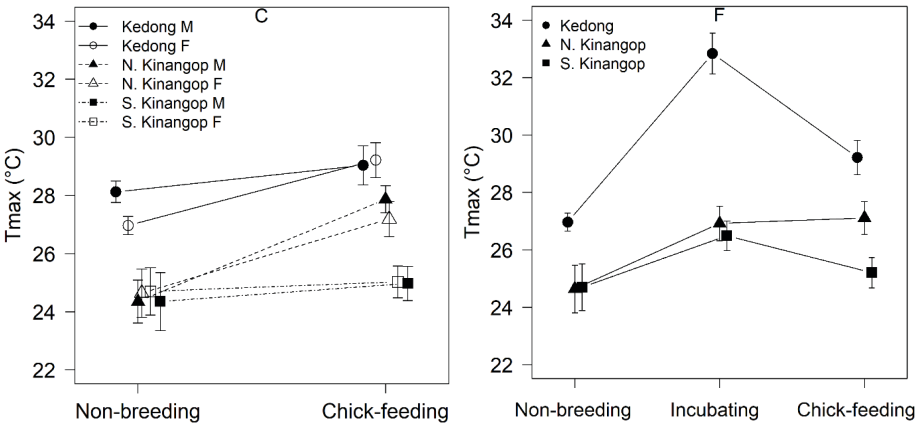


Figure 2. Relationships between total rainfall, average minimum (T_{min}) and average maximum (T_{max}) temperatures (^{0}C) and the different breeding statuses of male and female (A-C) and females only (D-F) Red-capped Larks *Calandrella cinerea* in South Kinangop, North Kinangop and Kedong in equatorial Kenya.

Subsequent post hoc tests revealed that rainfall did not differ significantly between chick-feeding and non-breeding periods within each location (all $t < 2.32$, all $P > 0.14$). Among locations, birds in South Kinangop were feeding chicks during periods with higher rain compared to birds in North Kinangop ($t = 4.29$, $P < 0.001$) and in Kedong ($t = 5.23$, $P < 0.001$), but rainfall did not differ significantly between North Kinangop and Kedong during chick-feeding ($t = 0.34$, $P = 0.99$). Similarly among locations when birds were not breeding, rain was higher in South Kinangop ($t = 4.01$, $P < 0.001$) and in North Kinangop ($t = 3.82$, $P = 0.002$) compared to Kedong, but rain did not differ significantly between South and North Kinangop ($t = 0.82$, $P = 0.94$).

On the main effect of breeding status, birds were feeding chicks during periods with significantly lower T_{min} (mean 10.56 ± 0.29 ^{0}C, SE) compared to non-breeding periods (mean 11.24 ± 0.27 ^{0}C, SE table 4). Birds in Kedong experienced significantly higher T_{min} compared to those in South ($t = 14.00$, $P < 0.001$) and North Kinangop ($t = 7.23$, $P < 0.001$), and those in North Kinangop experienced significantly higher T_{min} than those in South Kinangop ($t = 6.40$, $P < 0.001$).

Exploring the significant interaction between breeding status and location, post hoc tests revealed that T_{max} was higher during chick-feeding periods compared to when birds were not breeding in North Kinangop ($t = 4.43$, $P < 0.001$) and in Kedong ($t = 3.40$, $P = 0.01$) but not in South Kinangop ($t = 0.62$, $P = 0.98$). Among locations, birds in North Kinangop ($t = 4.04$, $P = 0.001$) and in Kedong ($t = 7.41$, $P < 0.001$) were feeding chicks during periods with higher T_{max} compared to South Kinangop; differences in T_{max} between when birds were feeding chicks in North

Kinangop and in Kedong were marginally non-significant, tending to be higher in Kedong (t = 2.72, $P = 0.05$). Similarly, T_{max} for non-breeding birds in Kedong was higher than that for non-breeding birds in South Kinangop (t = 4.00, $P < 0.001$) and North Kinangop (t = 5.04, $P < 0.001$). T_{max} for non-breeding birds in South and North Kinangop did not differ significantly (t = 0.11, $P = 0.99$).

Table 4. Results of models testing relationships between abiotic environmental factors (total monthly rain, mm) average minimum temperature ($T_{min,}$ ^{0}C) and average maximum temperature (T_{max} ^{0}C) and chick-feeding and non-breeding male and female Red-capped Larks *Calandrella cinerea* in South Kinangop, North Kinangop and Kedong in equatorial Kenya. Significant P values < 0.05 are in bold.

Environmental variable	Explanatory variable	DF	F	P
Rain (mm)	breeding status *x* location *x* sex	2, 139	0.50	0.61
	breeding status *x* sex	1, 141	0.0001	0.99
	location *x* sex	2, 142	0.14	0.87
	sex	1, 144	2.10	0.15
	breeding status *x* location	2, 144	3.87	**0.02**
T_{min} (^{0}C)	breeding status *x* location *x* sex	2, 139	1.31	0.74
	location *x* sex	2, 141	0.81	0.45
	breeding status *x* sex	1, 143	1.62	0.21
	breeding status *x* location	2, 144	2.67	0.07
	breeding status	1, 146	5.10	**0.02**
	location	2, 146	102.32	**< 0.001**
	sex	1, 146	0.05	0.82
Tmax (^{0}C)	breeding status x location x sex	2, 139	0.03	0.36
	location x sex	2, 141	0.20	0.82
	breeding status x sex	1, 143	0.76	0.39
	sex	1, 144	1.10	0.30
	breeding status x location	2, 144	3.09	**0.048**

Immune function and body mass in non-breeding, incubating and chick-feeding female Red-capped Larks in three locations

We also found little variation among breeding status but some variation among locations when comparing three breeding statuses in females only from the three locations (Fig 1 F-I). Breeding status had no significant effect on haptoglobin, agglutination and lysis, but again the interaction of breeding status *x* location significantly affected nitric oxide (Table 5). Subsequent post hoc tests on this interaction revealed one within location effect and several among location effects. Within South Kinangop, females had higher nitric oxide during incubation than during non-breeding (t = 3.05, $P = 0.04$). Among locations, non-breeding females in South Kinangop had significantly

lower nitric oxide than those in Kedong (t = 3.59, $P < 0.01$) and also tended to have a lower concentration compared to those in North Kinangop (t = 2.82, P = 0.08). In addition, chick-feeding females in South Kinangop had lower nitric oxide than those in North Kinangop (t = 3.27, $P = 0.02$). All other pair-wise comparisons for nitric oxide were not significant (all t < 2.11, $P > 0.36$). There were among location differences for haptoglobin (Table 5), with post hoc tests revealing lower concentrations in North Kinangop than in Kedong (t = 2.95, P = 0.01) but no other significant differences (all t < 1.94, $P > 0.13$).

Body mass of females gradually decreased from non-breeding to incubation to chick-feeding in South and North Kinangop, whereas in Kedong it increased from non-breeding to incubation but slightly decreased from incubation to chick-feeding (Fig 1 J). The interaction of breeding status *x* location and the effect of breeding status were borderline non-significant, but we found a highly significant effect of location on body mass (Table 5). Subsequent post hoc tests showed that females in South Kinangop (t = 5.18, $P < 0.001$) and North Kinangop (t = 5.43, $P < 0.001$) had higher body mass than those in Kedong but that females in South and North Kinangop did not significantly differ in body mass (t = 0.04, $P = 0.99$).

Table 5. Results of models examining variation in immune function between chick-feeding, incubating and non-breeding female Red-capped Lark *Calandrella cinerea* in South Kinangop, North Kinangop and Kedong in equatorial Kenya. Data of haptoglobin and nitric oxide were log transformed to obtain normality. P values < 0.05 are indicated in bold.

Immune index	**Response variable**	**DF**	**F**	**P**
Haptoglobin (mg/ml)	breeding status *x* location	4, 92	0.97	0.43
	breeding status	2, 96	0.46	0.63
	location	2, 96	4.42	**0.01**
Nitric oxide (mmol/ml)	breeding status *x* location	4, 98	3.24	**0.02**
Agglutination (titre)	breeding status *x* location	4, 90	1.75	0.15
	breeding status	2, 94	0.88	0.42
	location	2, 94	1.14	0.33
Lysis (titre)	breeding status *x* location	4, 92	7.47	0.11
	breeding status	2, 96	0.48	0.79
	location	2, 96	2.85	0.24
Mass (g)	breeding status *x* location	4,125	2.43	0.05
	breeding status	2, 129	2.88	0.06
	location	2, 129	20.95	**< 0.001**

Rainfall and temperature experienced by female Red-capped Larks during non-breeding, incubation and chick-feeding in the three locations

Females experienced differences in rainfall, T_{min}, and T_{max} between non-breeding, incubation and chick-feeding, but the pattern depended on location (Fig 2 D-F). Compared with non-breeding and incubation in each location, rainfall was highest during chick-feeding in South Kinangop and Kedong but lowest in North Kinangop (Fig 2 D). T_{min} was lowest and T_{max} was highest during incubation in all locations except North Kinangop (Fig 2 E-F). Rain had no effect on breeding status but it differed significantly between locations. Subsequent post hoc tests revealed that South Kinangop ($t = 4.45$, $P < 0.001$) and North Kinangop ($t = 2.89$, $P = 0.01$) received more rain than Kedong. Interactions of breeding status *x* location were significant for T_{min} and T_{max} (Table 6). Further analysis of the significant interaction of breeding status *x* location for T_{min} revealed within and among location effects. Within Kedong, females were feeding chicks at a higher T_{min} than those that were incubating, and T_{min} was higher when females were not breeding compared to when they were incubating (Table 7a). When females were not breeding, T_{min} was higher in North Kinangop and in Kedong compared to South Kinangop (Table 7a). Similarly, females in North Kinangop and in Kedong were incubating during periods with higher T_{min} compared to those in South Kinangop (Table 7a). Females in Kedong were feeding chicks at a higher T_{min} than females in South Kinangop and North Kinangop, whereas females in North Kinangop tended to feed chicks at a higher T_{min} than those in South Kinangop (Table 7a). All other pair-wise comparisons were not significant (all $t < 1.54$, all $P > 0.75$).

Similarly, post hoc tests to further explore the significant interaction between breeding status and location for T_{max} revealed within and among location effects. Within Kedong, females were incubating at a higher T_{max} than when they were feeding chicks, and than when they were not breeding, and were feeding chicks at a higher T_{max} than when they were not breeding (Table 5b). Among locations, females in Kedong were incubating at a higher T_{max} compared to females in South Kinangop and in North Kinangop (Table 7b). Additionally, females in Kedong were feeding chicks at a higher T_{max} than those in South Kinangop (Table 7b). All other pair-wise comparisons were not significant (all $t < 2.64$, all $P > 0.12$)

Table 6. Results of models testing relationships between abiotic environmental factors (total monthly rain, mm) average minimum temperature ($T_{min,}$ ^{0}C) and average maximum temperature (T_{max} ^{0}C) and chick-feeding, incubating and non-breeding female Red-capped Lark *Calandrella cinerea* in South Kinangop, North Kinangop and Kedong in equatorial Kenya. Significant P values < 0.05 are in bold.

Environmental variable	**response variable**	**DF**	**F**	**P**
Rain (mm)	breeding status *x* location	4, 116	1.71	0.15
	breeding status	2, 120	1.04	0.36
	location	2, 120	9.99	**< 0.001**
T_{min} (^{0}C)	breeding status *x* location	4, 116	3.03	**0.02**
T_{max} (^{0}C)	breeding status *x* location	4, 116	4.30	**0.003**

Table 7. Multiple pairwise comparison of only the significant post hoc results of the interaction of breeding status *x* location in females for T_{min} and $T_{max.}$ Numbers in the column-row intersection are the P-values and the associated t-values (in bracket) for each paired comparison. Abbreviations in the columns headers represent the significantly higher values (at $P < 0.05$) compared to those in the rows. Legend: CFKE – chick-feeding in Kedong; NBKE – non-breeding in Kedong; NBNK – non-breeding in North Kinangop; INCNK – incubating in North Kinangop; INCKE – incubating in Kedong; CFNK – chick-feeding in North Kinangop; NBSK – non-breeding in South Kinangop; INCSK – incubating in South Kinangop; CFSK – chick-feeding in South Kinangop.

a) T_{min}

	CFKE	NBKE	NBNK	NBKE	INCNK	INCKE	CFKE	CFNK
INCKE	<0.01 (4.24)	<0.01 (4.80)						
NBSK			0.02 (3.34)	<0.01 (5.15)				
INCSK					<0.01 (4.20)	<0.01 (3.90)		
CFSK							<0.01 (6.37)	
CFNK							<0.01 (3.55)	
CFSK								0.09 (2.77)

b) T_{max}

	INCKE	CFKE	INCKE	CFKE
CFKE	<0.01 (5.05)			
NBKE	<0.01 (8.57)	0.02 (3.25)		
INCSK			<0.01 (7.64)	
INCNK			<0.01 (7.30)	
CFSK				<0.01 (4.79)

Discussion

In contrast to the prediction that immune function is suppressed by resource demanding activities such as breeding, we found that immune indices of year-round breeding equatorial Red-capped Larks did not differ between breeding (chick-feeding and incubation) and non-breeding birds. This finding was mostly consistent across the three different equatorial populations. There were only two exceptions: higher concentrations of NOx in chick-feeding birds compared to non-breeding ones in North Kinangop, and in incubating females compared to non-breeding ones in South Kinangop. These reproduction-associated increases are the reverse of our expectation that reproduction would lead to reductions in immune indices as a result of trade-offs. Body mass also did not differ between breeding and non-breeding birds in any of the populations. Although we had expected that environmental conditions would not differ between breeding and non-breeding birds in each location, we found that this was the case only for rainfall. T_{min} and T_{max} did differ between chick-feeding and non-breeding stages in all three and two populations, respectively, contrary to our previous findings that showed that timing of breeding in these lark populations is not affected by rainfall, T_{min} or T_{max} (Ndithia et al. 2017a). Despite the consistent lack of differences in immune indices, except NOx, between breeding and non-breeding birds in all populations, we did find significant differences among locations that differ in climatic conditions for all four immune indices. The exact nature of these effects depended on the immune index and breeding status, but not sex. In general, we propose that in equatorial tropical birds, variation in immune function is possibly better explained by climate-induced environmental conditions than by breeding status. Our findings also raise the question how within-location unpredictable environmental variation is associated with and impacts on immune function.

The lack of the expected downregulation of immune function during breeding (chick-feeding and incubation) compared to non-breeding birds in all locations might point to an evolutionary link between clutch size and immune function. Red-capped Larks have a clutch size of two, a relatively small size compared to temperate and arctic zone birds. A small clutch size may allow this species to simultaneously maintain both reproduction and immune function. As opposed to short-and-fast lived birds in mid-to-high latitudes that supposedly invest in reproduction at the expense of maintenance of other functions, e.g., immune function, long lived birds with a slow pace of life are associated with well-developed immune defences (Martin et al. 2006, Lee et al. 2008) and are known to favour functions that increase survivorship, such as immune capacity, even under challenging conditions such as reproduction (Ardia 2005, Lee 2006, Lee et al. 2008, Tella et al. 2002). In line with this life history strategy and immune function, the unexpectedly high within-location levels of nitric oxide during incubation in females in South Kinangop and during chick-feeding in males and females in North Kinangop compared to the respective non-breeding periods in each location raise the question whether breeding activities caused this increase in NOx. In addition, it raises questions whether breeding individuals are more immunocompetent (see Saino et al. 1997) or challenged birds skipped breeding.

The three populations living in climatically distinct locations differed in their immune indices, but no index consistently differed in the same way among locations. The finding that among-location differences in immune function vary according to breeding stage suggests that immune function does not simply reflect overall long-term differences in climate, but also at least partly responds to current weather conditions, despite their unpredictability (Ndithia et al. 2017a). Although on average rainfall, T_{min} and T_{max} differed among locations (Ndithia et al. 2017a), these average conditions were not always reflected in the weather conditions during each of the breeding stages. For example, while rainfall was substantially lower in Kedong than in North Kinangop during non-breeding, it was not different between these locations during chick-feeding. This suggests that birds in Kedong feed chicks at relatively wet times for that particular location, while in North Kinangop they feed chicks at relatively dry times.

Our findings suggest that different immune indices were differently influenced by environmental conditions. Temperature (Shephard and Shek 1998, Bowden et al. 2007) and rainfall (Rubenstein et al. 2008) have been shown to influence different components of immune function. Moreover, temperature (Lowen et al. 2007, Demas and Nelson 1998, Altizer et al. 2006, Watts et al. 1987) and rainfall (Bicout and Sabatier 2004) may influence the general geographical pattern and short-term local dynamics of disease prevalence, which may have consequences on the pattern of variation in immune function (Horrocks et al. 2012, Christe et al. 2001, Møller et al. 2003). We propose that bird populations living in different environments develop immune strategies that are presumably shaped by the influence that environmental conditions have on the local dynamics of disease risks. The alternative hypothesis that resource availability influences immune function via trade-offs appears to be less relevant in our system where food is available independent of rain or temperature (Ndithia et al. 2017a, Mwangi et al. in prep).

In conclusion, in contrast to temperate and arctic zone birds that live in environments characterized by very predictable seasonality and that exhibit trade-offs between reproduction and immune function, equatorial tropical birds faced with unpredictable environmental conditions maintain their immune function during energetically demanding life cycle stages, such as reproduction. Furthermore, the temporal variation in immune function that is exhibited by equatorial tropical birds appears to be more influenced by prevailing environmental conditions rather than investments in reproduction or perhaps life history events more generally. Red-capped Larks living in different environments with contrasting climatic conditions seem to exhibit different immune strategies to maximize their protection. Future studies should focus on the role of different environmental conditions on spatio-temporal dynamics of pathogens and parasites and, presumably, their resulting influence on variation in immune function in equatorial birds. In addition, efforts to understand immunological variation in an ecological framework should focus on the mechanisms that allow some bird species to maintain, and sometimes even increase, their immune defences while engaged in costly activities like reproduction.

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Chapter 6

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General discussion and synthesis

Henry K. Ndithia

Introduction

This section discusses succinctly the findings of this thesis and synthesizes them in the context of the hypothesis and expectations we set out to achieve. It further provides support in literature that justifies or provides an explanation for the findings.

The overall goal of this thesis was to determine variation in reproductive strategies, in growth and in immune function in lark species living in equatorial tropical environments that potentially vary in their environmental conditions among and within locations. In **chapter two**, we found that environmental conditions and breeding in Red-capped Larks *Calandrella cinerea* within each of our three climatically-distinct locations were frequently unpredictable and highly variable across the year. Among locations environmental conditions and breeding differed, suggesting that these conditions vary over a small spatial scale. Surprisingly, nesting activity in each of these locations was unrelated to any of the environmental conditions we measured, leaving an open question on what factors are important in influencing the timing of reproduction. This question requires further investigation. In **Chapter three,** nestling body mass and size at hatching were lowest in the most arid location, Kedong, perhaps related to resources females allocated to their eggs, while nestlings from Kedong grew faster than in the other two wetter locations, possibly due to the abundant food resources in this location compared to the other two. At hatching only and not in the days after, nestlings in Kedong were heavier during periods when most individuals in the population were breeding, while nestlings grew faster with more rain post-hatch in Kedong, pointing to better female body condition and food quality and quantity during these periods. We neither found among locational differences nor within Kedong differences in the variation in nestling immune function, suggesting that nestling immune function develops at a later stage post-fledge.

Contrary to expectations in **Chapter four**, immune function in two sympatric species, Red-capped and in Rufous-naped Larks *Mirafra africana* was not downregulated during reproduction but nitric oxide was instead upregulated during chick-feeding. In Red-capped Larks nitric oxide was high during breeding periods when average maximum temperature (T_{max}) was high, suggesting that higher T_{max} promoted a conducive environment for growth, development and reproduction of microorganisms and parasites that in turn triggered the elevation of nitric oxide. Red-capped and Rufous-naped Larks had contrasting immune indices with the former having higher nitric oxide than the latter while the latter had higher haptoglobin than the former. This suggests differences in life history adaptations of sympatric species facing variable and unpredictable environmental conditions. Similarly in **chapter five**, and contrary to expectation, nitric oxide in Red-capped Larks increased during breeding and decreased during non-breeding, pointing to the species' capacity to maintain two physiological processes (reproduction and immune function) concurrently. This may indicate that the species has the capacity to upregulate its nitric oxide levels due to the compromised (reduced) reproductive effort (this species has a clutch size of two). Periods during which nitric oxide was high and birds were breeding, T_{max} was also high, indicating that patterns of nitric oxide may have responded to patterns of breeding or to changes in T_{max}. Our three climatically-distinct locations differed in multiple immune indices,

suggesting that different immune indices were influenced differently by different environmental conditions. We conclude that birds living in environments with contrasting climatic conditions seem to develop different immune strategies to protect themselves against infections, presumably based on the prevailing environmental conditions that put a selective pressure on the prevailing disease risks in their environments. In addition, we propose that equatorial tropical birds optimize survival (investment in immune defense) over reproduction (small clutch sizes).

1. **New insights into the timing of breeding in equatorial tropical birds**

a) Environmental conditions differ between proximate locations, are unpredictable and highly variable within the year and among years

In contrast to the temperate and arctic zones where predictable seasonal changes in day-length are accompanied by predictable changes in annual environmental conditions that together provide reproductive cues to birds in these zones (Visser et al. 2006, Demas and Nelson 1998, Wingfield et al. 1997, Gwinner 2003, Versteegh et al. 2014, Farner 1964, Colwell 1974), in **Chapter 2**, we observed year-round unpredictable and large variation in rainfall, T_{min} and T_{max}, ground and flying invertebrates, and breeding activity in Red-capped Larks living in the cool and wet South Kinangop, the warm and wet North Kinangop and the warm and dry Kedong. Although we had expected a bimodal pattern of rainfall (see **chapter 1** of this thesis), this pattern, whenever it occurred, was characterized by high variation and, due to climate change, seems to be completely unpredictable in recent years (Mwangi et al. 2018). These factors varied among months and among years in an unpredictable manner. Work by several other authors, some based on long-term data, have equally depicted biotic and abiotic environmental factors in tropical regions to reflect our findings of high unpredictability and irregular variations in environmental variables (Young 1994, Grant and Boag 1980, Boag and Grant 1984, Wrege and Emlen 1991, Scheuerlein and Gwinner 2002, Stutchbury and Morton 2001, Conway et al. 2005, Moore et al. 2005, Ndithia et al. 2007). In addition, environmental conditions varied spatially in great extents in these three locations whose farthest direct distance apart is only 34 km. As expected, these locations exhibited a gradient of rainfall and temperature that decreased and increased from South Kinangop, North Kinangop and Kedong respectively. Therefore, environmental conditions in our study system vary at a small spatio-temporal scale. However, contrary to expectation that increasing gradient in rainfall represents an increasing gradient in primary productivity (and thus food availability) and breeding activity, the drier and warmer Kedong had the highest invertebrate biomass and the highest intensity and occurrence of breeding activity of larks. This suggests that our three locations may not represent the expected aridity gradient of primary productivity with a decrease in primary productivity and an associated decrease in invertebrate biomass. It would be interesting to see, through further studies, how different the findings would be using locations which reflect a true representation of such a productivity gradient. Additionally, an important next step would be include more years of sampling so that we can do formal tests of rhythmicity.

b) Larks do not use environmental conditions to plan their breeding schedule

Due to the predictability of environmental conditions in temperate and arctic zone, birds in these regions can use these changes as reproductive cues (Visser et al. 2006, Demas and Nelson 1998, Wingfield et al. 1997, Gwinner 2003, Versteegh et al. 2014, Farner 1964, Colwell 1974) resulting in synchronized spring breeding within and among species living at the same location (Nager and van Noordwijk 1995, Lambrechts et al. 1996). However, despite the unpredictable and highly variable environmental conditions in equatorial tropical environments, we sought to determine which of the biotic and/or abiotic environmental factors influence breeding in Red-capped Larks living and breeding simultaneously in three environments with differing climatic conditions, an understudied topic in equatorial tropics.

Surprisingly, we did not find any evidence in **Chapter 2** that breeding was timed to co-occur with rainfall, T_{min}, T_{max}, ground or flying invertebrate biomass in any of the three environments. We inferred that other factors (that we did not measure) may be responsible for influencing reproduction in larks, prime candidates of which are nest predation pressure, female protein reserves or social factors. Our larks are likely to breed whenever environmental conditions are conducive and permissive, e.g., low nest predation pressure, resulting in opportunistic breeding or whenever females have sufficiently re-supplied their reserves (Scott et al. 1987). Our findings are in line with other studies of equatorial tropical birds that have bred opportunistically (Grant and Boag 1980, Zann et al. 1996, Grant et al. 2000, Tieleman and Williams 2002, Tieleman and Williams 2005), and where authors suggest that protein reserves of individual females determine when to breed (Ward 1969, Fogden 1972, Jones and Ward 1976, Fogden and Fogden 1979, Zann et al. 1996). This results in asynchronous year-round breeding at the population level.

Socially, some individuals in the population may time their reproduction to benefit from periods of peak food availability (Dittami and Gwinner 1985, Komdeur 1996, Hau et al. 2000, Scheuerlein and Gwinner 2002). Other individuals may select to breed during 'unpopular' times when food supply is insufficient to avoid competition for food or nesting space by conspecifics or other species (Minot 1981). Other segments of our larks may have applied the predation-avoidance strategy where males and females avoid being the only pair breeding at a particular time due to the high predation risk associated with that position (Sansom et al. 2009, Fontaine and Martin 2006) and due to the prolonged nestling dependence on parents (Langen 2000) e.g., due to insufficient food or nutrient-poor diet. Further investigations at the individual levels to complement this population-level study, need to be done to develop a better understanding of whether some or all of these factors are important in the reproductive decision of Red-capped Larks. An alternative explanation for lack of environmental factor(s) relevant for lark reproduction is that the spatial scale at which we measured our explanatory variables, rainfall, T_{min}, T_{max}, ground and flying invertebrate biomass may be different from that at which Red-capped Larks use to time their reproduction. While we measured abiotic factors at location level and biotic factors at the plot level, Red-capped Larks bred at the territory level. Although Red-capped Larks do not maintain a year-round territory (only maintained during breeding periods), future further studies should focus on incorporating territory-level data to complement this population level data to investigate

whether larks use the micro-environment around the nest territory during the breeding periods to take reproductive decisions. Also, long-term monitoring of nesting activities would allow for formal testing of seasonality for occurrence of nests, for nest success and for chick growth.

2. Environmental conditions among and within environments: consequences for nestling growth but not for immune function

a) Unexpectedly, faster nestling growth occurred in the more arid environment

Patterns of growth and development vary widely among populations, variations that are hypothesized to reflect adaptation to specific environmental conditions (Starck and Ricklefs 1998, Demas and Nelson 2012). We tested this hypothesis by comparing growth rates of three populations of nestling Red-capped Larks living in the three climatically-distinct environments, the cool and wet South Kinangop, the warm and wet North Kinangop and the warm and dry Kedong in **Chapter 3**. Using absolute and relative measures of body mass, wing and tarsus lengths we found that nestling body mass and size at hatching, except for tarsus length which was longest in Kedong and shortest in South Kinangop, were lowest in the most arid location Kedong and highest in the most mesic location South Kinangop. This points to the influence of resources that females allocate to their eggs and that of food availability (Arnold 1992, Houston and Donnan 1992, Nager et al. 1997, Christians 2002). However, contrary to expectations, nestlings in Kedong grew faster compared to nestlings in South and North Kinangop, again pointing to the role of food quality and quantity. To further confirm food limitation as evidence for lower growth constants K for nestlings in South and North Kinangop, we fitted growth curves by restricting data to individual nestlings for which we had complete sets of repeated measurements, i.e., days 1 – 10, therefore excluding nestlings that 'disappeared' as a result of starvation, nest predation and flooding. These growth curves from restricted data sets yielded increased K-values for South and North Kinangop but not for Kedong. We had observed nestlings in poor condition in these two locations, which we interpreted to have caused the 'disappearance' of this subset of nestling that might have negatively affected the K-values in South and North Kinangop. Our previous study had revealed that the most arid location Kedong had the highest invertebrate biomass compared to the wetter locations of South and North Kinangop (see **Chapter 2** of this thesis). This unexpected finding may be explained by the fact that the local ecology of our three study locations may not represent a typical aridity gradient with a decrease in primary productivity, and an associated decrease in invertebrate biomass. Additionally, the large amounts of precipitation in South and North Kinangop, led to frequent flooding, which presumably negatively impacted on the food quality and quantity for nestling larks, with negative consequences on growth. To confirm and deepen our understanding, future studies should replicate this nestling growth study in environments that represent a true gradient of aridity and select for nestlings that are successful to fledging, excluding those that 'fall off' on the way.

b) Environmental conditions unimportant in the timing of breeding but important for successful reproduction

For the within location variation in growth in part of **Chapter 3,** we investigated the consequences of hatching at different times of the year on growth by comparing growth rate during times of the year with more and less nesting activity and more and less rain in Kedong. We found that at hatching only and not in subsequent days, nestling Red-capped Larks had higher body mass when more individuals in the population were breeding, again pointing to female favorable body condition during this period, and food quality and quantity. Moreover, 7-days old nestlings grew better with more rain. These findings seemingly contrast with our previous outcomes that showed that rainfall, temperature and food availability had no effect on the timing of breeding (see details in **chapter 2**). The fact that periods with more rain did not coincide with periods when more females were breeding, raises the question why females did not preferentially breed at times that were best for nestling growth. Presumably, females made optimal use of factors that could provide favorable breeding environment, factors that never occurred simultaneously during the year, and key among which included female body condition, food quality and quantity and rainfall. It seems apparent that the timing of breeding by this lark species is not affected by food and rain, but successful breeding, i.e., young fledging, is at least partly determined by environmental factors, such as rain, that typically correlate with food availability. Multiple studies of breeding birds of the temperate and arctic zones have shown that females time their breeding such that nestlings benefit optimally from the food peak that is common during springs (Martin 1987, Lack 1950, Perrins 1970, Visser et al. 1998). Similarly in tropical environments, food has been considered by several studies as being important in determining when birds breed (Skutch 1950, Ward 1969, Fogden 1972, Gradwohl and Greenberg 1982). These variations in growth rate demonstrate the strong role of female body condition and that of food availability in defining the pace-of-life and variation in life-history strategies within tropical environments. However, if food availability were not the determining factor of growth rate, nestlings would grow fast due to the fact that this species has a small and typically constant clutch size of two eggs/nestlings and parents would still be able to find enough food for them even in an environment with food scarcity/unpredictability.

c) Nestling immune function neither varied among environments with different climates nor during the year within Kedong

Although avian immune function is hypothesized to vary with the pace-of-life (Martin et al. 2004, Tieleman et al. 2005, Ricklefs and Wikelski 2002), environmental conditions can play a major role in determining its variation (Møller et al. 2006, Horrocks et al. 2012, Versteegh et al. 2012, Versteegh et al. 2014, Horrocks et al. 2015). Additionally, within a given pace-of-life, immune function is thought to vary throughout the year in adult birds (Nelson and Demas 1996, Horrocks et al. 2013, Hegemann et al. 2012, Versteegh et al. 2014) and in nestlings (Dubiec and Cichoń 2001, Christe et al. 2001, Dubiec and Cichoń 2005). To investigate these hypotheses, we utilized the spatiotemporal variation in climates of three locations that are in close proximity to each other yet have distinct patterns of rainfall, T_{min} and T_{max}, and within location, have patterns of rainfall

and food that are unpredictable and highly variable (see **Chapter 2** of this thesis). Red-capped Lark nestlings occur simultaneously in these locations. Unexpectedly, although haptoglobin, agglutination, and nitric oxide showed more variation within than among locations, neither the among-location comparison, nor the within-location analysis in Kedong revealed any significant differences in nestling immune function. Furthermore, while varying substantially among individual nestlings, the indices did not significantly co-vary with nest index or rain within Kedong. One most plausible explanation is that nestling immune function is not yet fully developed and develops with time presumably after fledging, an indication that is consistent with other studies (Stambaugh et al. 2011, Hegemann et al. 2013). The fact that lysis was zero for 96% of nestlings further supports the proposition that nestling immune function is not yet fully developed. The large variation in immune indices among individuals may reflect variation in immunological status of their parents through maternally derived antibodies (Hasselquist and Nilsson 2009, Pihlaja et al. 2006, Stambaugh et al. 2011, Starck and Ricklefs 1998, Mauck et al. 2005, Pihlaja et al. 2006, Stambaugh et al. 2011) which may reflect maternal exposure to the local parasite pressures (Gasparini et al. 2001, Lemke et al. 2003). Immune function of nestlings may develop at different rates across individuals or populations (Matson et al. 2014). Measurement of immune indices at a single time point (i.e., day 10) may also have been a drawback in this study. To develop a better understanding of the ontogenesis of the immune function, future studies should focus on measurement of several immune indices systematically at different growth time points, including post-fledge monitoring to adulthood.

3. Sympatric species do not vary their immune function with reproduction but potentially with the environment

Some equatorial environments exhibit substantial temporal variation in environmental conditions throughout the year yet birds that inhabit them breed year-round. Avian immune function, particularly in the temperate and arctic zones, has been shown to vary with reproduction (O'Neal and Ketterman 2012, Bonneaud et al. 2003a, Ardia 2005a, Greenman et al. 2005) but can also vary with variation in environmental conditions (Nelson and Demas1996, Marra and Holberton 1998, Shepherd and Shek 1998, Ruiz et al. 2002, Tieleman et al. 2005). Since our previous study in three equatorial tropical environments including North Kinangop had shown that environmental conditions did not influence reproduction (see **Chapter 2** of this thesis), equatorial tropical North Kinangop provides a unique study system that allows for investigation of reproduction-induced interspecific and intra-sexual variation in immune function independent of environmental condition, a unique study system not available in the temperate and arctic zones. In **Chapter 4**, we utilized this uniqueness to investigate how immune function and body mass of males and females of Red-capped and Rufous-naped Larks, two closely related species that occupy different ecological niches and exhibit different reproductive strategies within the North Kinangop grasslands, differed between chick-feeding and non-breeding. To exclude the potential of confounding effects of environmental conditions on breeding in the variation in immune function

in the two species, we tested if rainfall, T_{min} and T_{max} differed between chick-feeding and non-breeding. By confirming that environmental conditions do not differ between periods at which we sampled breeding and non-breeding birds, we become sure that any differences in immune function between breeding and non-breeding birds do not result from environmental variation.

Contrary to popular hypothesis that immune function is compromised during periods of breeding, we actually found that nitric oxide was upregulated during chick-feeding periods in the two species, while haptoglobin, agglutination and lysis did not differ between breeding (chick-feeding) and non-breeding. This is a sharp contrast with how in temperate and arctic zone birds vary their immune function in light of the timing of reproduction. The elevation of nitric oxide in Red-capped Larks co-occurred with periods of higher T_{max}, suggesting that higher T_{max} promoted a conducive environment for growth, development and reproduction of microorganisms and parasites (Zamora-Vilchis et al. 2012, Sehgal et al. 2011). Therefore birds may have responded to the abundance of pathogens and parasites through elevation of nitric oxide as other similar studies have shown (Møller et al. 2003, Christe et al. 2001, Horrocks et al. 2012).

We also found interspecific differences between nitric oxide and haptoglobin: Red-capped Larks had higher nitric oxide than Rufous-naped Larks, which in turn had higher haptoglobin than Red-capped Larks. This points to differences in life history adaptations of sympatric species and how they differently utilize their highly variable environments to presumably maximize reproductive success and optimize their protection against diseases and parasites. The high variability in the pattern of rain in North Kinangop may have created distinct micro-habitats which the two species selectively occupied, and which potentially have different disease vulnerabilities (Knowles et al. 2010, Bensch and Åkesson 2003, Froeschke et al. 2010, Angel et al 2010). Other studies comparing birds living in the same environment have also found that some species are more susceptible to disease than others (Perkins and Swayne 2003, Tumpey et al. 2004). Similarly, immune function of different species may respond differently to the same parasite or microbial infection in their environment (Blount et al. 2003, Matson et al. 2005, Pap et al 2010a, Pap et al 2010b), while immune function may also differ among species due to body size differences (Hasselquist 2007). Interestingly, while during our previous study in this location we found that breeding activity was unrelated to rain, T_{min}, and T_{max} (see **Chapter 2** of this thesis), we now found that in Red-capped Larks, T_{max} was higher during breeding (chick-feeding) than during non-breeding, suggesting a confounding influence of T_{max} on the comparison of immune function between breeding and non-breeding birds. Besides studying year-round temporal patterns of pathogen and parasite pressures and investigating the potential role of environmental conditions, particularly T_{max}, in the temporal variation of immune function, future further studies should investigate if and how (mechanisms) the highly variable environmental conditions shape variation in immune function in this system.

4. Immune function of conspecific larks living in climatically-distinct environments is not downregulated but is instead upregulated during reproduction and is likely influenced by the environment

Although seasonal variation in immune function in birds from temperate and arctic zones has been attributed to resource trade-offs between immune function and annual cycle events such as reproduction (Buehler et al. 2008, Hegemann et al. 2012a, Hegemann et al. 2012b, Horrocks et al. 2012a, Ilmonen et al. 2000, Martin et al. 2008), several studies provide a contrasting picture where immune function and other supposedly competing physiological processes have co-occurred without immunosuppression (Møller et al., 2003, Alonso-Alvarez et al. 2007, Christe et al. 2000, Allander and Sundberg 1997, Vindevogel et al. 1985). Conversely, variation in immune function has also been attributed to variation in environmental conditions (Sheldon and Verhulst 1996, Tieleman 2018), two factors that co-vary in the temperate and arctic zones and whose separate contributions have so far not been determined. Despite being well studied in the temperate and arctic zones, factors that influence variation in immune function in equatorial tropical birds have not been studied even though this region harbors year-round breeding bird species that allow teasing apart of the effects of annual cycle stages, e.g., reproduction, and environmental conditions. By confirming that environmental conditions do not differ between periods at which we sampled breeding and non-breeding birds, we become sure that any differences in immune function between breeding and non-breeding birds do not result from environmental variation. Also, some equatorial tropical environments that are geographically close are characterized by unpredictable and large variations in environmental conditions (see **Chapter 2** of this thesis), and are therefore uniquely suited for studying environmental effects on immune function.

To understand the roles of reproduction and the environment in influencing variation in immune function, we exploited these unique characteristics of equatorial tropical systems in **Chapter 5**, to study three populations of year-round breeding Red-capped Larks that occurs and breeds simultaneously in three proximal locations with distinct climatic conditions, cool and wet South Kinangop, warm and wet North Kinangop and warm and dry Kedong, Kenya (see **Chapter 2** for details of locational differences in climatic conditions). This provided an opportunity for intraspecific comparison of reproduction-induced variation in immune function within each population - including considering the within-location variation in environmental conditions - in addition to intraspecific comparison of variation in immune function among these three environmentally different locations. We therefore investigated if immune function and body mass differed between chick-feeding and non-breeding (males and females), and incubation (females only) in Red-capped Larks, and compared immune function and body mass among our three climatically distinct environments.

Contrary to our prediction and to expectation that immune function would be suppressed by resource demanding activities such as reproduction (Bentley et al. 1998, Nelson and Demas 1996, Martin et al. 2008), nitric oxide in year-round breeding Red-capped Larks increased during chick-feeding and incubation compared to non-breeding in North Kinangop and South Kinangop

respectively, pointing to support for an evolutionary link between life history strategy and immune function. In contrast to temperate and arctic zone larks, Red-capped Larks have a small and consistent clutch size of two eggs/nestlings and the species may therefore be able to maintain both physiological processes (reproduction and immune function) concurrently without adjustment of either. Longer-and-slower lived species, e.g., Red-capped Larks, are known to manifest a strategy that favors the maintenance of functions that increase survivorship, such as immune capacity, even under challenging conditions such as reproduction (Ardia 2005, Lee 2006, Lee et al. 2008, Tella et al. 2002).

Again, contrary to our previous findings which showed that breeding was not influenced by rain, T_{min} and T_{max} in any of our three locations (see **Chapter 2** of this thesis), we found that except for rain which did not differ between chick-feeding and non-breeding, T_{min} was low during chick-feeding compared to non-breeding in all three locations, while T_{max} was high during chick-feeding compared to non-breeding in North Kinangop and Kedong but not in South Kinangop. Nitric oxide was high during breeding (chick-feeding and incubation) and when T_{min} was low and T_{max} was high in these locations. Patterns of nitric oxide may therefore have responded to patterns of breeding activities or to changes in environmental conditions (especially T_{max}). In line with other studies, temperature has been reported to influence down-or-up-regulation of immune function in birds (Cheville 1979, Mashaly et al. 2004, Garvin et al. 2006, Butler et al. 2009, Pigeon et al 2012). We therefore propose a possible role of T_{min} and T_{max} but not of rain in confounding the comparison of immune function between breeding and non-breeding birds. Future studies should focus on investigating the possible effect of environmental conditions on nitric oxide and the immune function in general.

Unexpectedly, and contrary to the antigen exposure hypothesis that predicts reduced microbial abundance in arid environments (Horrocks et al. 2012) and thus expected decrease in immune function along a gradient of aridity from South Kinangop to North Kinangop and Kedong, we found that the three climatically-distinct environments differed in multiple immune indices but not consistently so in any of the immune indices, suggesting that different immune indices were influenced differently by different environmental conditions. Nitric oxide, agglutination and haptoglobin were either high in warmer locations (relative to others), or were high where different breeding stages (chick-feeding and non-breeding) were associated with higher T_{max}. Conversely, NOx and lysis were low and high respectively during chick-feeding in South Kinangop, and during periods associated with low temperature and high rainfall respectively. This suggests that variation in different immune indices depended more on specific environmental conditions regardless of breeding phase. This is in line with studies that have shown that temperature (Shephard and Shek 1998, Bowden et al. 2007) and rainfall (Rubenstein et al. 2008) may suppress or enhance immune function, and that patterns of different immune indices may influence the general geographical pattern and short-term local dynamics of disease prevalence, and which may have consequences on the pattern of variation in immune function (Horrocks et al. 2012, Christe et al. 2001, Møller et al. 2003). It seemed apparent that birds living in environments with contrasting climatic conditions develop different immune strategies during e.g., reproduction, to protect themselves against

infections. These strategies are presumably based on the different prevailing environmental conditions that put a selective pressure on the prevailing disease risks in their environments. How (mechanism) birds from equatorial region are able to maintain increased immune response even during reproduction is a subject that should be investigated further. Besides that, it would be worthwhile to study year-round spatio-temporal dynamics of pathogens and parasites that this species encounters and their corresponding influence on immune function.

General conclusion

This thesis brings to the fore crucial scientific knowledge on life history adaptations of equatorial tropical birds and provides new perspectives that are in contrast to popular hypothesis. It draws a sharp contrast between how birds from temperate and arctic zones on the one hand, and those from equatorial tropics on the other, cope with their environments. We found that this particular East African equatorial tropical region experiences large variations and unpredictable environmental conditions among proximal environments and within environments, and birds living in these environments presumably breed opportunistically when breeding conditions are favorable. Surprisingly, where we had found that environmental conditions played an insignificant role in the timing of breeding (see **Chapter 2** of this thesis), we discovered new perspectives in subsequent chapters which suggest that different environmental conditions act during different reproductive phases in larks. At hatching, nestling body mass and size presumably depended on how much resources females allocated to their eggs, but subsequent growth depended on how much food was available in the environment. Additionally, nestlings hatched during periods when most females in a location were breeding were heavier at hatching - presumably being the period that provided favorable breeding conditions for females - and nestlings grew better with more rain post-hatch, suggesting the role of food supply indirectly through rain. Food availability, resources available for female body condition and rain seemed to play a crucial role during nestling growth. However, while considering chick-feeding and non-breeding phases in sympatric species and in chick-feeding, incubation and non-breeding phases in conspecifics in the three environments, it is T_{max} on the one hand and T_{min} and T_{max} on the other but not rain that influenced successful breeding (here defined as nestlings reaching the late nestling phase). This underscores our proposition that our larks do not plan their breeding but breed opportunistically when conditions are favorable.

Similarly, and contrary to expectations, neither did two sympatric lark species nor did conspecifics living and breeding simultaneously in three climatically-distinct environments downregulate their immune function during breeding (chick-feeding) but both groups up-regulated their nitric oxide levels instead. Our presumption is that immunosuppression as a result of the cost of reproduction does not apply to all birds. These longer-and-slower lived larks tend to have the capacity to maintain both of these energetically-demanding physiological processes perhaps because reproduction in both of these species may be less demanding (compared to their high latitude counterparts) due to their relatively small clutch sizes (of two eggs). Remarkably again, Red-capped and Rufous-naped Larks exhibited opposing patterns of nitric oxide and haptoglobin,

suggesting differences in life history adaptations of sympatric species, and highlighting the within-location variability in environmental conditions in equatorial tropics that may influence differences in disease risk and hence immune responses. Red-capped Larks had elevated nitric oxide during breeding (chick-feeding) which coincided with periods of higher T_{max}, which raises the possibility that high T_{max} provided conducive environment for growth, development and reproduction of microorganisms and parasites. Additionally, different immune indices of conspecifics differed inconsistently among locations with different climates and regardless of breeding phase. Nitric oxide, agglutination and haptoglobin were associated with higher T_{max} and were more robust under warmer conditions, while lysis was associated with rainy conditions and was enhanced in the low temperature environment. We interpreted this to be an indication that different immune indices were differently influenced by environmental conditions.

This thesis places variation in environmental conditions – food availability, rainfall, T_{min} and T_{max} – as the central elements around which reproduction, nestling growth and immune function varies. Although Chapter 2 did not reveal evidence of nesting activities being related to any of the biotic and abiotic environmental factors in the three environments, we found that nestlings in chapter 3 had higher body mass at hatching, suggesting conditions for breeding for females were favorable during this periods, and grew better during periods with more rain. We conclude that for these larks, breeding is not triggered by any particular biotic or abiotic factor (that we measured) but that breeding success (i.e., reaching chick feeding or even fledging) is. Whether or not a breeding attempt is successful is partly determined by a combination of environmental conditions. This thesis also supports the proposition that equatorial tropical birds, exhibiting a slow pace-of-life strategy, optimize survival (investment in immune function) over reproduction (small clutch sizes). Further, our findings contradict the generalized temperate and arctic zone bias concept of reproduction-induced immunosuppression and justifies why more of such research should be conducted in the tropics. Future further studies should, 1) investigate and compare factors that influence the timing of breeding in these two lark species by narrowing the scale of investigation to the territory level, and by including female body condition, nest-predation pressure and social factors as possible candidates, 2) investigate whether up-regulation of nitric oxide during breeding was as a result of breeding activities or of changes in temperature (T_{min} and/or T_{max}), 3) investigate how among-and-within-location dynamics of environmental variation influence variation in pathogen and parasite pressure in these environments and their potential influence on the variation in immune function, and 4) to test whether equatorial tropical birds optimize survival over reproduction, a future study should aim to experimentally vary the reproductive workload (e.g., increased clutch size) of one of the two sympatric species (Red-capped or Rufous-napped Larks) and compare the resulting investment in immune function of both.

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English summary

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English Summary

Mid-to-higher latitude zones have predictable seasonal changes in environmental conditions and day-length is the main driver of the timing of reproduction in birds from this region. Birds in this region can time their reproduction with regularity, with increased photoperiod during the temperate zone spring. Day-length in this region also plays a major role in determining seasonal changes in environmental factors such as temperature and food availability which birds use as supplementary cues to fine-tune the timing of reproduction to local environmental conditions. This results in synchronized spring breeding within and among species living at the same location. Growth rates in birds are associated with the pace-of-life and nestlings are known to grow faster at higher latitudes, in elevated locations and in less arid environments. However, patterns of variation in nestling growth and development are as well hypothesized to reflect adaptations to specific environmental conditions. Body functions of organisms, e.g., reproduction, growth, immune function among others, require resources that are often limited and that come at a fitness cost. Physiological processes, e.g., reproduction, immune function, particularly in mid-to-higher latitude birds where such studies have been carried out, often compete with one another for these limited resources, resulting in trade-offs. Immune function has therefore been proposed to be compromised during demanding life cycle events, most particularly during reproduction when animals allocate resources to production and care of offspring. Nonetheless, both reproduction and immune function in birds from mid-to-higher latitude zones vary seasonally. Besides being influenced by trade-off with reproduction, seasonal variation in immune function in birds in this system can also be influenced by environmental conditions, which also influences the timing of reproduction. This makes it difficult to decouple the effects of reproduction from those of environmental factors on the variation in immune function.

In contrast, environmental conditions in equatorial tropical environments and in the tropics generally are variable within a location and among locations that are close to one another, leading to a diversity of breeding systems and rates of growth. Biotic and abiotic factors in different climatic conditions have either not quantitatively been determined or are scattered through literature in a manner that cannot be useful to test their contribution to reproduction in equatorial tropical birds. To our knowledge, no other study has looked at multiple factors that potentially influence reproduction in birds, and in different climatic conditions in equatorial tropics. In chapter 2 of this thesis, we investigated and compared environmental factors - rainfall, average minimum temperature (T_{min}), average maximum temperature (T_{max}), and ground and flying invertebrate biomasses - and intensity and occurrence of nesting activity in Red-capped Larks *Calandrella cinerea* within and among each of three study environments, cool and wet South Kinangop, warm and wet North Kinangop and warm and dry Kedong. Furthermore, we tested if any of these factors influenced the timing of reproduction in this species. We found that environmental conditions and nesting activity in Red-capped Larks within each of our three climatically-distinct locations were frequently unpredictable and highly variable across the year. Moreover, environmental conditions and nesting activity differed spatially (among locations), suggesting that these conditions vary over

a small spatial scale. To our surprise, nesting activity in each of these locations was unrelated to any of the environmental conditions we measured, leaving a question on what other factors are important in influencing the timing of reproduction in this species, a question that requires further investigations.

Nestling growth and development and ontogeny of immune function in equatorial tropical regions have been associated with slow pace-of-life. Furthermore, variation in growth and immune function within and among populations in this region is often associated with specific environmental conditions. In the face of the frequently unpredictable and high variability in environmental conditions in this region, a knowledge gap exists on how nestling growth varies in different environments with distinct climatic conditions, and in the same environment across the year. We compared growth and immune function in nestlings of year-round breeding equatorial Red-capped Larks in South Kinangop, North Kinangop and Kedong, Kenya, three locations that are geographically close but climatically distinct. In addition, we studied growth and immune function of lark nestlings as a function of year-round variation in breeding intensity and rain within Kedong only. We monitored body mass, wing, and tarsus lengths at hatching (day 1) and at 4, 7, and 10 days post-hatch. Additionally, we quantified four indices of immune function (haptoglobin, nitric oxide, agglutination and lysis) using blood samples collected on day 10. We found that nestling body mass and size at hatching were lowest in the most arid location, Kedong, perhaps due to resources females allocated to their eggs. However, contrary to our predictions, nestlings in Kedong grew faster post-hatch compared to the other two cooler and wetter locations of South and North Kinangop, possibly due to the abundant food resources in this location compared to the other two. At hatching only and not in the days after, nestlings in Kedong were heavier during periods when most individuals in the population were breeding, while nestlings grew faster with more rain post-hatch in Kedong, pointing to better female body condition and food quality and quantity during these periods. We found no significant differences in nestling immune function, neither among locations nor with the year-round variation within Kedong, suggesting that nestling immune function develops at later stages post-fledge.

Equatorial tropical birds have been hypothesized to be long-lived with a slow pace-of-life. Unlike their mid-to-higher-latitude counterparts, these birds have been associated with well-developed immune defenses (that increase survivorship) even under challenging conditions such as reproduction. Equatorial tropical regions often lack a predictable intra-annual seasonal variation in environmental conditions characteristic of mid-to-higher-latitude system, but rather exhibit large variation in environmental conditions within locations and among locations that are close to each other. This means that reproduction is not tightly correlated with variation in environmental conditions, and birds can breed year round, making equatorial tropical systems ideally suited for disentangling the effects of reproduction from those caused by variation in environmental conditions. We exploited this equatorial tropical system to understand the role of reproduction in influencing variation in immune function and body mass, independent of environmental conditions, in chapters 4 and 5 of this thesis. In both of these chapters, we checked to confirm that any differences in immune function between breeding and non-breeding birds did not result from

variation in environmental conditions by testing if rainfall, T_{min} and T_{max} differed between breeding and non-breeding birds.

In chapter 4, we investigated how immune function and body mass of males and females Red-capped and Rufous-naped Larks *Mirafra africana* differed between breeding (chick-feeding) and non-breeding birds in these two species that live in the same equatorial grassland environment, yet occupy different niches within the grassland. We also investigated how immune function and body mass were affected by sex. We found that immune function in these sympatric species was not downregulated during reproduction but nitric oxide was instead upregulated during chick-feeding in both species. Additionally, nitric oxide was high during breeding periods when T_{max} was also high in Red-capped Larks, suggesting that higher T_{max} promoted a conducive environment for growth, development and reproduction of microorganisms and parasites that in turn triggered the elevation of nitric oxide. Further, we found that Red-capped and Rufous-naped Larks had contrasting immune indices with the former having higher nitric oxide than the latter while the latter had higher haptoglobin than the former, an indication of differences in life history adaptations of sympatric species facing variable and unpredictable environmental conditions. Body mass did not differ between breeding and non-breeding in any of the two species, while sex had an effect on body mass in Rufous-naped Lark, with heavier males than females. Rain and T_{min} did not differ between breeding and non-breeding in both species. T_{max} did not also differ between these two breeding statuses in Rufous-naped Larks but did in Red-capped Larks where T_{max} was higher during chick-feeding than during non-breeding. For Red-capped Larks therefore, we cannot rule out a confounding effect of T_{max} on the comparison of immune function during chick-feeding and non-breeding birds.

In chapter 5, we investigated the role of reproduction in influencing variation in immune function and body mass in three populations of year-round breeding Red-capped Larks living in our three equatorial Kenyan locations with distinct climates, South Kinangop, North Kinangop and Kedong, and which are geographically nearby one another. Each of these three locations is characterized by large intra- and inter-annual variations in quantity and timing of rainfall and food. Since only females incubate in this species, we investigated if immune function and body mass differed by sex among chick-feeding and non-breeding (males and females), and incubation (females only) from these three environments that are generally permissive of year-round breeding. Contrary to expectation, nitric oxide in Red-capped Larks increased during breeding (chick-feeding) and decreased during non-breeding, pointing to the species' capacity to maintain two physiological processes (reproduction and immune function) concurrently. Moreover, in periods during which nitric oxide was high and birds were breeding, T_{max} was also high, indicating that patterns of nitric oxide may have responded to patterns of breeding or to changes in T_{max}. Spatially, our three climatically-distinct locations differed in multiple immune indices during different breeding status, suggesting that different immune indices were influenced differently by different environmental conditions. Immune indices and body mass were not affected by sex. We deduce that birds living in environments with contrasting climatic conditions seem to develop different immune strategies to protect themselves against infections, presumably based on the prevailing

environmental conditions that put a selective pressure on the prevailing disease risks in their environments.

In conclusion, this thesis provides novel insights and new perspectives regarding how birds in equatorial tropics cope with their environment. It provides contrasting life histories between birds from mid-to-higher latitude zones and those from equatorial tropics, and how environmental conditions from these regions differ in the way they influence physiological processes, e.g., reproduction, growth and immune function, in birds. Within the equatorial tropical region, this thesis demonstrates the frequent unpredictability and high variability in environmental conditions within a location and among proximal locations. This results to high variability in the timing of reproduction, growth patterns and immune function within the year in a locations and among these proximal locations. To our surprise, this thesis brought to the fore new scientific knowledge that is in contrast to popular hypotheses. We found that environmental conditions that we measured – rain, T_{min}, T_{max}, and food availability - did not influence reproductive decisions in Red-capped Larks. We proposed that other factors, e.g., nest predation, female protein reserves, or social factors could be important in the timing of reproduction and further studies targeting these factors need to be carried out. Contrary to our expectation nestling Red-capped Larks from the driest location Kedong, which we had predicted would represent the location with the lowest primary productivity and associated low food availability, grew faster than those in the two cooler and wetter locations of South and North Kinangop; Kedong had actually the highest food availability. We proposed that our three locations may not have represented a typical aridity gradient with a decrease in primary productivity and associated decrease in invertebrate biomass. Future studies should focus on investigations of variation in growth rate in environments that represent a true gradient of aridity.

Unexpectedly, immune function of nestling Red-capped Larks neither varied among the three locations we studied nor within Kedong, suggesting that nestling immune function develops at later stages post-fledge. To develop a detailed understanding of ontogenesis in immune function, further studies should focus on measurement of several immune indices systematically at different growth time points, including post-fledge monitoring to adulthood. Contrary to popular hypothesis that avian immune function is compromised during periods of breeding, we actually found that nitric oxide was upregulated during chick-feeding periods compared to non-breeding in two sympatric species, Red-capped and Rufous-naped Larks. Nitric oxide also increased during chick-feeding and during incubation compared to non-breeding in North Kinangop and South Kinangop respectively in a study investigating variation in immune function of Red-capped Larks within and among three locations with distinct climatic conditions. These two outcomes point to support for an evolutionary link between life history strategy and immune function. Furthermore, the elevation of nitric oxide during breeding in Red-capped Larks co-occurred with periods of higher T_{max} in both studies, suggesting that patterns of nitric oxide may have responded to patterns of breeding activities or to changes in environmental conditions, which future studies should investigate.

Nederlandse Samenvatting

Veranderingen in omgevingsfactoren in gebieden die op hogere breedtegraad liggen zijn vaak afhankelijk van het seizoen en daardoor voorspelbaar. Daglengte valt hiermee samen en in deze regio is dit de belangrijkste aanwijzing voor de timing van de voortplanting bij vogels. Vogels in dit gebied kunnen hun voortplanting afstemmen op de omgeving door gebruik te maken van de langer wordende fotoperiode tijdens de lente. In deze regio speelt daglengte ook een belangrijke rol bij het bepalen van veranderingen in omgevingsfactoren zoals temperatuur en beschikbaarheid van voedsel, factoren die vogels gebruiken als aanvullende aanwijzingen om de timing van de voortplanting af te stemmen op lokale omgevingsomstandigheden. Dit resulteert in synchronisatie van het broeden in de lente binnen en tussen soorten die op dezelfde locatie leven. Bij vogels is er een verband tussen de groeisnelheid van de jongen en het tempo van leven (ook wel "pace of life" genoemd), en het is bekend dat nestjongen sneller groeien op hogere breedtegraden, in hooggelegen gebieden en in nattere omgevingen. Echter, variatie in de groei en ontwikkeling van jonge vogels wordt ook verondersteld aangepast te zijn aan specifieke omgevingsomstandigheden. Lichaamsfuncties, b.v. voortplanting, en groei, vereisen middelen die vaak schaars zijn en die fitness kosten met zich meebrengen. Fysiologische processen, zoals voortplanting en het afweersysteem, moeten gebruik maken van dezelfde beperkte middelen, en dit resulteert vaak in een afweging waarbij er niet in alle functies geïnvesteerd kan worden (zogenaamde trade-offs). Dit is vooral bestudeerd bij vogels die leven op hogere breedtegraden. Een hypothese is dat het afweersysteem tijdens veeleisende fases van de levenscyclus wordt onderdrukt, met name tijdens de voortplanting, wanneer dieren bronnen zoals voedsel, energie en tijd toewijzen aan het grootbrengen en verzorgen van het nageslacht. Niettemin hebben de seizoenen zowel invloed op de voortplanting als op het afweersysteem bij vogels op hogere breedtegraden. Behalve dat het wordt beïnvloed door trade-offs met de voortplanting, kan bij vogels seizoensvariatie in het afweersysteem ook worden veroorzaakt door veranderingen in omstandigheden in de omgeving, veranderingen die samenvallen met de voortplanting. Dit maakt het moeilijk om de effecten van reproductie op de variatie in het afweersysteem los te koppelen van die van omgevingsfactoren.

In tegenstelling hierop, zijn de omgevingsomstandigheden in tropische equatoriale omgevingen, en in de tropen in het algemeen, variabel binnen en tussen locaties die dicht bij elkaar liggen, wat leidt tot een diversiteit aan eigenschappen die met het broeden te maken hebben, zoals groeisnelheden van jongen. Biotische en abiotische factoren in verschillende klimatologische omstandigheden zijn niet kwantitatief opgetekend, of zijn her en der door de literatuur verspreid. Daardoor is het hiermee niet mogelijk om de bijdrage te onderzoeken van deze factoren aan de voortplanting in tropische vogels in equatoriale gebieden. Voor zover bekend heeft geen enkele studie gekeken naar de effecten van meerdere omgevingsfactoren op de voortplanting bij vogels in verschillende klimatologische omstandigheden in equatoriale tropen. In hoofdstuk 2 van dit proefschrift hebben we omgevingsfactoren - regenval, gemiddelde minimumtemperatuur (T_{min}), gemiddelde maximumtemperatuur (T_{max}) en voedsel (kruipende en vliegende ongewervelden) - en intensiteit en voorkomen van nestactiviteit in roodkapleeuweriken (*Calandrella cinerea*)

onderzocht, en vergeleken binnen en tussen elk van drie studieomgevingen: een koel en nat gebied - South-Kinangop -, een warm en nat gebied - North- Kinangop -, en een warm en droog gebied - Kedong. We hebben getest of een van deze factoren de timing van de voortplanting bij de roodkapleeuwerik beïnvloedde. We vonden dat zowel omgevingsomstandigheden als nestactiviteit van de leeuweriken binnen elk van onze drie klimatologisch verschillende locaties onvoorspelbaar en zeer variabel waren gedurende het jaar. Bovendien verschilden omgevingsomstandigheden en nestactiviteit ruimtelijk (tussen locaties), wat suggereert dat deze omstandigheden variëren op een kleine ruimtelijke schaal. Tot onze verbazing was de nestactiviteit op elk van deze locaties niet gerelateerd aan de omgevingscondities die we hebben gemeten, waardoor de vraag ontstaat welke andere factoren de timing van de voortplanting bij deze soort beïnvloeden, een vraag die verder onderzoek vereist.

De groei van nestjongen en de ontwikkeling en ontogenie van het afweersysteem in tropische equatoriale gebieden worden in verband gebracht met een traag tempo van leven. Bovendien wordt variatie in groei en afweersysteem binnen en tussen populaties in deze regio vaak geassocieerd met specifieke omgevingsomstandigheden. In het licht van de vaak onvoorspelbare en grote variabiliteit in omgevingsomstandigheden in deze regio, is er weinig bekend over hoe de groei van nestjonen varieert in verschillende omgevingen met verschillende klimatologische omstandigheden, en in eenzelfde omgeving gedurende het jaar. Wij hebben groei en afweersysteem in nestjongen van equatoriale roodkapleeuweriken, vogels die het hele jaar kunnen broeden, in drie locaties die geografisch dichtbij elkaar liggen maar klimatologisch verschillend zijn met elkaar vergeleken: Zuid-Kinangop, Noord-Kinangop en Kedong, Kenia. Daarnaast hebben we in Kedong gedurende een jaar onderzocht wat het effect van variatie in broedintensiteit en regen op groei en afweersysteem van de leeuwerik-jongen was. We hebben lichaamsgewicht, vleugel- en tarsuslengte bij het uitkomen (dag 1) en 4, 7 en 10 dagen na het uitkomen gemeten. Daarnaast kwantificeerden we vier maten van afweersysteem (haptoglobine, stikstofoxide, agglutinatie en lysis) met behulp van bloedmonsters die we verzamelden op dag 10. We ontdekten dat lichaamsgewicht en grootte bij het uitkomen het laagst waren op de meest droge locatie, Kedong, wat zou kunnen komen doordat vrouwtjes leeuweriken hier minder (konden) investeren in hun eieren. Echter, in tegenstelling tot onze voorspellingen groeiden jongen in Kedong na het uitkomen sneller dan de andere twee, koelere en nattere locaties, Zuid- en Noord-Kinangop, mogelijk vanwege de grote hoeveelheid voedsel in Kedong in vergelijking met de andere twee locaties. Uit de resultaten van de vergelijking over het jaar in Kedong bleek dat tijdens de periodes dat de meeste individuen broedden, de nestjongen tijdens het uitkomen maar niet in de dagen erna zwaarder waren, terwijl de nestjongen sneller groeiden na het uitkomen in periodes dat er meer regen viel. Dit wijst erop dat tijdens deze periodes vrouwtjesleeuweriken een betere lichaamsconditie hebben, en dat de kwaliteit en kwantiteit van het voedsel goed is. We vonden geen significante verschillen in het afweersysteem van nestjongen, noch tussen locaties, noch in de variatie gedurende het jaar binnen Kedong, wat suggereert dat het afweersysteem van nestjongen zich in een later stadium, nadat de jongen het nest verlaten, ontwikkelt.

De hypothese is dat equatoriale tropische vogels lang leven met een langzaam levenstempo. In tegenstelling tot hun tegenhangers die op hogere breedtegraad leven, worden deze vogels geassocieerd met een goed ontwikkeld afweermechanisme (dat de overlevingskans vergroot), zelfs tijdens uitdagende periodes zoals tijdens de voortplanting. Equatoriale tropische regio's hebben vaak niet de voorspelbare jaarlijkse seizoensvariatie van omgevingsomstandigheden die kenmerkend is voor regio's op hogere breedtegraden, maar vertonen eerder grote onvoorspelbare verschillen in de omgevingsomstandigheden over tijd, en tussen locaties die dicht bij elkaar liggen. Dit betekent dat de voortplanting niet nauw gecorreleerd is met de variatie in de omgevingsomstandigheden, en dat vogels zich het hele jaar door kunnen voortplanten, waardoor equatoriale tropische systemen bij uitstek geschikt zijn om de effecten van reproductie te ontwarren van de effecten die worden veroorzaakt door variatie in omgevingsomstandigheden. In hoofdstuk 4 en 5 van dit proefschrift hebben we deze eigenschap van het equatoriale tropische systemen benut om inzicht te krijgen in de rol die voortplanting speelt in de variatie in het afweersysteem en lichaamsgewicht, onafhankelijk van omgevingsomstandigheden. In beide hoofdstukken hebben we gekeken of verschillen in afweersysteem tussen broedende en niet-broedende vogels niet het gevolg waren van verschillen in de omgevingsomstandigheden door te testen of regenval, T_{min} en T_{max} verschilden tussen broedende en niet-broedende vogels.

In hoofdstuk 4 hebben we onderzocht hoe het afweersysteem en lichaamgewicht van mannetjes en vrouwtjes verschilden tussen broedende en niet-broedende roodkap- en roodnekleeuweriken (*Mirafra africana)*. Deze twee soorten leven in hetzelfde equatoriale grasland, maar bezetten verschillende niches binnen dit grasland. We onderzochten ook hoe immuunsysteem en lichaamsgewicht beïnvloed werden door sekse. We ontdekten dat het afweersysteem bij deze sympatrische soorten niet werd verzwakt tijdens de reproductie, maar dat de concentraties stikstofoxide in plaats daarvan omhoog gingen in de periode waarin de ouders de jongen voeren. Bovendien waren de concentraties stikstofoxide van de roodkapleeuweriken tijdens broedperioden hoog als T_{max} ook hoog was. Dit suggereert dat een hogere T_{max} een gunstige omgeving schiep voor groei, ontwikkeling en reproductie van micro-organismen en parasieten die op hun beurt de verhoging van stikstofoxide teweegbrachten. Verder ontdekten we dat roodkap- en roodkeelleeuweriken contrasterende immuun-maten lieten zien. De eerstgenoemde had een hogere stikstofoxide dan de laatste, terwijl de laatste hoger haptoglobine had dan de eerste, een aanwijzing voor verschillen in levensstijl aanpassingen aan variabele en onvoorspelbare milieuomstandigheden van twee sympatrische soorten. In beide soorten verschilde lichaamsgewicht niet tussen broedende en niet broedende vogels, terwijl de mannetjes zwaarder waren dan de vrouwtjes in roodnekleeuweriken. Bij beide soorten hadden regen en T_{min} geen effect op het broedgedrag. T_{max} verschilde ook niet tussen de twee broedstatussen bij roodnekleeuweriken, maar deed dit wel bij roodkapleeuweriken waar de T_{max} hoger was tijdens het voeren van de jongen dan wanneer de vogels niet broedden. Voor roodkapleeuweriken kunnen we daarom een effect van T_{max} op de vergelijking van het afweersysteem van vogels tijdens de jongenfase en niet broedende vogels niet uitsluiten.

In hoofdstuk 5 onderzochten we hoe de voortplanting variatie in het afweersysteem en lichaamsgewicht van roodkapleeuweriken beïnvloedt. Dit onderzoek deden wij in onze drie equatoriale Keniaanse locaties die geografisch dichtbij elkaar liggen, echter met verschillende klimaten: Zuid-Kinangop, Noord-Kinangop en Kedong, drie gebieden waar de vogels gedurende het hele jaar kunnen broeden. Elk van deze drie locaties wordt gekenmerkt door grote schommelingen in de hoeveelheid en periodes van regenval en voedsel binnen en tussen jaren. Omdat alleen vrouwtjes in deze soort incuberen, onderzochten we variaties in het afweersysteem en het lichaamsgewicht tijdens het voeren van de jongen en wanneer de vogels niet broeden in beide seksen, en tijdens de incubatie alleen in vrouwtjes. In tegenstelling tot wat verwacht werd, steeg stikstofoxide in roodkapleeuweriken tijdens het voeren van de jongen en nam het af wanneer de vogels niet broeden. Dit wijst op het vermogen van de soort om gelijktijdig twee fysiologische processen (reproductie en afweersysteem) te handhaven. Bovendien was de T_{max} in perioden waarin stikstofoxide hoog was en vogels broedden, ook hoog, wat aangeeft dat patronen van stikstofoxide kunnen reageren op broedpatronen of op veranderingen in T_{max}. We vonden verschillen tussen onze drie klimatologisch verschillende locaties in meerdere maten van het afweersysteem tijdens verschillende broedstatussen, wat suggereert dat verschillende maten van het afweersysteem anders werden beïnvloed door verschillende omgevingscondities. De maten van het afweersysteem en lichaamsgewicht werden niet beïnvloed door sekse. We concluderen dat vogels die leven in een omgeving met contrasterende klimatologische omstandigheden, verschillende afweer-strategieën lijken te ontwikkelen om zichzelf tegen infecties te beschermen, vermoedelijk gebaseerd op de heersende omgevingsomstandigheden die een selectiedruk uitoefenen door de heersende ziekterisico's in hun omgeving.

Concluderend biedt dit proefschrift nieuwe inzichten en nieuwe perspectieven met betrekking tot hoe vogels in equatoriale tropen omgaan met hun omgeving. Het laat zien dat de levensgeschiedenissen van vogels die leven op hogere breedtegraadzones en die van equatoriale tropen anders zijn, en hoe de omgevingsomstandigheden van deze gebieden verschillen in de manier waarop ze fysiologische processen, bijvoorbeeld reproductie, groei en afweersysteem, beïnvloeden bij vogels. Dit proefschrift toont de onvoorspelbaarheid en grote variabiliteit in omgevingsomstandigheden binnen een locatie en tussen dichtbij elkaar liggende locaties binnen het equatoriale tropische gebied. Deze variatie in omstandigheden heeft tot gevolg dat er een hoge variabiliteit is over het jaar in de timing van reproductie, groeipatronen en afweersystemen zowel op één locatie, en tussen de drie dicht bij elkaar liggende locaties.

Dit proefschrift heeft nieuwe wetenschappelijke kennis opgeleverd die in contrast staat met de in zwang zijnde hypothesen. We ontdekten dat de omgevingscondities die we hebben gemeten - regen, T_{min}, T_{max} en voedselbeschikbaarheid – de beslissing om zich wel of niet voort te planten in roodkapleeuweriken niet beïnvloeden. We denken dat andere factoren, zoals nestpredatie, vrouwelijke eiwitreserves of sociale factoren belangrijk zouden kunnen zijn in de timing van de voortplanting en dat studies gericht op deze factoren moeten worden uitgevoerd. In tegenstelling tot onze verwachting groeiden jonge roodkapleeuweriken sneller in de droogste locatie, Kedong, waarvan we voorspeld hadden dat deze de laagste primaire productiviteit en de

bijbehorende lage voedselbeschikbaarheid zou hebben, dan die op de twee koelere en nattere locaties in Zuid- en Noord-Kinangop. Kedong bleek de hoogste voedselbeschikbaarheid te hebben van de drie gebieden. Onze drie locaties geven mogelijk niet een typische droogtegradiënt weer, met een daling van de primaire productiviteit en de bijbehorende afname van biomassa van ongewervelde dieren. Toekomstige studies moeten zich richten op onderzoeken naar variatie in groeisnelheid in omgevingen die een echte gradiënt in droogte vertegenwoordigen.

Tegen onze verwachting in, varieerde het afweersysteem van nestjongen van de roodkapleeuwerik niet tussen de drie locaties die we bestudeerden, noch binnen Kedong, wat suggereert dat het afweersysteem van de nestjongen zich in latere stadia na het uitvliegen ontwikkelt. Om een gedetailleerd inzicht in de ontogenese van het afweersysteem te krijgen, zouden verdere studies gericht moeten zijn op het systematisch meten van verschillende maten van het afweersysteem, op verschillende tijdstippen in de groei, inclusief het volgen van de jongen tot volwassenheid. In tegenstelling tot de populaire hypothese dat het afweersysteem van vogels tijdens de broedperiode verzwakt wordt, constateren we in dit proefschrift dat in twee sympatrische soorten, roodkap- en roodnekleeuweriken, de hoeveelheid stikstofoxide toeneemt tijdens het voeren van de jongen in vergelijking met periodes dat er niet gebroed wordt. Stikstofoxide nam ook toe tijdens het voeren van de jongen en de incubatie in vergelijking met niet broedende vogels in respectievelijk Noord-Kinangop en Zuid-Kinangop in het onderzoek naar de variatie in het afweersysteem van roodkapleeuweriken binnen en tussen drie locaties met verschillende klimatologische omstandigheden. Deze twee uitkomsten wijzen op ondersteuning voor een evolutionaire link tussen levensgeschiedenisstrategie en het afweersysteem. Verder is de verhoging van stikstofoxide tijdens het broeden in roodkapleeuweriken samen met perioden van hogere T_{max} in beide onderzoeken opgetreden, wat suggereert dat patronen van stikstofonoxide kunnen hebben gereageerd op patronen van broedactiviteit of op veranderingen in milieuomstandigheden, factoren die toekomstige studies zou moeten onderzoeken.

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Addresses of Co-authors

B. Irene Tieleman[1]
Kevin D. Matson[2]
Maaike A. Versteegh[1]
Muchane Muchai[3]
Samuel N. Bakari[1, 4]

[1]Groningen Institute for Evolutionary Life Sciences, University of Groningen, P.O. Box 11103, 9700 CC Groningen, The Netherlands

[2]Resource Ecology Group, Department of Environmental Sciences, Wageningen University, Droevendaalsesteegh 3a, 6708 PB Wageningen, The Netherlands

[3]Department of Clinical Studies (Wildlife and Conservation), College of Agriculture and Veterinary Sciences, University of Nairobi, Box 30197-00100, Nairobi, Kenya

[4]Ornithology Section, Department of Zoology, National Museums of Kenya, P.O. Box 40658 – 00100 GPO, Nairobi, Kenya

List of Publications

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