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# Strive to survive

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# Strive to survive:
## The Skylark's ecology and physiology in an annual-cycle perspective

**Arne Hegemann**

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# Strive to survive:
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**PART III: Blood parasites and brood parasites**

**PART IV: Combing ecology, physiology and conservation**


staatsbosbeheer
Zaans Huisje
Zaans Huisje


LED HEADLIGHT

# CHAPTER 1

# General introduction

Arne Hegemann

## Annual-cycle perspectives in avian research

The ecology, physiology and behaviour of birds, and most other vertebrates, is characterized by different phases of their annual cycle. Individuals need to carry out activities like reproduction, moult or migration, and time them with biotic and abiotic environmental changes to optimise their fitness. It seems intuitive that events during one annual-cycle stage can influence subsequent annual-cycle stages. For example, unfavourable weather conditions during spring migration may delay the arrival date on the breeding grounds. In turn, this may delay the onset of reproduction, which usually leads to reduced reproductive output. Already in the early 1970s, Fretwell (1972) pointed out the need to consider such seasonal interactions in avian research. Indeed, early work on geese suggested carry-over effects between different annual-cycle stages. For example, Brent Geese (*Branta bernicla*) with a high dominance status profit from enhanced feeding opportunities, resulting in accumulation of more body reserves in spring. These birds also have the highest probability of successful breeding (Teunissen *et al.* 1985). Finally, one of the core assumptions in ecology and evolution, a trade-off between current and future reproduction (Williams 1966, Stearns 1992), also suggests the involvement of carry-over effects between annual-cycle stages. Despite the evident connection between annual-cycle stages, the integration and combined analyses of multiple parts of the annual cycle remained the exception, rather than the rule in avian research for many decades.

One reason was our inability to track individual (migratory) birds beyond a few kilometres, which made it challenging to follow individuals throughout the annual cycle. With the establishment of and recent advances in tracking technology (e.g. colour-ringing, satellite transmitters, light level geolocators or stable isotope analyses) it becomes increasingly possible to follow individuals through (a great part of) their annual cycle and over several years. Results from studies using such techniques have well established that the different phases of the annual cycle are tightly connected by carry-over effects on the individual level (Marra *et al.* 1998, Webster *et al.* 2002, Drent *et al.* 2003, Norris *et al.* 2004, Norris 2005, Webster & Marra 2005, Marra *et al.* 2006). These studies targeted on migratory birds and focussed on carry-over effects in relation to individual movement patterns and behavioural ecology. Evidence for the importance of carry-over effects between different annual-cycle stages in resident species is much scarcer, but has been demonstrated particularly with respect to trade-offs between current and future reproduction (e.g. Nilsson & Svensson 1996). However, most studies that do not focus on migration still study only a single season. This holds particularly true for studies on physiology. As a result, mechanistic links that mediate carry-over effects are still poorly understood (but see Piersma & van Gils 2011). One classic example is the increased winter mortality of Kestrels (*Falco tinnunculus*) that raised experimentally enlarged broods in the preceding summer (Daan *et al.* 1996). Almost two decades later, the underlying physiological mechanisms that causes the death of a bird months after increased workload, remains unknown. Clearly, there is an urgent need for a better understanding of the physiological mechanisms linking different annual-cycle stages.

## The role of immune function in an annual-cycle perspective

Survival is one of the most important components of lifetime reproductive success (Clutton-Brock 1988). Besides starvation and predation, disease is the most important cause of mortality. The immune system is a major physiological component of self-maintenance and promotes survival by reducing the probability of disease-related mortality (Roitt *et al.* 1998). Because the immune system also incurs costs in terms of its production, maintenance and activation (Schmid-Hempel 2003, Klasing 2004), one of the central hypotheses in ecological immunology proposes that immune defences are traded off against competing physiological and behavioural processes (Sheldon & Verhulst 1996, Lochmiller & Deerenberg 2000, Norris & Evans 2000). Its costs and benefits make the immune system a potential candidate for (i) a physiological mechanism that may be involved in shaping the exact timing and outcome of annual-cycle events like reproduction and migration, and (ii) for mediating carry-over effects between stages. While the benefits of a functioning immune system - increased survival - are obvious, the costs of becoming sick can be paid in various currencies like physiological and behavioural changes (reviewed by Owen-Ashley & Wingfield 2007). Such changes can impact different annual-cycle activities, like changing territorial behaviour, (Owen-Ashley & Wingfield 2006, Owen-Ashley *et al.* 2006), reducing parental effort (Bonneaud *et al.* 2003), postponing moult (Martin 2005) or delaying migration (van Gils *et al.* 2007). Consequently, a better understanding of immune function in wild animals may help to gain better insights in the underlying mechanisms that are responsible for ecological and evolutionary processes involved in shaping the annual cycle and the life history of individuals.

If the outcome of the trade-off between immune function and other physiological and behavioural processes differs among annual-cycle stages, seasonal modulations in immune function may result. Additionally, the selective pressures exerted by pathogens and parasites, which are also expected to shape these trade-offs (Horrocks *et al.* 2011), may differ spatially (Piersma 1997, Mendes *et al.* 2006b) and are known to vary temporally (Dowell 2001, Cosgrove *et al.* 2008). The outcome of such trade-offs between immune function and other physiological and behavioural processes could very well explain findings of reduced immune responses during reproduction (e.g. Ilmonen *et al.* 2000, Bonneaud *et al.* 2003, Ardia 2005), during migration (e.g. Owen & Moore 2006, Owen & Moore 2008, Buehler *et al.* 2010b) or during winter (e.g. Svensson *et al.* 1998). In addition to trade-offs between the immune system and other physiological systems, trade-offs within the immune system itself may also occur. For example, organisms may shift from more to less costly defences during times of high energy demand or low resource availability (Hasselquist 2007, Buehler & Piersma 2008, Martin *et al.* 2008). More specifically, Lee (2006) hypothesizes a switch from costly inflammatory responses to highly specific but less costly antibody responses. However, few studies have investigated multiple immune indices over the entire annual cycle in free-living or captive birds (Buehler *et al.* 2008, Pap *et al.* 2010a, Pap *et al.* 2010b), and none has investigated the consistency of seasonal patterns across multiple years.

Furthermore, it remains difficult to distinguish a reallocation of resources away from the immune system from a reallocation or redistribution within the immune system. Reductions in one or more elements of the immune system do not necessarily equate to a net reduction in immune function, because other parts of the immune system may be boosted simultaneously (Adamo 2004). To understand trade-offs and interactions within the immune system, experimental challenges of the immune system and subsequent quantification of the responses using multiple indices are required (Martin *et al.* 2006b, Martin *et al.* 2008, Boughton *et al.* 2011, Pedersen & Babayan 2011).

## Physiology, ecology and behaviour in the annual cycle of Skylarks: An integrative approach

Studying details of immune function throughout the annual cycle in free-living birds is crucial to understand how birds stay healthy year-round (Buehler *et al.* 2010a). Linking these studies with ecological and behavioural data can shed light on how birds trade off immune function with other annual-cycle events such as reproduction or migration.

In this thesis, I combine natural history and immune function throughout the annual cycle of free-living Skylarks (*Alauda arvensis*), a temperate zone breeding bird. I apply a variety of tracking methods (colour-ringing, radio telemetry and stable isotope analyses) to follow birds throughout the annual cycle and for multiple years. We apply a variety of tools and techniques (e.g., immunological tests, genetic tools, metabolic measurements) to measure aspects of physiology in general and of immune function in particular. Furthermore, experimental manipulations in the field and under lab settings help us to reveal mechanistic links. This unique integrative and multifaceted approach allows me to combine physiology, ecology and behaviour in order to gain a better ecological and evolutionary understanding of links between different stages of the annual cycle in particular and of life-history evolution in general.

## The study species: The Skylark (*Alauda arvensis*)

The Skylark is a widespread, temperate zone passerine occurring in open habitats in most of Eurasia. Its preferred habitat contains a mixture of short and sparse vegetation with open ground to forage (20–50% vegetation cover, 15–25 cm vegetation height). Vertical structures like trees are avoided year-round (Glutz von Blotzheim & Bauer 1985, Donald 2004). While natural steppes are the primary habitat, most birds of Europe nowadays breed in modern agricultural farmland (Donald 2004). Birds from northern Europe migrate, whereas populations from southern Europe are resident year-round.

In the Netherlands, Skylarks experience several annual-cycle stages characterised by seasonal changes in energy and time budgets, social structure and diet (Fig. 1.1). Dutch Skylarks arrive by mid or end of February on the breeding grounds to establish the territories and most pairs start reproduction in early May. Skylarks build their nests on the

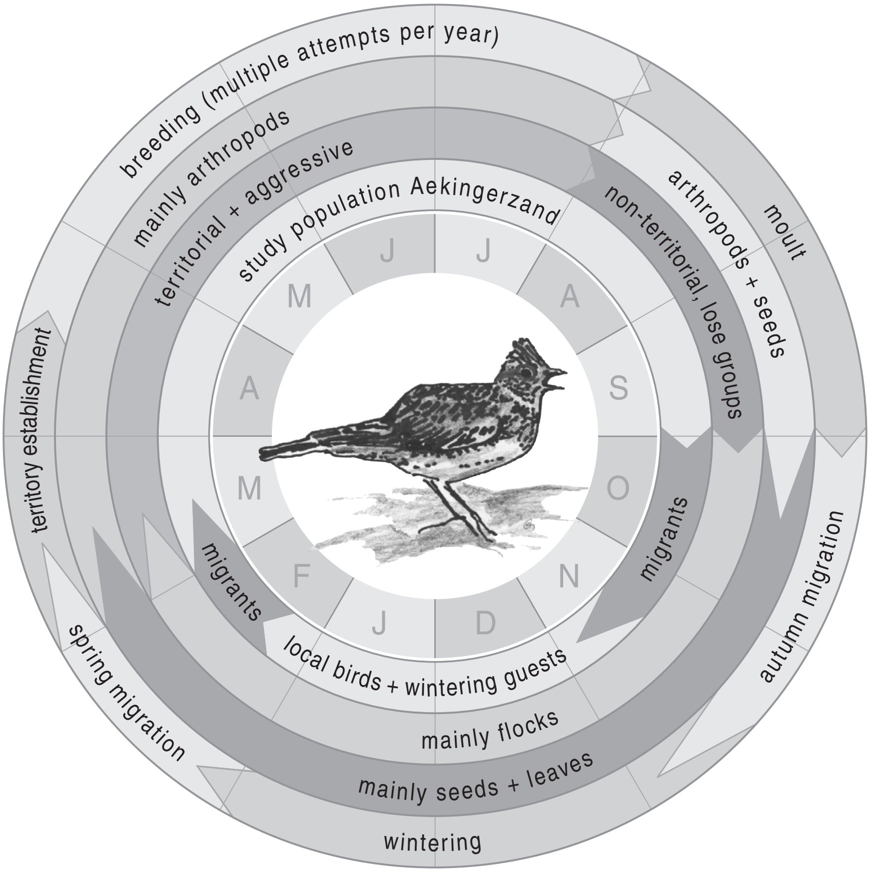

**Figure 1.1:** The annual cycle of a Skylark in the northern Netherlands indicating duration and characteristics of each annual-cycle stage. Length of arrows in the transition between two stages indicates the amount of variability between years and/or individuals. Data on diet are based on Green (1978), Donald *et al.* (2001) and Geiger *et al.* (in prep.). All other data are based on results presented in this thesis and further own unpublished data.

ground and face high predation rates (Donald 2004). Females produce several clutches per year; each clutch contains 3–5 eggs. A single female can have three successful nests in a breeding season, but five unsuccessful attempts in one season also occur (own observations). Skylark nestlings develop quickly and leave the nest at an age of about 8 days, which is well before they can fly (at an age of 14 days). Parents continue feeding fledglings until they reach independence at 30 days of age (Delius 1964, Delius 1965, Glutz von Blotzheim & Bauer 1985). The multiple breeding attempts make the Skylarks' breeding season relatively long compared to other passerines. The last fledglings leave the nest at the end of July, in some years even in early August. In August and September, adults and young undergo a complete moult on their breeding grounds. October and the first half of November as well as February and March are characterised by strong Skylark migration. During these periods, also many birds from northern and eastern breeding populations pass through the Netherlands (LWVT/SOVON 2002). Skylarks also commonly winter in most parts of the Netherlands (e.g. Bijlsma *et al.* 2001).

During the breeding season, Skylarks are highly territorial and aggressive, but outside the breeding season they form flocks, especially during migration and winter. They are mainly diurnally active throughout the year, but night migration is common as well. Outside the breeding season, their diet is dominated by seeds and green parts of plants. During summer, arthropods take up a high percentage of the diet. Nestlings are almost exclusively fed with arthropods (Green 1978, Glutz von Blotzheim & Bauer 1985, Jenny 1990a, Donald *et al.* 2001, Donald 2004).

## Declining populations

As many other farmland birds, the Skylark has been rapidly declining in (western) Europe (BirdLife International 2004, EBCC 2009). Since 1980, European populations declined by 48% and an approximate 39 million Skylarks were lost in this period (PECBMS 2012). In the Netherlands, the decline is even much steeper: numbers dropped by almost 95% from around 700,000 breeding pairs in the 1970s to about 38,000 in 2005 (Teixeira & Alleijn 1979, SOVON 2002, van Dijk *et al.* 2008). The decline is tightly associated with the general intensification of agricultural practices (Chamberlain *et al.* 2000, Newton 2004, Donald *et al.* 2006). Specifically, the switch from spring cereals to winter cereals has been suggested as the main cause for the decline: Winter cereals grow taller and denser in an early phase of the breeding season and become unsuitable for Skylark nesting in early summer. As a result, the number of breeding attempts per season significantly decreased and the number of fledglings is not sufficient anymore to maintain population levels (Chamberlain *et al.* 1999, Chamberlain *et al.* 2000, Donald *et al.* 2002, Donald 2004, Donald & Morris 2005). The change from summer to winter cereals also resulted in a loss of overwintering stubble fields, which is thought to reduce food supply in winter, and could reduce survival rates (Donald *et al.* 2001, Newton 2004, Siriwardena *et al.* 2007, Siriwardena *et al.* 2008). Furthermore, the use of pesticides and herbicides negatively impacts population levels (Topping & Odderskaer 2004, Henderson *et al.* 2009). Despite the clear links to changes in agricultural intensification, the exact causes and underlying (physiological) mechanisms of the population declines in Skylarks are still poorly understood.

## The study area: The Aekingerzand

In this study, I focussed on a breeding population of Skylarks in the “Aekingerzand”, part of the National Park Drents-Friese Wold in the northern Netherlands (52°55'N, 6°18'E). The Aekingerzand contains a mixture of open sand, groups of trees, heath- and grasslands on nutrient-poor soil and surrounded by a thin belt of forest (Fig. 1.2). About 500

**Figure 1.2:** Map of the study area Aekingerzand. The Aekingerzand is part of the National Park Drents-Friese Wold in the northern Netherlands. The study area includes different habitats like sand dunes, forest edges, heath- and grassland.

sheep graze the area year-round, keeping the vegetation short and limiting the extent of succession. The Aekingerzand covers about 386 ha, but less than 240 ha are suitable habitat for Skylarks. The unsuitable parts include open sand or areas dominated by trees. About 80–100 pairs of Skylarks (own data) and 60–80 pairs of Woodlarks (S. Waasdorp personal communication) breed in the area. The National Park Drents-Friese Wold is surrounded by intensive farmland with maize, potatoes and wheat as the main crop types and to a lesser extent by intensive grasslands. During the migratory seasons I caught Skylarks together with Kees van Eerde using his fascinating set-up of large clapnets at the National Park Dwingelderveld, about 15 km SW of the Aekingerzand.

The scarce and short vegetation, open sandy patches and numerous small hills make the Aekingerzand a particularly suited place for Skylark research. These habitat structures closely resemble the natural habitat of the species and host a high density of breeding pairs. These habitat structures also provide the unique opportunity to read colour-rings in a species that is rather cryptic. Thus studying Skylarks in the Aekingerzand offers the opportunity to closely follow colour-ringed individuals and study their ecology and behaviour. As Skylarks show high annual return rates (Delius 1965, Jenny 1990b), we can follow birds in the Aekingerzand over multiple years: adult males have annual return rates of 71–81%, adult females of 60–72%, male nestlings of 16–32% and female nestlings of 13–23% (own unpublished data). In contrast, the high and dense vegetation in the modern agricultural landscape usually allows observations only when birds are seen flying. This makes it very challenging to follow many individual birds throughout their life in such habitats.

Studying Skylarks in a high-density natural population as the Aekingerzand offers several more advantages. An integrative approach combining aspects of ecology, behaviour and physiology is labour-intensive and requires for example (repeated) catching of individual birds or carrying out experiments. This is logistically only feasible in a high density population, where many individual birds can be studied in close detail at a time. Accomplishing such studies in areas with low Skylark densities like modern agricultural landscapes would be impossible. Still, Skylark conservation might be able to benefit from fundamental evolutionary and ecological studies in a natural high-density population. For example, extrapolating mechanistic links between environmental conditions and survival in natural populations may help understanding causes of population declines in farmland, where birds face changed ("manipulated") conditions. Moreover, very few studies of Skylarks in natural habitat exist, with the pioneering work of Juan Delius on his study population in dunes of northwest England being the only one published in detail (Delius 1964, Delius 1965, but see also Wolfenden & Peach 2001). But how do we want to thoroughly understand mechanisms underlying population declines when we lack reference data from natural populations? Taken together, conservation efforts for Skylarks in agricultural landscapes can also benefit from fundamental evolutionary and ecological studies in a natural high-density population like the Aekingerzand. Applied correctly, these studies may help to improve conservation plans also in different habitats (see also Sutherland *et al.* 2004).

## Thesis outline

This thesis aims to integrate aspects of ecology and behaviour with aspects of physiology in an annual-cycle perspective of Dutch Skylarks. Part I focuses on the natural history of Dutch Skylarks that forms the basis and provides key background information for most subsequent chapters. Part II focuses on the annual cycle of immune function. Part III sheds light on blood and brood parasites. Part IV combines ecological and physiological approaches, identifying mechanistic links and carry-over effects. I conclude with a synthesis in which I link the results of different chapters with each other and discuss them in a broader context. I also point out how conservation management plans for Skylarks in European farmland may benefit from our results.

More specifically, in chapter 2 my co-workers and I studied the wintering strategy of Dutch Skylarks, in order to obtain a complete picture of the annual cycle. While we know much about natural history of Skylarks during breeding, it remained unclear whether Skylarks that breed in the Netherlands are resident or migratory. It has been hypothesized that a part of the Dutch breeding population winters locally and is joined by birds from Scandinavia and Russia (e.g. Venema 2001, SOVON 2002). Other sources suggest that most breeding birds migrate (Lensink 1993, Vergeer & van Zuylen 1994, Hustings *et al.* 2006, van Dijk *et al.* 2008), with France being the most important wintering destination (van Beusekom 2006). Yet, these competing ideas had not yet been verified. We analysed a 100 year dataset from the Dutch Ringing Centre and radio-tagged Skylarks from the Aekingerzand to verify which wintering strategy Dutch Skylarks follow. This combination of approaches suggests coexistence of migration and local wintering among Dutch Skylarks in general and specifically also in our study population.

The Skylark is one of few European passerine species where sexes do not show plumage dimorphism. Additionally, Skylarks have a complete postnuptial moult in adults and a complete post-juvenile moult in young birds on their breeding grounds (Pätzold 1975, Glutz von Blotzheim & Bauer 1985, Svensson 1992). Traditionally, wing length is used to sex Skylarks and tongue spots have been suggested as a tool to age birds. In chapter 3, we put these theories to a test.

After having established that Dutch Skylarks are partial migrants (chapter 2), and with the additional knowledge of how to sex them (chapter 3), I now make the step to part II in which we investigated the physiology of Skylarks, with a focus on the immune system. We present patterns of baseline immune function and of induced immune responses across the complete annual cycle. In chapter 4, we tested two hypotheses of ecoimmunology by measuring components of innate and acquired arms of the immune system through two complete annual cycles and within and among four breeding seasons. If patterns of immune function are due to trade-offs with other fitness enhancing activities that are consistently cyclical, then seasonal immune modulation is predicted to be consistent across years. Alternatively or additionally, allocation to immune function could be a consequence of encountered environmental conditions that change not only throughout the year but may also vary between years (e.g. resource availability and pathogen pressure). We also explored if, (i) body mass varied in a manner similar to the immune indices at the population level, and (ii) if body mass correlated with these indices on the individual level. We found significant variation in immune indices and body mass across the annual cycle, and these patterns differed between years, suggesting a substantial influence of environmental conditions on baseline immune function.

After having established annual patterns of baseline (non-induced) immune function, we tested in chapter 5 and 6 if induced immune responses show the same patterns. In chapter 5, we first describe patterns of metabolic rate and body mass throughout the annual cycle. We then quantified the energetic costs of an induced immune response (acute phase reaction to an LPS injection) and measured metabolic rate, body mass loss, body temperature, as well as ketone and glucose concentrations. We again did this throughout the annual cycle to test if the energetic costs of an immune reaction are modulated across the annual cycle. Despite clear variation in energy budgets, the magnitude of the acute phase response was similar in all annual-cycle stages, contradicting the hypothesis that energetically expensive immune responses are compromised during energetically demanding times (Lee 2006, Hasselquist 2007, Martin *et al.* 2008).

In chapter 6, we then investigated and characterised the immunological part of the induced immune response. We experimentally challenged the immune system of Skylarks and measured multiple immune indices to quantify the immune response. Such a design is important to understand trade-offs and interactions within the immune system (Martin *et al.* 2006b, Boughton *et al.* 2011, Pedersen & Babayan 2011). This experimental study further allowed us to answer the question whether induced responses are modulated among annual-cycle stages (following patterns of baseline immune function, chapter 4) or if they are maintained throughout the year (reflecting patterns of energetic costs, chapter 5). The result demonstrates that immune responses involve multiple immune parameters and that immune responses are consistent among the annual-cycle stages of Skylarks.

In chapter 7, we changed perspective and studied the counterpart of immune function: parasite infections. Together with my colleague Pavel Zehtindjiev and others we looked at haemosporidian infections in Skylarks from the Aekingerzand and in Skylarks caught in Italy. The prevalence of infections was lower in Skylarks from the Aekingerzand compared to birds caught in Italy.

Even though Cuckoos (*Cuculus canorus*) do not challenge the immune system, they form another potential type of parasite: brood parasites. Skylarks have rarely been reported as host for Cuckoos (Glutz von Blotzheim & Bauer 1985, Moksnes & Roskaft 1995, Davies 2000). In chapter 8 we document a possible case of Cuckoo nestling ejection by its host parents and raise the question if Skylarks can discriminate a parasite nestling. Such behaviour would be remarkable because I know of no published case of Cuckoo nestling ejection by host parents. Furthermore Antonov *et al.* (2010) showed in an experimental study that Skylarks are completely unable to recognize eggs. These authors suggest that anti-brood parasite defences never evolved in this species.

The last part of this thesis (part IV) aims for an integration of all individual results derived from studies reported in previous chapters. In chapter 9, we experimentally handicapped Skylarks and investigated the effects on immune function, survival and reproduction in order to understand mechanistic links between current and future reproduction, one of the keystones of life-history theory. We conclude that the balance between current reproduction and survival shifts from affecting nestlings to affecting parents as the reproductive season progresses, and that immune function is the physiological mechanism mediating this trade-off.

In chapter 10, we link the wintering strategy of individual birds (migration versus residency) to their immune function, reproduction and survival. Partial migratory populations offer the unique opportunity to study carry-over effects of migration on reproduction and survival within a single population. We tried to take the understanding of carry-over effects to a higher level by studying immune function as underlying mechanism. We used stable deuterium analyses from claw samples to determine the migration strategy of individual birds in the previous winter. We found that the wintering strategy (migration vs. residency) has carry-over effects on immune function, body mass and future return rates.

I end this thesis with a general discussion (chapter 11), in which I synthesise all findings of this thesis on physiological and ecological aspects throughout the annual-cycle of Skylarks. I will discuss how the work presented in this thesis has shaped our ideas on how birds adjust their immune system throughout the annual cycle in order to stay alive and reproduce. To illustrate our ideas, I introduce a new concept on immune function. I also point out how our fundamental ecological and evolutionary studies can be used to improve conservation management plans. I further identify gaps in our current knowledge and formulate ideas for future research. The outlined research ideas will help to further increase our understanding of how environmental factors shape the interplay between ecological, physiological and behavioural aspects in the annual cycle of free-living birds. Such knowledge will also increase our ability to successfully protect and conserve (threatened) species.

### Acknowledgements

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# PART I

## Natural history


PH-THS

# CHAPTER 2

# Are Dutch Skylarks partial migrants? Ring recovery data and radio-telemetry suggest local coexistence of contrasting migration strategies

Arne Hegemann, Henk P. van der Jeugd, Merlijn de Graaf,
Lotte L. Oostebrink & B. Irene Tieleman

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**Abstract** In recent years, Skylarks (*Alauda arvensis*) have undergone dramatic population declines in many European countries. Evidence exists for deteriorating conditions during the breeding season, but little is known about the situation during the rest of the annual cycle. Here we use two approaches to test if the Dutch breeding population of Skylarks consists of resident and/or migratory individuals. First, we present an analysis of ring recoveries from the Dutch Ringing Centre "Vogeltrekstation". Out of 25 recoveries, 12 Skylarks were resident in winter, 10 migrated and three were classified as probable migrants. Resident birds were accompanied during winter by birds from northern and eastern Europe. Very limited natal and breeding dispersal recorded in the same dataset suggests that our results were not influenced by long dispersal distances. Next, we compared these results to a local radio-telemetry study in the northern Netherlands. During two different years we equipped a total of 27 Skylarks from a breeding population with radio-transmitters and followed them during the subsequent winter. Four birds were found to winter locally. Out of 23 individuals that we did not find in winter, 14 returned in the following breeding season to the study area, all with a working transmitter, suggesting that they wintered outside our study area. Two ring recoveries of birds from the same study population indeed showed migration to south-west Europe. Based on these two lines of evidence, we conclude local coexistence of a resident and a migrant strategy in Dutch Skylarks. The findings of our study are important for the planning of conservation efforts, as we can only protect this rapidly declining species when we know their behaviour and whereabouts throughout the entire annual cycle.

## Introduction

In western Europe, farmland birds have declined in recent decades by almost 50% (BirdLife International 2004, EBCC 2009, PECBMS 2009). While declines are frequently associated with changed or changing conditions during the breeding season and deterioration of the breeding habitat (Newton 2004, Donald *et al.* 2006), the importance of factors operating outside the breeding season is less clear. However, the general change in agricultural practise from summer to winter cereal and the subsequent loss of overwintering stubble fields is thought to reduce food supply in winter and could reduce survival rates (Donald *et al.* 2001, Newton 2004, Siriwardena *et al.* 2007, Siriwardena *et al.* 2008).

In the Netherlands, the Skylark (*Alauda arvensis*) is one of the farmland species with the steepest decline: numbers dropped by almost 95% from around 700,000 breeding pairs in the 1970s to only about 38,000 in 2005 (Teixeira & Alleijn 1979, SOVON 2002, van Dijk *et al.* 2008). In the Netherlands, Skylarks are also common during migration (LWVT/SOVON 2002), and in winter (e.g. Vergeer & van Zuylen 1994, Poelmans & van Diermen 1997, Bijlsma *et al.* 2001, Venema 2001, Hustings *et al.* 2006). From the patterns of passage and from ringing recoveries it is well established that Skylarks from northern breeding populations migrate through the Netherlands on their way to the wintering grounds in south-west Europe (Speek & Speek 1984, Spaepen 1995, LWVT/SOVON 2002). However, it is unclear whether Skylarks that breed in the Netherlands are resident or migratory. It has been hypothesized that part of the population of Dutch breeding birds winter locally and are joined by birds from Scandinavia and Russia (e.g. Venema 2001, SOVON 2002), but also that most breeding birds migrate (e.g. Lensink 1993, Vergeer & van Zuylen 1994, Hustings *et al.* 2006, van Dijk *et al.* 2008) with France as the most important wintering destination (van Beusekom 2006). Yet, these ideas have not been verified using ringing data, stable isotopes, radio-telemetry or other tracking methods, even though such knowledge is essential to develop a more powerful conservation strategy (Sutherland *et al.* 2004, van Beusekom 2006, Bos *et al.* 2009).

Skylark populations from northern Europe migrate to south-west Europe, mainly to France and Spain, whereas southern European and British populations are thought to be resident (Spaepen & Van Cauteren 1962, Spaepen & Van Cauteren 1968, Zink 1975, Glutz von Blotzheim & Bauer 1985, Spaepen 1995). Birds that are wintering on the northern edge of the wintering range react to cold spells, and winter movements occur especially when snow fall restricts access to food (Glutz von Blotzheim & Bauer 1985, Donald 2004). However, the geographical border between resident and migratory populations remains unclear (Glutz von Blotzheim & Bauer 1985, Bauer *et al.* 2005).

Futhermore, it is unclear whether resident and migratory strategies occur within a single Skylark population. Partial migration is defined as occurring when one part of the population remains in the breeding area year round and another part migrates (Gauthreaux 1982, Terrill & Able 1988, Alerstam 1990, Berthold 1993, Newton 2008). The mechanism for partial migration is thought to be a mixture of environmental and genetic factors, with the latter being more prominent in obligate partial migrants and environmental factors like food supply being more important in facultative partial migration (Schwabl & Silverin 1990, Berthold 1993, Newton 2008). Partial migration has been described for several temperate breeding birds, with European Robin (*Erithacus rubecula*), Blackbird (*Turdus merula*) and Blue Tit (*Cyanistes caeruleus*) being the most prominent examples (e.g. Schwabl *et al.* 1984, Smith & Nilsson 1987, Adriaensen & Dhondt 1990, Nilsson *et al.* 2006, Partecke & Gwinner 2007). Data on Skylarks are lacking so far.

In this study we investigated migration strategies of Skylarks in the Netherlands using two different approaches. First, we analysed all ring recoveries of Skylarks from the database of the Dutch ringing centre "Vogeltrekstation". Second, we conducted a radio-telemetry study in a local breeding population of Skylarks in the northern Netherlands. We combine the results of both approaches to evaluate whether Skylarks that breed in the Netherlands migrate or winter locally. Additionally, we analyse the ring recoveries of Skylarks that spend the winter in the Netherlands, to trace their origin. Finally, we present data on natal and breeding dispersal of Skylarks ringed in the Netherlands during the breeding season to verify that our results on the migratory strategies of Skylarks are not in fact influenced by long-distance dispersal.

## Methods

### ***Ring recoveries***

Since 1911 more than 88,000 Skylarks have been ringed in the Netherlands (Vogeltrekstation data until November 2008), of which 497 were recovered. The database contains an additional 35 recoveries of birds ringed in other countries and found in the Netherlands.

We selected all cases where distinction between migration strategies (resident in winter versus migrant) is possible ($n$ = 25, Appendix 1). These include birds that were (1) ringed during the breeding season in the Netherlands (either as nestling or as breeding adult) and recovered in any winter, or during any autumn or spring migration; (2) ringed during winter and reported during the breeding season in the Netherlands; and (3) ringed during migration and recovered during the breeding season within the Netherlands. We define migration based on distance between ringing and recovery site by visually deriving a divide in travelled distance between residents and migrants (see Figure 2.1). Skylarks recovered closer to the inferred breeding area than the divide are regarded as residents, and birds that flew further as migrants (see Helm *et al.* 2006). Birds either ringed or recovered during active migration in the main migration period in October were classified into a third category "probable migrants" ($n$ = 3).

We defined the migration periods as 30 Jan – 25 Mar for spring migration and 24 Sep – 19 Nov for southward migration. These periods are based on diurnal migration as observed during more than 67,000 observation hours in the years 1976–1993 at 121 observation stations throughout the Netherlands (LWVT/SOVON 2002). Accordingly, the wintering season is defined as the period 20 Nov – 29 Jan and the breeding season (including the moulting period) as 26 Mar – 23 Sep.

To evaluate whether, in addition to Dutch birds, Skylarks from northern origins also winter in the Netherlands, we selected all birds that were ringed during winter (20 Nov – 29 Jan) in the Netherlands and were later recovered further north. We additionally checked for the origin of late migrants (1 Nov – 19 Nov).

If long-distance natal or breeding dispersal occurs in Skylarks, this could potentially influence our results given the selection criteria we used to classify migrants. Therefore we performed an analysis of natal and breeding dispersal by selecting all recoveries from birds ringed during the breeding season as either nestlings or adults and that were recovered during any subsequent breeding season ($n = 43$). Of these 43 recoveries, 23 birds were found by the ringer and 20 by another person, and thus estimated dispersal is not only based on birds recaptured by a ringer within a study population.

### *Study area for radio-telemetry*

We obtained detailed information on a breeding population in the "Aekingerzand", part of the National Park Drents-Friese Wold in the northern Netherlands (N 52°55'; E 6°18'). The area is a mixture of open sand, groups of trees, heath- and grasslands on nutrient-poor soil and surrounded by a thin belt of forests. The wider surroundings are characterised by agricultural fields and small villages. The study population consists of about 100 pairs; the vast majority of individuals are colour-ringed.

### *Radio-tracking*

We equipped 28 Skylarks with radio-transmitters. Eight birds (3 adult males, 4 adult females, 1 juvenile female) received the transmitter in the period 13 Jul – 26 Sep 2007 and another 20 (9 adult males, 4 adult females, 4 juvenile males, 2 juvenile females and 1 male of unknown age) in the period 03 Aug – 22 Sep 2008 when all birds showed active moult. Ageing of the birds was based on previous ringing or on plumage characteristics; sex was determined by wing length and in doubtful cases confirmed by molecular sexing.

The radio-transmitters (172 MHz-band) were specifically designed for this project by JDJC Corp. d.b.a. Sparrow Systems, United States. The life-time was assumed by the manufacturer to be at least 6-7 months. In fact, in all cases where birds returned to the breeding grounds, transmitters continued to work for longer than this expected life-span and one transmitter was still working one year after attachment. Transmitters were fixed on the back of a bird using figure-eight-harnesses (Rappole & Tipton 1991) made from elasticated cotton thread. Transmitters, including harness, ranged in weight from 1.29 to 1.50 g, equalling 3.1 – 5.2% of a bird`s body weight at time of attachment. During the period when transmitters were attached, Skylarks in this study population are close to their minimum weight in the annual cycle (A. Hegemann unpubl.). From when transmitters were attached until the end of September we carried out repeated searches for birds in the study area to check if birds were still present and to determine if transmitters produced regular signals on the supposed frequency. One transmitter attached to a bird in 2007 failed to work properly due to an antenna that was broken two weeks after attachment. Therefore we excluded this bird from further analysis.

Starting in October when birds left the Aekingerzand, we searched for radio-tagged birds using two portable ICOM IC-R20-receivers with hand-held 5-element yagi-antennas. In a radius of up to 8 km around the Aekingerzand, the agricultural landscape was checked for the presence of radio-tagged birds by one person searching for 4–5 days per week and about five hours per day. In the open landscape (excluding villages and wooded areas) we scanned for all used frequencies, conducting scans at regular distances of not more than 1 km apart. In addition we used a dipole antenna mounted on the roof of a car with a automatically scanning receiver to search for birds while driving around the search area. During winter 2008/09 the hand-held antenna was mounted on a 4-m long plastic pole to increase reception. Furthermore we conducted telemetry from a small aircraft (Cessna skyhawk), flying for 2–4 hours at a height of 250–300 m and with a speed of 100–110 km/h in circles over the study area. Flights were completed once in winter 2007/08 (on 8 December) and twice in winter 2008/09 (on 16 January and 6 February). With these flights we covered an area with a radius of about 12 km around the Aekingerzand. During the flight on 8 December 2007 we received signals from one bird we did not find previously by ground telemetry. During the two flights in winter 2008/09 we did not find any additional birds. During all flights radio-tagged birds with known location, or one additional transmitter that was not attached to a bird, were used as references values. From the air we were able to detect these references signals from a distance of at least 2 km. Another search flight was flown in spring 2009 (on 3 March) to search for potentially dispersed birds, but none were found.

## Results

### *Migration strategies as shown by ringing data*

Based on 25 ring recoveries, we classified 12 birds as residents, 10 as migrants and three as probable migrants (Figure 2.1). Eight birds had been ringed as nestlings and six of these were migratory, with three being recovered in their first winter and three in their second or third winter respectively (Table 2.1). All birds ringed as adults ($n$ = 17) had been ringed outside the breeding season, and four were classified as migrants, and another two as probable migrants. Migratory birds ($n$ = 10) travelled on average 713.0 km ± SD 531.3 (median 437 km) between ringing and recovery location. Most likely these migrants were still on migration when caught/recovered (eight out of ten during the migratory period), and therefore this is not an estimate of the total migration distance. Migratory birds ringed as nestlings and recovered as first-year birds or as adults did not differ in the distance travelled between ringing and recovery location (Wilcoxon-Test, $W$ = 4, $P$ = 0.18). The furthest distance was covered by a bird ringed as a nestling in 1968 and hunted the same autumn in Spain at 1900 km.

### *Origin of birds wintering in the Netherlands based on ringing data*

Eleven birds ringed in winter in the Netherlands were recovered in or on their way towards breeding areas outside the Netherlands, in Denmark ($n$ = 8), Russia ($n$ = 1),

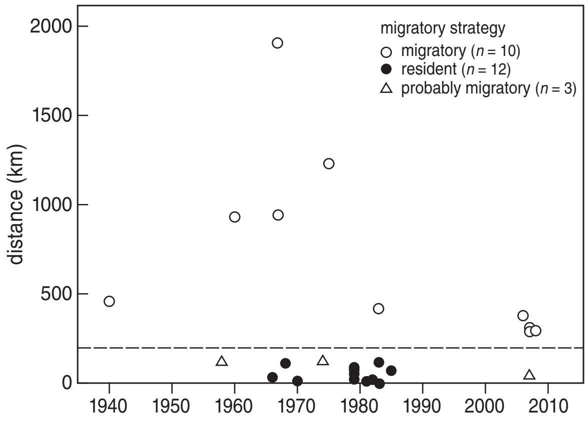


**Figure 2.1:** Distance between ringing and recovery location in Skylarks from the Netherlands by year of recovery. The dotted line (visually drawn) separates resident and migratory birds. For further details see Methods.

Sweden (*n* = 1) and Norway (*n* = 1) (Appendix 2). Additionally two birds ringed during the breeding season in Denmark and Norway, respectively, were found in winter in the Netherlands. Furthermore, birds that were ringed during the last part of the southward migration period in the Netherlands (1 Nov – 19 Nov) were later reported in Denmark (*n* = 8) and Russia (*n* = 1) (Appendix 2).

### *Natal and breeding philopatry based on ringing data*

Thirty Skylarks ringed as nestlings were reported during a later year as breeding birds. Twenty-six of these birds (1 male, 18 female, 7 unknown) were found within 4 km of the ringing site, and four (all unknown sex) had dispersed further than 10 km from the place of hatching. The furthest recovery was found in a fresh pellet of a Montagu`s

**Table 2.1:** Distance between ringing and recovery location for Skylarks of the Dutch breeding population with known migration strategy (*n* = 25) by age and sex. All adults were ringed outside the breeding season.

| | | | Sex | | | Distance | | | |
|---|---|---|---|---|---|---|---|---|---|
| Ringed as | Recovered as | Migration strategy | Male | Female | Unknown | Max | Min | Median | Average |
| Nestling | First-year | Migratory | | | 3 | 1900 | 295 | 459 | 884.7 |
| | First-year | Resident | | | 1 | | | 73 | 73.0 |
| | Adult | Migratory | | 1 | 2 | 937 | 415 | 925 | 759.0 |
| | Adult | Probably migratory | | | 1 | | | 47 | 47.0 |
| Adult | | Migratory | | 2 | 2 | 1219 | 294 | 343 | 549.8 |
| | | Resident | | | 11 | 116 | 0 | 28 | 48.7 |
| | | Probably migratory | | | 2 | 122 | 46 | 84 | 84.0 |

Harrier (*Circus pygargus*) one year after being ringed as a nestling, at a distance of 41 km from its place of hatching (H.J. Ottens pers. comm.). Because Montagu's Harriers of this specific population hunt over distances of more than 18 km from their nest site (C. Trierweiler pers. comm.), the actual dispersal distance of this bird remains uncertain. Of the 13 birds ringed as adults (5 males, 7 females, 1 unknown) during the breeding season all were reported in subsequent years within 2 km of the site of ringing.

***Radio-telemetry results of Aekingerzand study population***

In the winter of 2007/08 all radio-tagged birds had left the Aekingerzand by 2 October. In the course of the winter we detected signals from three of the seven radio-tagged birds (43%) at distances of 0 – 9 km from the Aekingerzand. These included two adult males and one adult female (Table 2.2). One of these males stayed from 9 October till 12 November on an agricultural field just outside the Aekingerzand before it moved a few kilometres further. Both males returned to the breeding site in the following breeding season, while we lost track of the female during winter. Two birds (both adult females; 29% of all birds) were not detected during the winter in the surroundings of the Aekingerzand, but returned to the area in the following breeding season. One of them stayed from 12–20 October on an agricultural field 3.5 km from the original breeding site, before moving out of our study area. Two birds were never located during winter and did not return to the breeding site. Their migratory strategy remains unknown (Table 2.2).

In the winter of 2008/09 we located only one of the 20 radio-tagged birds in the search area around the Aekingerzand (5%, Table 2.2). This adult male (transmitter frequency 172.475) was still in the Aekingerzand on 3 October, but was not detected in the breeding area after this date (Table 2.3). He was located again on 24 November in a group of about 60 Skylarks on harvested potato fields 4 km from the breeding site. He stayed in this vicinity for several weeks and the last signal was detected from this bird on 16 January 2009. However, this bird did not return to the original breeding area the following spring. All other radio-tagged Skylarks left the Aekingerzand in October, with

**Table 2.2:** Radio-tagged Skylarks from the Aekingerzand population. Numbers in brackets give percentage of birds in relation to total number of tagged birds.

| Age | Sex | Total 2007/08 | Detected winter 2007/08 | Not detected but returned summer 2008 | Total 2008/09 | Detected winter 2008/09 | Not detected but returned summer 2009 |
|---|---|---|---|---|---|---|---|
| Adult | Male | 3 | 2 | | 9 | 1 | 7 |
| Adult | Female | 3 | 1 | 2 | 4 | | 3 |
| Juvenile | Male | | | | 4 | | 2 |
| Juvenile | Female | 1 | | | 2 | | 0 |
| Unknown | Male | | | | 1 | | 0 |
| Total | | 7 | 3 (43%) | 2 (29%) | 20 | 1 (5%) | 12 (60%) |

**Table 2.3:** Presence of radio-tagged Skylarks in the Aekingerzand during autumn 2008 and in early spring 2009. Grey cells indicate days when particular transmitters were not applied yet. X marks presence of birds. M=male, F=female, ad=adult, juv=juvenile. During winter no birds were detected in the Aekingerzand on 3 and 12 December 2008 and 15 January 2009. The male with transmitter frequency 172.475 wintered outside the Aekingerzand.

| Frequency 172 MHz + KHz | 183 | 214 | 564 | 495 | 593 | 187 | 335 | 539 | 517 | 132 | 475 | 697 | 575 | 661 | 669 | 636 | 414 | 611 | 437 | 460 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sex | M | M | M | F | F | M | M | M | M | M | M | M | M | M | M | F | F | F | F | M |
| Age | Juv | Juv | Juv | Juv | Juv | Juv | Ad | Ad | Ad | Ad | Ad | Ad | Ad | Ad | Ad | Ad | Ad | Ad | Ad | ? |
| Date of capture | 03/08 | 04/08 | 13/09 | 09/08 | 10/09 | 05/08 | 09/09 | 09/09 | 10/09 | 13/09 | 19/09 | 19/09 | 20/09 | 20/09 | 22/09 | 04/08 | 09/09 | 10/09 | 19/09 | 13/09 |
| Date of control | | | | | | | | | | | | | | | | | | | | |
| 06/08/2008 | X | X | ▨ | ▨ | ▨ | X | ▨ | ▨ | ▨ | ▨ | ▨ | ▨ | ▨ | ▨ | ▨ | ▨ | ▨ | ▨ | ▨ | ▨ |
| 10/08/2008 | X | X | ▨ | ▨ | ▨ | X | ▨ | ▨ | ▨ | ▨ | ▨ | ▨ | ▨ | ▨ | ▨ | X | ▨ | ▨ | ▨ | ▨ |
| 08/09/2008 | X | X | ▨ | X | ▨ | X | ▨ | ▨ | ▨ | ▨ | ▨ | ▨ | ▨ | ▨ | ▨ | X | ▨ | ▨ | ▨ | ▨ |
| 09/09/2008 | | X | ▨ | X | ▨ | X | ▨ | ▨ | ▨ | ▨ | ▨ | ▨ | ▨ | ▨ | ▨ | X | ▨ | ▨ | ▨ | ▨ |
| 17/09/2008 | X | X | X | | X | X | X | X | X | X | ▨ | ▨ | ▨ | ▨ | ▨ | X | X | X | ▨ | X |
| 03/10/2008 | X | | | | | | | X | | | X | | X | | | | | | X | X |
| 24/10/2008 | X | | | | | | | X | | | | | | X | | | | | X | X |
| 27/10/2008 | X | | | | | | | X | | | | | | | | | | | | |
| 28/10/2008 | X | | | | | | | X | | | | | | | | | | | | |
| 29/10/2008 | | | | | | | | | | | | | X | | | | | | | |
| 30/10/2008 | | | | | | | | | | | | | X | | | | | | | |
| 12/11/2008 | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | ** | | | | | | | | | |
| 12/02/2009 | | | | | | | | | | | | | X | | | | | | | |
| 14/02/2009 | | | | | | | | | | X | | | X | | | | | | | |
| 07/03/2009 | | X | | | | | X | X | | X | | X | X | X | X | | | X | | |
| 13/03/2009 | | X | | | | | X | X | | X | | X | X | X | X | | | X | X | |
| 29/03/2009 | | X | | | | | X | X | | X | | X | X | X | X | | X | X | X | |
| 02/04/2009 | | X | X* | | | | X | X | | X | | | X | X | | | X | X | X | |

*This bird established a territory outside the study area. Even though we often were able to receive signals in the study area, we likely missed it during earlier controls.
**This bird wintered a few km outside the study area on agricultural fields. We received the last signal on 16 Jan 2009.

the last radio-tagged male detected there on 30 October (Table 2.3). Twelve of the 19 birds (60%) which we did not detect in winter returned during the following breeding season, with the first two males being in their territories on 12 February and one more male being there on 24 February. On 7 March a total of nine birds (8 males, 1 female) were present, and two more females on 13 March and on 29 March, respectively (Table 2.3). We did not receive any signals from seven birds (35%) after they left the Aekingerzand and their migratory strategy remains unknown.

Both winters combined, we found three of 17 males and one of 10 females to winter locally (Fisher's Exact Test, $P = 1$). Four adults were found to winter locally, while we did not detect any of the seven juveniles in winter (Fisher's Exact Test, $P = 0.55$). The proportion of birds we found in our study area during winter, differed between the two years (Fisher's Exact Test, $P = 0.04$).

## Discussion

Ring recoveries from the entire Netherlands for the past 100 years clearly demonstrate that Dutch Skylarks are partial migrants; some birds migrate to the southwest and others winter close to their breeding grounds. Our radio-telemetry study on a local breeding population in the northern part of the Netherlands confirmed that some birds winter very close to the breeding location while others were not found in winter in the immediate surroundings, but returned in spring. Our results provide the first evidence for the simultaneous occurrence of migratory and resident strategies in the Dutch Skylark population. Our data suggest that both natal and breeding dispersal occur over limited distances in Dutch Skylarks and thus our conclusions on migration strategies are unlikely influenced by long dispersal distances. High site fidelity in Skylarks to both natal and breeding area has been described earlier (Duncan 1987, Wolfenden 1990 cited in Dougall 1996, Paradis *et al.* 1998, Dougall 1999, Wernham 2002).

Locally-wintering Dutch-breeding Skylarks are accompanied during winter by birds from the north and east, as shown by recoveries of birds ringed in winter. Eleven such recoveries were of birds breeding in the Netherlands and 11 were of birds found in breeding areas to the north or east. Thus the Netherlands also serve as wintering grounds for Skylarks from the north-east. Resident birds and wintering birds from the north probably use the same habitats and potentially join the same flocks. Indeed, we captured birds with a wing length of up to 122 mm in fields where colour-ringed and radio-tagged birds from our local study population were also present. Birds from the study population have a maximum wing length of 117 mm (A. Hegemann unpubl.) suggesting that these longer-winged birds are coming from northern populations (Glutz von Blotzheim & Bauer 1985). Our data do not allow us to make quantitative statements about the frequencies of the different migration strategies because we lack the crucial information on ringing efforts, recovery rates and reporting rates in the Netherlands relative to those in other countries (see Körner-Nievergelt *et al.* 2010).

We are also not yet able to say whether the migration strategy of an individual bird is fixed or flexible between years because we did not track any individual twice. Nonetheless, different proportions of birds undertaking local wintering among the radio-tagged birds in the two years of our study could indicate that migration strategies are not fixed. Our study population thus might consist of facultative partial migrants, where some individuals migrate in some years and not in others, depending on conditions at the time (Terrill & Able 1988, Newton 2008). A switch in migratory strategy in individual birds has been shown for European Robins (Harper 1985, but see Adriaensen & Dhondt 1990), rarely occurred in American Dippers (*Cinclus mexicanus*) (Gillis *et al.* 2008), and was age and sex-dependant in Blackbirds (Schwabl & Silverin 1990).

Our telemetry study showed that some birds wintered very close to their breeding territory and made use of the surrounding agricultural fields. This is also confirmed by the sighting of three colour-ringed individuals from the Aekingerzand breeding population on 26 November 2007 and two resightings on 30 January 2008. Further resightings are lacking because of the difficulty in reading colour-rings on Skylarks in winter.

Combining data from both winters, 15 of the 23 birds with radio-transmitters that were undetected during winter returned to the original breeding grounds during the subsequent breeding season. Since all transmitters still worked in the spring, this suggests that these birds migrated out of our search area. We cannot exclude the possibility that we missed some birds or that birds wintered just outside of the area that we searched. Despite this uncertainty, we have unequivocal evidence for true migration from our local study population, because two individuals captured during breeding were originally ringed during migration in Belgium. The disappearance of the radio-tagged birds in the autumn also fits the timing of migration of the two birds from our study population captured in Belgium during southward migration. We therefore assume that at least a subset of the birds that we did not find during winter truly migrated.

Our sample sizes are currently too small to draw any definite conclusions about age-differences in migration strategies. Age-related migration patterns are hard to document, as both adult and young Skylarks undergo a complete post-breeding moult and are impossible to age in autumn based on field characteristics. Nevertheless, Van Dobben & Mörzer Bruyns (1939) suggested that first-year birds migrate later than adults based on dissection of birds killed at lighthouses. Different timing and travel distances between age classes have been shown for a number of other short-distant migratory species in Europe (reviewed by Newton 2008).

We found both males and females to winter locally, but sample sizes are small. In the 1970s it was suggested that Skylarks wintering in north-western Europe were mainly males (Niethammer 1970, Senk *et al.* 1972, Vauk 1972, Zink 1987) and during cold weather movements in winter in the Netherlands more than 70% of all caught Skylarks were males (Levering & Keijl 2008). Females were significantly over-represented in migrating Skylarks in southern Italy (Scebba 2001) suggesting that female Skylarks migrate further south as shown for many other European partial migrants (reviewed by e.g. Gauthreaux 1982, and Newton 2008). Only in Scotland the sex ratio among Skylarks is equal during winter (Dougall 1997). If such a sex-bias in migration strategies exists, this could have important implications for conservation measures.

In light of ongoing discussions about reversing negative population trends and how to improve the winter situation for farmland birds, our findings provide important information for conservation plans and activities. It has been suggested that the change in agricultural practices from summer to winter cereals and thus the loss of overwintering stubble fields as a food source for Skylarks in winter might be – alongside an insufficient reproductive output – another important problem associated with the decline in Skylark numbers (Donald *et al.* 2001, Newton 2004, Siriwardena *et al.* 2008). It is interesting to note that despite the enormous decline in the Dutch breeding population, the migratory tendency has not disappeared, which might have been expected if migration depends on competition for limited available winter food. Identifying where Skylarks from the Dutch breeding population spend the winter is therefore an essential question to answer if we are to develop more effective conservation measures (see van Beusekom 2006). Our data suggest that a two-pronged wintering conservation strategy is necessary for the Dutch breeding population: the situation for wintering birds needs to be improved not only in south west Europe, where Skylarks are still hunted (European Communities 2007) but also in the Netherlands itself.

## Acknowledgements

We are grateful to all ringers as well as to all the people who reported recoveries of ringed Skylarks during the past 100 years. Catching Skylarks to apply radio-transmitters and radio-telemetry would not have been possible without many volunteers, especially Rob Voesten, Martin Keiser, Richard Ubels, Flavia Geiger and Chris Trierweiler. Jos Hooijmeijer, Juliana Dänhardt and Sophie Brasseur provided equipment. Marco van der Velde performed the molecular sexing of some birds. This work was partially supported by a grant from BirdLife Netherlands to BIT. The telemetry-study was further financially supported by grants from the Schure-Beijerinck-Popping Fonds, the Dr. J.L. Dobberke Stichting and the Deutsche Ornithologen-Gesellschaft to AH. Comments by Christiaan Both, Theunis Piersma, the associate editor and two anonymous reviewers improved the manuscript. Nick Horrocks improved the English.

**Appendix 1 (next page):** List of all 25 birds that were used to distinguish between resident and migratory strategies. M=migrant, R=resident, PM=presumably migrant, M=Male, F=female, ?=unknown; accuracy of finding: 0=accurate to day, 1=accurate within 3 days either side of the date coded, 4=accurate within 2 weeks either side of the date coded; recovery condition: 0=unknown, 1=dead, but no information on how recently, 2=freshly dead, within about a week, 3=not freshly dead, more than about a week, 5=sick, wounded, fate unknown, 7=alive and probably healthy and certainly released, 8=alive and probably healthy and released by a ringer; finding circumstances: 1= found, bird or body mentioned, 10=shot, 19='hunted', likely shot, 20= hunted, trapped, poisoned intentionally by man, 28=ring read in the field, 40=killed by traffic, 43=collision with wire, 44=collision with windows, 47=attracted to light, 61=taken by cat, 64=taken by owl or raptor, identity predator available, 99=no information at all; direction: direction between ringing and recovery site in degrees.

| Ringing | | | | | | Finding | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Strategy | Sex | Age at ringing | Ringing date | North | East | Finding date | Accurancy | Country | North | East | Condition | Circum-stances | Distance (km) | Direction |
| M | ? | Full-grown | 15/10/1974 | 43°24' | 01°36' | 12/05/1975 | 0 | Netherlands | 52°48' | 06°54' | 2 | 44 | 1219 | 31 |
| M | ? | Full-grown | 29/10/2002 | 51°07' | 02°41' | 30/05/2006 | 0 | Netherlands | 53°12' | 07°04' | 2 | 64 | 380 | 52 |
| M | F | Full-grown | 13/10/2003 | 50°53' | 03°18' | 01/05/2007 | 0 | Netherlands | 52°55' | 06°18' | 8 | 20 | 306 | 42 |
| M | F | Full-grown | 19/10/2007 | 51°04' | 03°16' | 14/05/2008 | 0 | Netherlands | 52°56' | 06°19' | 8 | 20 | 294 | 45 |
| M | ? | Nestling | 17/07/1939 | 53°00' | 04°48' | 11/02/1940 | 0 | England | 52°36' | 02°00' | 0 | 99 | 459 | 264 |
| M | ? | Nestling | 17/05/1960 | 51°54' | 05°06' | 06/10/1960 | 0 | France | 44°42' | 01°12' | 1 | 1 | 925 | 210 |
| M | ? | Nestling | 01/06/1966 | 51°19' | 06°06' | 25/10/1967 | 0 | France | 44°12' | 00°54' | 7 | 20 | 937 | 214 |
| M | ? | Nestling | 09/05/1968 | 51°00' | 05°48' | 15/09/1968 | 4 | Spain | 36°00' | -05°36' | 0 | 19 | 1900 | 209 |
| M | ? | Nestling | 25/05/1982 | 52°17' | 06°54' | 11/12/1983 | 0 | France | 49°53' | 02°21' | 2 | 10 | 415 | 230 |
| M | F | Nestling | 27/06/2006 | 52°53 | 06°37 | 08/10/2007 | 0 | Belgium | 50°43' | 04°12' | 8 | 20 | 295 | 215 |
| PM | ? | Full-grown | 08/10/1972 | 51°06' | 04°00' | 15/09/1974 | 0 | Netherlands | 51°54' | 05°12' | 1 | 40 | 122 | 43 |
| PM | ? | Full-grown | 17/10/2003 | 51°20' | 04°58' | 02/09/2007 | 0 | Netherlands | 51°19' | 05°38' | 3 | 0 | 46 | 92 |
| PM | ? | Nestling | 15/06/1956 | 52°36' | 04°42' | 12/10/1958 | 2 | Netherlands | 51°42' | 03°42' | 2 | 47 | 121 | 214 |
| R | ? | Full-grown | 15/01/1966 | 52°24' | 04°30' | 30/03/1968 | 0 | Netherlands | 53°18' | 05°06' | 8 | 20 | 108 | 22 |
| R | ? | Full-grown | 19/01/1966 | 51°24' | 03°24' | 22/05/1966 | 0 | Netherlands | 51°24' | 03°48' | 3 | 1 | 28 | 90 |
| R | ? | Full-grown | 07/01/1970 | 52°24' | 043°6' | 01/04/1970 | 0 | Netherlands | 52°18' | 04°24' | 1 | 1 | 18 | 231 |
| R | ? | Full-grown | 29/12/1976 | 52°25' | 04°34' | 23/07/1979 | 0 | Netherlands | 52°15' | 04°27' | 3 | 64 | 20 | 203 |
| R | ? | Full-grown | 01/01/1979 | 52°33' | 04°37' | 07/04/1979 | 0 | Netherlands | 51°59' | 04°07' | 2 | 64 | 72 | 208 |
| R | ? | Full-grown | 04/01/1979 | 52°25' | 04°34' | 15/04/1979 | 4 | Netherlands | 52°03' | 04°13' | 3 | 1 | 47 | 210 |
| R | ? | Full-grown | 27/01/1979 | 52°33' | 04°37' | 30/05/1979 | 0 | Netherlands | 51°49' | 04°08' | 1 | 1 | 88 | 202 |
| R | ? | Full-grown | 07/12/1980 | 52°08' | 04°20' | 23/06/1981 | 0 | Netherlands | 52°15' | 04°28' | 3 | 64 | 16 | 35 |
| R | ? | Full-grown | 16/12/1981 | 52°33' | 04°37' | 08/05/1982 | 0 | Netherlands | 52°21' | 04°32' | 1 | 64 | 23 | 194 |
| R | ? | Full-grown | 08/01/1982 | 52°21' | 04°32' | 21/05/1983 | 0 | Netherlands | 52°21' | 04°32' | 1 | 64 | 0 | 0 |
| R | ? | Full-grown | 10/02/1982 | 52°25' | 04°34' | 03/05/1983 | 0 | Netherlands | 53°11' | 05°44' | 3 | 1 | 116 | 43 |
| R | ? | Nestling | 05/07/1985 | 53°04' | 04°44' | 18/11/1985 | 0 | Netherlands | 52°25' | 04°34' | 8 | 20 | 73 | 189 |

**Appendix 2:** List of all 12 birds that were used to evaluate the origin of birds wintering in the Netherlands (classification W= wintering) and the 10 birds that were present in the Netherlands during the late autumn migration period (classification LM=late migration period). For abbreviations see Appendix 1.

| | | Ringing | | | | | Finding | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Classification | age at ringing | Sex | Ringing date | Ringing date | North | East | Finding date | Country | North | East | Condition | Circumstances | Distance (km) | Direction |
| LM | Full-grown | M | 02/11/1967 | Netherlands | 51°42' | 03°42' | 25/07/1969 | Denmark | 56°24' | 10°54' | 2 | 40 | 702 | 42 |
| LM | Full-grown | ? | 13/11/1967 | Netherlands | 52°24' | 04°36' | 28/07/1969 | Denmark | 56°12' | 08°06' | 0 | 1 | 479 | 28 |
| LM | Full-grown | ? | 06/11/1969 | Netherlands | 52°24' | 04°30' | 27/07/1970 | Denmark | 57°06' | 10°24' | 1 | 1 | 645 | 36 |
| LM | Full-grown | M | 01/11/1977 | Netherlands | 52°08' | 04°20' | 09/04/1979 | Denmark | 56°00' | 08°24' | 1 | 1 | 505 | 32 |
| LM | Full-grown | ? | 17/11/1977 | Netherlands | 52°24' | 04°36' | 16/06/1978 | Denmark | 56°30' | 10°30' | 1 | 40 | 594 | 40 |
| LM | Full-grown | ? | 04/08/1978 | Norway | 70°09' | 28°52' | 15/11/1978 | Netherlands | 53°05' | 06°35' | 1 | 1 | 2214 | 211 |
| LM | Full-grown | ? | 11/11/1979 | Netherlands | 52°25' | 04°34' | 21/03/1981 | Denmark | 55°39' | 09°10' | 3 | 1 | 468 | 40 |
| LM | Full-grown | M | 02/11/1989 | Netherlands | 52°51' | 04°32' | 12/07/1992 | Denmark | 57°15' | 11°01' | 1 | 43 | 684 | 37 |
| LM | Full-grown | M | 03/11/1994 | Netherlands | 51°51' | 05°59' | 28/05/1996 | Denmark | 55°12' | 09°32' | 1 | 64 | 347 | 42 |
| LM | Full-grown | M | 01/11/2002 | Netherlands | 51°40' | 03°41' | 15/03/2004 | Russia | 54°57' | 19°47' | 7 | 20 | 1128 | 71 |
| W | Full-grown | ? | 10/12/1967 | Netherlands | 52°24' | 04°30' | 03/04/1969 | Norway | 59°18' | 09°12' | 5 | 1 | 821 | 21 |
| W | Full-grown | ? | 12/01/1968 | Netherlands | 52°24' | 04°30' | 02/04/1969 | Denmark | 56°36' | 09°36' | 1 | 1 | 571 | 35 |
| W | Full-grown | ? | 30/12/1968 | Netherlands | 52°24' | 04°36' | 28/09/1972 | Denmark | 55°36' | 10°06' | 2 | 19 | 505 | 45 |
| W | Full-grown | F | 24/12/1970 | Netherlands | 52°36' | 04°36' | 04/06/1971 | Sweden | 59°18' | 15°24' | 1 | 1 | 1002 | 42 |
| W | Full-grown | ? | 29/12/1970 | Netherlands | 52°24' | 04°36' | 30/08/1971 | Russia | 57°48' | 29°30' | 1 | 1 | 1691 | 69 |
| W | Full-grown | ? | 30/11/1973 | Netherlands | 52°24' | 04°36' | 14/05/1974 | Denmark | 55°30' | 11°12' | 1 | 1 | 552 | 51 |
| W | Full-grown | M | 01/12/1973 | Netherlands | 52°36' | 04°36' | 10/07/1974 | Denmark | 55°24' | 09°00' | 1 | 1 | 424 | 43 |
| W | Full-grown | ? | 02/12/1973 | Netherlands | 52°24' | 04°30' | 11/09/1974 | Denmark | 55°18' | 09°36' | 2 | 43 | 464 | 46 |
| W | Full-grown | ? | 27/11/1977 | Netherlands | 52°25' | 04°34' | 03/07/1980 | Denmark | 56°10' | 09°21' | 2 | 61 | 519 | 37 |
| W | Full-grown | ? | 03/01/1985 | Netherlands | 52°21' | 04°32' | 11/05/1986 | Denmark | 57°22' | 09°43' | 7 | 28 | 648 | 31 |
| W | Nestling | ? | 28/05/1993 | Denmark | 56°18' | 10°30' | 25/11/1993 | Netherlands | 52°24' | 04°32' | 8 | 20 | 578 | 222 |
| W | Full-grown | M | 23/12/2001 | Netherlands | 52°21' | 04°31' | 22/07/2002 | Denmark | 56°31' | 09°37' | 2 | 61 | 568 | 35 |

OCEAN
WEST
5 6 7 8 9 10 11 12 13 14 15
FRANCE

SPI 2000
16 ths
mm
0 10 20 30
0.1 mm
TIELEN

# CHAPTER 3

# The use of tongue spots for aging and wing length for sexing Skylarks (*Alauda arvensis*) - A critical evaluation

Arne Hegemann, Rob Voesten, Kees Van Eerde,
Marco van der Velde & B. Irene Tieleman

**Ringing & Migration 27: 7–12 (2012)**

**Abstract** The Skylark (*Alauda arvensis*) is a European passerine species lacking plumage dimorphism and having a complete post-nuptial moult in adults, and a complete post-juvenile moult in young birds. Tongue spots were thought to allow age discrimination after moult. Based on data from a study population in the Netherlands, where the age of many birds was known, we show that tongue spots are not useful for ageing Skylarks. Some nestlings lack tongue spots and half of all birds had tongue spots when three years or older. Regarding sex discrimination, males and females can usually be distinguished from a bimodal wing length distribution, but the threshold and amount of overlap in wing length between sexes vary greatly between studies. Here, we show that 98% of all individuals from our Dutch breeding population could be sexed correctly by wing length. However, during migration and winter, when populations from other regions co-occur in the same area, molecular analyses revealed that the overlap in wing length between sexes increased. Therefore, ringers need to be cautious when assigning sex, and small males especially are often misclassified. For example, based on criteria used in France, 14% of Dutch male Skylarks, which are partial migrants, would be misclassified as females.

## Introduction

The Skylark (*Alauda arvensis*) is one of the few European passerine species where sexes do not differ in plumage characteristics, and that undergoes a complete post-nuptial moult in adults and a complete post-juvenile moult in birds of the year in August-September (Pätzold 1975, Glutz von Blotzheim & Bauer 1985, Donald 2004). From the end of September onwards, when most Skylarks have completed their moult, age classes can usually not be distinguished based on plumage characteristics (Glutz von Blotzheim & Bauer 1985, Svensson 1992, Winkler & Jenni 2007). Ageing based on skull ossification is also difficult from October onwards as most young birds have completed the ossification by the end of moult (Svensson 1992, Winkler & Jenni 2007). Therefore any birds ringed outside the breeding season can only be aged as "fully grown". This is an unsatisfactory situation for many ringers and has led to speculations about characteristics that still allow age determination after a completed moult. Tongue spots have been suggested as a potential characteristic for age determination in autumn and winter: birds still showing tongue spots have been suggested to be first-year birds, whereas birds without tongue spots were suggested to be adults (Demongin 2006, Staal 2010). This would require that all nestlings have tongue spots and that these remain at least until the first winter and disappear no later than during moult in their second year. However, a critical evaluation of the reliability of tongue spots as age characteristic has been missing until now.

While males and females can usually easily be sexed by behaviour during the breeding season, accurately sexing them in the field outside the breeding season remains impossible (Glutz von Blotzheim & Bauer 1985). In the hand the wing length has proven to be a reliable characteristic to sex birds due to the strong bimodal distribution (Svensson 1992). However, wing length differs among populations depending on their migratory distance and consequently the critical wing length to distinguish between males and females varies as well (Glutz von Blotzheim & Bauer 1985). Across Europe, different studies have revealed different thresholds between sexes, but the range of wing lengths that do not allow sex classifications is usually small (Table 3.1). This brings the potential for wrong sex classifications around the threshold. Yet no study has investigated the shift between thresholds, especially when different populations occur at the same location, for example during migration or in wintering areas.

The aims of this study were to 1) investigate if tongue spots can be used to reliably age birds; 2) report differences between males and females in wing length from a Dutch breeding population and 3) report wing length of both sexes during migration and winter in the Netherlands to highlight how the threshold changes when looking at different populations even at the same geographical location. Taken together this article should clear up some myths surrounding the aging and sexing of Skylarks, and to give ringers a reliable guideline for their fieldwork.

## Methods

### *Study sites and data collection*

We studied Skylarks in the northern Netherlands in 2006-2010, focusing on a population in the Aekingerzand (N 52°55'; E 6°18'). We collected data during the breeding season in five consecutive years (2006–2010). We ringed nestlings in the study population with individual colour-ring combinations and metal rings and captured adults feeding nestlings with mist-nests or traps on the nest. We also collected data outside the breeding season in the years 2007–2009 during autumn migration, wintering and spring migration. During winter (10 Dec 2007 – 22 Jan 2008; 09 Dec 2008 – 15 Jan 2009), we caught birds ($n = 25$) during the night on agricultural fields surrounding the Aekingerzand, where part of the study population winters (Hegemann *et al.* 2010); two persons walked steadily over the fields carrying a 30 m long mist-net horizontally and lighting the area under the net with a big torch. We dropped the net on birds that slept on the ground but flew off when getting into the cone of the light. During spring (14 Mar – 24 Mar 2008; 25 Feb – 01 Mar 2009; $n = 27$) and autumn (08 Oct – 4 Nov 2007 and 11 Oct – 02 Nov 2008, $n = 97$), actively migrating birds were caught during the day by luring them with playback (from an mp3 player) of conspecific songs to large clap-nets at a location 15 km southeast of the Aekingerzand.

### *Age determination*

During the breeding seasons 2006-2009 a total of 166 adults and 585 nestlings were ringed. Of the nestlings, 47 were recaptured at least once during a subsequent year, and for these local recruits we know their exact age. Of the adults, 66 were recaptured at least

**Table 3.1:** A few literature examples of wing length range in male and female Skylarks across Europe. Sexing was based on dissection of dead birds ('Dead' or 'Skins') or on field measurements on live birds ('Live'). Note that some of the references are important guides for ringers across Europe.

| Wing length (mm) | | | | | |
|---|---|---|---|---|---|
| Male | Female | left unsexed | Country (population) | Sexing based on | Reference |
| 107-121 | 98-110 | | Denmark (spring migrants) | Dead | (Glutz von Blotzheim & Bauer 1985) |
| 107-122 | 101-108 | | Switzerland (Oct - Mar) | Dead | (Glutz von Blotzheim & Bauer 1985) |
| 109-118 | 97-106 | | Germany | Dead | (Glutz von Blotzheim & Bauer 1985) |
| 104-116 | 97-108 | | Sweden | Skins | (Svensson 1992) |
| 104-118 | 97-111 | | UK (all year) | Skins | (Dougall 1998) |
| >111 | <110 | 110-111 | Scotland (winter) | Live | (Dougall 1997, Dougall 1999) |
| >114 | <110 | 110-114 | Italy (migrants) | Live | (Scebba 2001) |
| >109 | <104 | 104-109 | Netherlands (?) | ? | (Speek 1994) |
| >111 | <110 | 110-111 | France (migration) | ? | (Demongin 2006) |
| 114-129 | 96-109 | 110-113 | Netherlands (migration) | Live | (Levering & Keijl 2008) |

**Figure 3.1:** Tongue spots in Skylarks. A) Nestlings a few days old showing their tongue spots when begging. The bird on the bottom right of the picture has medium tongue spots, the two in the middle small tongue spots. The tongue spots of the fourth nestling are not clearly visible on this photo. 12 June 2008, Aekingerzand, the Netherlands. Photo taken by Mauro Varaschin. B) Large tongue spots in an adult Skylark. Note that tongue spots in adults are always less contrasting than in nestlings. 14 December 2009, Vredepeel, the Netherlands. Photo taken by Jan Staal.

once during a subsequent year. At the moment of ringing these adults were, for the purpose of this study, assumed to be 1 year old, but might have been older. For these birds we have therefore a minimum age estimate.

### *Wing length and tongue spots*

Wing length was measured to the nearest 1mm using the maximum chord method (Svensson 1992). Dark spots at the end of the tongue (hereafter tongue spots) of adult birds (Fig. 3.1B) were classified into one of three groups (no tongue spots, small tongue spots, large tongue spots). During breeding all birds captured were measured by AH and RV, who trained beforehand to get high consistency among measurements and between ringers. All birds outside the breeding season were measured only by AH. In 2010 RV classified tongue spots of all nestlings ($n = 122$) into four categories (no tongue spots, small tongue spots, medium tongue spots, large tongue spots, Fig. 3.1A) when birds were ringed (age 7–8 days).

### *Sex determination*

During the breeding season, we sexed birds based on the presence or absence of a brood patch. The sexes can be reliably distinguished on this feature because only females incubate and thus only this sex has a brood patch (Glutz von Blotzheim & Bauer 1985). Two more reasons make us confident the sexing was reliable: all Skylarks ringed as nestlings have been sexed molecularly and of all nestlings recaptured later as adults only "molecular" females showed brood patches ($n = 20$) and "molecular" males never ($n = 28$).

Furthermore observed behavioural differences (like singing, courtship behaviour) of the colour-ringed birds always supported the sex already assigned by us to the bird.

Outside the breeding season birds were sexed according to wing length, based on the data obtained during the breeding season and in the literature (Glutz von Blotzheim & Bauer 1985, Svensson 1992). To confirm if sexing was done correctly birds in a potential overlap zone, as well as a subset of birds from the complete range of wing lengths, were sexed using molecular techniques. For molecular sexing, small blood samples were collected by puncturing the brachial vein with a sterile needle. Red blood cells were stored in 95% alcohol at -20°C until the time of laboratory analyses. In the laboratory DNA was extracted from red blood cell samples using a chelex- (Walsh *et al.* 1991) or salt-extraction method (Richardson *et al.* 2001), subsequently DNA samples were sexed using the method of Griffiths *et al.* (1998).

## Results

### *Tongue spots and age*

115 of 122 nestlings showed tongue spots (94.3%). The remaining 7 nestlings (5.7%) did not have any tongue spots. Of the 115 nestlings showing tongue spots, 31 (27.0%) had small, 37 (32.2%) medium and 47 (40.9%) large tongue spots. Of eight Skylarks caught during their first moult (six of them were previously ringed as nestlings, the other two were aged based on remaining juvenile feather characteristic) between 3 August and 22 September four of them had no tongue spots, one small tongue spots and three large tongue spots.

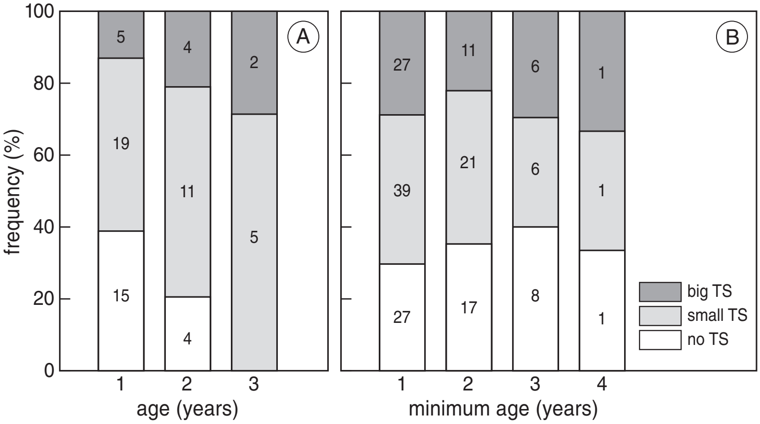


**Figure 3.2:** Percentage of adult Skylarks showing tongue spots (TS) in different age categories. a) Birds of known age: ringed as nestlings and recaptured in a following breeding season. b) Birds with minimum age estimate: ringed as adults during the breeding season (and assumed to be one year old) and recaptured during a following breeding season. Numbers in bars represent sample size. Only one recapture per year is shown, but multiple data points are used when birds were recaptured in more than one year.

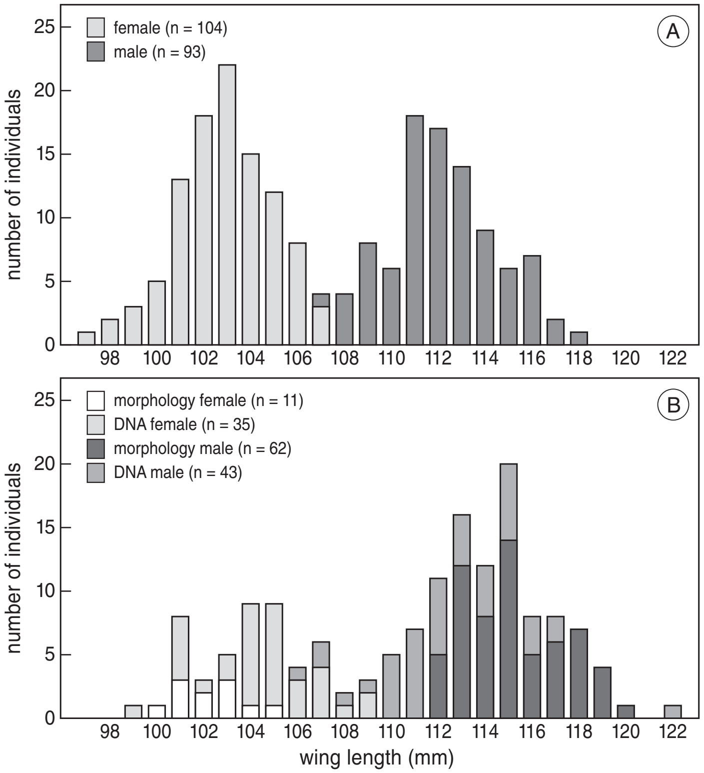


**Figure 3.3:** Wing length of adult Skylarks separated by sex. A) Birds caught during the breeding season when feeding nestlings in our study population at the Aekingerzand, the Netherlands. Birds were sexed on the absence/presence of brood patches. B) Skylarks caught during the two migration periods and winter in the northern Netherlands. All birds with a wing length of 104–111 mm and a subsample of birds with both longer and shorter wings have been sexed molecularly (“DNA male/female”). The exception is two birds with a wing length of 104 mm and 105 mm were we are lacking a blood sample. These and the remaining birds with either shorter or longer wings were sexed on morphology as they were in the exclusive range of either males or females (“Morphology male/female”).

Of 585 Skylark nestlings ringed during 2006–2009, a total of 47 were recaptured as adults during the breeding season in later years. Among these 47 birds, the percentage of birds having no, small or large tongue spots did not differ among 1, 2 or 3 year old birds (Fisher's Exact $P = 0.21$; $n = 65$; Fig. 3.2A) although the sample size ($n = 7$) for three-year olds is small. The same pattern occurred in birds ringed as adults during the breeding season (and assumed to be in their 1st year when ringed) and recaptured in later years (Fisher's Exact $P = 0.89$; $n = 165$; Fig. 3.2B) although the sample size ($n = 3$) of birds at least four years old is very small. From either group at least half of the birds of three years or older still had tongue spots and about one third of these even had large tongue spots. One individual ringed as a nestling in 2002 by BIT still had small tongue spots when recaptured at an age of six years in 2008.

### *Wing length and sex*

The wing lengths of Skylarks of known sex from our breeding population showed a bimodal distribution. Females had wings ranging from 97 to 107 mm (mean = 102.9 mm, SD 2.06, median = 103 mm, *n* = 104) and males from 107 to 118 mm (mean = 112.2 mm, SD 2.33, median = 112 mm, *n* = 93). Thus 98% of all Skylarks from our study population could be sexed correctly based on wing length alone. Only four individuals (2%) fell within the overlap range, having a wing length of 107 mm (Fig. 3.3A).

Skylarks caught during autumn migration, winter and spring migration had slightly longer wings than local breeding birds (Fig. 3.3B). Based on molecular sexing (*n* = 78: 35 females, 43 males), there was overlap in wing length between the sexes from 106 to 109 mm. Birds from 110 mm onwards were males and birds smaller than 106 mm females (Fig. 3.3B). Thus wing lengths of females ranged from 99 to 109 mm (mean = 103.9 mm, SD 2.38, median = 104 mm, *n* = 46) and of males from 106 to 122 mm (mean = 114.1 mm, SD 2.89, median = 114 mm, *n* = 105). 90% of all birds during migration could be sexed correctly using wing length alone.

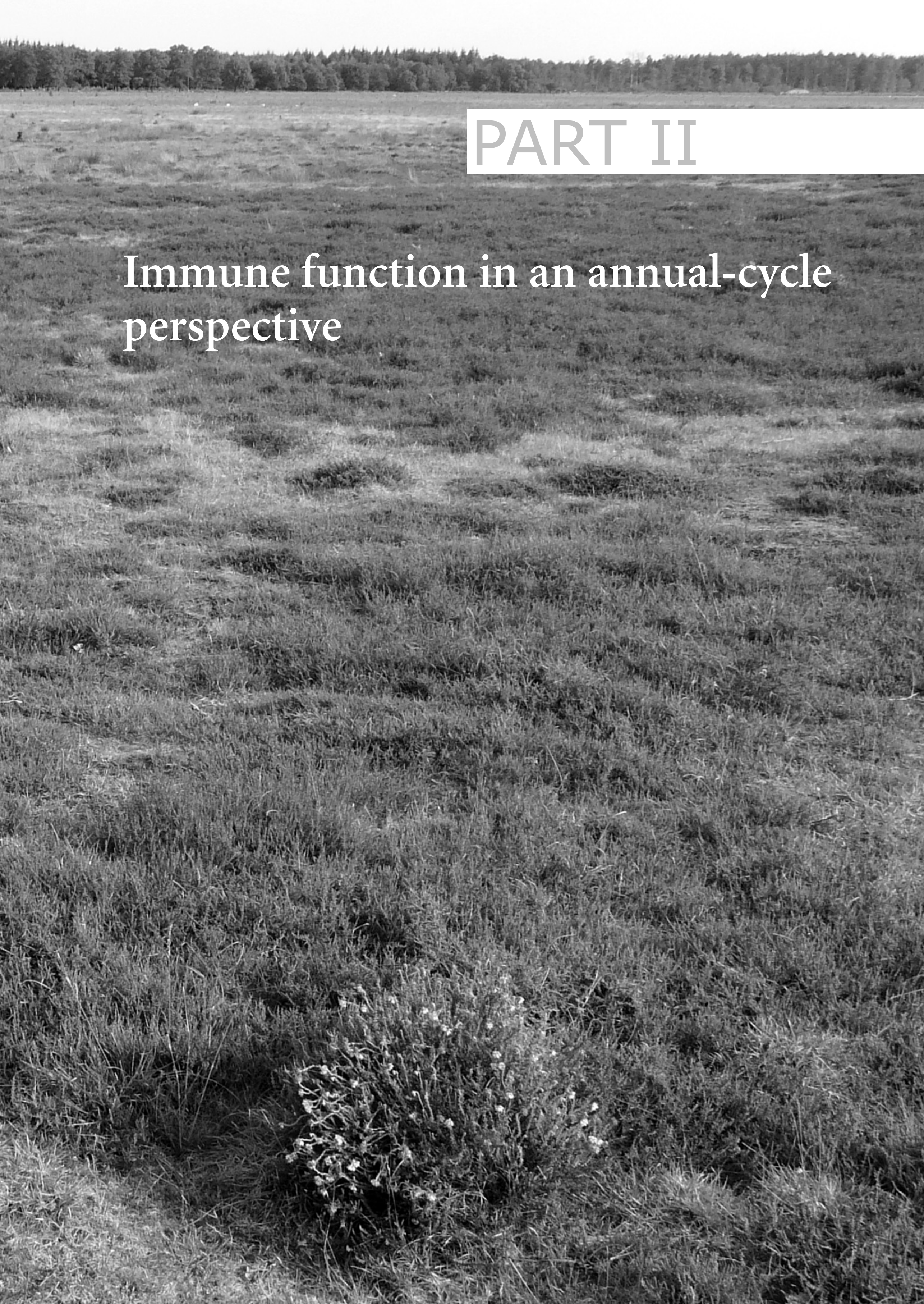
## Discussion

Our study showed that tongue spots can not be used to age Skylarks accurately. A small percentage (5.7%) of nestlings did not show tongue spots, a phenomenon already described by Pätzhold (1975) who noticed that some nestlings lacked tongue spots. Furthermore, 50% of the birds caught as juveniles during their first moult had no tongue spots. Even though we do not know if these birds had ever had tongue spots, given the fact that less than 6% of the nestlings lack tongue spots, it seems likely that tongue spots had been present and had already disappeared. More importantly, more than half of the Skylarks three years or older still had tongue spots. Our findings clearly demonstrate that tongue spots are no reliable indicator of age. Consequently Skylarks caught after the completed moult (especially during migration or winter) can not be aged unless any other characteristic is proven to allow age discrimination. A potential candidate to investigate is the iris colour, as changes in colouration with age are well established in raptors (Forsman 1997) and are also known for several passerines species (Svensson 1992, King & Muddeman 1995, Winkler & Jenni 2007).

Our study supports earlier work showing that within a breeding population, males and females overlap only marginally in their wing length (see summary in Glutz von Blotzheim & Bauer 1985). Wing lengths of Skylarks from our breeding population are very similar to those described by Delius (1964, 1965) for a population in northwest England. He found female wing length to range from 95–108 mm and male wing length from 105–118 mm. Among our breeding population only birds with a wing length of 107 mm could not be sexed based on this character. However, Skylarks caught in the same region outside the breeding season showed considerable overlap in wing length between males and females. Outside the breeding season, birds with wing length 106–109 mm would have been impossible to sex without molecular methods, while birds from our breeding population with a wing length of 106 mm were certainly females and birds with wings of 108 mm or longer certainly males. This indicates that, during migration and winter periods, we caught populations with at least partly different origin (see also Hegemann *et al.* 2010). It further highlights the difficulties in reliably sexing Skylarks caught during migration or winter. If the results of our study also apply to other regions then a small proportion of birds might have been sexed wrongly in other studies when the category of "unsexed" birds was based on too narrow range of overlapping wing lengths (Dougall 1997, Scebba 2001), although other discriminant measures (tail length and third primary length) were also utilised by these authors. Also, for other species, problems and pitfalls of relying on biometric data for sexing have been described, with sexual dimorphism varying in time and space (van De Pol *et al.* 2009). To the best of our knowledge only Delius (1964, 1965) showed the existence of males with short wings on live birds in the field, even though other studies revealed their existence on skins (Svensson 1992, Dougall 1998). Given the criteria many ringers across Europe use for sex determination (e.g. Svensson 1992, Speek 1994, Demongin 2006, Winkler & Jenni 2007), particularly small males are likely to be mis-classified. For example, Dutch Skylarks are partial migrants with the non-sedentary individuals migrating to and/or through France (Hegemann *et al.* 2010). Given that French ringers classify birds with a wing shorter than 110 as females (Demongin 2006), about 14% of Skylark males originating from the Netherlands are likely to be mis-classified as females. Therefore our results suggest that studies on Skylarks of unknown origin need to leave birds with a wider range of wing lengths unsexed. We specifically recommend that only birds with wings of 106 mm or shorter should be classified as females. In our dataset Skylarks with wings of 110 mm or longer were exclusively males, but we stress that females with even longer wings have been described from museum skins, despite the fact that these wings might have already undergone shrinkage (Dougall 1998). We furthermore stress that our recommendations are applying to birds from Northern, Western and Central Europe only and that data from Asia and non-migratory populations in Southern Europe are necessary to verify whether this also applies to other populations.

## Acknowledgements

We are grateful to the many volunteers who helped catch Skylarks at various times of the year. The late Bernard van Acker encouraged AH in 2006 to systematically record tongue spots and thus had an important influence on the origin of this study. Moana Grysan provided French literature. Maurone Varaschin and Jan Staal kindly provided the images. Raymond Klaassen and two anonymous reviewers provided useful comments to an earlier version. Staatsbosbeheer Drents-Friese Wold and Staatsbosbeheer Dwingelder Veld kindly allowed us to work on their land. This study was partly funded by a grant from Birdlife Netherlands. The study was performed under a ringing permit from the Dutch Centre for Avian Migration and Demography ("Vogeltrekstation"). Taking blood samples was done under license D4743A and DEC5219B of the Institutional Animal Care and Use Committee of the University of Groningen.



# PART II

## Immune function in an annual-cycle perspective


QUICK USER GUIDE
Counting 25/50/75/100 samples
Calibration:
Handicap experiment:
Radio transmitter
LPS experiment
Mist net with
Baseline
Added
Control
Short
Medium
Tongue spots:
Feathers mites:
Tarsus short
Tarsus long
Mass (g):
Wing (mm):
Ketone:
Blood
7 8 9 10 11 12 13 14 15

# CHAPTER 4

# Immune function in a free-living bird varies over the annual cycle, but seasonal patterns differ between years

Arne Hegemann, Kevin D. Matson, Christiaan Both & B. Irene Tieleman

**Oecologia: 170: 605–618 (2012)**

**Abstract** A central hypothesis of eco-immunology proposes trade-offs between immune defences and competing physiological and behavioural processes, leading to immunological variation within and among annual-cycle stages; and such has been revealed for some species. However, few studies investigated simultaneously patterns of multiple immune indices over the entire annual cycle in free-living birds, and none has investigated the consistency of seasonal patterns across multiple years. We quantified lysis, agglutination, haptoglobin, leukocyte profiles, and body mass in free-living Skylarks (*Alauda arvensis*) through two complete annual cycles and within and among four breeding seasons. The Skylarks' annual cycle is characterised by annually repeated changes in energy and time budgets, social structure and diet. If trade-offs relating to these cyclic changes shape evolution, predictable intra-annual immune patterns may result. Alternatively, intra-annual immune patterns may vary among years if fluctuating environmental changes affect the cost-benefit balances of immune function. We found significant variation in immune indices and body mass across the annual cycle, and these patterns differed between years. Immune parameters differed among four breeding seasons, and in all years, lysis and agglutination increased as the season progressed independent of average levels. Population-level patterns (intra-annual, inter-annual, within breeding season) were consistent with within-individual patterns based on repeated measurements. We found little evidence for sex differences, and only haptoglobin was correlated (negatively) with body mass. We conclude that immune modulation is not simply a pre-programmed phenomenon that reflects predictable ecological changes. Instead, fluctuating environmental conditions that vary among years likely contribute to the immunological variation that we observed.

## Introduction

The immune system is a major physiological component of self-maintenance and promotes survival by reducing the probability of disease-related mortality (Roitt *et al.* 1998). Because the immune system also incurs costs in terms of its production, maintenance and activation (Schmid-Hempel 2003, Klasing 2004), organisms likely adjust the amount of resources allocated to the system relative to other activities in order to maximize fitness. One of the central hypotheses in ecological immunology proposes that immune defences are traded off against competing physiological and behavioural processes (Sheldon & Verhulst 1996, Lochmiller & Deerenberg 2000, Norris & Evans 2000). If the outcome of this trade-off differs among annual-cycle stages depending on resource availability and/or fitness benefits, then seasonal modulations in immune function might result. Additionally, the selective pressures exerted by pathogens and parasites, which are also expected to shape these trade-offs (Horrocks *et al.* 2011), may differ spatially (Piersma 1997, Mendes *et al.* 2006b) and are known to vary temporally (Dowell 2001, Cosgrove *et al.* 2008).

Seasonal variation in immune function has been described for a number of vertebrate taxa (reviewed by Nelson & Demas 1996, Nelson *et al.* 2002, Martin *et al.* 2008). This variation can manifest itself as an overall reduction in investment in the immune system or as a reallocation within the immune system (Lee 2006, Hasselquist 2007, Buehler *et al.* 2008, Martin *et al.* 2008). Studies report reductions in immune indices during reproduction (e.g. Ilmonen *et al.* 2000, Bonneaud *et al.* 2003, Ardia 2005), migration (e.g. Owen & Moore 2006, Owen & Moore 2008), moult (Martin 2005, Moreno-Rueda 2010) and winter (Svensson *et al.* 1998). Thus far, in free-living birds most studies of immune function are restricted to part of an annual cycle. Only two studies examined immune indices over more than two annual-cycle stages, and both found seasonal modulation of the immune system. In Great Tits (*Parus major*) the heterophil/lymphocyte ratio varies seasonally and peaks after breeding (Pap *et al.* 2010b). Among four annual-cycle stages, House Sparrows (*Passer domesticus*) showed significant variation in six of eight measured immunological variables with patterns varying depending on the variable (Pap *et al.* 2010a).

Additional data on seasonal variation of different immune parameters over the complete annual cycle and data over multiple years are needed to determine if trade-offs lead to consistent intra-annual immune patterns. Two main hypotheses can be distinguished: 1) If seasonal patterns are due to trade-offs with other fitness enhancing activities that are consistently cyclical, then seasonal immune modulation is predicted to be consistent across years. For example, a shift in energy allocated to reproduction, moult or migration can result in smaller energy investments into the immune system and can lead to seasonally-characteristic changes that are independent of year. 2) Alternatively or additionally, allocation to immune function could be a consequence of encountered environmental conditions that change not only throughout the year but may also vary between years (e.g. resource availability and pathogen pressure). Overall, relationships between annual-cycle stage and immune function may be inconsistent among years if inter-annual variation in environmental conditions is strong or if seasonal trade-offs are weak. Such high flexibility would indicate broad reaction norms of immune function (see also Pedersen & Babayan 2011).

Beyond helping to disentangle the two main hypotheses outlined above, studying multiple complete annual cycles has an array of broader biological and methodological implications. For example, understanding within- and between-annual cycle variation is crucial when interpreting studies that investigate only a limited part of the annual cycle or a single year. Furthermore, such studies can help identify and explain immunological differences across annual-cycle stages and/or years, between the sexes and in relation to body condition. Males and females are hypothesized to differently allocate resources to their immune system since the sexes can differ in terms of energy expenditure and parasite exposure (Hasselquist 2007, Martin *et al.* 2008). Additionally it has been suggested that energy stores are a proximate mechanism for seasonal modulations in immune responses (Owen-Ashley & Wingfield 2006, Owen-Ashley & Wingfield 2007), but energy stores can change among annual-cycle stages and between years, depending on environmental conditions. Lastly, seasonal and annual patterns could arise because of changes in population composition or due to individual flexibility. Repeatedly measuring individuals within and among seasons and years can shed light on this possibility and will help validate single time-point measurements.

We tested these hypotheses on free-living Skylarks (*Alauda arvensis*) by measuring components of the innate and acquired arms of the immune system across the entire annual cycle. Skylarks experience six distinct annual-cycle stages (territory settlement, breeding, moult, autumn migration, winter and spring migration), which vary in social structure, diet, activities, habitat choice (Fig. 4.1) and basal metabolic rate (Hegemann *et al.* 2012c). These stages therefore could represent differences in resources and in pressures from pathogens and parasites, affecting the trade-off for optimal investment in immune function and other fitness enhancing traits.

We measured three general categories of immune defence: 1) the abilities of plasma to agglutinate and lyse foreign cells (Matson *et al.* 2005), processes which involve natural antibodies and complement and that are thought to be unaffected by previous exposure (Ochsenbein & Zinkernagel 2000); 2) acute phase protein (haptoglobin) concentrations, which usually increase in response to inflammation or infection (Thomas 2000, Matson *et al.* 2012); and 3) the relative abundances of five leukocyte types, which reflect both innate and acquired components and change in response to immunological stimulation (Feldman *et al.* 2000). The latter includes the ratio of heterophils and lymphocytes (H/L ratio) which is related to different types of stressors, including immunological ones (reviewed by Davis *et al.* 2008). We took biological and methodological factors into consideration when choosing to focus mainly on measures of innate immunity: This sub-system is an important first line of defence (Janeway *et al.* 2005), and this importance might translate into consistency over longer time scales, a point that coordinates with our main hypotheses. Additionally, while measures of innate immunity can vary over shorter scales (e.g. reflecting current "health status" or "physiological condition," (Van de Crommenacker *et al.* 2010), the absence of immunological memory in verte-

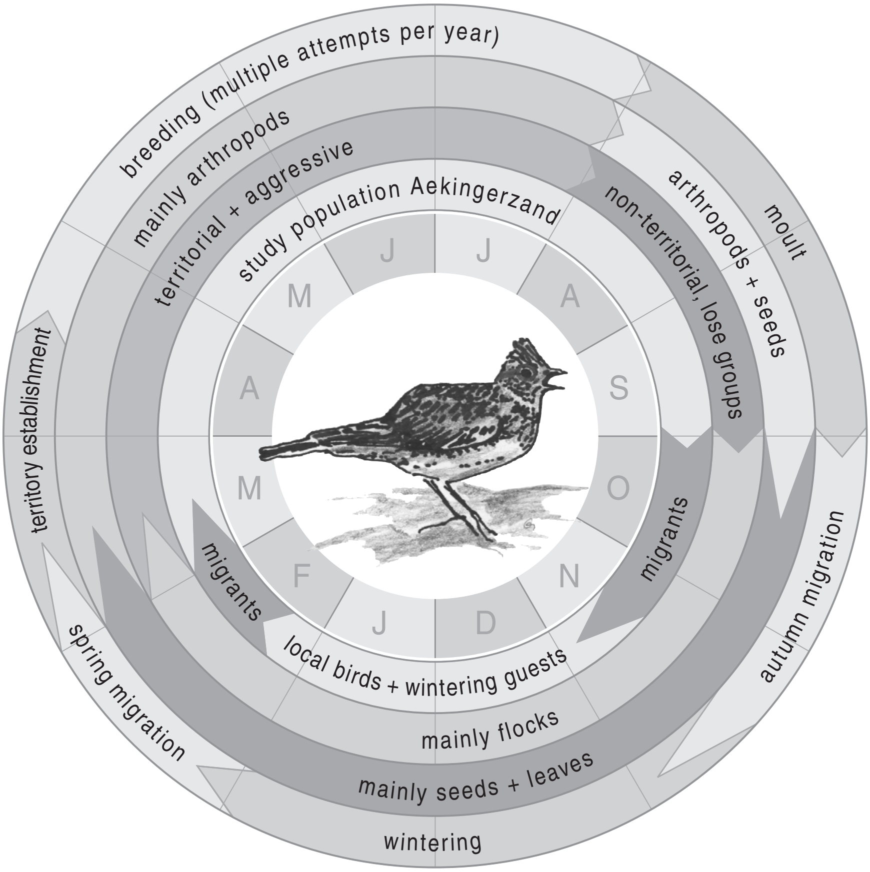


**Figure 4.1:** The annual cycle of a Skylark in the northern Netherlands. Length of arrows in the transition between two stages indicates the amount of variability between years and/or individuals. Data on diet are based on Green (1978), Donald *et al.* (2001) and Geiger *et al.* (in prep.). All other data are based on own unpublished data and Hegemann *et al.* (2010).

brate innate sub-systems allows for interpretation of repeated samples without confounding the magnitude of an index and the exposure to a particular disease (Janeway & Medzhitov 2002, Kurtz 2004).

We explored three potential sources of variation in immune indices: 1) among annual-cycle stages, 2) between years, 3) within one annual-cycle stage (breeding). For all three categories we also explored if, at the population level, body mass varies in a manner similar to the immune indices and if, at the individual level, body mass correlates with these indices. Because sexes differ in behaviour and physiology, we included sex in our analyses. Lastly, we used intra-individual repeated measures to investigate whether seasonal and annual patterns within individuals were consistent with population mean seasonal and annual patterns. Because Skylarks undergo characteristic seasonal changes in their ecology that are repeated on a regular basis, we expected corresponding intra-annual patterns of immune function that are repeatable among years, as a result of trade-offs between immune function and other physiological and behavioural demands. Alternatively, if fluctuating environmental conditions exert a substantial influence, then intra-annual patterns in immune function may differ among years. Fluctuating environmental conditions may affect immune patterns either qualitatively (i.e. the shape of seasonal patterns) or quantitatively (i.e. the magnitude of differences between seasons).

## Methods

### ***Study system***

Skylarks are widespread, temperate zone passerines that breed on the ground in open habitats ranging from natural steppes to modern agricultural farmland across Eurasia (Donald 2004). Birds from northern populations migrate, whereas southern populations are resident year-round, and Western European birds are partial migrants (Glutz von Blotzheim & Bauer 1985, Donald 2004, Hegemann *et al.* 2010).

We studied Skylarks throughout the annual cycle in the northern Netherlands (N 52°55'; E 6°18') in 2006–2009, focusing on a population in the Aekingerzand (Fig. 4.1). Skylarks in our study population are partial migrants. Some birds migrate, while others winter locally on agricultural fields that surround their breeding site, where they are accompanied by birds from northern and eastern Europe (Hegemann *et al.* 2010). During migration, birds from northern and eastern populations pass the Netherlands (LWVT/SOVON 2002). During all parts of the annual cycle Skylarks forage and sleep exclusively on the ground.

We collected data during the breeding season in four consecutive years (2006–2009), and during two successive years (2007–2008) for moult, autumn migration, wintering and spring migration. We also collected samples during the territory establishment period in one year (2007). During territory establishment, breeding and moult we sampled birds at the Aekingerzand. During territory establishment (27 Feb – 22 Apr 2007), we lured birds ($n = 14$) into mist nets by using recordings of territorial songs and calls. During the four breeding seasons (21 Apr – 3 Aug 2006–2009), we caught adults ($n = 189$) feeding nestlings with mist-nests or traps on the nest. During moult (14 Aug – 27 Sep 2007; 2 Aug – 22 Sep 2008), birds ($n = 33$) were caught at night by flushing them into nets. During winter (10 Dec 2007 – 22 Jan 2008; 9 Dec 2008 – 15 Jan 2009), we caught birds ($n = 25$) during the night by flushing them into nets on agricultural fields surrounding the Aekingerzand. During winter, we were unable to exclusively target birds from our study population, and we possibly caught a mix of local breeders and birds from more-northern breeding sites. During spring (14 Mar – 24 Mar 2008; 25 Feb – 1 Mar 2009; $n = 27$) and autumn (8 Oct – 4 Nov 2007 and 11 Oct – 2 Nov 2008, $n = 97$), actively migrating birds were caught during the day by luring them with conspecific songs to large clap-nets at a location 15 km southeast of the Aekingerzand. When tape-lured, migrants interrupt their migratory flight. Skylarks not actively migrating rarely respond to a tape lure during these seasons (Hegemann *et al.* unpublished observations); hence, we are confident about the migratory status.

Migrating and wintering birds were sampled only once, but a subset of Skylarks was sampled repeatedly during other annual-cycle stages. We used these repeated measures to investigate whether seasonal and annual patterns at the population level were consistent with patterns at the individual level. During breeding, 44 birds were sampled twice; 19 birds, three times; 5 birds, four times; and 2 birds, six times. Repeated sampling occurred either within a single breeding season ($n$ = 19) or in two ($n$ = 38), three ($n$ = 12) or four ($n$ = 1) different breeding seasons. Some individuals were also caught in two different stages: 11 birds during territory establishment and breeding and 18 birds during breeding and moult.

### *Sample and data collection*

Blood samples (~100-150 µl) were collected into heparinised capillary tubes by puncturing the brachial vein with a sterile needle. Samples were collected immediately after capture (median: 5 min; range: 2–35 min) and before any expected impacts of handling stress (Buehler *et al.* 2008). Blood smears for leukocyte enumeration were made from fresh blood. Blood was stored on ice until centrifuged in the lab (10 min, 7000 rpm). Plasma and packed cell fractions were stored frozen for future analyses. Structural measurements (body mass, tarsus length, wing length) were taken after blood collection, and all birds were ringed with a metal ring from the Dutch Ringing Centre; birds from the study population Aekingerzand were additionally ringed with a unique combination of color rings. Birds were sexed biometrically and some doubtful cases were sexed molecularly (Glutz von Blotzheim & Bauer 1985, Hegemann *et al.* 2012a). All individuals included in this dataset were fully grown. Because Skylarks undergo a complete postnuptial moult in August-September, age classes could not be distinguished.

### *Immune assays*

We analysed the preserved plasma samples using two immunological assays. A hemolysis-hemagglutination assay was used to quantify titres of complement-like lytic enzymes and non-specific natural antibodies (Matson *et al.* 2005). Scans of individual samples were randomized among all plates and scored blindly to season, year and individual (by AH). A plasma standard was run in duplicate in all plates. The average within-plate variation (standard deviation) was 0.18 lysis titres and 0.32 agglutination titres. The average among-plates variation (calculated per batch) was 0.52 lysis titres and 0.80 agglutination titres. These values are only slightly higher than the variation originally described by Matson *et al.* (2005). A commercially available colorimetric assay kit (TP801; Tri-Delta Diagnostics, NJ, USA) was used to quantify haptoglobin concentrations (Matson *et al.* 2006). We followed the instructions provided by the kit manufacturer with a few minor modifications. Specifically, we extended the standard curve to a more diluted range. We also normalized all final haptoglobin values according to a plate-specific pool to control for variation within and among plates and batches (Matson *et al.* 2012). Both assays were carried out in four batches (July 2007, February 2009, March

2009 and September 2009). Samples from 2006 were analysed in batch 1, samples from the first annual cycle in batch 1 and 2, from the second annual cycle in batch 2 and 3 and samples from 2009 in batch 4.

Blood smears were prepared for microscopic observation (Campbell 1995) and smears were randomized and examined by one person (C. Gottland), who was blind to season. Leukocyte proportions were determined for the first 100 white blood cells (WBC) counted; in rare cases where cells were highly dispersed and WBCs were difficult to find, proportions were based on fewer than 100 cells. Cells where classified as lymphocytes, heterophils, basophils, monocytes or eosinophils (Latimer & Bienzle 2000).

### *Data analysis and statistics*

We present analyses of several data subsets: 1) two annual cycles (2007 and 2008) with five stages each (breeding, moult, autumn migration, winter, spring migration), 2) one annual cycle (2007) that included a sixth annual-cycle stage (territory establishment), and 3) four breeding seasons (2006–2009). We defined an annual cycle (or bird year) as starting with territory establishment and ending with spring migration. We refer to these as 2006, 2007, 2008 and 2009 even though they slightly differ from calendar years.

We used linear mixed models and generalized linear models in the program R, version 2.9.2 (R Development Core Team 2009). Sex, annual-cycle stage and year and all interactions were included when applicable. To test if immune parameters are related to body mass at the individual level, we included a mass index (in g) in our models that was independent of season-, sex- and year-specific variation. We derived this mass index by calculating each individual's deviation from the corresponding season-, sex- and year-specific population mean. Individual identity was included in all analyses as a random effect to avoid pseudo-replication. When comparing the four breeding seasons, we included Julian day and its square to test effects related to day length. Final models were achieved via backwards elimination (log likelihood ratio test, $P < 0.05$) using the "drop1"-function of *R*.

Data on white blood cells are only available for 2008 with the exception of spring migration where we have data from 2008 and 2009. T-tests revealed that none of the WBC types differed between the two spring migrations (all $t < 1.61$, all $P < 0.12$), and thus data for this stage were pooled. We have no repeated measures for the WBC data. WBC data were analysed with generalized linear models with a quasi-binomial approach and *F*-tests. White blood cell types were analysed separately using binomial approaches that incorporated the counts of one cell type and the total remaining WBC number (i.e. Basophils against the sum of heterophils, lymphocytes, monocytes and eosinophils). Additionally heterophils and lymphocytes were tested binomially as H/L ratio. Assumptions of all models were checked on the residuals of the final model.

For the plasma parameters and body mass for which we had repeated measures within individuals, we investigated whether patterns found within individuals were consistent with the average population level or were instead the result of other phenomena such as selective catching, individual differences in timing or changes in population composition. To accomplish this, we used the method described by van de Pol & Wright (2009) to test if population-level patterns differed from within-individual patterns. These analyses were restricted to within-breeding season patterns and the three annual-cycle stages (territory establishment, breeding and moult) with repeated measurements.

Significant interactions could result if large differences in annual means are paired with proportional changes among annual-cycle stages. If a year × annual-cycle stage interaction remained significant, we log-transformed response variables to investigate this possibility. In all cases the interaction remained significant. To determine which annual-cycle stages (within year) and which breeding seasons (among years) differed from each other, we used Tukey posthoc tests ("multcomp" package, Hothorn *et al.* 2008).

## Results

### *Variation among stages in a single annual cycle*

Lysis, agglutination, haptoglobin and body mass varied among the six annual-cycle stages (2007) and three of the six cellular parameters (leukocyte profiles) varied among the five stages measured (2008) (Fig. 4.2A-E). Lysis peaked during breeding, agglutination declined from breeding to winter, and haptoglobin concentrations peaked during spring migration (Fig. 4.2A-C). Monocytes and basophils were highest during autumn migration, and eosinophils peaked during spring migration (Fig. 4.2E). Birds were heaviest during winter and lightest during breeding (Fig. 4.2D, 4.3D). Variation between annual-cycle stages was statistically significant for six immune parameters and body mass (Table 4.1). The proportions of heterophils and lymphocytes and the H/L ratio showed no significant seasonal variation (Table 4.1). Males had significantly higher lysis titres but a lower H/L ratio and lower proportions of heterophils than females throughout the annual cycle (Table 1). Males (37.14 ± 0.33 g, $n = 94$) were heavier than females (32.45 ± 0.25 g, $n = 83$).

### *Inconsistent seasonal patterns between years*

The seasonal variation in immune parameters and body mass was not consistent between years. When we compared 2007 and 2008, we found a significant interaction between year and annual-cycle stage for all three immune variables (lysis, agglutination, haptoglobin) and body mass (Table 4.2, Fig. 4.3A-D). The interactions were not simply the result of one year that was higher and characterized by proportionally greater changes between stages (Fig. 4.3A-D), because they remained significant after log-transforming the response variables. The interaction sex × year was highly significant for lysis titres but not significant for agglutination, haptoglobin concentrations and body mass (Table 4.2). Males showed higher lysis than females in the first annual cycle, but lower than females in the second annual cycle. After removing the non-significant interactions from the model, there was no significant effect of sex on agglutination and haptoglobin ($df = 1{,}290$, $F = 3.06$, $P = 0.08$ and $df = 1{,}292$, $F = 0.00$, $P = 0.97$, respectively).

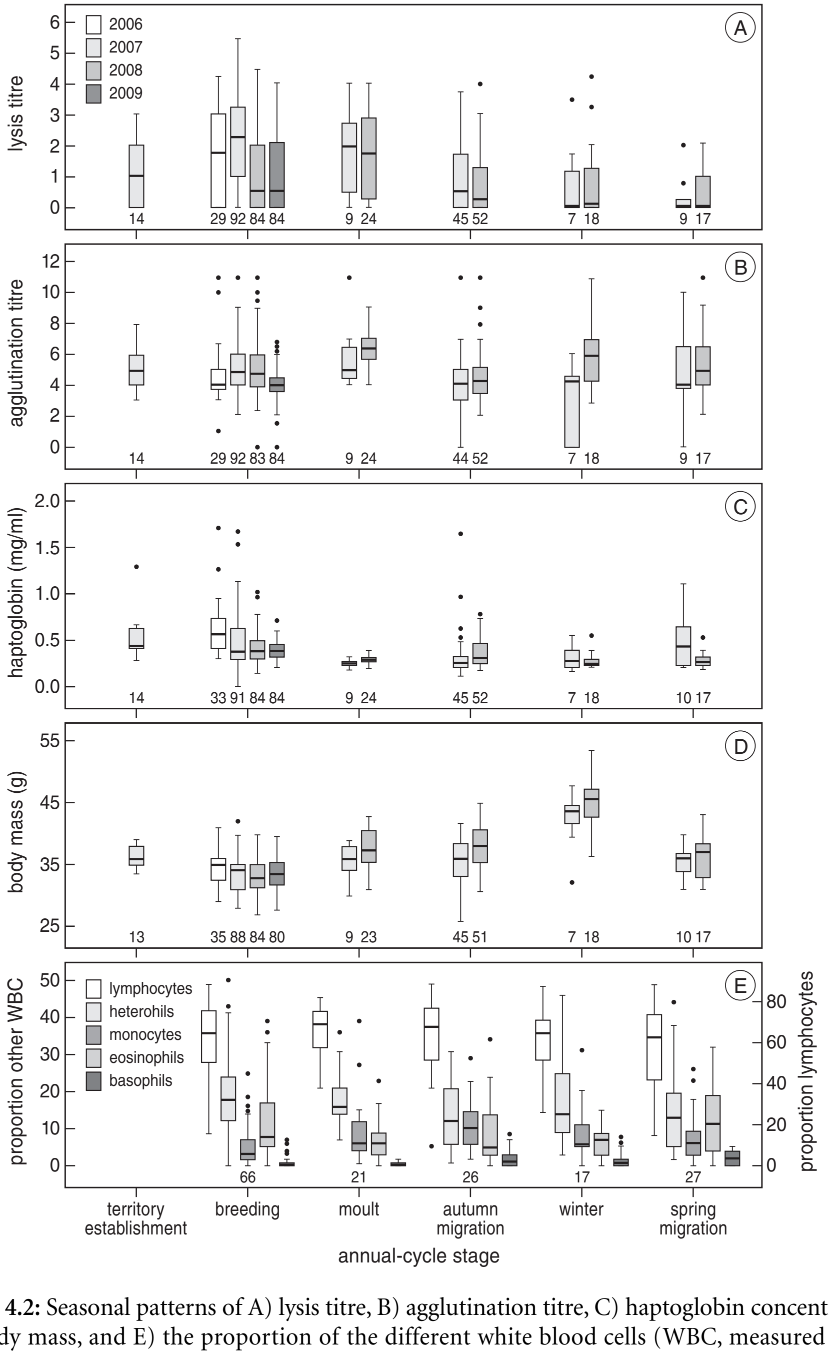


**Figure 4.2:** Seasonal patterns of A) lysis titre, B) agglutination titre, C) haptoglobin concentrations, D) body mass, and E) the proportion of the different white blood cells (WBC, measured only in 2008) of free-living Skylarks in six annual-cycle stages. Note that lymphocytes are scaled on the right y-axis, while the other, less numerous WBC types are scaled on the left y-axis. Numbers under box-plots represent sample size. The horizontal lines in the box gives the median, the box covers the 25–75% range, and vertical lines the 5-95% range. Dots show outlying data points, which were included in the analyses.

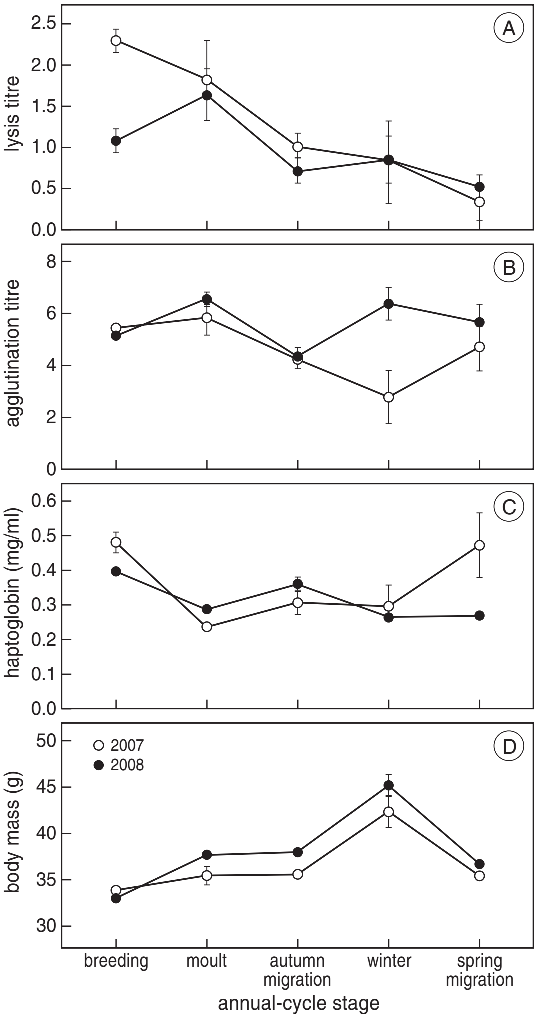


**Figure 4.3:** Patterns of average immune function throughout two annual cycles to illustrate the variation between immune patterns in two annual cycles. A) lysis titre, B) agglutination titre, C) haptoglobin concentrations and D) body mass of Skylarks. Shown are means with s.e. (please note the difference to Figure 4.2 where medians are shown).

### *Consistent patterns among annual cycles and individuals*

The annual cycle as measured within single individuals (repeated measures) has the same basic pattern as at the population level. Seasonal patterns between territory establishment, breeding and moult measured on different individuals did not statistically differ from seasonal patterns established from repeated measurements within individuals for all immune parameters and body mass (always $P > 0.22$).

**Table 4.1:** Statistics and coefficients of the linear mixed models (innate immune parameters and body mass) and generalized linear models (leukocyte profiles) of measures of immune function in Skylarks throughout one annual cycle (2007 for innate immune parameters and 2008 for leukocyte profiles). Individual bird identity was included as random effect to avoid pseudo replication whenever applicable. Results are from linear models after removing all non-significant terms ($P > 0.05$). $P$-values < 0.05 are bold. BMd is a body mass index that is calculated as each individual's deviation from its corresponding season-, sex- and year-specific mean and thus independent of these three sources of variation. All rows reporting data from generalized linear models are marked with an asterix.

**Single annual cycle (2007 for innate immune parameters and 2008 for leukocyte profiles)**

| | Sex | | | | Annual-cycle stage | | | BMd | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | *df* | *F* | *β* | *P* | *df* | *F* | *P* | *df* | *F* | *β* | *P* |
| Lysis | 1,147 | 9.51 | 0.66 | **0.002** | 5,17 | 10.88 | <**0.001** | 1,16 | 0.11 | | 0.740 |
| Agglutination | 1,146 | 2.91 | | 0.090 | 5,16 | 3.85 | **0.018** | 1,16 | 3.52 | | 0.079 |
| Haptoglobin | 1,147 | 0.35 | | 0.554 | 5,16 | 4.38 | **0.011** | 1,16 | 9.47 | -0.03 | **0.007** |
| Body mass | 1,148 | 113.57 | 4.40 | <**0.001** | 5,17 | 9.89 | <**0.001** | | | | |
| *H/L ratio | 1,150 | 4.67 | -0.28 | **0.032** | 4,153 | 0.91 | 0.462 | 1,152 | 0.04 | | 0.850 |
| *Heterophils | 1,150 | 5.84 | -0.28 | **0.017** | 4,153 | 1.80 | 0.133 | 1,152 | 0.11 | | 0.745 |
| *Lymphocytes | 1,150 | 1.42 | | 0.235 | 4,153 | 0.28 | 0.888 | 1,152 | 0.01 | | 0.932 |
| *Basophils | 1,150 | 0.07 | | 0.794 | 4,153 | 6.19 | <**0.001** | 1,152 | 0.50 | | 0.479 |
| *Monocytes | 1,150 | 0.26 | | 0.609 | 4,153 | 4.83 | **0.001** | 1,152 | 0.01 | | 0.936 |
| *Eosinophils | 1,150 | 0.01 | | 0.934 | 4,153 | 2.50 | **0.045** | 1,152 | 0.20 | | 0.652 |

**Table 4.2:** Statistics and coefficients of the linear mixed models of measures of immune function and body mass in Skylarks over two repeated annual cycles (2007 and 2008). Individual bird identity was included as random effect to avoid pseudo replication. Results are from linear models after removing all non-significant terms ($P > 0.05$). $P$-values < 0.05 are bold. For explanation of BMd see legend of Table 4.1.

**Repeated annual cycle (2007,2008)**

| | Sex × year | | | Annual-cycle stage × year | | | BMd | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *df* | *F* | *P* | *df* | *F* | *P* | *df* | *F* | *β* | *P* |
| Lysis | 1,47 | 9.41 | **0.004** | 4,47 | 5.27 | **0.001** | 1,46 | 0.74 | | 0.393 |
| Agglutination | 1,46 | 0.34 | 0.564 | 4,48 | 3.44 | **0.015** | 1,47 | 0.89 | | 0.350 |
| Haptoglobin | 1,42 | 0.73 | 0.399 | 4,47 | 2.86 | **0.033** | 1,47 | 7.22 | -0.01 | **0.010** |
| Body mass | 1,43 | 0.04 | 0.843 | 4,44 | 5.42 | **0.001** | | | | |

**Table 4.3:** Statistics and coefficients of linear mixed models of three plasma measures of immune function in Skylarks during four consecutive breeding seasons (2006–2009) and of generalized linear models for leukocyte profiles which were measured only in one breeding season (2008). Individual bird identity was included as random effect to avoid pseudo replication whenever applicable. Results are from linear models after removing all non-significant terms ($P > 0.05$). $P$-values $< 0.05$ are bold. For explanation of BMd see legend of Table 4.1. All rows reporting data from generalized linear models are marked with an asterix.

| **Breeding** (2006-2009) | Sex | | | Julian day | | | | Year | | | BMd | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *df* | *F* | *P* | *df* | *F* | *β* | *P* | *df* | *F* | *P* | *df* | *F* | *β* | *P* |
| Lysis | 1,178 | 0.32 | 0.570 | 1,101 | 41.24 | 0.02 | **<0.001** | 3,101 | 24.98 | **<0.001** | 1,90 | 1.75 | | 0.190 |
| Agglutination | 1,177 | 0.23 | 0.630 | 1,101 | 11.74 | 0.02 | **0.001** | 3,101 | 10.38 | **<0.001** | 1,95 | 0.24 | | 0.628 |
| Haptoglobin | 1,181 | 0.74 | 0.391 | 1,96 | 0.88 | | 0.350 | 3,97 | 13.19 | **<0.001** | 1,97 | 3.79 | -0.01 | 0.054 |
| Body mass | 1,183 | 135.2 | **<0.001** | 1,98 | 30.01 | -0.02 | **<0.001** | 3,98 | 5.86 | **0.001** | | | | |
| *H/L ratio | 1,65 | 1.75 | 0.190 | 1,65 | 2.17 | | 0.146 | | | | 1,65 | 0.83 | | 0.373 |
| *Heterophils | 1,65 | 1.55 | 0.217 | 1,65 | 1.85 | | 0.178 | | | | 1,65 | 0.73 | | 0.395 |
| *Lymphocytes | 1,65 | 1.97 | 0.166 | 1,65 | 2.26 | | 0.138 | | | | 1,65 | 0.9 | | 0.347 |
| *Basophils | 1,65 | 0.06 | 0.813 | 1,65 | 3.79 | | 0.056 | | | | 1,65 | 0.34 | | 0.563 |
| *Monocytes | 1,65 | 0.02 | 0.889 | 1,65 | 4.05 | 0.02 | **0.048** | | | | 1,65 | 0.32 | | 0.573 |
| *Eosinophils | 1,65 | 1.64 | 0.206 | 1,65 | 0.06 | | 0.801 | | | | 1,65 | 1.25 | | 0.267 |

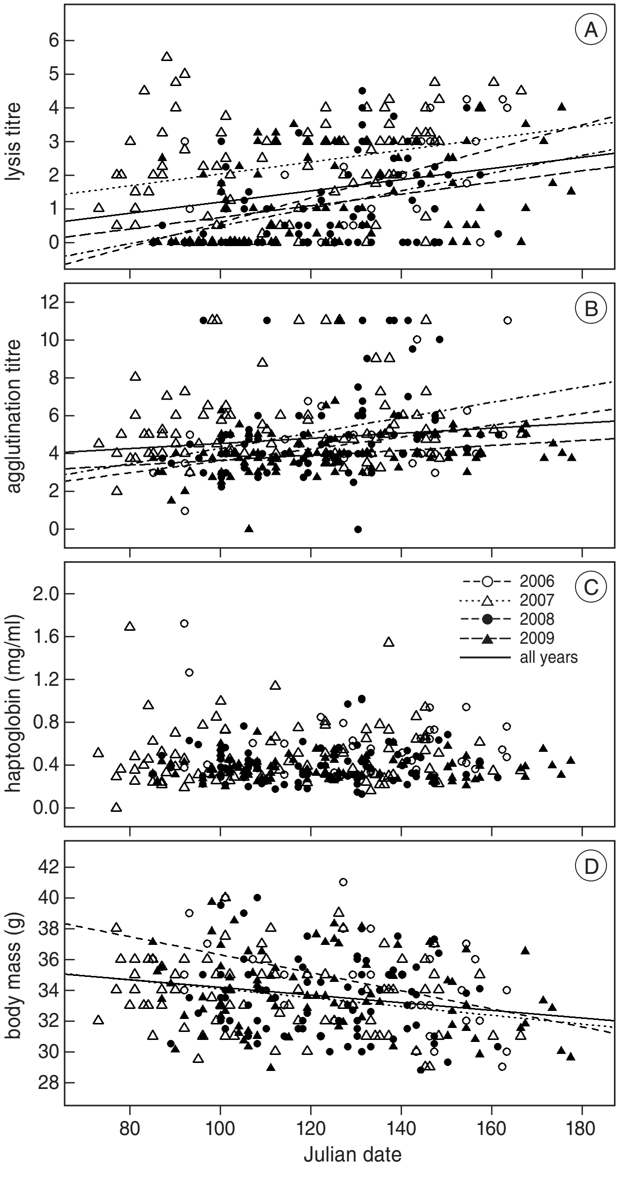


**Figure 4.4:** Patterns of A) lysis titre, B) agglutination titre, C) haptoglobin concentrations and D) body mass in the course of four different breeding seasons in free-living Skylarks. Regression slopes are presented if $P < 0.05$.

Regardless of which dataset was used (i.e. the complete 6-stage annual cycle of 2007, the repeated 5-stage annual cycles of 2007 and 2008, or the entire dataset including 2006–2009), post-hoc analyses on models including season and year (if applicable) as main effects revealed that immune function differed consistently between breeding and autumn migration with respect to all five immune parameters that varied by stage

(Tukey test always $P < 0.029$). Lysis and agglutination titres and haptoglobin concentrations were higher during breeding than during autumn migration; proportions of basophils and monocytes exhibited the opposite pattern. Also consistent among datasets, lysis titres were significantly higher during breeding than during spring migration (Tukey test always $P < 0.001$), and haptoglobin concentrations were significantly higher during breeding than during moult (Tukey test always $P < 0.05$).

### Variation among breeding seasons

Lysis, agglutination, haptoglobin, and body mass showed significant differences among the four successive breeding seasons (Table 4.3). Lysis was significantly higher in 2007 than in all other years (Tukey test always $P < 0.003$, Fig. 4.2A). Agglutination was lower in 2009 than in 2007 and 2008 (Tukey test both $P < 0.001$), but did not differ from 2006 (Tukey test $P = 0.66$, Fig. 4.2B). Haptoglobin concentrations in 2006 were significantly higher than in all subsequent years (Tukey test always $P \leq 0.003$) and concentrations in 2007 were significantly higher than in 2009 (Tukey test $P = 0.008$, Fig. 4.2C). Body mass in 2008 was lower than in 2006 and 2009 (Tukey test both $P < 0.006$, Fig. 4.2D). For the three immune variables and body mass, the differences among breeding seasons at the population level were qualitatively similar to the differences within individuals that were sampled in more than one year (always $P < 0.18$).

### Variation within the breeding season

Over the course of four pooled breeding seasons, lysis and agglutination titres both increased, body mass decreased, and haptoglobin concentration showed no trend (Fig. 4.4A-D). The effect of Julian day was significant for lysis, agglutination and body mass (Table 4.3). This effect differed marginally between years for body mass (Julian day × Year interaction $df = 3{,}94$, $F = 2.78$, $P = 0.045$) but did not differ between years for lysis and agglutination (Julian day × Year interaction $df = 3{,}87$, $F = 2.00$, $P = 0.12$ and $df = 3{,}92$, $F = 2.18$, $P = 0.10$ respectively). In all four cases the within-season pattern did not differ between birds sampled repeatedly and birds sampled only once (always $P > 0.39$). As the 2008 breeding season progressed, proportions of monocytes and basophils increased. The increase in monocytes was marginally significant, while the increase in basophils was marginally non-significant (Table 4.3). There was no relationship between Julian date and the H/L ratio or the proportions of heterophils, lymphocytes or eosinophils. During breeding, males and females did not differ significantly in any of the plasma indices or WBC counts (Table 4.3). All effects of date did not deviate from linearity, since date squared was never significant.

### Correlations between immune indices and body mass index

Individuals with a relatively high body mass had relatively low haptoglobin concentrations regardless of which dataset was used (Fig. 4.5). This relationship was significant for both the single and the repeated annual cycle (Table 4.1, 4.2) and borderline non-significant ($P = 0.054$) during breeding (Table 4.3). We found no relationship with body mass index for lysis, agglutination or any of the WBC measures (Table 4.1, 4.2, 4.3).

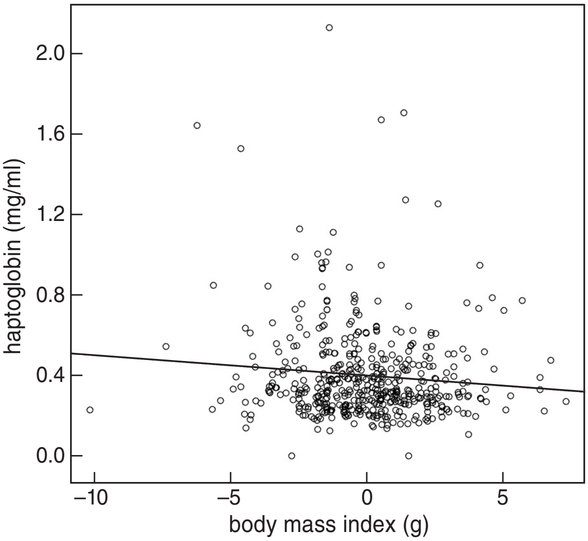


**Figure 4.5:** Relationship between a body mass index (in g) and haptoglobin concentrations in free-living Skylarks. The body mass index is calculated as each individual's deviation from its corresponding season-, sex- and year-specific mean and thus independent of these three sources of variation. Data from all years and annual-cycle stages pooled. Statistics for the separate data sets can be found in Table 4.1A-C.

## Discussion

We found variation in indices of innate and acquired immune defence among six annual-cycle stages in free-living Skylarks, but the shape of the seasonal patterns varied with immune index and differed between years. Body mass also showed variation among annual-cycle stages, and as with the immune indices, the pattern differed between years. The among-year differences in body mass suggest that environmental factors such as food availability, temperature or pathogen pressure varied. Such factors could also lead to the differences in seasonal immune patterns among years. If prevailing environmental conditions and immune patterns are linked, then seasonal and annual variation within individuals (repeated measures) should parallel seasonal and annual variation measured at the population level; our results supported this. This finding made it also unlikely that aging explained any differences between years. Furthermore we found little evidence for differences between the sexes, and only haptoglobin concentrations were (inversely) related to body mass. Overall, our study supports the premise that birds seasonally modulate their immune system during the annual cycle. Because years differ, we conclude that this modulation is not a result of trade-offs dictated only by energetically-demanding, regularly-occurring annual-cycle activities, but that environmental variation among annual-cycle stages and years is important.

### *Seasonal patterns of immune function are flexible*

Our finding of inter-annual differences in seasonal patterns of immune indices raises the question if other species are similarly flexible between years and if it depends on a species'ecology. Other studies have suggested trade-offs between certain annual-cycle activities and immune function: reduced immune function has been described during migration (Owen & Moore 2008), moult (Moreno-Rueda 2010), winter (Svensson *et al.* 1998) and breeding (e.g. Ilmonen *et al.* 2000, Bonneaud *et al.* 2003, Tieleman *et al.* 2008), and Pap *et al.* (2010a, 2010b) report seasonal variation over a complete annual cycle. These studies all considered only a single year, and the observed seasonal differences might also result from particular ecological conditions rather than physiological trade-offs that differed consistently between annual-cycle stages. Because we found differences in seasonal variation between years, we hypothesize that immune function is influenced by the combined effects of resource availability and parasite pressure, which in turn affect trade-offs between immune function and other fitness-related physiological systems. This process could lead to immunological flexibility among annual-cycle stages and among years. The fact that other fitness related parameters like clutch size, breeding success and recruitment rate, which are known to be largely influenced by food availability and weather conditions, also vary considerably between years (Hegemann *et al.* unpublished data), supports our argument that environmental conditions differ between years. At the same time it highlights that studies of trade-offs between immune function and other fitness-related processes need to incorporate variation in environmental conditions within and among annual-cycle stages and years.

During territory establishment, breeding and moult we exclusively sampled birds from our local study population, but during winter, spring migration and autumn migration we caught a mixture of local birds and birds from more northern and eastern locations. One might therefore ask if the variation in seasonality of immune function between years stems from sampling different proportions of birds from different origins during winter and migration in the two years. We cannot completely rule out this possibility, but it is unlikely that it explains the between-year variation, for two reasons: 1. Also within the breeding season and during moult we observed variation between years, confirmed by repeated measurements on the same individuals. 2. The three immune parameters and body mass were never consistently different within one stage between the two years. Only a subset of measured parameters differed, similar to patterns expressed by our local study population during breeding and moult.

Despite the flexibility of immune patterns, we also found consistent differences in immune function between some annual-cycle stages, indicating that some regularity in seasonal patterns of immune function exists. All five immune parameters that showed significant seasonal variation showed differences between breeding and autumn migration independent of which dataset we considered: lysis, agglutination, and haptoglobin concentrations were higher during breeding, while monocyte and basophil proportions were higher during autumn migration. These consistent differences between breeding and autumn migration could indicate that some immune patterns are more dependent on seasonal changes in the species' ecology. Going from breeding to autumn migration, Skylarks switch their diet, become less aggressive, and form flocks. The changes in WBC proportions might reflect this and other processes. For example, flocking may enhance the transmission of infectious diseases and/or parasites which could explain higher concentrations of basophils that are involved in parasite elimination (Latimer & Bienzle 2000). Likewise phagocytic monocytes can play a role in removal of damaged cells (Latimer & Bienzle 2000), which might accumulate while actively migrating.

In summary, we see a tight connection between the hypothesis of immune responses being traded off with other annual-cycle demands (Owen-Ashley & Wingfield 2007, Buehler & Piersma 2008, Martin *et al.* 2008) and the hypothesis that seasonal modulations result from seasonal environmental fluctuations in e.g. disease risk and parasite exposure, food availability or weather conditions (Nelson *et al.* 2002, Møller *et al.* 2003, Hasselquist 2007). To further resolve the processes underlying seasonality in immune parameters, longer term monitoring of immune function in free-living birds is required. Additionally, experimental studies that manipulate environmental conditions or trade-offs in several annual-cycle stages (see Buehler *et al.* 2008 for an example on captive birds) and/or across multiple years can help us better understand the intra-annual changes in immune function. Because pathogen and parasite exposure can also shape the immune system, and their influence can exert effects over a wide range of timescales (among days or years, through evolutionary time, etc.), measures of pathogen pressure should be included where possible (Horrocks *et al.* 2011).

### *Interpretation of immune indices and trade-offs within the immune system*

In the current study, we focused on measures that are relatively insensitive to pathogen challenge (e.g. hemolysis and hemagglutination, Matson *et al.* 2005), that are known to change over the shortest of timescales but that typically return to baseline levels after a response is resolved (e.g. haptoglobin, van de Crommenaker *et al.* 2010; and leukocyte distributions, Latimer and Bienzle 2000), or that are repeatable and bear some capacity to predict future responses (e.g. habtoglobin, Matson *et al.* 2012). Most of these measures represent the first lines of defence against infection. Lymphocytes are most closely associated with acquired immunity (second line of defence; Roitt *et al.* 1998), and natural antibodies, the main driver of agglutination, are best viewed as a link between the innate and acquired immune sub-systems (Ochsenbein & Zinkernagel 2000). Overall, this approach of measuring a suite of different indices that appear to be relatively insensitive to cumulative effects of exposure has proven useful in our current application and in other recent studies (Boughton *et al.* 2011, Buehler *et al.* 2011, Demas *et al.* 2011, Palacios *et al.* 2012).

The seasonal modulation and possible reorganisation of immune indices in the current study contrast with the year-round consistency of the inflammatory responses (acute phase responses to an endotoxin injection) exhibited by the same Skylark population (Hegemann *et al.* 2012c). This contrast hints at different levels of regulation of the immune system. It also suggests that, while the ability to always respond by a certain magnitude to an infection may be crucial to survival, the starting points (i.e. the levels of immunological maintenance) required to initiate these responses differ among stages and indices. On the one hand, variable starting points may reflect a time-specific balance between the availability of resources and the likelihood of encountering a challenge. On the other hand, such responses may reflect the evolutionary pressure to address dire situations (e.g. a breach of initial defences by an infectious agent) with acute redirection of resources in an attempt to prevent mortality. In terms of methodology, this contrast highlights the utility of quantifying both maintenance and response components when studying wild animals.

### *Variation within breeding seasons*

As the breeding season progressed, lysis, agglutination and the proportion of monocytes and basophils increased and body mass decreased, but there was no trend in haptoglobin or the other white blood cell types. Increases in lysis and agglutination were measured in all four years and the slope of the increase did not differ between years even though the mean values did. These patterns were also established from individuals measured repeatedly; a result that diminishes the possibility that these trends result from different timing by individuals with different immune characteristics. Further studies are required to determine if the increases in lysis and agglutination with the progression of the breeding seasons result from increases in pathogen and parasite pressure, from a shift in the balance between current and future reproduction or are linked to other changes.

### *Immune variation with sex and body condition*

Some studies present hypotheses (Hasselquist 2007, Martin *et al.* 2008) or data (Parejo & Silva 2009, Pap *et al.* 2010a) suggesting that males and females differently allocate resources to the immune system due to their different behaviours and physiology. Other studies find little or no evidence for sex differences in immune parameters (Raberg *et al.* 2003, Lee *et al.* 2006, Martin *et al.* 2006a). Our results are in line with the second group: we found few sex differences in general, and we did not find any significant interaction between sex and annual-cycle stage, despite differences in physiology (e.g. reproductive organs, hormone profiles) and behaviour (male song flights and territorial defense, female incubation) between sexes in Skylarks, especially during territory establishment and breeding.

Birds with relatively high haptoglobin concentrations had relatively low values of a body mass index, a measure of condition independent of season, sex, and year. The slope of this relationship appears shallow, but changes of 0.1 mg/ml are biologically relevant (van de Crommenacker *et al.* 2010). Acute increases in haptoglobin concentrations normally signify an infection-associated inflammation (Thomas 2000). Thus, underweight individuals with high haptoglobin concentrations might be suffering from systemic inflammation and its indirect behavioural or physiological effects that limit food intake or its direct effects of elevated metabolic rate (Owen-Ashley *et al.* 2006, Owen-Ashley & Wingfield 2007, Hegemann *et al.* 2012c). At the population level, haptoglobin concentrations were highest during territory establishment and breeding. During these stages, Skylarks are territorial and aggressive (Glutz von Blotzheim & Bauer 1985, Donald 2004). The high risk of injury during territorial fights (little skin scratches when birds attack each other with their feet or bill) may be one of the causes for high haptoglobin concentrations, even though heterophil concentrations, another important line of first defence, were not specifically higher during these stages.

### *Conclusion and future perspective*

To conclude, we found that indices of innate immunity and leukocyte distributions differ among the six distinct annual-cycle stages experienced by a free-living temperate zone bird. These differences in immune function and differences in body mass among annual-cycle stages were inconsistent between two study years. However, all within and among season patterns were similar at the levels of individual and population. Overall our results suggest that immune function is sensitive to prevailing environmental conditions, which can differ not only from stage to stage but also from year to year. Our study highlights the need for measurements across multiple years in order to draw sound conclusions about seasonal and annual variation in physiology. Going forward, studies of this sort should strive to identify influential environmental factors (e.g. resource availability and pathogen pressure) and incorporate relevant measurements of these factors. Hence, we stress the importance of a detailed ecological knowledge of the studied species. Lastly, experimental studies that manipulate immune function will be useful for revealing and understanding any trade-offs that occur during an annual cycle.

## Acknowledgements

Many volunteers helped catch Skylarks at various times of the year. We are thankful to everyone, especially Rob Voesten, Kees van Eerde, Martin Keiser, Richard Ubels, and Chris Trierweiler. Marco van der Velde performed the molecular sexing; Cecile Gottland and Emmanuelle Gilot did the blood slide counts. Ido Pen and Maaike Versteegh gave valuable advice on statistics. Dick Visser made Figure 4.1 and Chris Trierweiler draw the Skylark used in this figure. Staatsbosbeheer Drents-Friese Wold kindly allowed working in their area. Discussions with lab group members and comments on earlier drafts by Bob Mauck, Debbie Buehler, Theunis Piersma and Luisa Mendes as well as suggestions by anonymous reviewers improved the manuscript. This work was supported by a grant from BirdLife Netherlands, a Rosalind Franklin Fellowship and a Veni fellowship (863.04.023) from the Netherlands Organization for Scientific Research to BIT, by grants from the Schure-Beijerinck-Popping Fonds to AH and a Veni fellowship (863.08.026) to KDM. The study was performed under license D4743A and DEC5219B of the Institutional Animal Care and Use Committee of the University of Groningen.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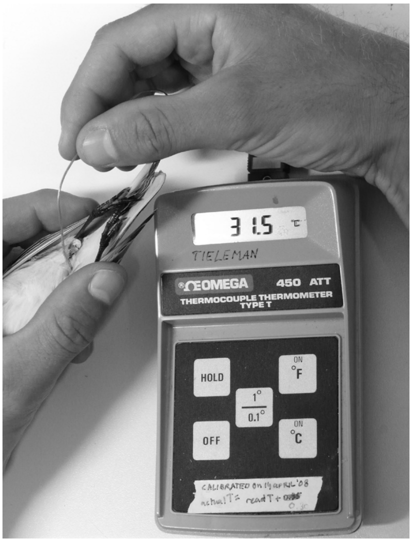
# CHAPTER 5

# Wild Skylarks seasonally modulate energy budgets but maintain energetically costly inflammatory immune responses throughout the annual cycle

Arne Hegemann, Kevin D. Matson, Maaike A. Versteegh & B. Irene Tieleman

**PLoS ONE 7(5): e 36358 (2012)**

**Abstract** A central hypothesis of ecological immunology is that immune defences are traded off against competing physiological and behavioural processes. During energetically demanding periods, birds are predicted to switch from expensive inflammatory responses to less costly immune responses. Acute phase responses (APRs) are a particularly costly form of immune defence, and hence, seasonal modulations in APRs are expected. Yet, hypotheses about APR modulation remain untested in free-living organisms throughout a complete annual cycle. We studied seasonal modulations in the APRs and in the energy budgets of Skylarks (*Alauda arvensis*), a partial migrant bird from temperate zones that experiences substantial ecological changes during its annual cycle. We characterized throughout the annual cycle changes in their energy budgets by measuring basal metabolic rate (BMR) and body mass. We quantified APRs by measuring the effects of a lipopolysaccharide injection on metabolic rate, body mass, body temperature, and concentrations of glucose and ketone. Body mass and BMR were lowest during breeding, highest during winter and intermediate during spring migration, moult and autumn migration. Despite this variation in energy budgets, the magnitude of the APR, as measured by all variables, was similar in all annual-cycle stages. Thus, while we find evidence that some annual-cycle stages are relatively more energetically constrained, we find no support for the hypothesis that during these annual-cycle stages birds compromise an immune defence that is itself energetically costly. We suggest that the ability to mount an APR may be so essential to survival in every annual-cycle stage that Skylarks do not trade off this costly form of defence with other annual-cycle demands.



## Introduction

Seasonal variation in immune function has been found in a variety of vertebrate taxa and has been attributed to seasonally changing annual-cycle demands, resource availability and exposure to pathogens and parasites (Nelson & Demas 1996, Martin *et al.* 2008). Because production, maintenance and use of the immune system require energy (Bonneaud *et al.* 2003, Mendes *et al.* 2006a), a central hypothesis states that immune defences, particularly those components that have high costs, are traded off against other competing physiological and behavioural processes (Sheldon & Verhulst 1996, Lochmiller & Deerenberg 2000, Norris & Evans 2000). Such trade-offs putatively explain findings of reduced immune responses in relation to reproduction (e.g. Ilmonen *et al.* 2000, Bonneaud *et al.* 2003, Ardia 2005), during migration (e.g. Owen & Moore 2006, Owen & Moore 2008) or during winter (e.g. Svensson *et al.* 1998). Furthermore the outcome of such trade-offs is affected by the evolutionary pressures exerted by pathogens and parasites (Piersma 1997), which may also change throughout the year. In addition to trade-offs between the immune system and other physiological systems, trade-offs within the immune system may also occur. For example, organisms may shift from more to less costly defences during times of high energy demand or low resource availability (Hasselquist 2007, Martin *et al.* 2008). More specifically, Lee (2006) hypothesizes a switch from costly inflammatory responses to highly specific but less costly antibody responses.

The acute phase response (APR), an innate response that is initiated minutes after detecting an inflammatory agent, is an early defence against threats that have already breached physical barriers like the skin. APRs involve an array of physiological and behavioural changes, including fever and anorexia (reviewed by Owen-Ashley & Wingfield 2007), and these responses incur costs from metabolic upregulation and tissue degradation (Lochmiller & Deerenberg 2000, Klasing 2004, Sorci & Faivre 2009). In birds, potential proximate mechanisms underlying seasonal changes in APRs are hypothesized to include hormonal suppression and seasonal differences in energy stores (Owen-Ashley & Wingfield 2006, Owen-Ashley & Wingfield 2007). Thus far, studies of seasonal modulations in APRs consider only two annual-cycle stages (Owen-Ashley & Wingfield 2006) and have been done on either captive birds or wild birds that have been in captivity for at least several weeks (e.g. Owen-Ashley *et al.* 2006, Burness *et al.* 2010). While offering some insight, the conclusions of these studies are limited by the lack of a complete year-round perspective on immune function and by lack of simultaneous measurements of the energy budget.

To identify which annual-cycle stages are energetically demanding, ecologists quantify indices of energy metabolism (McKechnie 2008, Swanson 2010). Basal metabolic rate (BMR) is the most standardized measure (King 1974), and BMR relates to many other ecologically-important variables including activity level (Deerenberg *et al.* 1998, Nudds & Bryant 2001), food availability and diet (Mcnab 1988, Mueller & Diamond 2001), organ sizes and body composition (Daan *et al.* 1990, Piersma *et al.* 1996, Tieleman *et al.* 2003), and daily energy expenditure (Daan *et al.* 1990, Nilsson 2002, Tieleman *et al.* 2008). These relationships make BMR an interesting trait for ecological studies of seasonal variation. Metabolism represents only part of the energetic balance, and energetic challenges can also result from limitations on resource availability. Thus, data on body mass and biochemical markers can provide critical information about whether birds obtain nutrients from available food or from body reserves. Two such biochemical markers are glucose and ketone. Glucose is one of the main sources for energy production in birds (Braun & Sweazea 2008) and the primary carbohydrate absorbed by the avian intestine (Klasing 1998). Ketone concentrations reflect lipid catabolism during fasting (Robinson & Williamson 1980, Cherel *et al.* 1988, Jenni-Eiermann *et al.* 2002).

Most temperate zone birds experience substantial changes in their ecology over the course of a year. Energy and time budgets change in association with seasonal activities like migration and reproduction and with variable environmental conditions like temperature and precipitation. The Skylark (*Alauda arvensis*), is a typical temperate zone passerine and a partially migratory species, with migration dependent on breeding location (Glutz von Blotzheim & Bauer 1985, Donald 2004). During an annual cycle, Skylarks go through five distinct annual-cycle stages: spring migration, breeding, moult, autumn migration, winter. With transitioning stages, Skylarks face changes in environmental conditions, social structure (pairs during breeding, flocks outside the breeding season), and diet (predominantly insectivorous during summer, predominantly granivorous during winter) (Hegemann *et al.* 2012b).

To understand how seasonal changes in energy budgets relate to seasonal changes in immune function, simultaneous measurements of both are needed throughout the year in the same species. So far, only components of this design have been investigated. For example, studies of the energy budget of Skylarks are restricted to the breeding season. These studies show that the field metabolic rate of breeding Skylarks is 11% below allometric predictions and that the ratio between field metabolic rate and BMR is 1.7 (Tieleman *et al.* 2002, Tieleman *et al.* 2004), which is substantially below the optimal working capacity of four-times-BMR for birds tending broods proposed by Drent and Daan (1980). Measurements during other annual-cycle stages are required to determine if metabolism levels during breeding are comparatively low. Studies of baseline immune function in Skylarks and other birds show differences in indices among different annual-cycle stages, but these studies have been measured on un-challenged birds and in isolation of energetic measurements (Buehler *et al.* 2008, Pap *et al.* 2010a, Pap *et al.* 2010b, Hegemann *et al.* 2012b). Nevertheless, these studies suggest that patterns of immune function are linked to changing environmental conditions (Buehler *et al.* 2008, Hegemann *et al.* 2012b). To our best knowledge no studies linking modulations in the energy budgets over a complete annual cycle to modulations in energetically-costly immune responses have been carried out in any free-living vertebrate.

We studied seasonal modulations of energy budgets and APRs in wild Skylarks across the species' entire annual cycle. To characterize changes in the energy budget, we measured body mass and basal metabolic rate. To quantify the energetic and nutritional costs of activating the APR, we measured the effects of a lipopolysaccharide injection on metabolic rate, body mass, body temperature, and concentrations of glucose and ketone.

Based on the hypothesis that birds should compromise expensive immune responses during energetically-demanding times of the annual cycle, we expected seasonal modulations in the magnitude of the APR to occur in relation to changes in the energy budget. Since males and females are hypothesized to differently allocate resources to their immune system (Hasselquist 2007, Martin *et al.* 2008), we also investigated if the sexes mount APRs of different magnitudes.

## Material and methods

### *Ethics Statement*

The study described here was specifically approved by the Institutional Animal Care and Use Committee of the University of Groningen under license DEC5219B. The populations study in the Aekingerzand was done under licence D4743A and DEC5219B of the same committee and all their guidelines and conditions were strictly followed.

### *Birds and field capture*

We caught Skylarks during five annual-cycle stages in the northern Netherlands in 2008 and 2009 focusing on our study population at the Aekingerzand (N 52°55'; E 6°18'). Skylarks in our study population are partial migrants. Some birds migrate; others winter locally and are accompanied by birds that breed further north and east (Hegemann *et al.* 2010). During breeding (15 Jun – 7 Jul 2008), we caught birds (9 m, 6 f ) that were feeding nestlings with mist-nests or traps on the nest from our study population at the Aekingerzand. Birds were caught in the afternoon and released early next morning to minimize the time adults were absent from their nest. From three nests we took both parents but at different times to help ensure continued food provisioning for the nestlings. During molt (3 Aug – 22 Sep 2008), we caught birds (12 m, 7 f) from the same population by flushing birds into nets during the night. During winter (9 Dec 2008 – 15 Jan 2009) birds from the study population use agricultural fields that surround the core study area (Hegemann *et al.* 2010). We caught birds (14 m, 3 f) on these fields by flushing them into nets at night. During migration in spring (14 Mar – 24 Mar 2008 and 25 Feb – 1 Mar 2009; 12 m, 12 f) and autumn (9 Oct – 2 Nov 2008; 17 m, 9 f), we caught actively migrating birds with clap-nets during periods of visible diurnal Skylark migration at a location about 15 km southeast of the Aekingerzand. We are confident about the migratory status: when tape-lured, migrants interrupt their migratory flight, but local birds that are not currently migrating do not respond.

Upon capture, we punctured the brachial vein with a sterile needle and collected blood samples into heparinised capillary tubes before taking structural measurements. Birds were sexed biometrically and some doubtful cases were sexed molecularly (Hegemann *et al.* 2012a). All individuals were fully grown. Because Skylarks undergo a complete post-nuptial moult in August-September, age classes could not be distinguished. Since Skylarks breed in their first year (Hegemann unpublished data) and both young and adult birds are known to migrate (van Dobben & Mörzer Bruyna 1939, Hegemann *et al.* 2010), we have no indications that an age bias between stages exists and could influence the interpretation of the results.

### *Experimental protocol and respirometry setup*

After capture, we brought birds into captivity and, assigned each one to either the experimental or control group. We balanced these groups for sex. Because birds were caught at different times of the day and because the respirometry setup could measure a maximum of three individuals per night, time in captivity varied. Of the 101 Skylarks, 76 spent <24 h in captivity before the experiment started (median: 17:40 h; minimum: <2 h ($n = 2$); maximum: 69 h ($n = 2$)). We housed up to three birds per cage (30×40×60 cm) during all annual-cycle stages except the breeding season, when Skylarks were territorial and housed individually. Prior to initiation of the experimental protocol, birds had access to water, mealworms and seeds *ad libitum*.

At the start of the experimental protocol at 16.30 food and water were withdrawn and birds were isolated in a dark box for 1 h. At 17:30, we collected baseline pre-metabolic body temperatures and masses of all birds, and we injected the experimental birds with LPS. We inserted a thermocouple about 1 cm into the cloacae and recorded the temperature (OMEGA ATT thermometer) to the nearest 0.1°C once the temperature was stable for 10 sec., and we measured body mass to the nearest 0.1 g. Experimental birds were injected intra-abdominally with 2.5 mg LPS in 10 mL PBS per kg body mass.

The LPS dose was based on results of a pilot study in Skylarks and rooted in published data. In Japanese Quail (*Coturnix coturnix japonica*), a dose of 0.5 mg LPS per kg body mass does not lead to a significant response, and doses higher than 2.5 mg/kg do not lead to additional increases in the response magnitude (Koutsos & Klasing 2001). Thus, we first tested the effects of 1.0 mg/kg ($n = 3$) and 2.5 mg/kg ($n = 3$) in Skylarks. These birds responded more strongly to the higher dose in terms of average mass-corrected metabolic rate (2.75 vs. 2.52 ml $O_2$ / hr / g) and body temperature 13 hours post-injection (40.8 vs. 40.0°C). No birds died following injection with either dose of LPS. Combined, these results suggested to us that the higher dose (2.5 mg/kg) was appropriate for our current study and that it led to a greater response than would be possible from the vehicle (PBS) alone.

LPS injections mimic bacterial infection without resulting in infection. Control birds remained un-injected because puncturing the skin and other tissues and injecting a vehicle (e.g. PBS only) can also result in inflammation (K. Klasing and B. Helm, personal communications). Consequently, the experimental responses must be viewed as a result of both the LPS and the injection procedure. This combination of effectors does not pose interpretational problems for our study since our central interest is inflammation versus the absence of inflammation and not the effects of LPS per se.

Immediately after measuring body temperature and body mass from all birds and injecting the experimental birds, birds were sealed individually in airtight metabolic chambers. The metabolic chambers sat inside a larger environmental chamber that was set to 30°C, which is within the thermoneutral zone of Skylarks (Tieleman *et al.* 2002). The first 1.5 hours in the chamber served as acclimation and equilibration period. We recorded $O_2$-consumption and $CO_2$-production from 19:00 to 6:30 the following morning using standard flow-through respirometry (Gessaman 1987). Briefly, compressed ambient air was dried and pumped through calibrated mass-flow controllers (model 5850S; Brooks Instrument, PA, USA) at 40 L $h^{-1}$ and through the metabolic chambers. After leaving the chambers, the air passed through silica gel filters to remove the moisture added by the birds (e.g. through respiration). Then, the percentages of $O_2$ in the air were measured with gas analyzers ($O_2$: Servomex Xentra 4100, Crowborough, UK). A reference stream of dried air that bypassed the metabolic chambers was analysed at least once every two hours.

We calculated $O_2$ consumption (mL $h^{-1}$ $g^{-1}$) using equations adapted from Hill (1972). Nightly metabolic rate was calculated as the average $O_2$ consumption per bird between 19:00 and 6:30. BMR was calculated as the lowest average $O_2$ consumption during any 12 min interval during the night.

At 6:30 next morning (14 h after the start of the experimental protocol and after 11.5 h of metabolic measurements) we took birds out of the chambers, immediately measured body temperature, collected a blood sample and re-measured body mass. All data and samples were collected < 10 min after opening the metabolic chamber. We used ~15 µL of fresh blood to measure glucose and ketone concentrations with a handheld diagnostic device (CardioChek PA Analyzer 1708 with glucose test strips 1713 and ketone test strips 1718; Polymer Technology Systems, IN, USA). Ketone concentrations were not measured during autumn migration. Upon completion of the entire protocol, birds were released at the site of capture.

### *Effects of duration in captivity*

We conducted a pilot study to compare effects of short term and longer term captivity. Three Skylarks were held in captivity for 8 hours (short); four other Skylarks were acclimated to captivity over 55 days (long). Following these captivity periods, we subjected the birds to the protocol of this study. We found no difference between these two groups in their responses to a challenge with 1.0 mg LPS / kg body mass (e.g., mass loss: short = 9.0 ± 1.1% (SD), long = 9.9 ± 2.2%, $t(5) = 0.6$, $p = 0.6$; $O_2$ consumption: short = 3.3 ± 0.5 mL/hr/g, long = 3.5 ± 0.8, $t(5) = 0.4$, $p = 0.7$). While the stress of short term captivity did not appear to affect these metabolic parameters of APRs, captive birds generally differ from their wild counterparts in many other ways (e.g., nutrition, activity). As a result, we favoured studying birds that were in captivity for as short a period as possible. We also explored if stress from short-term captivity affected birds differently in different annual-cycle stages. We used heterophil/lymphocyte ratios and concentrations of heat shock protein 70 as indicators for stress (Gross & Siegel 1983, Davis 2005, Merino *et al.* 2006), and we found no differences in the effects of captivity among annual-cycle stages.

### *Statistics*

We compared experimental and control groups for each response variable using linear models analysed with the program R version 2.9.2 (R Development Core Team 2009). We included sex, annual-cycle stage, treatment and all possible interactions as explanatory variables. We always started with the full model and simplified it using backwards elimination based on log likelihood ratio test with $P < 0.05$ as selection criterion ("drop1" in R) until reaching the minimal adequate model. Assumptions of all models were checked on the residuals of the final model. Graphs were made using the package "gplots" (Warnes 2009). Sample sizes differ slightly among response variables due to technical problems (e.g. thermometer failure).

Experimental and control groups did not differ significantly in body mass ($\chi^2_{1,99}=1.95$, $P = 0.17$) or glucose concentration ($\chi^2_{1,87} = 0.03$, $P = 0.86$) when measured in the field just after catching and thus well in advance of the LPS injection. We also found no significant pre-metabolic differences in body mass ($\chi^2_{1,99} = 1.42$, $P = 0.24$) and body temperature ($\chi^2_{1,99} = 0.65$, $P = 0.42$) between the two treatment groups immediately prior to the LPS injection.

## Results

### *Seasonal modulation in body mass and basal metabolic rate*

Body mass and BMR of Skylarks varied among annual-cycle stages ($\chi^2_{4,46} = 34.66$, $P < 0.001$; $\chi^2_{4,46} = 31.46$, $P < 0.001$; Figure 5.1), suggesting a seasonal modulation of the energy budget. Both body mass and BMR were lowest during the breeding season and highest during winter (Figure 5.1). During spring migration, moult and autumn migration values of body mass and BMR were comparable and intermediate.

### *Seasonal modulation of the acute phase response*

The experimentally-induced APR led to increases in metabolic rate, body temperature, mass loss and ketone concentrations, but we found no evidence for different effects of

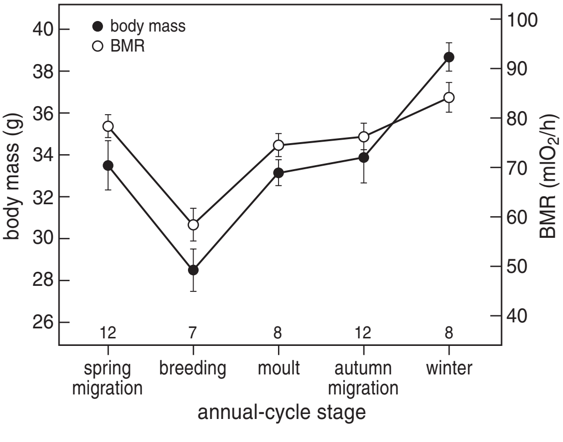


**Figure 5.1:** Body mass and basal metabolic rate (BMR) of control Skylarks throughout the annual cycle. Body mass measurements were taken in the mornings upon completion of the metabolic measurements. Numbers represent samples sizes.

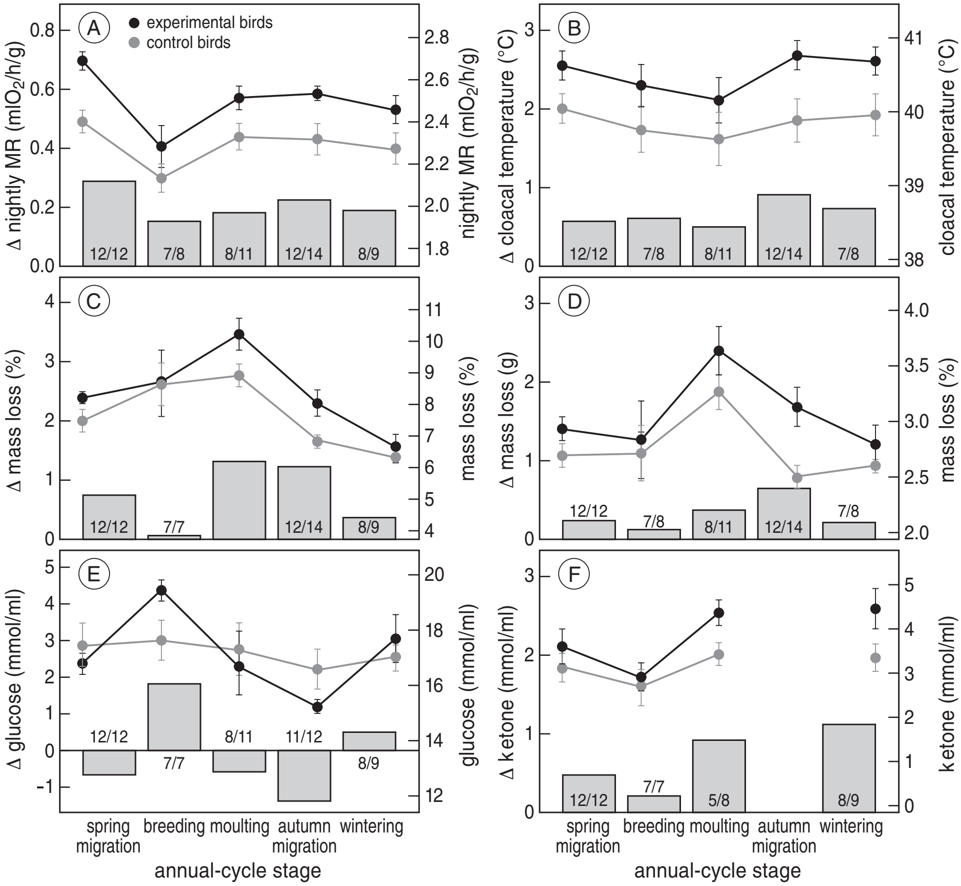


**Figure 5.2:** Effect of LPS injection on A) mass-specific nightly metabolic rate, B) body temperature, C) relative mass loss during the night, D) absolute mass loss during the night, E) glucose concentration and F) ketone concentration of Skylarks after 13 hours. Experimental birds were injected with LPS; control birds were un-injected. Black symbols represent experimental birds (LPS-injected), grey symbols control birds (un-injected). Bars represent the difference between these two groups. Means and standard errors are shown; numbers in bars represent sample sizes per group (control/experimental). The graphs show raw data without correcting for sex effects. There was never a significant treatment × annual-cycle stage interaction (all $P > 0.084$). See Table 5.1 for statistics.

LPS in different annual-cycle stages (Table 5.1, Figure 5.2A-F; body masses relevant for interpretation of metabolic measures are provided in Table 5.2). For every variable, we removed the non-significant interaction term (treatment × annual cycle-stage) before testing the main effects of LPS injection and annual-cycle stage. Both were significant (Table 5.1).

LPS injection caused a significant increase in mass-specific nightly metabolic rate, mass-specific BMR, body mass loss, body temperature and ketone concentrations (Table

**Table 5.1:** Statistics and coefficients of the linear models of measures of metabolism, body mass and temperature in Skylarks. Experimental birds were injected with LPS; control birds were un-injected. Results are from linear models after removing all non significant terms ($P > 0.05$). All tests are based on chi-square statistics. $P$-values $< 0.05$ are bold.

| Trait | Annual-cycle stage | | | Sex | | | | Treatment | | | | Treatment × Annual-cycle stage | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *df* | Chi$^2$/$F$ | $P$ | *df* | Chi$^2$/$F$ | $P$ | $\beta$ † | *df* | Chi$^2$/$F$ | $P$ | $\beta$ ‡ | *df* | Chi$^2$/$F$ | $P$ |
| Nightly metabolic rate° | 100,4 | 27.56 | <**0.001** | 100,1 | 13.36 | <**0.001** | 0.144 | 100,1 | 28.04 | <**0.001** | 0.205 | 100,1 | 2.54 | 0.639 |
| Basal metabolic rate° | 100,4 | 37.41 | <**0.001** | 100,1 | 6.17 | **0.013** | 0.087 | 100,1 | 25.66 | <**0.001** | 0.176 | 100,1 | 3.35 | 0.502 |
| Body mass loss, grams* | 100,4 | 25.77 | <**0.001** | 100,1 | 15.16 | <**0.001** | -0.441 | 100,1 | 12.16 | <**0.001** | 0.371 | 100,1 | 3.66 | 0.454 |
| Body mass loss, %* | 100,4 | 45.48 | <**0.001** | 100,1 | 0.02 | 0.884 | | 100,1 | 8.18 | **0.004** | 0.796 | 100,1 | 3.32 | 0.506 |
| Body temperature | 98,4 | 5.72 | 0.221 | 98,1 | 1.80 | 0.180 | | 98,1 | 15.49 | <**0.001** | 0.668 | 98,1 | 1.23 | 0.873 |
| [Glucose] | 96,4 | 12.10 | **0.017** | 96,1 | 0.47 | 0.495 | | 96,1 | 0.31 | 0.575 | | 96,1 | 5.69 | 0.223 |
| [Ketone] | 67,3 | 14.39 | **0.002** | 67,3 | 7.48 | **0.006** | 0.717 | 67,3 | 5.69 | **0.017** | 0.590 | 67,3 | 2.26 | 0.521 |

† Reference category is 'male';
‡ Reference category is 'control';
° mass-specific;
* Calculated over the 13 h experimental period

**Table 5.2:** Body mass (in g ± s.e.) of Skylarks per annual-cycle stage after 13 h of nightly metabolic measurements. Experimental birds were injected with LPS; control birds were un-injected.

| | Control | Experimental |
|---|---|---|
| Spring migration | 33.5 ± 1.17 | 32.7 ± 0.80 |
| Breeding | 28.5 ± 1.00 | 29.9 ± 1.13 |
| Moult | 33.1 ± 0.62 | 31.7 ± 0.87 |
| Autumn migration | 33.9 ± 1.16 | 35.5 ± 0.97 |
| Winter | 38.6 ± 0.67 | 39.7 ± 1.26 |

5.1). On average, the LPS injection increased mass-specific nightly metabolic rate by 9.06% (Figure 5.2A), body temperature by 0.7°C (Figure 5.2B), increased mass loss by 0.6 g or 13.6% (Figure 5.2C, D). Glucose concentrations did not significantly change in response to LPS injection (Table 5.1, Figure 5.2E).

Annual-cycle stages differed in mass-specific nightly metabolic rate, body mass loss, glucose and ketone concentrations (Table 5.1). Moreover, the shape of the seasonal patterns differed among these variables (Figure 5.2). Mass-specific nightly metabolic rate (Figure 5.2A) followed the patterns of BMR (Figure 5.1) with a dip during breeding. Nightly mass loss in percentage or absolute terms was highest during moult and lowest during winter (Figure 5.2C, D). Glucose concentration was highest during breeding and lowest during autumn migration (Figure 5.2E); ketone concentration was lowest during breeding and high during moult and winter (Figure 5.2F).

### *Sex differences*

Males and females differed significantly in mass-specific nightly metabolic rate, nightly body mass loss in grams and ketone concentrations (Table 5.1). We found no differences between the sexes among annual-cycle stages (interaction sex × annual-cycle stage always $F < 5.56$ and $P > 0.23$) or in their response to the LPS injection (sex × treatment always $F < 1.32$ and $P > 0.25$). Compared with males, females had a 7.2% higher mass-specific nightly metabolic rate and a 18.9% higher ketone concentration. Males lost more grams of body mass during the night than females (males 3.06 g, female 2.68 g), but this effect was proportional to the difference in body mass between the sexes, and disappeared when relative mass loss was considered (males 7.9%, female 8.1%).

## Discussion

We found that the acute phase responses of Skylarks was consistent among five annual-cycle stages (spring migration, breeding, moult, autumn migration, winter). This constancy of inflammatory responses contrasted sharply with the observed seasonal variability in the Skylark energy budget, which was reflected by changes among annual-cycle stages in terms of energy metabolism, body mass and concentrations of glucose and ketone. Thus, while we find evidence that some annual-cycle stages are relatively more energetically constrained, we find no support for the hypothesis that during these annual-cycle stages birds compromise an immune defence that is itself energetically costly.

### *Testing the trade-off hypothesis*

Our results clearly demonstrate that the inflammation caused by an LPS injection was energetically costly for Skylarks, but we have no evidence for seasonal modulation of the inflammatory response in this species. A lack of seasonal modulation contradicts current hypotheses relating compromised inflammatory responses with other life-history demands (Owen-Ashley & Wingfield 2007, Buehler & Piersma 2008, Martin *et al.* 2008). Consistent APRs throughout the annual cycle might signal that this innate defence is simply too important to be compromised. Maintenance of this response, however, does not rule out possible trade-offs with other physiological and behavioural processes. For example, some birds change their territorial behaviour in response to an inflammatory challenge (Owen-Ashley & Wingfield 2006, Owen-Ashley *et al.* 2006). If inflammatory responses are indeed linked to broader physiological functioning via a resource budget and the responses are seasonally consistent as our data suggest, then birds undergoing an APR might be forced to reduce the resources they spend on other traits, for example by delaying migration (van Gils *et al.* 2007), reducing parental effort (Bonneaud *et al.* 2003) or postponing moult (Martin 2005). These types of adjustments support the life-history trade-off hypothesis (Owen-Ashley & Wingfield 2007) in the sense that, instead of the immune system being compromised, other annual-cycle events are suppressed, which in turn may negatively impact individual fitness.

Skylarks in our study population are partial migrants, with some birds migrating and others wintering locally (Hegemann *et al.* 2010). We chose to focus our study on the year-round inflammatory responses of Skylarks in the breeding location, reflecting that part of our study population that winters locally. Because birds from northern and eastern populations join the local Skylark population outside breeding and moult, we potentially caught a mixture of birds from different populations during winter and migration. We explored if this led to larger coefficients of variation (CV) for the various response variables, but found no difference in the CV during winter and migration in comparison with breeding and moult. This is in agreement with our expectation that inflammatory responses (e.g. APRs) relate to the local and current conditions experienced by birds, because they are mounted over very short periods of time (minutes to hours). We therefore conclude that the APR for Skylarks experiencing the annual environmental variation in the Netherlands is similar in magnitude in all seasons, and crucial enough to be not traded off against other annual-cycle functions.

### *Consistent induced responses but variable baseline levels*

The lack of seasonal modulation in the magnitude of an APR contrasts with the literature and with the results of a related study in which we measured constitutive immunological parameters in unchallenged Skylarks. In this study, lysis and agglutination titres, haptoglobin concentrations, and proportions of eosinophils, basophils and monocytes differed among annual-cycle stages when measured directly upon capture in the field (Hegemann *et al.* 2012b). Studies on other species also find reductions in particular immunological components during specific annual-cycle stages (Svensson *et al.* 1998, Ilmonen *et al.* 2000, Bonneaud *et al.* 2003, Deerenberg *et al.* 2003, Ardia 2005, Owen & Moore 2006, Hasselquist *et al.* 2007, Owen & Moore 2008). Taken together, these studies point out that seasonal modulations of immune systems differ among species, environments, life-histories, and, importantly, immune parameters. Our Skylark results develop this further by showing that within a species baseline values of some immune indices were seasonally variable, but the magnitude of response to a standard inflammatory challenge was seasonally consistent. Overall, these studies identify some interpretational limitations of different approaches as well as the importance of distinguishing between baseline values and induced responses when studying ecological immunology. Furthermore, the contrasting results, in effect, make the case for measuring both baselines and response when linking ecology, evolution and immunology.

### *Physiological responses to LPS injection*

Significant changes in six of the seven measured parameters indicated that the LPS injection successfully triggered an inflammatory response. The increase in nightly metabolic rate, BMR and body temperature fall within the expected costs of fever (Baracos *et al.* 1987). The average increase in metabolic rate (regardless of stage) was similar to the increases reported in other studies after immune challenges in single annual-cycle stages (Svensson *et al.* 1998, Ots *et al.* 2001, Mendes *et al.* 2006a, Nilsson *et al.* 2007). Thus, inflammation-associated metabolic costs may be fairly conserved among avian species and not simply consistent among annual-cycle stages within Skylarks.

LPS-injected Skylarks lost on average 13.6% more body mass over night than control birds. It has been suggested that mass losses following an LPS injection are mainly due to sickness-related anorexia (Owen-Ashley & Wingfield 2006, Owen-Ashley & Wingfield 2007) rather than metabolic costs per se. As we measured mass loss over the night while all birds were resting and none had access to food, our estimates of mass loss reflect a true metabolic cost. Likewise, experimental birds had significantly higher ketone concentrations, which reflect lipid catabolism during fasting (Cherel *et al.* 1988), and this elevation can be seen as a direct consequence of the LPS-induced metabolic changes. Body mass at the point of capture in the field did not predict mass loss over the metabolic measurement period, even though body mass showed strong seasonal variation. Thus, our data do not support the idea that energy stores are a proximate mechanism for seasonal modulations in immune defences (Owen-Ashley & Wingfield 2006, Owen-Ashley & Wingfield 2007).

Although we found sex differences in most parameters, we found no evidence that the LPS injection had different effects in males and females. We acknowledge that sample sizes per sex are limited and that a lack of sex × treatment interactions could be due to low statistical power. However, Owen-Ashley *et al.* (2006) also find little evidence for sex differences in LPS-induced sickness behaviour in White-crowned Sparrows

(*Zonotrichia leucophrys*). These results contrast with the idea that the two sexes allocate resources to the immune system differently (Hasselquist 2007, Martin *et al.* 2008) but support our hypothesis that the APR is critically important and always maintained.

### *Conclusions*

We found no evidence for seasonal modulation of acute phase responses among the five distinct annual-cycle stages of a wild temperate zone bird, even though energy budgets show strong seasonal variation. Skylarks undergoing an experimentally triggered inflammatory response exhibited increases in metabolic rate, body mass loss, body temperature and ketone concentration, and these changes demonstrate energetic costs of an APR. The consistent lack of interaction between treatment and annual-cycle stage suggests that the acute phase response is an essential immunological defence, one that is too crucial for survival to be compromised through trade-offs with other annual-cycle activities despite the response's clear costs.

## Acknowledgements

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# CHAPTER 6

# Immune response to an endotoxin challenge involves multiple immune parameters and is consistent among the annual-cycle stages of a free-living temperate zone bird

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**Submitted**

**Abstract** Trade-offs between immune function and other physiological and behavioral processes are central in ecoimmunology, but one important problem is how to distinguish a reallocation of resources away from the immune system from a reallocation or redistribution within the immune system. While variation in baseline values of individual immune parameters is well established, studies in wild animals on multiple parameters during an immune response are lacking. It also remains to be tested if and how immune responses correlate with baseline values that vary e.g. over the course of an annual cycle. We studied immunological responses to an endotoxin challenge in Skylarks (*Alauda arvensis*), a partial migrant bird breeding in temperate zones. We compared birds injected with the endotoxin LPS with un-injected controls, characterizing immunological responses with leukocyte profiles, titres of lytic enzymes and natural antibodies, and concentrations of haptoglobin and heat shock proteins. We did this in five annual-cycle stages to test if the response varied throughout the year. The endotoxin challenge affected 6 of 10 measured parameters. Lysis titres and proportions of heterophils increased; haptoglobin concentrations and proportions of lymphocytes, basophils and eosinophils decreased. The variable effects on different immune components demonstrate the complexity of an immune response. We found no evidence that the response differed between annual-cycle stages. The response was independent of baseline measures taken directly upon capture in the field, indicating that birds were facing no ceiling when mounting an immunological response. Values of five parameters collected under field conditions were significantly related to values taken under standardised lab conditions. We conclude that multiple parts of the immune system are modulated during an immunological response and that responses are not re-organised throughout the annual cycle.

## Introduction

A central premise in ecological immunology is that animals trade off investment into immune function against other competing physiological and behavioral processes (Sheldon & Verhulst 1996, Lochmiller & Deerenberg 2000, Norris & Evans 2000). However, one important problem that ecoimmunologists face is how to distinguish a reallocation of resources away from the immune system from a reallocation or redistribution within the immune system. Reductions in one or more elements of the immune system do not necessarily equate to a net reduction in immune function because other parts of the immune system might be boosted simultaneously (Adamo 2004). Simultaneous measurements of multiple immune indices can help address this problem (Adamo 2004, Matson *et al.* 2006, Boughton *et al.* 2011, Buehler *et al.* 2011, Demas *et al.* 2011). Yet, understanding trade-offs and interactions within the immune system requires an experimental challenge of the immune system and subsequent quantification of the response using multiple indices (Martin *et al.* 2006b, Martin *et al.* 2008, Boughton *et al.* 2011, Pedersen & Babayan 2011).

The immune system can be experimentally challenged by injection of an endotoxin like lipopolysaccharide (LPS) (Owen-Ashley & Wingfield 2007). LPS initiates an immune response by mimicking the first stages of a bacterial infection without actually resulting in sustained disease. This innate response begins minutes after endotoxin detection and defends against threats that breach physical barriers like the skin. Most experimental studies on induced immune responses in free-living birds so far focus on hormonal, behavioural or metabolic changes (Bonneaud *et al.* 2003, Owen-Ashley & Wingfield 2006, Owen-Ashley *et al.* 2006, Owen-Ashley & Wingfield 2007, Adelman *et al.* 2010, Hegemann *et al.* 2012c, reviewed by Hasselquist & Nilsson 2012). Studies in free-living birds that characterise multiple immunological responses and subsystems simultaneously are lacking so far.

In addition to quantifying which parts of the immune system are affected by a simulated infection, experimental immune challenges can also be used to investigate the consistency of responses through time at the population level. Immune responses may be constant among annual-cycle stages, or responses may be seasonally reorganised as a result of trade-offs with other physiological and behavioural demands. Hypotheses relate increased energy demands and decreased resource availability to compromises in costly immune functions and shifts towards less costly immune components (Nelson & Demas 1996, Nelson 2004, Hasselquist 2007, Martin *et al.* 2008). Immunological mechanisms aimed at avoiding autoimmunity (Råberg *et al.* 1998) and preventing oxidative stress (Sorci & Faivre 2009) might further influence this process. Several studies on non-induced (baseline) immune function indeed show that different indices express different seasonal patterns among and within annual-cycle stages (Nelson & Demas 1996, Buehler *et al.* 2008, Pap *et al.* 2010a, Pap *et al.* 2010b, Hegemann *et al.* 2012b). Thus, reorganisation of baseline immune function appears to depend on both environmental conditions and competing biotic processes. Data on seasonal variation in induced immune responses are scarce. However, these are the data needed to verify the hypothesis that free-living birds switch from costly inflammatory responses to highly specific but less costly antibody responses during demanding times (Lee 2006). A study on captive Red Knots (*Calidris canutus*) provides evidence for saved costs on inflammatory responses during demanding times (Buehler *et al.* 2009). In contrast, wild Skylarks do not modulate the energetic investment in the acute phase response despite the existence of energetically constraint seasons. They maintain similar response throughout the annual cycle as measured by metabolic rate, body temperature, body mass loss, ketone and glucose concentrations (Hegemann *et al.* 2012c). These results provide a good starting point to study the response of multiple immune indices during an immune challenge in different annual-cycle stages. Such a study will also allow to test if induced responses are modulated among annual-cycle stages (following patterns of baseline immune function) or if they are maintained throughout the year (reflecting patterns of energetic costs).

Variability in baseline immunological values might also represent important constraints for responses because the ability to mount an immune response might depend on baseline values. For example, baseline haptoglobin concentrations in pigeons (*Columba livia domestica*) have some capacity to predict post-challenge response concentrations (Matson *et al.* 2012). Great tits (*Parus major*) with high pre-immunisation H/L-ratios mount weaker antibody responses (Krams *et al.* 2012). However, it remains to be tested in free-living birds if particularly high (or low) baseline values of a given immune parameter limit the responsiveness of that parameter to an immunological stimulus (i.e. 'immunological ceiling'). In other words, do individuals with relatively high baseline values respond differently to an immune challenge than birds with relatively low levels? The existence of immunological ceilings can have important implications for the interpretation of values collected from field samples.

In this study, we challenged wild Skylarks (*Alauda arvensis*) with LPS and compared them with un-injected controls during five annual-cycle stages to test 1. which immune parameters are affected by an endotoxin challenge, 2. if the immunological response varies among annual-cycle stages, and 3. if baseline values present constraints to the magnitude of the immune response. To capture a broad picture of the immune response we measured different components of immune defence. Natural antibodies and complement to agglutinate and lyse foreign cells (Matson *et al.* 2005), two measures that are thought to be unaffected by previous exposure (Ochsenbein & Zinkernagel 2000). The acute phase protein haptoglobin, which limits the role of plasma iron as nutrient for pathogens and is a initiator of oxidative damage (Murata *et al.* 2004, Quaye 2008). The relative abundances of leukocytes, which reflect both innate and acquired components of immune function. Leukocytes are circulating continuously through the blood to maintain a state of readiness and are redistributed in response to immunological stimulation (Feldman *et al.* 2000). Leukocyte analyses include the heterophil/lymphocyte (H/L) ratio which is related to immunological and other stressors (reviewed by Davis *et al.* 2008). Heat shock proteins, which indicate stress (Martinez-Padilla *et al.* 2004) and have been suggested to be a potential indicator for autoimmune risk (Hasselquist & Nilsson 2012). Furthermore they play an important role in modulating innate and acquired immunity (Pockley 2003, Pockley *et al.* 2008) through their capacity to activate complement and trigger the release of inflammatory cytokines (Calderwood *et al.* 2007).

## Methods

### *Study subjects*

We caught adult Skylarks during five annual-cycle stages in the northern Netherlands in 2008 focusing on our study population at the Aekingerzand (N 52°55'; E 6°18'; (Hegemann *et al.* 2012b). Some Skylarks in our study population migrate; others winter locally and are accompanied by birds that breed further north and east (Hegemann *et al.* 2010). We caught birds during breeding in June and July (9 m, 6 f ), molt in August and September (12 m, 7 f), autumn migration in October (12 m, 12 f), winter in December and January (14 m, 3 f), and spring migration in March (17 m, 9 f). Birds were sexed biometrically, and in some doubtful cases molecularly (Hegemann *et al.* 2012a). For details on catching see Hegemann *et al.* (2012c).

### *Sampling protocol*

When catching Skylarks in the field we collected blood (~150uL) into heparinised capillary tubes from the brachial vein immediately after capture (median: 5 min; range: 2.25–30 min) to minimize any impacts of handling stress (Buehler *et al.* 2008). We then took structural measurements. We refer to measurements from these samples as "field values".

After capture, birds were brought into captivity and housed with up to three birds per cage (30×40× 60 cm). In the breeding season when Skylarks are territorial, birds were housed individually. Birds had access to *ad libitum* water and food (mealworms and seeds) until 16:30 on the experimental-protocol start day (for details, see Hegemann *et al.* 2012c).

We started the experimental protocol with isolating birds in a dark box for 1 h without food and water. At 17:30 we injected experimental birds with 2.5 mg LPS in 10 ml PBS per kg body mass in their abdominal cavities (Hegemann *et al.* 2012c). Control birds remained un-injected, because puncturing the skin and underlying tissues for injecting only a vehicle (i.e. PBS) can result in inflammation (K. Klasing and B. Helm, personal communications). Consequently, the experimental responses must be viewed as a result of both LPS and injection procedure. This combination does not pose interpretational problems for our study since our central interest is immune response and not the effects of LPS per se. After injection the experimental birds and their corresponding controls were put into dark boxes (metabolic chambers) where they spent the night at thermo neutral conditions (Hegemann *et al.* 2012c). The next morning at 6:30 (13 h after injecting experimental birds) we collected another blood sample (150 uL) within 10 min of removing birds from boxes.

From each blood sample (field and lab), we used a small drop to make blood smears for leukocyte enumeration. The remainder of each sample was centrifuged at 7000 rpm for 10 min. Plasma and red blood cells were separated and stored at -20°C. Upon completion of the protocol, birds were released at the site of capture. Experiments were performed under license DEC5219B of the Institutional Animal Care and Use Committee of the University of Groningen.

### *Immune assays*

We used a hemolysis-hemagglutination assay (rabbit red blood cells, B-0009H; Harlan, Leicestershire, United Kingdom) to quantify titres of complement-like lytic enzymes (i.e., lysis) and non-specific natural antibodies (i.e., agglutination) in plasma (Matson *et al.* 2005, Hegemann *et al.* 2012b). Scans of individual samples were randomized among all plates and scored blindly to treatment and season (by AH). We used a commercially available colorimetric assay kit (TP801; Tri-Delta Diagnostics, NJ, USA) to quantify haptoglobin concentrations (mg ml-1) in plasma samples (Hegemann *et al.* 2012b, Matson *et al.* 2012). Blood smears were examined by one person (C. Gottland), who was blind to treatment and season. The first 100 white blood cells (WBC) per slide were identified and counted as lymphocytes, heterophils, basophils, monocytes or eosinophils (Hegemann *et al.* 2012b).

### *Heat shock proteins (Hsp70)*

Cell lysates were obtained as in Tomas *et al.* (2004) and total protein concentration was determined by the Bradford method using bovine serum albumin (BSA) as the standard. Concentrations of Hsp70 were determined from the cell lysates by means of an enzyme linked immunosorbent assay (ELISA) using the protocol described by Mahmoud and Edens (2003). Briefly, 100 ul of samples (dilution 1:10), standards (0-50 ng recombinant human Hsp70) and a positive control (HeLa Cell Lysate) were coated in duplicate in 96-well immunoplates at 4°C overnight. After blocking non-specific binding sites, plates were incubated 1h with100 ul of anti-Hsp70 monoclonal antibody (H5147; Sigma) diluted 1:1000. Following washing, plates were incubated with 100 μl of 1:5000 alkaline phosphatase conjugated goat anti-mouse IgG polyclonal antibody (SAB-101; Stressgen) for 1h. Finally we added 1 mg ml$^{-1}$ pNPP in coating buffer for 30 min, and read the absorbance of individual wells at 405 nm with a microplate reader (PowerWave; BioTek). Hsp70 concentration was calculated from the standard curve. All final Hsp70 values were standardized by dividing Hsp70 concentration by total protein and normalized according to plate-specific positive controls to facilitate inter-plate comparisons.

### *Statistics*

We compared experimental and control groups for each response variable using linear models using R, version 2.9.2 (R Development Core Team 2009). We included treatment, annual-cycle stage, sex and all possible interactions as explanatory variables. White blood cell types were analysed with generalized linear models with a quasi-binomial approach and F-tests. These tests incorporated the counts of one cell type and the total remaining WBC number (e.g. basophils against the sum of heterophils, lymphocytes, monocytes and eosinophils using the ‘c-bind’ function in R). H/L ratios were tested in a linear model.

To test if the field values affected the outcome of the experiment, we calculated the individual deviation from season- and sex-specific means. We included this sex- and season-independent term and the interaction with treatment in all analyses. A ceiling in the ability to respond would be indicated by a significant interaction. A significant main effect would indicate that individuals express consistent immune parameters in the field and in the lab after having gone through a standardised experimental protocol in the preceding 14 hour period.

We always started with the full model and simplified it using backwards elimination based on log likelihood ratio test with $P < 0.05$ as selection criterion ("drop1" in R) until reaching the minimal adequate model. Model assumptions were checked using the residuals of the final model. Sample sizes, which are provided in the figures, differ among response variables due to insufficient plasma volume. Graphs were made using the package "gplots" (Warnes 2009). Experimental and control groups did not differ significantly in any of the 10 parameters (always $P > 0.25$) when measured in the field just after catching.

## Results

### *Immunological responses after endotoxin challenge*

Compared with control birds, injected Skylarks exhibited increased lysis 13 hours after an endotoxin challenge (Figure 6.1A), but experimental and control birds did not differ in terms of agglutination (Figure 6.1B). Concentrations of haptoglobin were significantly lower in endotoxin-challenged birds (Figure 6.1C). Experimental birds had significantly higher proportions of heterophils than control birds (Figure 6.1E). The proportions of lymphocytes, basophils and eosinophils were lower in experimental birds (Figure 6.1F,B,I). The H/L-ratio, the proportion of monocytes and concentrations of Hsp70 were not affected by the endotoxin challenge (Figure 6.1D,H,J). Thus, experimental birds differed significantly from control birds in 6 of the 10 physiological parameters (Table 6.1). We never found a significant difference between the sexes in their response to the endotoxin challenge (interaction treatment × sex always $\chi^2/F < 1.27$, $P > 0.26$). Independent of treatment, males and females differed significantly in 2 of the 10 parameters in the morning after the injection (Table 6.1). Females exhibited significantly higher proportions of eosinophils among their WBCs (females 10.6%, males 5.9%). Males had significantly higher haptoglobin concentrations (15.9%) and statistically-marginally higher lysis titres (16.1%).

### *Seasonal variation in immune response*

Skylarks showed significant differences among annual-cycle stages in 8 of the 10 measured parameters (Table 6.1, Figures 6.1A-J), but the immune response after the endotoxin challenge did not differ among annual-cycle stages: the interaction between annual-cycle stage and endotoxin challenge was not significant for any of the measured parameters (Table 6.1, Figures 6.1A-J).

### *Immunological ceiling and individual consistency*

The response to the endotoxin challenge was independent of field immune values. Changes in immune parameters after the endotoxin challenge were always independent

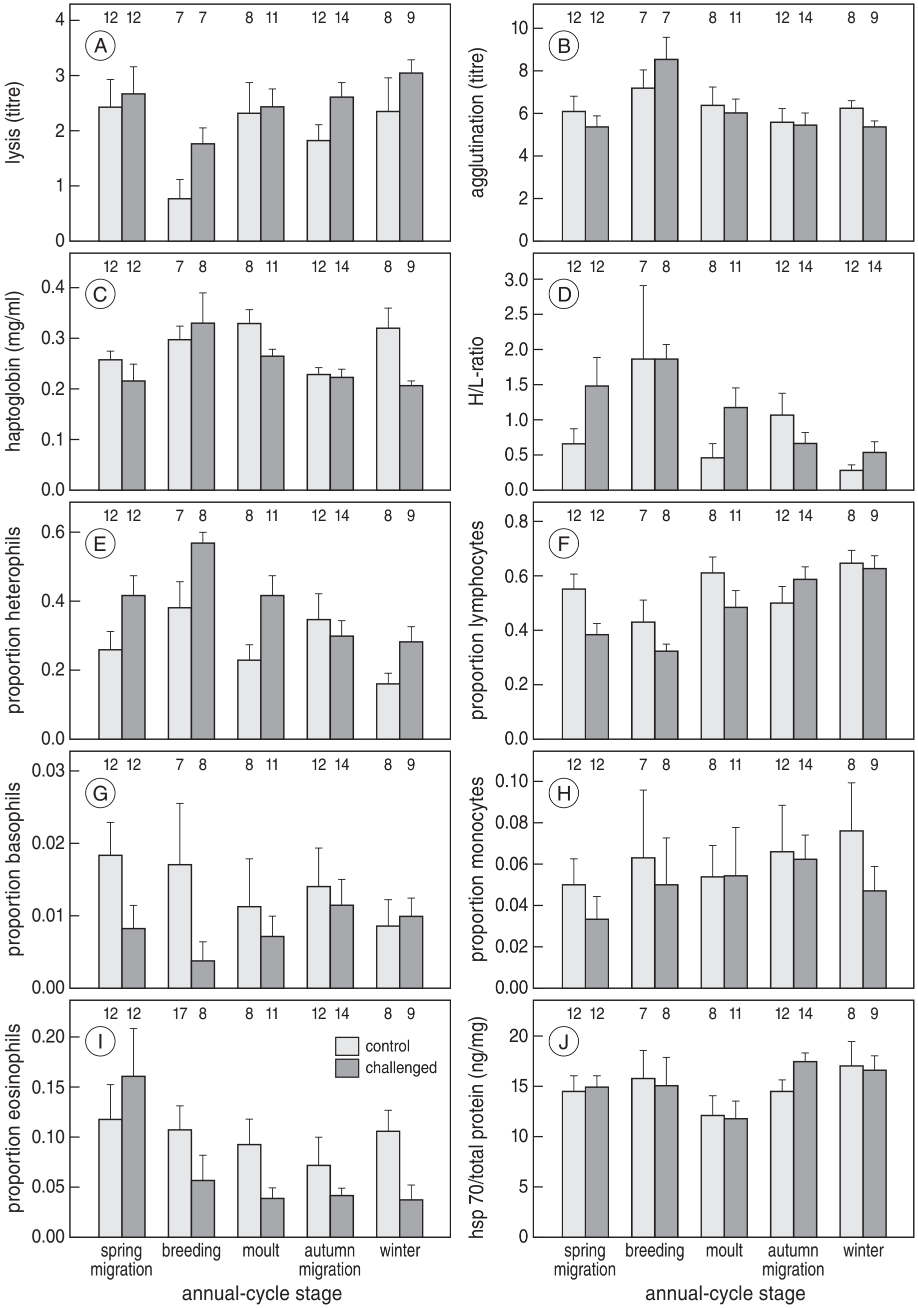

**Figure 6.1:** Effects of an endotoxin challenge on 10 immune parameters in Skylarks as measured from the blood after 13 h after the experimental start. Experimental birds were injected with LPS; control birds were un-injected. Means and standard errors are shown; numbers in bars represent sample sizes. There was never a significant treatment × season interaction (all $P > 0.08$). LPS injection had a significant effect on lysis titres, haptoglobin concentrations and the proportion of lymphocytes, basophils and eosinophils. Full statistical details can be found in Table 6.1.

**Table 6.1:** Statistics and coefficients of linear models for 10 measured parameters in Skylarks. Experimental birds were injected with LPS; control birds were un-injected. Results are from linear models after removing all non-significant terms ($P > 0.05$). $P$-values $< 0.05$ are bold.

| Trait | Treatment | | | | Season | | | Sex | | | | Field value deviation[§] | | | | Treatment × season | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *df* | Chi$^2$/*F* | *P* | $\beta$ [‡] | df | Chi$^2$/*F* | *P* | *df* | Chi$^2$/*F* | *P* | $\beta$ [†] | *df* | Chi$^2$/*F* | *P* | $\beta$ | *df* | Chi$^2$/*F* | *P* |
| Lysis titre | 98,1 | 8.35 | **0.004** | 0.679 | 98,4 | 13.68 | **0.008** | 98,1 | 3.83 | 0.050 | -0.481 | 98,1 | 9.14 | **0.003** | 0.365 | 98,1 | 4.39 | 0.356 |
| Agglutination titre | 99,1 | 0.38 | 0.535 | | 99,4 | 13.33 | **0.010** | 99,1 | 2.16 | 0.142 | | 99,1 | 0.42 | 0.516 | | 99,1 | 3.53 | 0.474 |
| [Haptoglobin] | 100,1 | 12.36 | <**0.001** | -0.172 | 100,4 | 22.99 | <**0.001** | 100,1 | 7.54 | **0.006** | -0.140 | 100,1 | 8.87 | **0.003** | 0.514 | 100,1 | 8.26 | 0.083 |
| Heterophil: Lymphocyte | 100,1 | 2.42 | 0.120 | | 100,4 | 15.12 | **0.004** | 100,1 | 0.01 | 0.924 | | 100,1 | 2.72 | 0.099 | | 100,1 | 4.79 | 0.309 |
| Heterophils | 100,1 | 12.71 | **0.005** | 0.589 | 100,4 | 4.59 | **0.002** | 100,1 | 0.01 | 0.931 | | 100,1 | 8.61 | **0.004** | 0.026 | 100,1 | 1.99 | 0.103 |
| Lymphocytes | 100,1 | 4.59 | **0.035** | -0.311 | 100,4 | 5.30 | <**0.001** | 100,1 | 1.15 | 0.286 | | 100,1 | 11.10 | **0.001** | 0.014 | 100,1 | 2.23 | 0.072 |
| Basophils | 100,1 | 4.37 | **0.039** | -0.520 | 100,4 | 0.48 | 0.747 | 100,1 | 0.93 | 0.337 | | 100,1 | 1.27 | 0.263 | | 100,1 | 1.30 | 0.278 |
| Monocytes | 100,1 | 0.68 | 0.412 | | 100,4 | 0.58 | 0.676 | 100,1 | 0.35 | 0.852 | | 100,1 | 0.07 | 0.788 | | 100,1 | 0.19 | 0.943 |
| Eosinophils | 100,1 | 7.25 | **0.008** | -0.505 | 100,4 | 2.80 | **0.031** | 100,1 | 9.19 | **0.003** | 0.576 | 100,1 | 22.57 | <**0.001** | 0.033 | 100,1 | 1.84 | 0.129 |
| [Heat Shock Protein 70] | 99,1 | 0.50 | 0.481 | | 99,4 | 9.50 | 0.049 | 99,1 | 0.16 | 0.686 | | 99,1 | 0.47 | 0.495 | | 99,1 | 1.75 | 0.781 |

[†] Reference category is 'male'.
[‡] Reference category is 'control'.
[§] A derived covariate calculated per individual as follows: (individual trait value) - (sex- and season-specific trait mean).

from the corresponding value measured in the field (interaction treatment × deviation of the field value always $\chi^2/F < 3.12$, $P > 0.08$).

After accounting for treatment, individual Skylarks showed values that were consistent between deviation of the field values and morning samples for 5 parameters. With lysis titre, haptoglobin concentration, H/L ratio, and eosinophil proportion, we found significant positive relationships between the field values (corrected for sex and season-variation) and the morning values (Table 6.1). There was no significant relationship between deviation of the field values and morning values for agglutination titre, the Hsp70 concentration, the H/L-ratio and the proportion of basophils and monocytes (Table 6.1).

## Discussion

Skylarks exhibited complex and multifaceted responses when experimentally challenged with endotoxin. Thirteen hours post-injection, some parameters increased (lysis titre, heterophil proportion), others decreased (haptoglobin concentration, proportion of lymphocytes, basophils and eosniophils) and others were unchanged (agglutination titres, H/L-ratio, Hsp70 concentration and proportion of monocytes). The complexity of the immune response to endotoxin highlights methodological complications for ecoimmunologists trying to interpret samples and data collected from birds in the field. For example, relatively high or low values of one immune parameter should be interpreted cautiously when measured in isolation from other parameters. If birds are responding to a natural immunological challenge (e.g., an infectious disease) while being sampled, then incorrect conclusions might be drawn regarding the immunological status of an individual when only a single immune parameter is measured. Despite its complexity, we found no evidence for seasonal reorganization of the immune response, which was consistent among five annual-cycle stages. Furthermore, we found no evidence for an immunological ceiling; birds showed similar responses to an immunological challenge regardless of their baseline values measured from values collected in the field. After accounting for treatment, individuals showed consistent values in samples from the field and samples from the lab with lysis titre, haptoglobin concentration, H/L ratio, and eosinophil proportions.

### *Physiological responses after immune challenge*

One particularly surprising result that highlights the complications associated with assigning a single index, relates to haptoglobin. LPS-injected Skylarks exhibited 13 hours post-challenge significantly lower concentrations of haptoglobin compared to uninjected control birds. Haptoglobin is an acute phase protein that is released from the liver during a pathogenic challenge. Normally in birds, concentrations of haptoglobin or iron-binding functional equivalents increase in association with inflammation (Thomas 2000, van de Crommenacker *et al.* 2010). Our finding suggests that in Skylarks haptoglobin might be more appropriately classified as a negative, rather than a positive, acute phase protein when measured 13 hours after the endotoxin challenge. Any functional relevance of the observed reductions in concentrations of this protein, which sequesters iron, remains to be elucidated. Notably, compared to other species that have been similarly assayed, Skylarks maintain relatively high circulating concentrations of baseline haptoglobin (Matson 2006). The decrease we observed in Skylarks following LPS injection may relate to dissimilar rates of haptoglobin production and consumption in this species. This result also suggests a greater reliance of Skylarks on constitutive (rather than induced) production of this bacteriostatic and antioxidant molecule. Testing these possibilities will require more detailed studies (e.g. with more frequent sample time points) of the LPS-induced inflammation time-course in Skylarks and other species of birds. However, results of a pilot study showed that haptoglobin concentrations in Skylarks decreased by 13 hours and remained low at 24 hours after an LPS injection (unpublished data of KDM).

Lysis titres of Skylarks increased following endotoxin challenge, but there was no difference in agglutination (natural antibody) titres between control and experimental birds. Antibody production normally requires days not hours. Thus it is not surprisingly that agglutination titres did not differ between groups 13 hours post challenge. The increase in lysis titres during infection points to another important complication of ecoimmunology: High values are not necessarily better (De Coster *et al.* 2011). Instead, values should be viewed in relation to the immunological status (Pap *et al.* 2011).

Circulating leukocytes are important for the protection against invading microorganisms. During immune responses redistributions of leukocytes populations occur (Gehad *et al.* 2002). In Skylarks, proportions of heterophils increased and proportions of lymphocytes decreased following endotoxin challenge. Since heterophils relate to innate immunity and lymphocytes relate to acquired immunity, the innate inflammatory response we elicited could primarily affect heterophil concentrations (De Boever *et al.* 2009). However, upon an immune challenge a redistribution of peripheral blood lymphocytes to secondary lymphoid organs occurs (Gehad *et al.* 2002), and this process could contribute to reduced numbers of lymphocytes in the circulating blood. Basophils are one of the first leukocyte types to enter tissue during an early inflammatory response in birds (Katiyar *et al.* 1992). The decreased proportion of basophils suggests that these cells are no longer circulating in the peripheral blood and have migrated into the tissue at the LPS injection site.

We found no difference in concentrations of intracellular Hsp70 concentrations between control and experimental Skylarks. Autoimmune reactions caused by physiological stress during an immune response might be an important cost of immunity (Råberg *et al.* 1998) and heat shock protein quantification could be an indirect way to assess the potential risks of autoimmune reactions (Hasselquist & Nilsson 2012). Our data do not provide any evidence for increased physiological stress during the immune response to an endotoxin. Heat shock proteins also have more direct immunological functions. Extracellular levels of certain types, such as Hsp60 and Hsp70, exhibit modulating effects on innate and acquired immunity (Pockley 2003, Pockley *et al.* 2008) and these proteins are involved in the activation of complement and release of cytokines

(Calderwood *et al.* 2007). In rats intra- and extracellular heat shock protein concentrations are correlated (Fleshner *et al.* 2004), but the lack of change in intracellular heat shock protein concentrations in immunologically challenged Skylarks, despite increased complement activity, might indicate different relationships in wild birds. Detailed studies of both extracellular and intracellular heat shock protein concentrations at multiple time points following an immunological challenge are required to reveal the causes and consequences of heat shock protein variation in wild birds.

### *Consistent responses throughout the annual cycle*

We found no evidence that the response of the immune system to endotoxin differed among 5 annual-cycle stages experienced by Skylarks. The reaction to the endotoxin challenge also did not differ between the sexes. These findings are in line with our previous finding that energetic components of an acute phase response (as measured by metabolic rate, body temperature, body mass loss, ketone and glucose concentrations) are not seasonally modulated in this species (Hegemann *et al.* 2012c). After statistical correction of the treatment effects, it is noteworthy that we found seasonal differences in eight of ten immunological parameters that we measured using samples collected in the lab. These data support our earlier findings that free-living Skylarks modulate their baseline immune function among annual-cycle stages (Hegemann *et al.* 2012b). Taken together, our results suggest that Skylarks do modulate baseline values of immune function, as has been described for other species (Buehler *et al.* 2008, Pap *et al.* 2010a, Pap *et al.* 2010b). However, both the energetic (Hegemann *et al.* 2012c) and the immunological (this study) consequences of an endotoxin challenge are constant throughout the year, independent of other annual-cycle demands and equal for both sexes. This strongly suggests that mounting this type of immune response is crucial to survival and cannot be compromised. Only baseline defences can be traded off with other demands. This conclusion further highlights the interpretational limitations and the importance of distinguishing between baseline values and induced responses when studying ecological immunology (Adamo 2004, Hegemann *et al.* 2012b, Hegemann *et al.* 2012c).

### *Immunological ceiling and individual consistency*

The strength of the immunological response as measured by 10 parameters was independent of the corresponding values measured upon capture in the field. Thus birds did not face an immunological ceiling, and similar immune responses can be mounted regardless of an individual's baseline values. A corresponding pattern also exists at the population level as the immune response was independent from seasonal patterns of baseline values (see above).

Several immune indices show a significant correlation between values from samples collected in the field and values from samples collected in the lab after birds had gone through a standard 14 hour protocol. After correction for treatment, lysis titre, haptoglobin concentration, H/L ratio, and eosinophil proportion showed a positive correlation between the field and morning values. These results indicate that individuals exhibit consistent values in the face of variable environmental and physiological conditions.

Birds sampled in the morning in the lab exhibited highly standardized conditions (temperature and light regime, food and water availability). However, in the field, at least some conditions varied, like time of the day and previous activity. While many factors are known to affect immune indices (e.g. diurnal patterns, (Navarro *et al.* 2003, Martinez-Padilla 2006); flight behavior (Matson *et al.* 2012)), Skylarks showed consistent values for five parameters independent of the conditions under which they were taken. This suggests that these indices are robust against short term (hours) biotic and abiotic environmental variation. Consequently they are suitable for ecoimmunologits interested in longer term environmental variation or in the immunological status of their study subjects.

## Acknowledgements

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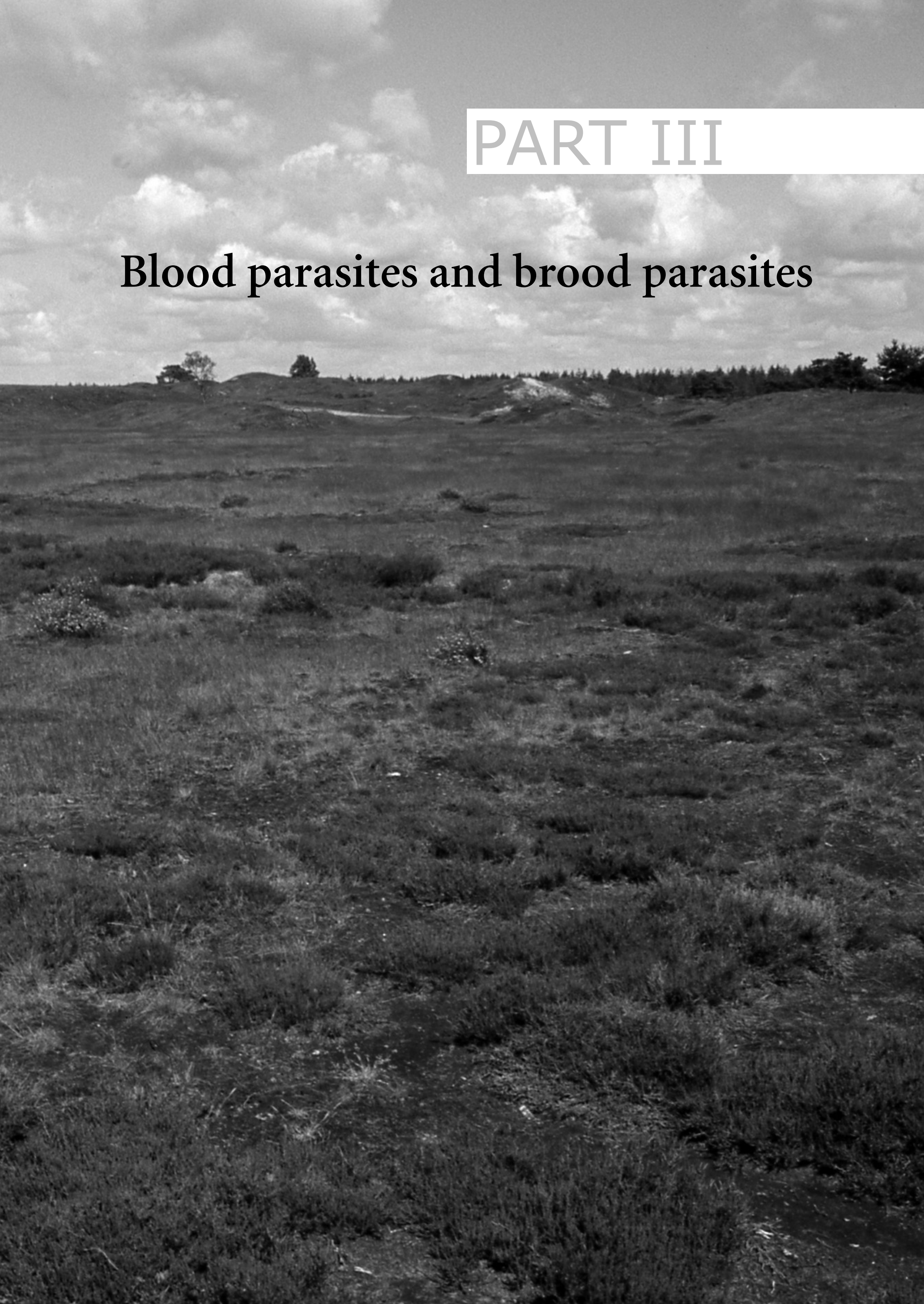
# PART III

# Blood parasites and brood parasites



# CHAPTER 7

# Haemosporidian infections in Skylarks (*Alauda arvensis*): a comparative PCR-based and microscopy study on the parasite diversity and prevalence in southern Italy and the Netherlands

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**Abstract** Changes in agricultural management have been identified as the most probable cause for the decline of Skylark (*Alauda arvensis*) populations in Europe. However, parasitic infections have not been considered as a possible factor influencing this process. Four hundred and thirty-four Skylarks from the Southern Italy and the Netherlands were screened for haemosporidian parasites (Haemosporida) using the microscopy and polymerase chain reaction (PCR)-based methods. The overall prevalence of infection was 19.5%; it was 41.8% in Italian birds and 8.3% in Dutch birds. The prevalence of *Plasmodium* spp. was 34.1% and 6.5% in Skylarks from Italy and Netherlands, respectively. Approximately 15% of all recorded haemosporidian infections were simultaneous infections both in Italian and Dutch populations. Six different mitochondrial cytochrome *b* (cyt *b*) lineages of *Plasmodium* spp. and three lineages of *Haemoproteus tartakovskyi* were found. The lineage SGS1 of *Plasmodium relictum* was the most prevalent at both study sites; it was recorded in 24.7% of birds in Italy and 5.5% in the Netherlands. The lineages SYAT05 of *Plasmodium vaughani* and GRW11 of *P. relictum* were also identified with a prevalence of <2% at both study sites. Two *Plasmodium* spp. lineages (SW2 and DELURB4) and three *H. tartakovskyi* lineages have been found only in Skylarks from Italy. Mitochondrial cyt *b* lineages SYAT05 are suggested for molecular identification of *P. vaughani*, a cosmopolitan malaria parasite of birds. This study reports the greatest overall prevalence of malaria infection in Skylarks during the last 100 years and shows that both *Plasmodium* and *Haemoproteus* spp. haemosporidian infections are expanding in Skylarks so it might contribute to a decrease of these bird populations in Europe.

## Introduction

Pigment-forming haemosporidians (Haemosporida) are a clearly phylogenetically defined group of obligate heterogeneous parasites, which inhabit birds all over the world except Antarctic (Greiner *et al.* 1975, McClure *et al.* 1978, Atkinson & van Ripper III 1991, Bishop & Bennett 1992). Over 45% of bird species of the world fauna have been currently investigated with respect to infection with haemosporidians (Valkiūnas 2005). *Haemoproteus* spp. was recorded in approximately 50% and *Plasmodium* and *Leucocytozoon* spp. in approximately 30% of the investigated bird species (Valkiūnas 2005). Although these parasites are wide-spread geographically, their prevalence varies markedly between different regions, host species and populations (Greiner *et al.* 1975, Valkiūnas 2005).

The Skylark (*Alauda arvensis*) is largely distributed in the temperate zone of Europe, Asia and Northern Africa, with 13 subspecies described (Cramp 1988, Donald 2004). In Western Europe, the Skylark is undergoing a rapid population decline in recent years, as are many other farmland birds (BirdLife International 2004, Donald *et al.* 2006). The decline of farmland birds is frequently associated with changed conditions during the breeding season and deterioration of the breeding habitat (Donald 2004, Newton 2004). Although the impact of parasites on bird populations is often overlooked in wildlife ornithology, parasitism certainly is an important factor in conservation biology and should therefore be considered in biodiversity preservation studies (van Riper III 1991, Valkiūnas 2005, Parker *et al.* 2006). The establishment of parasites in new hosts and geographic areas is often associated with changes in virulence and might lead to devastating outbreaks among resident bird populations, which is particularly well documented in relatively simple island ecosystems in Hawaii islands (Atkinson *et al.* 2001) and recently recorded in Galápagos (Levin *et al.* 2009). A possible role of blood parasites as a factor influencing the decline of Skylark populations has not been considered because of limited knowledge about haemosporidian infections in this bird species (Bennett *et al.* 1982, Valkiūnas 2005). The aim of this study was to describe distribution, diversity, prevalence and intensity of haemosporidian parasites in Skylarks from two sites in Europe.

## Materials and methods

### *Study sites and collection of blood samples*

One hundred and forty six Skylarks were caught in the Volturno Plain (41°02'N 13°55'E) located 40 km north of Naples, Italy between 1 and 30 of October in 2006 and 2007. Two hundred and eighty eight Skylarks were sampled in the northern part of the Netherlands (N 52°55 E 006°18) between May 2006 and December 2007: 12 of them were caught during the period of establishment of breeding territory, 145 adult birds - during the breeding season, 9 - during moult, 45 - during autumn migration, and 2 - during winter. Additionally, 71 nestlings have been sampled in nests; they were 5–7 day old. Skylarks were caught using mist nets or traps. The birds were banded to avoid resampling. Blood samples were taken by puncturing the brachial vein. Blood films were air-dried, fixed in absolute methanol in the field and stained with Giemsa solution in the laboratory as described by Valkiūnas *et al.* (2008).

A complementary blood sample (20–50 μl) was collected using heparinized microcapillaries and stored in non-lysis SET- buffer (Waldenstrom *et al.* 2004) or in 96% ethanol (only Dutch samples). In the field, the samples were stored at ambient temperature (Italy) or on ice (the Netherlands) and later stored at –20°C in the laboratory. The blood samples were analysed by molecular methods between 1 – 24 months after their collection. In total 434 samples were collected at both study sites (Table 7.1).

### *Examination of blood films and parasite morphology*

An Olympus BX51 light microscope equipped with an Olympus DP12 digital camera and imaging software DP-SOFT was used to examine blood slides, prepare illustrations and to take measurements. Blood films were examined for 10–15 min at low magnification (×400), and then at least 100 fields were studied at high magnification (×1,000), as described by Valkiūnas *et al.* (2008). We used the morphometric features (Table 7.2) and identified parasites according to Valkiūnas (2005). Intensity of infection was estimated

**Table 7.1:** Prevalence of haemosporidian infections in Skylark (*Alauda arvensis*) at two study sites in Europe, 2006–2007.

| Parasite species and mitochondrial cytochrome *b* lineage | Prevalence | |
|---|---|---|
| | Italy (*n*=146) | The Netherlands (*n*=288) |
| *Haemoproteus tartakovskyi* | | |
| hALARV1 | 1 (0.7)* | 0 |
| hALARV2 | 1 (0.7) | 0 |
| hALARV3 | 2 (1.4) | 0 |
| Total | 4 (2.7) | 0 |
| *Plasmodium* (*Haemamoeba*) *relictum* | | |
| pSGS1 | 36 (24.7) | 16 (5.5) |
| pDURB4 | 1 (0.68) | 0 |
| pGRW11 | 4 (2.7) | 0 |
| pMOTALB1 | 0 | 1 (0.3) |
| Total | 41 (28.0) | 17 (5.9) |
| *Plasmodium* (*Novyella*) *vaughani* | | |
| pSYAT5 | 3 (2.0) | 2 (0.6) |
| *Plasmodium* (*Novyella*) sp. | | |
| pSW2 | 6 (4.1) | 0 |
| Unidentified simultaneous infections | 8 (5.5) | 5 (1.7) |
| Grant total | 61 (41.8) | 24 (8.3) |

* Percentage is given in parentheses.

as a percentage by actual counting of the number of parasites per 1,000 red blood cells or per 10,000 red blood cells if infections were light (i.e., <0.1%), as recommended by Godfrey *et al.* (1987).

The morphology of gametocytes of *Haemoproteus tartakovskyi* from Skylarks was compared with the type and voucher specimens of *H. tartakovskyi* from its type host the Common Crossbill (*Loxia curvirostra*) (accession no. 413.91) at the Collection of the Institute of Ecology, Nature Research Centre, Vilnius. Morphology of *Plasmodium* (*Novyella*) *vaughani* from the Skylark was compared with type and voucher material of the same parasite from its type vertebrate host the American Robin (*Turdus migratorius*) and additional vertebrate host the Blackbird (*Turdus merula*) (accession nos. 635, 639, 654, 655) in the Garnham Collection at the Natural History Museum, London.

**Table 7.2:** Morphometric parameters of mature blood stages of *Haemoproteus tartakovskyi* (lineage hALARV1) and *Plasmodium* (*Novyella*) *vaughani* (lineage pSYAT05).

| Feature | Measurements (μm)[a] | |
|---|---|---|
| | *Haemoproteus tartakovskyi* | *Plasmodium vaughani* |
| Meront | | |
| Length | | 3.7-7.5 (5.4 ±0.8) |
| Width | | 1.6-3.3 (2.3 ± 0.5) |
| Area | | 6.2-12.7 (9.7 ± 1.5) |
| Area of globule | | 0.5-1.2 (0.8 ± 0.2) |
| No. of pigment granules | | 1-2 (1.2±0.4) |
| No. of merozoites | | 4-8 (5.6 ± 1.3) |
| Macrogametocyte | | |
| Length | 10.6-12.3 (11.6±0.5) | 9.6-12.2 (11.3±0.8) |
| Width | 3.1-4.8 (4.1±0.4) | 2.5-3.3 (2.8±0.2) |
| Area | 39.3-53.9 (49.4±4.1) | 23.2-39.2 (28.6±4.5) |
| Gametocyte nucleus | | |
| Length | 2.1-2.9 (2.5±0.2) | 2.2-4.2 (2.9±0.5) |
| Width | 1.4-2.6 (1.9±0.4) | 1.3-2.8 (2.1±0.4) |
| Area | 2.3-5.6 (3.7±0.8) | 2.9-6.3 (4.2±1.1) |
| Number of pigment granules | 13-22 (16.1±2.4) | 3-8 (5.3±1.3) |
| Microgametocyte | | |
| Length | 10.7-12.7 (11.5±0.5) | 10.5-12.8 (12.1±0.7) |
| Width | 3.9-5.3 (4.4±0.4) | 2.8-3.7 (3.2±0.3) |
| Area | 40-57.9 (50.6±3.8) | 29.8-43.6 (35.4±3.9) |
| Gametocyte nucleus | | |
| Length | 6.1-9.2 (7.6±1) | 5.8-7.9 (6.8±0.8) |
| Width | 3-4.6 (4±0.4) | 2.5-3.2 (2.9±0.3) |
| Area | 18.7-35.3 (27.7±4.1) | 11.7-22 (16.8±2.8) |
| Number of pigment granules | 9-20 (14.8±2.6) | 4-9 (5.8±1.2) |

[a] All measurements ($n = 31$) are given in micrometers. Minimum and maximum values are provided, followed in parentheses by the arithmetic mean and standard deviation.

### *Extraction of DNA, PCR, sequencing and analysis of molecular data*

The DNA was extracted using a standard ammonium acetate method (Sambrook *et al.* 2002). Diluted total DNA was used as the template in PCR assays for detection of the parasites, using primers and temperature profiles as in Hellgren *et al.* (2004). The method consists of a nested PCR assay that amplifies a part of the parasites mitochondrial cyt *b* gene in two steps, first an initial PCR (primers HaemNFI/HaemNR3; 570 bp excluding primers) that amplifies parasites from all of the three genera followed by a second step that separates *Leucocytozoon* spp. (primer HaemFL/HaemR2L; 478 bp excluding primers) from parasites of the genera *Plasmodium* and *Haemoproteus* spp. (primers HaemF/HaemR2; 480 bp excluding primers). By amplifying the parasite DNA in two PCRs the sensitivity of the screening is increased (Hellgren *et al.* 2004, Waldenstrom *et al.* 2004). Positive or negative infections were seen as presence or absence of bands on a 2% agarose gel using 1.5 μl of the final PCR product. Samples which showed positive amplification where sequenced, using the procedures described by Bensch *et al.* (2000). Amplified fragments were sequenced from the 5'end with the primer HaemF. We used dye terminator cycling sequencing (big dye) kit and the samples were loaded on an ABI PRISM TM 3100 sequencing robot (Applied Biosystems, Florida, USA).

The obtained sequences were edited and aligned using the BioEdit program (Hall 1999). The appearance of double peaks in the sequence was considered as mix infection. All unique lineages, i.e. sequences differing by one or more nucleotide basepare, were sequenced in the reversed direction with the complement primer HaemR2. The 38 taxa (total 474 nucleotides) in the final alignment were used for Bayesian analysis. Bayesian phylogeny was constructed using mrBayes version 3.1.2 (Ronquist & Huelsenbeck 2003). We used the General Time Reversible model including invariable sites and variation among sites (GTR+I+G) as suggested by the software mrModeltest 2.2 (Nylander 2004 software available from <http://www.ebc.uu.se/systzoo/staff/nylander.html>). Two simultaneous runs were conducted with a sample frequency of every 100th generation over 3 million generations. Convergence in phylogeny estimation for each analysis was assessed using the programe Tracer (Rambaut, A. & Drummond, A., available at http://evolve.zoo.ox.ac.uk) and used to indicate the appropriate "burn-in" period. The 25% of the trees were discarded as burn-in period. The remaining trees were used to construct a Majority rule consensus tree. The phylogenies were visualized using Tree View 1.6.6. (Software available from <http://evolution.genetics.washington.edu/phylip/software.html>). We used sequences of haemosporidian parasites, which species were positively identified (for linkage of parasite lineages with their morphospecies, see Krizanaskiene *et al.* 2006, Palinauskas *et al.* 2007, Krone *et al.* 2008, Martinsen *et al.* 2008, Valkiunas *et al.* 2008, Valkiunas *et al.* 2008, Zehtindjiev *et al.* 2008, Bensch *et al.* 2009, Krizanauskiene *et al.* 2010). GenBank accession numbers and MalAvi reference names (see Bensch *et al.* 2009) of all lineages mentioned in this article are given in Figure 7.1.

The sequence divergence between the different lineages was calculated with the use of a Jukes-Cantor model of substitution, with all substitution weighted equally, implemented in the program MEGA version 4 (Tamura *et al.* 2007) where the pairwise deletion was selected.

Student's *t*-test for independent samples was used to determine statistical significance between mean linear parameters. Prevalences were compared by Yates corrected Chi-square test. A *P*-value of 0.05 or less was considered significant.

The representative blood slides were deposited in the Nature Research Centre, Vilnius, Lithuania (accession nos. 47733 – 47736 NS). Sequences of new parasite lineages were deposited in GenBank (nos. GU289671, GU289672, GU289673).

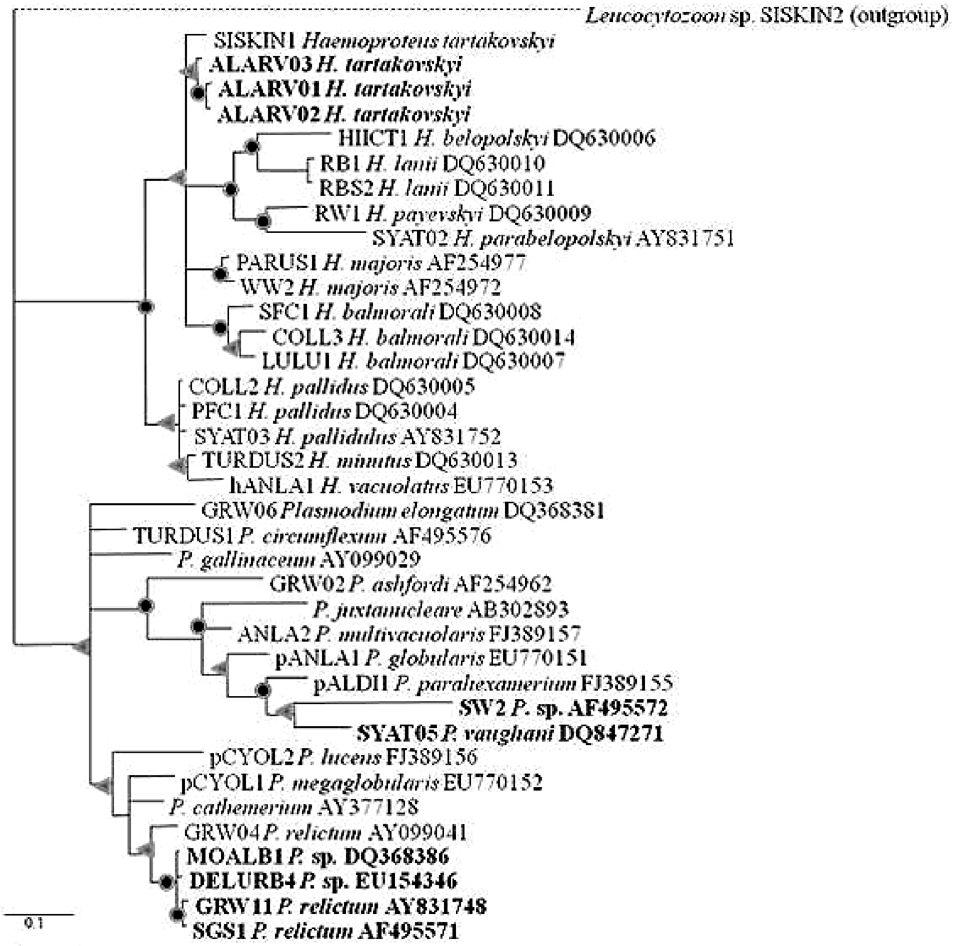

**Figure 7.1:** Bayesian phylogeny of cytochrome *b* gene lineages of positively identified species of avian pigment-forming haemosporidian parasites. Lineages recorded in the Skylark (*Alauda arvensis*) are given in bold. Names of the lineages (when available) are given before the species names of parasites; GenBank accession numbers of the lineages are provided after the parasite species names. Nodal support values (circles - 80–100%, and triangles - 60–70%) indicate posterior clade probabilities.

## Results

### *Molecular analysis of blood samples*

Only pigment-forming haemosporidian parasites of the genera *Haemoproteus* and *Plasmodium* were found using molecular techniques; that is in accord to microscopy data (see below) (Table 7.1). The overall prevalence of haemosporidian infection was 19.5%; it was 41.8% in Italian and 8.3% in Dutch Skylarks ($P < 0.001$). We detected 3 lineages of *Haemoproteus* spp. and 6 lineages of *Plasmodium* spp. in 85 infected Skylarks (Fig. 1). *Plasmodium* parasites were found in 34.1% of Italian and 6.5% of Dutch Skylarks ($P < 0.001$). Haemoproteids were present in 2% of the Italian birds and absent from the Dutch Skylarks. In Dutch Skylarks, the prevalence of *Plasmodium* spp. was 4.6% for adults and 9.9% in nestlings ($P < 0.05$, not significant), indicating the active malaria transmission at the study site. According to the PCR diagnostics, 5.5% and 1.7% simultaneous infections have been detected in Italian and Dutch populations, respectively. All recorded malarial infections were simultaneous in nestlings.

At both study sites, the lineage SGS1 of *Plasmodium relictum* was most prevalent; it was recorded approximately in 24.7% of birds in Italy and 5.5% in the Netherlands (Table 7.1). This lineage together with the lineages pGRW11, pMOALB1, pDURB4 and pGRW4 form well supported clade of *P. relictum* morphospecies (Fig. 7.1). Lineages pGRW11, pDURB4 and pSW2 has been recorded only in Italian birds and lineage pMOALB1 was recorded only in Dutch Skylarks; these lineages were rare (prevalence < 5%). *Plasmodium* lineage pSYAT05 was found in both studied populations (Table 7.1).

Three *Haemoproteus* lineages (hALARV1, hALARV2 and hALARV3) were found only in Skylarks from Italy. These lineages cluster together with lineage hSISKIN1 of *Haemoproteus tartakovskyi* (the *p*-distances between these lineages ranged from 0.2% to 2.3% with a total mean distance of 1.25%) and form well supported clade with the latter parasite (Fig. 7.1).

### *Microscopic investigation*

All samples were examined microscopically, and the PCR-based diagnostics (both positive and negative results) was confirmed by microscopic observations. Microscopic examination revealed undetected by PCR simultaneous haemosporidian infections, which were present approximately in 15% of infected birds in both Italian and Dutch populations. Over 60% of all recorded infections were light (< 0.001%), so could be regarded as chronic. For some of the detected cyt *b* lineages (pSW2, pMOTALB1 and pDURB4) we were unable to identify species due to low intensity of parasitemia and absence of all necessary blood stages on the slides.

*Haemoproteus tartakovskyi* (Figs 7.1, 7.2 E-H) (lineages hALARV1, hALARV2 and ALARV3), *Plasmodium* (*Novyella*) *vaughani* (Figs 7.1, 7.2 I-L) (lineage pSYAT05) and *Plasmodium relictum* (lineage pSGS1) were identified using morphological features of blood stages of the parasites. Lineages pMOALB1 and pDURB4 of *Plasmodium* spp. are closely related to the lineages pSGS1, pGRW11 and pGRW4 of *P.* (*Haemamoeba*) *relictum* (Palinauskas *et al.* 2007; Knowles *et al.* 2010) with genetic difference among them between 0.2% and 2.3% (Fig. 7.1); these lineages probably belong to this morphospecies. However, our material is incomplete for these parasites' unequivocal identification using morphological characters because the recorded infections were too light. The unidentified *Plasmodium* lineage (pSW2) is genetically distant from other *Plasmodium* spp. lineages (from 6.6% to 12.3%); based on available morphological features it belongs to subgenus *Novyella*. Additional material is needed for identification of this parasite species.

The parasites of the lineages hALARV1, hALARV2 and hALARV3 (Figs. 7.1, 7.2 E-H) are indistinguishable morphologically among each other and from *H. tartakovskyi*; they are also genetically similar to the lineage pSISKIN1 of *H. tartakovskyi* (Figs. 7.1, 7.2 A-D) from its type host, the Common Crossbill (*Loxia curvirostra*) and additional host the Siskin (*Spinus spinus*). Genetic divergence between these lineages varies between 0.2% and 2.1%. We consider all these lineages as intraspecies genetic variation of the same morphospecies, i. e. *H. tartakovskyi*.

Intensity of parasitemia was light in all infected birds; it was < 0.05% in the great majority of our samples. The highest intensity of malaria infection (0.77%) was recorded in one birds infected with the *P. relictum* lineage SGS1 in Italy on 27 October 2006. All *Haemoproteus* infections were of low intensity (between 0.015% and 0.018%). Because 1) *Haemoproteus tartakovskyi* has been recorded in Skylarks for the first time, and 2) lineages for molecular identification of *P. vaughani*, a widespread agent of avian malaria, have not been determined, we describe recorded in this bird parasites briefly and link these morphospecies with their cyt *b* lineages.

### *Description of parasites*

*Plasmodium* (*Novyella*) *vaughani* Novy and MacNeal, 1904 (Fig. 7.2 I-L, Table 7.2)

*Avian host*: Skylark (*Alauda arvensis*) (Passeriformes, Alaudidae).

*DNA sequences*: Mitochondrial cyt *b* gene lineage pSYAT05 (479 bp), GenBank accession no. DQ847271

*Prevalence*: 5 of 434 (1.2%) (Table 7.1).

*Additional hosts*: the lineage pSYAT05 of *P. vaughani* has been recorded in 7 bird species: American Robin (*Turdus migratorius*), Blackbird (*Turdus merula*), Blackcap (*Sylvia atricapilla*), Sardinian Warbler (*Sylvia melanocephala*) and Tomtit (*Petroica macrocephala*) (Hellgren *et al.* 2007, Martinsen *et al.* 2008, Bensch *et al.* 2009).

*Geographical distribution*: the lineage pSYAT05 was recorded in the USA, Europe and New Zealand (Hellgren *et al.* 2007, Martinsen *et al.* 2008, Bensch *et al.* 2009), so seems to be cosmopolitan in distribution.

*Representative blood films*: Voucher specimen (accession number 47736 NS *Alauda arvensis*, 4 October 2007, collected by P. Zehtindjiev) is deposited in the Nature Research Centre, Vilnius, Lithuania. Simultaneous infection of *P. relictum* is present in the voucher slide 47736 NS.

*Erythrocytic meronts* (Fig. 7.2 L): Develop in mature erythrocytes; they were seen anywhere in the host cells. Fully grown meronts are variable in form, most frequently are roundish, oval or irregular; mature meronts contain between 4 and 8 merozoites

(Fig. 7.2, L, Table 7.2); one clearly defined round refractive colourless globule is present in each meront (Fig. 7.2, L, Table 7.2); it is frequently located close to a clump of pigment granules. The influence of meronts on infected erythrocytes is not pronounced.

*Macrogametocytes* (Fig. 7.2 J): Develop in mature erythrocytes, are elongated in form. The cytoplasm is homogeneous in appearance, sometimes contains large vacuoles. Parasite nucleus is prominent (Table 7.2), variable in shape, usually median in position (Fig. 7.2 J). Pigment granules are few (Table 7.2), elongated or sometimes roundish, of medium size (0.5-1.0 µm), randomly scattered throughout the cytoplasm or clumped in small groups. The influence of gametocytes on infected erythrocytes is not pronounced.

*Microgametocytes* (Fig. 7.2 I, K). The general configuration is as for macrogametocytes with the usual haemosporidian sexual dimorphic characters. The parasite nucleus is diffuse, and its size is markedly variable in different gametocytes (Fig. 7.2 I, K).

*Haemoproteus tartakovskyi* Valkiūnas, 1986 (Fig. 7.2 E-H, Table 7.2)

*Avian host*: Skylark (*Alauda arvensis*) (Passeriformes, Alaudidae).

*DNA sequences*: Mitochondrial cyt *b* gene lineages hALARV1 (479 bp, GenBank accession no. GU289671), hALARV2 (479 bp, GenBank accession no. GU289672) and hALARV3 (479 bp, GenBank accession no. GU289673).

*Prevalence*: 4 of 434 (1.0%) (Table 7.1).

*Additional hosts*: The lineages hALARV1, hALARV2 and hALARV3 have been recorded only in Skylarks so far. Closely related lineage hSISKIN1 is prevalent in crossbills and siskins in Europe (Fig. 7.1).

*Geographical distribution*: The lineages hALARV1, hALARV2 and hALARV3 have been recorded only in Italy.

*Representative blood films*: Voucher specimens (accession numbers 47733 NS, 47734NS and 47735 NS, *Alauda arvensis*, 23 - 26 October 2006, collected by P. Zehtindjiev) are deposited in the Institute of Ecology, Nature Research Centre, Vilnius, Lithuania.

*Young gametocytes* (Fig. 7.2 E, F): The earliest forms (Fig. 7.2 E) can be seen anywhere in the infected erythrocytes; they are roundish or oval, each possesses a large nucleus and prominent cytoplasm. As parasite develops, gametocytes adhere to the erythrocyte nuclei and extend longitudinally along the nuclei markedly displacing them laterally (Fig. 7.2 F).

*Macrogametocytes* (Fig. 7.2 G): The cytoplasm is homogeneous in appearance, sometimes contains small vacuoles. Gametocytes markedly displace the nucleus of erythrocytes laterally, frequently to the envelope of the host cells (Fig. 7.2 C, G). Parasite nucleus is oval or roundish usually more or less median in position (Fig. 7.2 G); pigment granules are numerous (Table 7.2), oval and roundish, of medium size (0.5-1.0 µm), usually randomly scattered throughout the cytoplasm.

*Microgametocytes* (Fig. 7.2 H): The general configuration is as for macrogametocytes with the usual haemosporidian sexual dimorphic characters. The parasite nucleus is diffuse, and its size is variable in different gametocytes (Fig. 7.2 H).

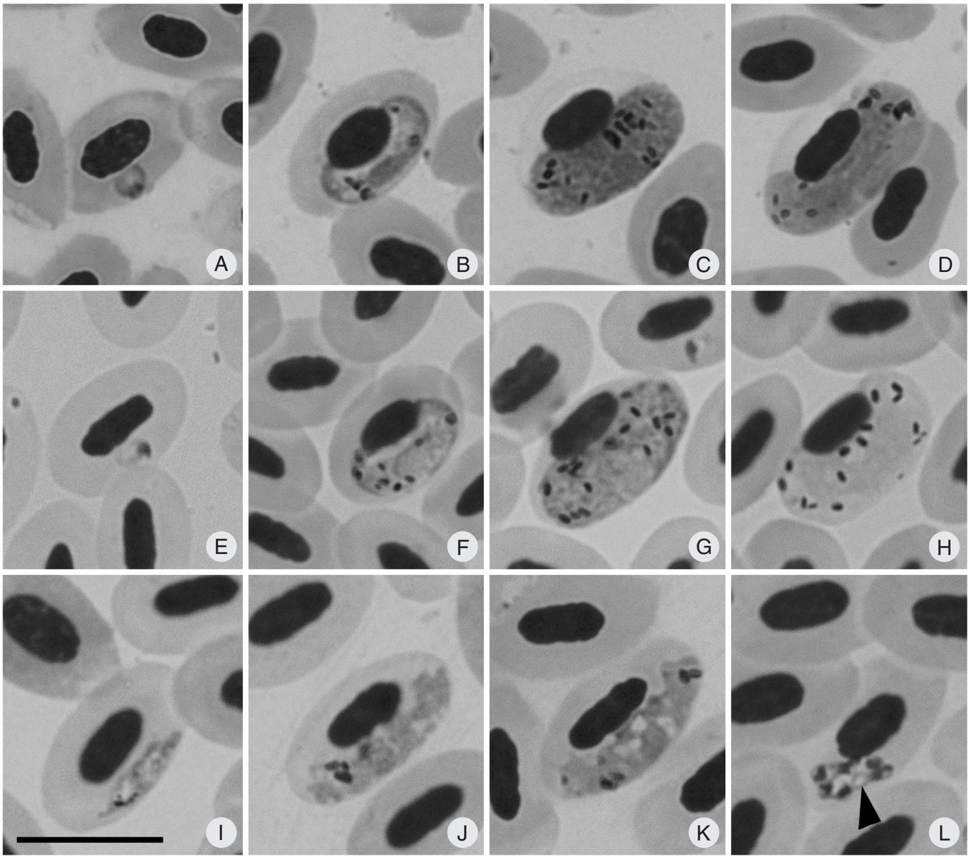

**Figure 7.2:** *Haemoproteus tartakovskyi* from the blood of its type vertebrate host, the Crossbill (*Loxia curvirostra*) (A-D, lineage hSISKIN1) and the Skylark (*Alauda arvensis*) (E-H, lineage ALARV03), and *Plasmodium* (*Novyella*) *vaughani* (I-L, lineage pSYAT05) from the Skylark: A-B, I, E young gametocytes, C, G, J – macrogametocytes, D, H, K – microgametocytes; L – erythrocytic meront). Arrow – a refractive colourless globule. Giemsa-stained thin blood films. Bar = 10 µm.

## Discussion

Molecular and microscopy approaches were combined in investigations of haemosporidian parasites of Skylarks for the first time during this study. The PCR-based methods were less sensitive in determining simultaneous infections than microscopic examination of blood films. That should be taken in consideration in field studies of blood parasites using general primers, as previously discussed by Valkiūnas *et al.* (2006) and Martinez *et al.* (2009).

*Plasmodium relictum* (lineages pSGS1) was the most prevalent haemosporidian parasite in Skylarks. That was expected because this lineage is widespread and actively transmitted in the Old World (Palinauskas *et al.* 2007, Bensch *et al.* 2009). Unidentified to species level *Plasmodium* lineages pDURB4 and pMOALB1 are closely related to pSGS1 and pGRW11 (Fig. 7.1), so probably belong to the same morphospecies *P. relictum*, but further morphological studies are needed to prove that.

Linkage between DNA sequences and identifications based on traditional morphological species can provide important information about life-history strategies for parasitologists and evolutionary biologists studying phylogenetic relationships of these organisms; it can also be used for molecular identification of parasites (Krizanauskiene *et al.* 2010). Unfortunately, the number of incorrectly identified species is increasing in GenBank (Valkiūnas *et al.* 2008, Krizanauskiene *et al.* 2010). To ensure parasites' species identification, we used museum type specimens in our identifications and also provided brief description of reported parasites in this study.

*Plasmodium vaughani* is second only to *P. relictum* in frequency of occurrence in birds. According to the previous studies, this avian malaria parasite has been reported in numerous bird species belonging to many host families and even orders, but is particularly common in passerines (Garnham 1966, Valkiūnas 2005), so the record of *P. vaughani* in Skylarks was not unexpected. In spite of worldwide distribution, molecular identification of *P. vaughani* has not been developed yet. That is important to do because the majority of natural malarial infections are light, so frequently are difficult to identify to species level in single blood films. We suggest using the lineage pSYAT05 for molecular identification of *P. vaughani*. Morphological features and measurements of blood stages of parasites of the lineage pSYAT05 are indistinguishable from *P. vaughani* from its type vertebrate hosts, the American Robin. Gametocytes (Fig. 7.2I-K) and erythrocytic meronts (Fig. 7.2L), which are typical for *P. vaughani*, predominate among parasites of the pSYAT05 lineage in our material. Importantly, the same lineage was found in the USA in the American Robin, the type host of *P. vaughani* (Martinsen *et al.* 2008); it also present in the Black Bird, which is a common host of this parasite in Europe (Valkiūnas 2005, Hellgren *et al.* 2007). These data are in accord with former microscopic investigations, which showed cosmopolitan distribution and broad avian host range of *P. vaughani* (Garnham 1966, Corradetti & Scanga 1973, Bennett *et al.* 1982, Valkiūnas 2005). Formerly, *P. vaughani* was found in Skylarks only in Kazakhstan (prevalence is 3%, see Yakunin & Zhazyltaev 1977). This parasite has been reported in European Skylarks for the first time during this study.

We found the lineage SW2 of *Plasmodium* (*Novyella*) sp. only in Italian birds. This lineage is common in Sedge Warblers (*Acrocephalus shoenobaenus*) in Africa (Waldenstrom *et al.* 2002); it was found in Skylarks for the first time during this study. We were unable to identify this parasite to species level because intensity of infection was light.

Gametocytes of the lineages hALARV1, hALARV2 and hALARV3 are indistinguishable from each other in all their main qualitative and morphometric parameters. Comparison of blood stages of these Skylark parasites with the type specimens of *H. tartakovskyi* (lineage hSISKIN1, Fig. 7.2A-D) from its type vertebrate host, the Common Crossbill (Valkiūnas 1986) showed that all these haemoproteids are indistinguishable. We consider all these lineages as intraspecies variation of *H. tartakovskyi* and attribute them this species. That is in accord to our phylogenetic analysis and is similar to the level of intaspecies variation reported, for instance in *Haemoproteus balmorali* (Fig. 7.1). Lineages of *H. tartakovskyi* are prevalent in Common Crossbills, Hawfinches and Siskins in Europe (Bensch *et al.* 2009). This haemoproteid has been reported in Skylarks for the first time during this study. Because *H. tartakovskyi* normally is prevalent in fringillid birds (Valkiunas 1986), it might be that our report of this parasite is a case of new emerging haemosporidian infection in European Skylarks. *Haemoproteus tartakovskyi* is transmitted by biting midge *Culicoides impunctatus* (Valkiunas *et al.* 2002); its development in avian host and virulence remain unknown.

*Plasmodium* (*Novyella*) sp. (lineage pSW2) infection has been recorded in African migrating Sage Warbler (*Acrocephalus schoenobaenus*) (Waldenstrom *et al.* 2002). According to our study this malaria parasite has been reported for the first time in Skylarks, so should be considered as a possible new pathogen in this bird species, so might contribute to decrease of Skylarks' population in Europe. It is interesting that the lineage pSW2 have been reported in non-migrant Tawny Owl (*Strix aluco*) in Germany (Krone *et al.* 2008), so transmission certainly takes place in Europe.

Only two haemosporidian parasite species have been identified in Skylarks in Western Europe in the 20th century: *Haemoproteus alaudae* and *Plasmodium* (*Haemamoeba*) *supraecox* (Bennett *et al.* 1982, Valkiūnas 2005). Malaria parasites have been recorded incidentally in this bird, and the overall prevalence of haemoproteids was reported to be < 10% before massive decline of Skylarks populations in Western Europe (Peirce 1981). Interestingly, these 2 haemosporidian parasites were not recorded during this study. However, we found at least 4 additional species (3 *Plasmodium* and 1 *Haemoproteus*) and 9 different mitochondrial DNA-lineages of haemosporidians, which have not been reported in Skylarks in Western Europe before. It is worth noting that *P. relictum* has been reported in 1 of 5 examined Skylarks in Georgia (Burtikashvili 1978) and *P. vaughani* was seen in 2 of 65 examined Skylarks in Kazakhstan (Yakunin & Zhazyltaev 1977), so these parasites have broad range of distribution in Skylarks. Recently, *Plasmodium* spp. were found in 20% of Skylarks in France (Chavatte *et al.* 2009). However, during this study we report highest prevalences and genetic diversity of malaria parasites and haemoproteids than ever been reported in this bird before. Additionally, *H. tartakovskyi* seems to be an emerging parasite in Italian Skylarks because it has been reported only in birds belonging to the Fringillidae so far; it is prevalent in Siskins, Common Crossbills and Hawfinch in Europe (Valkiūnas *et al.* 2003, Valkiūnas 2005). These data indicate possible ongoing colonization of Skylarks by blood parasites in changing environment conditions. That warrants further investigation, particularly due to recent reports on mortality of birds caused by emerging *Haemoproteus* spp. infections in Europe (Olias *et al.* 2011). It worth noting that transmission of the same lineages of haemoproteids between passerines of different families certainly occurs in Europe (Križanaskienė *et al.* 2006, 2010), but epidemiological significance of this phenomenon remains insufficiently understood.

It is important to note that significantly greater prevalence of pigment-forming haemosporidian infections was recorded in Skylarks sampled in Italy than in the Netherlands (Table 7.1). The birds sampled in Italy where caught during their seasonal migration; they probably came from North-eastern Europe to the study site and were on their way to wintering quarters in the Mediterranean.

Importantly, malarial parasites were prevalent in 5-7 day old nestlings during this study. Furthermore, all recorded *Plasmodium* spp. infections were simultaneous in nestlings; such infections have been reported to be particularly virulent in birds (Zehtindjiev *et al.* 2008, Palinauskas *et al.* 2009). Because *Plasmodium* spp. are particularly virulent and even lethal in juveniles (Garnham 1966, Valkiūnas 2005), it seems probable that malaria might be a factor contributing to mortality in European Skylarks due to direct or indirect (via predators) elimination of nestlings and/or fledglings.

The Skylarks are "species of Conservation Concern in Europe", owing to the populations' decline of over 50%, particularly in West Europe since 1960s and mainly since 1980s (BirdLife International 2004). Although there are examples of dramatic impact of malaria parasites and other haemosporidians on wildlife, particularly in ecosystems where malaria has been emerging (Garnham 1966, Atkinson *et al.* 2001, Valkiūnas 2005, Levin *et al.* 2009), blood parasites have not yet been considered in understanding the decline of Skylark populations. This study does not provide final answer if blood parasites contribute to decline of Skylarks populations; additional field studies comparing distribution and virulence of certain species of haemosporidians between areas where Skylarks are decreasing and where populations are stable are needed to answer this questions satisfactorily. However, our data indicate that haemosporidian parasites are likely emerging in Skylarks, so should be considered in conservation programs. That is particularly true due to recent reports about mortality in birds caused by emerging haemosporidian infections in Europe (Olias *et al.* 2011, Valkiūnas 2011). Thus, recent decline of Skylark populations in Western Europe is accompanied by increased prevalence and diversity of haemosporidian parasites, which should be considered as possible factors contributing to the population decline process and need further research.

## Acknowledgements

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## CHAPTER 8

# Can Skylarks (*Alauda arvensis*) discriminate a parasite nestling? Possible case of nestling Cuckoo (*Cuculus canorus*) ejection by its host parents

Arne Hegemann & Rob Voesten

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**Abstract** The Common Cuckoo (*Cuculus canorus*) is an obligate brood parasite and many studies have dealt with egg rejection by host species. However, evidence for ejection of Cuckoo nestlings by host parents has not been reported. Here we describe an observation of a Skylark (*Alauda arvensis*) pair that probably ejected a young Cuckoo and subsequently raised their own offspring. This was the only case we detected Cuckoo parasitism among 348 Skylark nests in our study area in the northern Netherlands, while we found about 21% of 43 Meadow Pipit (*Anthus pratensis*) nests being parasitized by Cuckoos.

## Introduction

The Common Cuckoo (*Cuculus canorus*; hereafter, Cuckoo) is an obligate brood parasite which breeds across Eurasia (Glutz von Blotzheim & Bauer 1985, Davies 2000). More than 125 bird species are known as hosts, but only less than 20 are commonly used (Wyllie 1981, Moksnes & Roskaft 1995, Davies 2000). Many studies have dealt with egg rejection by host species (e.g. Moksnes & Roskaft 1989, Moksnes *et al.* 1991a, Moksnes *et al.* 1991b, Honza & Moskat 2008, Vikan *et al.* 2010), but evidence for nestling rejection is very scare. There are several hypotheses to explain the lack of nestling discrimination (reviewed by Davies 2000, Grim 2006). The basic idea is that parents need to learn in their first breeding year how their own eggs and chicks look like, to be able to discriminate between own and foreign eggs/young. If parasitized in the first year, parents learn that their eggs are more variable (including the cuckoo egg), and in future years may not eject cuckoo eggs. In contrast, if they would imprint during the chick phase to find out how their own chicks look, a parasitized parent in the first breeding attempt would not accept its own young in future attempts, because cuckoo chicks eject host eggs and chicks (Lotem 1993). Chick discrimination therefore is more difficult and costly to evolve than egg discrimination, especially if parents need to learn the appearance of their own chicks. However, across the different avian host–parasite systems around the world, three ways that lead to nestling rejection have been identified. The nestling 'discrimination without recognition' hypothesis is based on tentative support that Eurasian Reed Warblers (*Acrocephalus scirpaceus*) might be able to discriminate own versus parasite nestlings by the length of the feeding period, i.e. Cuckoo nestlings need to be fed for a longer period than own offspring. As the time span of parental care seems to be preprogrammed parents reduce or even stop feeding Cuckoo nestlings at their late nest stage (Grim *et al.* 2003, Grim 2007). However, experimental tests on other species found no evidence for discrimination against a nestling of a different species in their nest (Davies & Brooke 1989). Rejection of nestling parasites by deserting the nest in an early feeding stage has been rarely described for other parasite-host systems (Grim 2006 and see references therein). Direct nestling ejection (the physical removal of a live parasite nestling) has only recently been described for one host–parasite system. In Australia, two species of Gerygone (*Gerygone laevigaster*) and (*G. magnirostris*) regularly eject young of the Little Bronze-Cuckoo (*Chalcites minutillus*) (Sato *et al.* 2010, Tokue & Ueda 2010).

To our best knowledge, there is no record of a live nestling Common Cuckoo being ejected by its host parents (Davies 2000, see also Grim *et al.* 2003, Grim 2006). Here, we describe an observation of a Eurasian Skylark (*Alauda arvensis*) pair that most likely ejected a young Cuckoo and subsequently raised their own offspring.

### *Study population*

In 2006–2009 we studied a breeding population of Skylarks in the "Aekingerzand", part of the National Park Drents-Friese Wold in the northern Netherlands (52°55'N; 6°18'E). The area is a mixture of open sand, groups of trees, heath- and grasslands on nutrient-poor soil and surrounded by a thin belt of forests. Beside 80–100 pairs of Skylarks and

**Figure 8.1:** Clutch of four Skylark eggs and one Cuckoo egg (based on coloration and size supposedly middle left). Picture taken by Rob Voesten, 27 May 2009.

about 50 pairs of Meadow Pipit (*Anthus pratensis*), several other passerine species (including Woodlark (*Lullula arborea*), European Stonechat (*Saxicola rubicola*) and Tree Pipit (*Anthus trivialis*)) breed in the area. Every breeding season several male and female Cuckoos are present in the area.

### *Rate of parasitism in our study area*

We found in four successive breeding seasons 2006–2009 a total of 348 Skylark nests (163 were found during egg stage, but 62 failed before hatching; 184 were found when already in the feeding stage). We detected parasitism by a Cuckoo only once (0.3%), the case described here. Since Skylarks do not seem to reject foreign eggs (Antonov *et al.* 2010), it is unlikely that we missed parasitation events due to eggs being rejected before we found the nest. In the study area the Meadow Pipit is a common breeding bird and we checked some nests in 2008 and 2009. We found a total of 43 nests of which nine were parasitized by Cuckoos (20.9%). Of the 34 unparasitized nests we found 12 with eggs of which six failed before hatching and 22 nests contained nestlings when found. Of the nine nests that were parasitized five contained at the moment of finding eggs (of which three hatched, and two were depredated), and four a young Cuckoo.

### *Observation of nestling Cuckoo rejection*

On 27 May 2009 we discovered an incubated Skylark nest with 5 eggs (Fig. 8.1). On 3 June (around noon) three young Skylarks had hatched (day 0); two eggs were still remaining, and we did not realise at that time that one was a Cuckoo egg. We did not visit the nest on 4 and 5 June. At 5:45 on 6 June, we found a young Cuckoo in the nest,

**Figure 8.2:** Two young Skylarks and one young Cuckoo. Same nest as in Figure 8.1. Picture taken by Rob Voesten, 6 June 2009.

and two of the four Skylarks chicks (age of all day 3) were out of the nests, probably ejected by the Cuckoo chick. The two young out of the nest were dead, the two inside still alive (Fig. 8.2). At around 14:00 the young Cuckoo was found about 30 cm out of the nest, being still alive as were the two nestling Skylarks in the nest. We returned the young Cuckoo into the nest. At 19:50 all three nestlings were still alive in the nest. At 6:30 next morning the female Skylark was brooding one nestling Skylark. The other nestling and the Cuckoo were found dead about 10 cm out of the nest. Both carcasses were already cold, but had very fresh and thus probably post-mortem injuries likely caused by a rodent. Small bites were visible on the back of one and the belly of the other bird. We ringed the remaining young Skylark on 10 June and it left the nest on 13 June 2009.

## Discussion

We interpret the observation that the young Cuckoo was twice found outside the nest as an ejection (physical removal) by the host parents. The first time we moved it back to see if it got ejected again, and indeed the next day we found it outside the nest for the second time. It was probably ejected from the nest in the early morning of that day after it presumably managed to eject one more Skylark nestling.

Two alternative explanations why the young Cuckoo was outside the nest seem very unlikely to us. (1) The young Cuckoo fell out of the nest while trying to eject a nestling Skylark. Such cases have rarely been described for Reed Warbler nests (Wyllie 1981), but seem unlikely for a ground nest in a shallow depression. (2) A predator took the Cuckoo nestling out of the nest. Predators would not leave nestlings next to the nest without eating any. Furthermore, during our study we never observed any similar cases of nestlings being dead or alive just outside the nests. We therefore believe that both alternative hypotheses are unlikely and instead postulate an active physical ejection of the live parasite nestling by one of the parent Skylarks.

Such behaviour is not only astonishing because we know of no published case of Cuckoo nestling ejection by host parents, but also because Antonov *et al.* (2010) showed in an experimental study a complete lack of egg recognition abilities in Skylarks and suggested that anti-parasite defences never evolved in this species. Skylarks are only rarely reported as host for Cuckoos (Glutz von Blotzheim & Bauer 1985, Moksnes & Roskaft 1995, Davies 2000) and the few known cases have been attributed to Cuckoo females that were unable to find a suitable nest of the preferred Meadow Pipit (Davies 2000). The 'spatial habitat structure hypothesis' suggests that Skylarks are usually breeding too far away from trees to be a potential Cuckoo host (Roskaft *et al.* 2002, Antonov *et al.* 2010). In our study area Skylarks and Meadow Pipits share the same meadows for breeding, both equally close to single trees, hence the chances for a female Cuckoo finding nests should be similar for these species. However, Meadow Pipits were commonly parasitized in our study, whereas we discovered only a single case of Cuckoo parasitism among 348 Skylark nests over four years. Meadow Pipits are one of the most common Cuckoo hosts in Europe (Glutz von Blotzheim & Bauer 1985, Moksnes & Roskaft 1989, Davies 2000). We also noted that Meadow Pipits strongly reacted to the presence of an adult Cuckoo and expressed intensive antagonist and alarming behaviour, while we never observed a similar reaction in Skylarks.

Why then the egg was laid into a Skylark nest in the first place remains speculative. Individual Cuckoo females usually specialize in parasitizing one particular host species (Lack 1968, Davies 2000, Honza *et al.* 2001) or a group of species that have similar eggs and nests (Moksnes & Roskaft 1988). Laying in a nest of a species that is not commonly parasitized but that has similar nests and eggs in the same habitat has been suggested when a nest of the preferred host species is unavailable (Davies 2000). For the case described here, we suggest the same mechanism. A suitable Meadow Pipit nest was unavailable at the moment of laying and thus the female Cuckoo chose for a Skylark nest.

Our observation is anecdotal, and it remains to be tested experimentally if Skylarks have a more general ability to reject Cuckoo nestlings from their nest. This would be spectacular, because current models of evolutionary host–parasite arms races are to a large extent based on the idea that egg discrimination evolves more easily than chick discrimination (Lotem 1993). If egg discrimination can evolve more easily it might also explain why species that are recently not parasitized show advanced egg recognition abilities. These species probably have a history of Cuckoo parasitism that led to the evolution of egg discrimination (Schulze-Hagen *et al.* 2009). However, there may be (few) species that have evolved chick discrimination rather than egg discrimination as a viable anti-parasitism strategy. This may not be through discrimination of own offspring against a young parasite, but rather by rejecting nestlings that eject other offspring from the nest. In this light, it is interesting that in our case the Cuckoo nestling hatched not first as it is usually the case (Davies 2000), but later than some of the Skylark nestlings. It is remarkable that the incubation period of Skylark and Cuckoo eggs is similar with 11-12 days (Wyllie 1981, Glutz von Blotzheim & Bauer 1985), and thus the head start Cuckoo eggs usually have because of the internal incubation (Birkhead *et al.* 2010) is not bringing any advantage in Skylark nests. As a result, the Cuckoo chick hatched later than the first Skylark nestlings, and at this moment the host parents were more likely to discriminate against the Cuckoo chick that started to eject the other nestlings from the brood.

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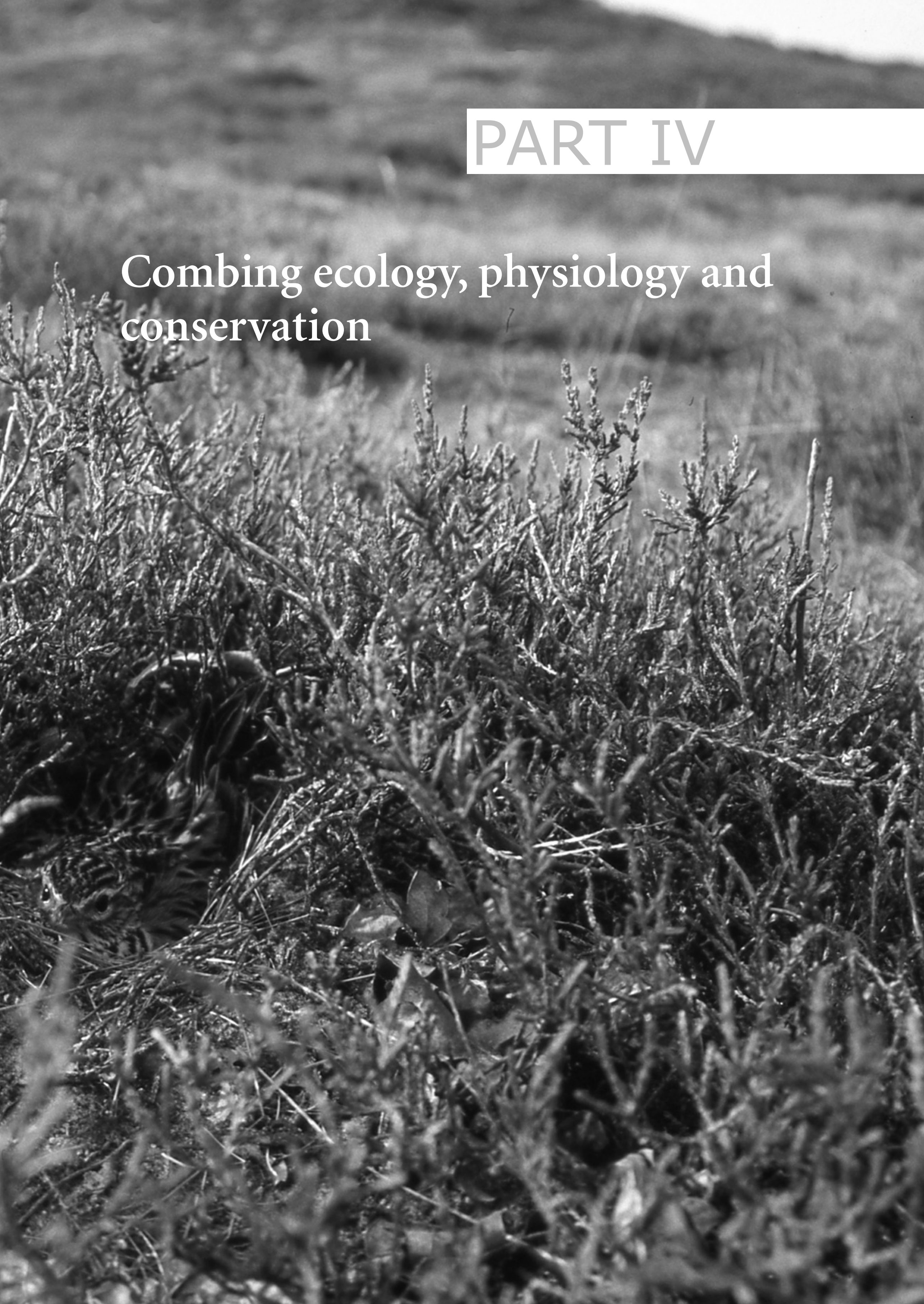

# PART IV

## Combing ecology, physiology and conservation

# CHAPTER 9

# Offspring pay sooner, parents pay later: Experimental evidence that immune function mediates trade-offs between parental effort and survival

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**Abstract** Life-history theory predicts that organisms trade off survival against reproduction, but the time scales on which various physiological mechanisms act remains unclear. We investigated three time scales at which immune function can mediate this trade-off: within broods, within season and between years. We attached extra weight to adult Skylarks (*Alauda arvensis*) and measured effects on body mass, immune function and survival. We also measured nest success, feeding rates, diet composition, nestling size, nestling immune function and recruitment rates. Parent birds were handicapped during first broods. When nestlings of these broods fledged, parent body condition had not changed, but experimental birds experienced higher nest failure. Depending on the year, immune parameters of nestlings from experimental parents were either higher or lower than in control nestlings. Five weeks later, when parents were feeding their second brood, the balance between self-maintenance and nest success had shifted and this shift depended partly on year. Independent of year, lysis titres were higher in control birds than in experimental birds. In 2007 experimental adults had higher agglutination values but lower nest success than control birds; this pattern was reversed in 2008. Mass and immune function of their nestlings did not differ from controls in either year. Although weights were removed after breeding, winter mortality was higher in experimental birds. Mortality could be predicted by immune measurements. Control birds that survived had a low H/L-ratio and a reduced agglutination titre compared to non-survivors, while this pattern was reversed for experimental birds. Among birds that returned the next year, body condition and reproductive performance a year after the experiment did not differ between treatment groups. We conclude that the balance between current reproduction and survival shifts from affecting nestlings to affecting parents as the reproductive season progresses and that immune function is the physiological mechanism mediating this trade-off.

## Introduction

The trade-off between investment into reproduction and self-maintenance (*e.g.* immune function) can act on different time scales. Manipulations of reproductive effort can have direct effect on nestlings, resulting in, for example, reduced mass gain or increased mortality (Mauck & Grubb 1995, Moreno *et al.* 1999). Effects on the condition of adults might take more time to develop and may become visible only after the breeding season (Nilsson & Svensson 1996). Increased adult mortality often occurs in the subsequent winter (Daan *et al.* 1996) and decreased investment into the immune system may be an important mechanism (Gustafsson *et al.* 1994, Norris *et al.* 1994). Understanding the processes that act on different time-scales and the mechanisms that mediate them is crucial because the trade-off between current and future reproduction is central in life-history theory (Williams 1966, Stearns 1992).

Despite the evidence that consequences of a shift in the trade-off between reproduction and self-maintenance can occur on different time scales, to our best knowledge no study has actually studied the underlying physiological mechanisms at multiple time levels. Furthermore, experimental studies of the trade-off between reproduction and self-maintenance that link changes in immune function to subsequent survival probabilities in both adults and their offspring, are lacking. Many studies on the trade-off between current reproduction and self-maintenance focus only on one time point, the current reproduction (*e.g.* Tieleman *et al.* 2008, Harding *et al.* 2009, Beaulieu *et al.* 2010, Leclaire *et al.* 2010) and only few studies include a second time point, either on reproductive parameters of second broods (*e.g.* Smith *et al.* 1987, Tinbergen 1987, Linden 1988) or on adult condition and performance in the following year (*e.g.* Wernham & Bryant 1998, Hanssen *et al.* 2005). Changes in parental effort that affect subsequent survival probabilities (*e.g.* Daan *et al.* 1996, Wernham & Bryant 1998), may be mediated by changes in immune function. Trade-offs between reproduction and immune function are well established (*e.g.* Norris *et al.* 1994, Deerenberg *et al.* 1997, Nordling *et al.* 1998, Hasselquist *et al.* 2001, Hanssen *et al.* 2005, Knowles *et al.* 2009) and increased parasite infection rates in birds raising enlarged broods have been described (Gustafsson *et al.* 1994, Norris *et al.* 1994, Knowles *et al.* 2009).

Studying the cost of reproduction and its underlying mechanism requires an experimental approach. Manipulations of brood size, the most typical approach, have been criticized because these manipulations alter the proximate stimulus for the parents by increasing the number of begging nestlings, which might influence the motivation to provide food (Moreno *et al.* 1999). An alternative is to directly manipulate the costs of locomotion (*e.g.* walking and flying). Handicapping birds with extra weight causes an increase in locomotion costs (Kvist *et al.* 2001, Engel *et al.* 2006, Schmidt-Wellenburg *et al.* 2008) that is independent from the motivation stimulus (Moreno *et al.* 1999, Tieleman *et al.* 2008). Thus, this approach provides the opportunity to study reallocations in trade-offs between parental investment and self-maintenance.

We present a comprehensive immunological and behavioural dataset on Skylarks (*Alauda arvensis*) to study trade-offs between parental investment in reproduction and self-maintenance along a time axis. We manipulated movement costs in free-living birds by handicapping them with an extra weight, and we measured a variety of fitness-related parameters over three different time scales: a) short-term effects within a breeding attempt and b) medium-term effects on second broods within the same season. We then removed the experimental handicap to measure c) carry-over effects on survival, immune system and reproductive performance in the subsequent year. We measured multiple indices of immune function in the parents to quantify investment into self-maintenance at each of these time points and link these to survival rates into the subsequent year. We quantified current reproduction by measuring number and size of offspring. To explore whether nestlings differ beyond size and fledgling rate, we quantified feeding rates, diet composition, immune parameters and recruitment rates. We expected that adults reduce investment into immune function, which might negatively impact survival, or reduce investment into reproductive effort, which might lead to declines in nestling quality affecting recruitment.

## Methods

### *Birds and experimental treatments*

We studied Skylarks in the Aekingerzand, the Netherlands (N 52°55’; E 6°18’) between 2007 and 2009 (Hegemann *et al.* 2010, Hegemann *et al.* 2012b). To initiate the experiment, adults were caught feeding 1-8 day old nestlings during the first half of the breeding season (21 April 2007- 31 May 2007; and 4 May 2008 – 10 June 2008). We refer to the data collected at this initial capture as baseline values. Pairs were assigned to control and experimental groups, and individuals were measured up to three different times (within-brood, within-season, carry-over). We attached an extra weight (10% of body mass) to experimental birds with a figure-eight harness (Rappole & Tipton 1991, Schmidt-Wellenburg *et al.* 2008). Consequently, birds were handicapped by the combined effects of carrying the extra weight and the harness. Control birds remained without harness or extra weight. We included experimental birds in this study only when both parents of a nest received a handicap to avoid the possibility that an unhandicapped partner compensated for a handicapped one (Harding *et al.* 2009, Wiebe 2010). For control birds we included birds when the partner was not captured.

To evaluate the short term effects of the handicap, we initiated the experiment before the nestlings of the focal birds were 3 days old and resampled the adults when nestlings were 7-11 days old (“within-brood” measurements, $n$ = 6 experimental, $n$ = 8 controls).

To evaluate the longer-term effects of the handicap, we recaptured and resampled 16 control and 16 experimental birds 5 weeks later (median = 36.5 days, range: 27–73 days) when they were attending their second brood (“within-season” measurements). At this time, we removed the extra weight of experimental birds.

To evaluate carry-over effects of the handicap on survival to the breeding season following the treatment (2008 and 2009, respectively), we examined return rates of adults (survival) and young (recruitment) by ring reading. To evaluate reproduction and physiology of the survivors (of which we had within-season measurements), we measured reproductive output (see below) and recaptured birds (5 control, 6 experimental) to re-measure body mass and immune parameters. Two experimental and two control pairs stayed together from first to second breeding season; all other birds had a new partner.

### *Sample and data collection*

Adults were sampled upon capture, and nestlings were sampled around 8 days of age. Blood samples (~100–150 μl from adults, ~ 70–100 μl from nestlings) were collected into heparinised capillary tubes from the brachial vein. Adults were bled immediately after capture (median: 5 min; range: 3–15 min) and before impacts of handling stress on immune parameters (Buehler *et al.* 2008). Blood smears for leukocyte enumeration (adults only) were made from a drop of fresh blood. The remaining blood was stored on ice until centrifuged in the lab (10 min, 7000 rpm). Plasma was frozen for future analyses. Structural measurements and body mass were taken after blood collection, and birds were ringed with metal and colour rings. Adult birds were sexed biometrically (Hegemann *et al.* 2012a), nestlings were sexed molecularly.

We used plasma to quantify titres of complement-like lytic enzymes and non-specific natural antibodies (Matson *et al.* 2005, Hegemann *et al.* 2012b), and concentrations of haptoglobin, an acute phase protein that binds hemoglobin (Hegemann *et al.* 2012b, Matson *et al.* 2012). Blood of 8 day old nestlings did not show lytic activity (Hegemann *et al.* unpublished).

Leukocyte proportions (lymphocytes, heterophils, basophils, monocytes or eosinophils) were determined from blood smears for the first 100 white blood cells (WBC) by one person (C. Gottland), who was blind to year and treatment. In most blood smears (61%) no basophils were detected, so we did not analyse this cell type.

Nest success rates (at least one fledged nestlings vs. nest failure) and number of fledglings were measured just before fledglings left the nest at day 8. We measured feeding rates on first broods in 2008 ($n$ = 14 nests) by observing nests with binoculars for one hour in the morning. Feeding rates were measured when nestlings were 4 days ($n$ = 9) and 6–7 days ($n$ = 11) old.

Skylark nestlings usually produce a dropping during ringing. These were collected per nest, preserved with table salt and frozen until analyses. Droppings of 27 nests (first broods: 9 control, 3 experimental; second broods: 9 control, 6 experimental) were analysed (Flinks & Pfeiffer 1987) by HF, who was blind to brood and treatment. We summarized the dropping analyses in three variables per nest: number of animal remains, average length of animal prey, and number of different prey types. Invertebrate length was estimated using a reference collection and information from literature (Calver & Wooller 1982, Ralph *et al.* 1985, Flinks & Pfeiffer 1987, Jenni *et al.* 1990).

### *Statistics*

We analysed data using R version 2.14.0 (R Development Core Team 2011). Treatment groups did not differ by chance in brood size, body mass or any immune parameter at the initiation of the experiment (always $P > 0.21$). For within-brood and within-season measurements, we used linear models and the differences between the two measurements as the dependent variables. Nest success rates, number of fledglings and number of recruits per fledgling were analysed on the nest level with generalized linear models and a binomial error structure. Body mass and immune parameters of nestlings were analysed with linear mixed models and feeding rates were analysed with generalized linear mixed models, both including nest as a random effect. Adult survival was analysed with generalized linear models with binomial error structure. We tested if survival could be predicted by any measurement at the end of the experiment. We did this by sequentially including the interaction of treatment with each measured immune parameter, with body mass and with their change during the experiment.

We included treatment, year, sex, baseline value, age of nestlings, number of nestlings and length of experiment if applicable in each model. We also included two-way-interactions involving treatment. We always started with the full model and simplified it using backward elimination based on likelihood-ratio test and F-Statistics (Chisq-Statistics for generalized linear models with binomial or poisson error structure and mixed models) and with $P < 0.05$ as the selection criterion ("drop1"-function of R) until reaching the minimal adequate model. Assumptions of all models were checked on the residuals of the final model. We report interactions only when significant. A detailed description of statistical methods and full statistics of main effects can be found in Appendix 1.

## Results

### *Within-brood effects*

The short-term handicap did not lead to significant differences between treatment groups of adults with respect to body mass, lysis titres, agglutination titres, haptoglobin concentrations, proportions of heterophils, lymphocytes, eosinophils, monocytes and the H/L-ratio (always $P > 0.18$, $F < 2.06$; Fig. 9.1A-F, Appendix 2).

Short-them handicap had moderate effects on nest success measure. Control nests had a success rate of 76% compared with 47% for experimental nests, but this difference was borderline non-significant ($\chi^2{}_{1,40} = 3.69$, $P = 0.055$). As a consequence control pairs produced more fledglings ($\chi^2{}_{1,39} = 4.14$, $P = 0.042$). Restricting the comparison to successful nests only, we found no difference in fledgling numbers between treatment groups ($\chi^2{}_{1,26} = 0.02$, $P = 0.89$). Control nests produced 0.22 recruits per fledgling and experimental nests 0.11, a non-significant difference ($\chi^2{}_{1,25} = 0.82$, $P = 0.37$).

Short-term handicap impacted nestling quality. Nestlings raised by experimental parents had higher haptoglobin concentrations and agglutination titres than those raised by control parents in 2007, but this pattern was reversed in 2008 (year*treatment interaction $\chi^2{}_{1,62} = 4.05$, $P = 0.044$; Fig. 9.1G and $\chi^2{}_{1,62} = 4.84$, $P = 0.028$, Fig. 9.1H). Nestlings of experimental parents were 7.9% lighter than control nestlings (Fig. 9.1F), but this difference - consistent in 2007 and 2008 – was not significant ($\chi^2{}_{1,69} = 1.66$, $P = 0.19$). Feeding rates equaled 9.9 ± 1.34 visits per hour in control ($n = 11$ nests) and 11.8 ± 2.20 visits per hour in experimental group ($n = 9$ nests), a non-significant differ-

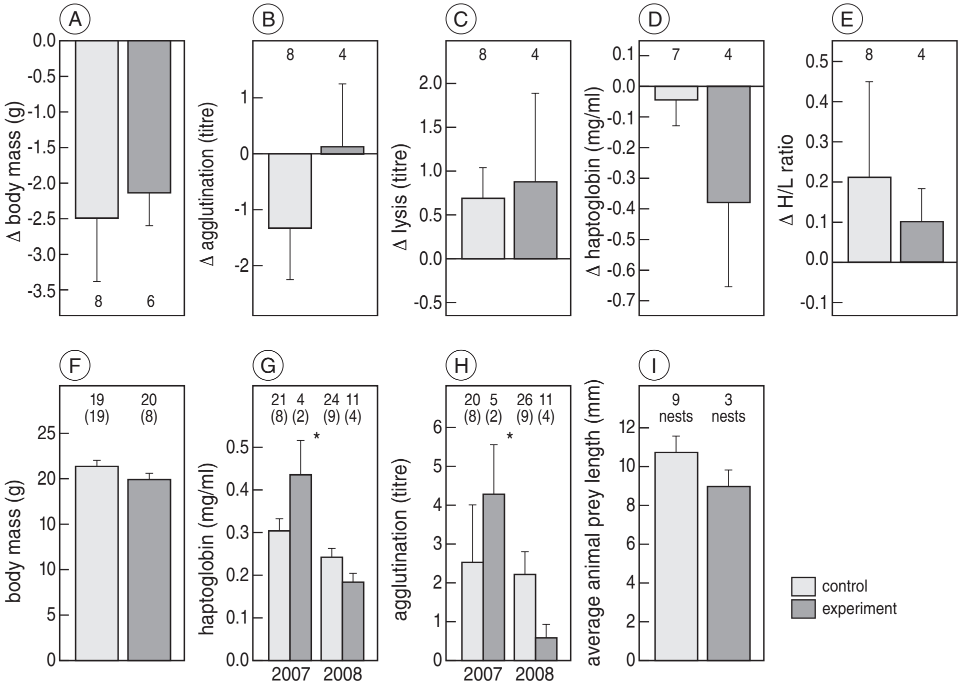

Figure 9.1: Short-term (within-brood) effects of an experimental handicap on the trade-off between reproduction and self-maintenance in Skylarks. A) – E) body mass and immune parameters in adult Skylarks. Values are expressed as the difference between the baseline measure taken when their nestlings were small, and the final measure taken when their nestlings were about to fledge. F)-H) body mass and immune measures of nestling Skylarks from control and experimental parents; the latter were assigned to treatment groups 0-7 days earlier. I) Average length of animal prey in droppings of nestling Skylarks. Bars depict mean and standard error. Numbers represent sample size of individual birds. For nestlings the number of nests is given in parentheses. If both years are plotted the interaction between year and treatment was significant. Statistical analyses can be found in Results and Appendix 2.

ence ($\chi^2_{1,20} = 0.50$, $P = 0.48$, Fig. 9.1J). We found no significant differences between treatment groups in size ($F_{1,13} = 0.51$, $P = 0.49$, Fig. 9.1I) number ($\chi^2_{1,9} = 0.43$, $P = 0.57$) or diversity ($\chi^2_{1,9} = 0.25$, $P = 0.61$) of prey fed to nestlings.

### *Within-season effects*

We found a second nest for 66% of all birds. The chance to find a second nest did not differ between treatment groups ($\chi^2_{1,43} = 0.46$, $P = 0.50$) or years ($\chi^2_{1,42} = 0.05$, $P = 0.83$). The experimental treatment had a significant effect on lysis and agglutination titres of adult Skylarks with the effect on agglutination being dependent on year (Fig. 9.2 B,C). Lysis titres increased in both groups from first to second broods but the increase was weaker in experimental birds than in control birds ($F_{1,27} = 4.79$, $P = 0.037$). Agglutination titres decreased in 2007 in control birds, but increased in experimental birds,

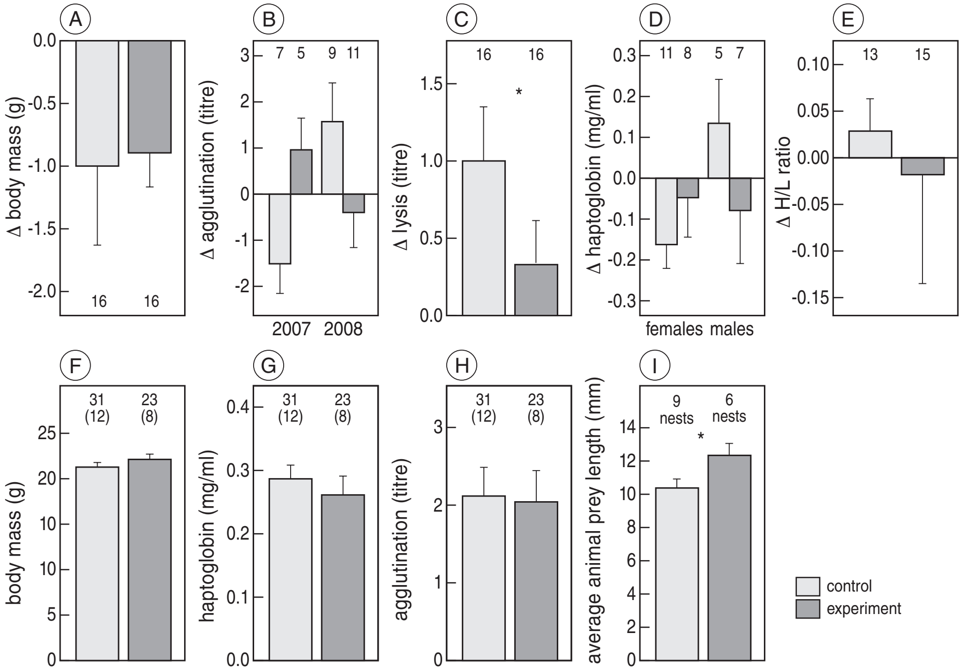

**Figure 9.2:** Medium-term (within-season) effects of an experimental handicap on the trade-off between reproduction and self-maintenance in Skylarks. A) – E) body mass and immune parameters in adult Skylarks 27-73 (median 36.5) days after the experiment started. Values are expressed as the difference between second and first broods. F)-H) body mass and immune measures of nestling Skylarks of control and experimental parents; the latter were assigned to treatment during first broods. I) Average length of animal prey in droppings of nestling Skylarks. Bars depict mean and standard error. Numbers represent sample size of individual birds. For nestlings the number of nests is given in parentheses. Stars denote statistically significant differences. If both years are plotted the interaction between year and treatment was significant. For haptoglobin the interaction sex × treatment was significant and hence results are plotted per sex and treatment. Statistical analyses can be found in Results and Appendix 2.

while this pattern was reversed in 2008 (treatment × year interaction: $F_{1,26} = 5.27$, $P = 0.030$). Haptoglobin concentrations were affected by treatment and sex (treatment × sex interaction: $F_{1,24} = 5.85$, $P = 0.023$; Fig. 9.2D). In females, haptoglobin concentrations increased more strongly in experimental birds than in control birds, while concentrations in control males increased and in experimental males decreased. In both groups the change in proportion of lymphocytes and eosinophils was negatively correlated with baseline values. However this correlation was stronger in experimental birds (treatment × baseline $F_{1,23} = 7.41$, $P = 0.012$ for lymphocytes and $F_{1,23} = 5.24$, $P = 0.031$ for eosinophils). From first to second brood adult Skylarks exhibited decreased body mass, increased proportions of heterophils and stable H/L-ratios and proportions of monocytes, but experimental and control birds did not differ in any of these changes (always $P > 0.23$, $F < 1.53$, Figure 9.2A,E, Appendix 2).

The probability of nest success during the second half of the season differed between treatment groups depending on year. In 2007, 78% of control nests were successful compared with 25% of experimental nests. In 2008, 62% of control and 87% of experimental nests were successful (interaction year × treatment $\chi^2_{1,25} = 4.52$, $P = 0.033$). Restricted to successful nests, number of fledglings did not differ between control and experimental nests ($\chi^2_{1,17} = 0.11$, $P = 0.74$). The number of recruits per fledgling was 0.10 for control nests and 0.15 for experimental nests, a non-significant difference ($\chi^2_{1,17} = 0.41$, $P = 0.52$).

Body mass ($\chi^2_{1,52} = 0.89$, $P = 0.34$), agglutination titre ($\chi^2_{1,53} = 0.60$, $P = 0.44$) and haptoglobin concentration ($\chi^2_{1,53} = 0.05$, $P = 0.82$) of nestlings did not differ between treatments (Fig. 9.2F-H). The droppings of experimental nestlings contained remains of longer animal prey than control groups ($\chi^2_{1,13} = 4.37$, $P = 0.037$, Fig. 9.2I). The number of animals ($F_{1,13} = 1.81$, $P = 0.20$) and the diversity of prey ($F_{1,13} = 2.28$, $P = 0.13$) did not differ between groups.

### *Carry-over effects*

In 2008 return rates of previously handicapped birds were considerably lower than of control birds (40.0% versus 85.7%). In 2009 72.7% of experimental birds and 66.6% of control birds returned (interaction treatment × year $\chi^2_{1,26} = 2.22$, $P = 0.14$). Combining both years, previously handicapped birds showed lower return rates than control birds

**Table 9.1:** Carry-over effects on body mass, immune parameters and reproductive measures in the year following the experiment. Shown are mean values, standard errors and sample sizes per treatment group. Statistical analyses can be found in Results and Appendix 2.

| Parameter | Control mean ± se | Experimental mean ± se | N (Control/ Experimental) | F/Chisq | *P* |
|---|---|---|---|---|---|
| Body mass (g) | 32.5 ± 1.3 | 34.6 ± 1.1 | 11(5/6) | 2.54 | 0.15 |
| Lysis (titre) | 0.45 ± 0.23 | 1.54 ± 0.43 | 11(5/6) | 4.43 | 0.06 |
| Agglutination (titre) | 3.8 ± 0.2 | 4.5 ± 0.4 | 11(5/6) | 1.77 | 0.22 |
| Haptoglobin (mg/ml) | 0.33 ± 0.04 | 0.39 ± 0.03 | 11(5/6) | 0.65 | 0.44 |
| H/L ratio | 0.27 ± 0.06 | 0.61 ± 0.23 | 11(5/6) | 0.98 | 0.36 |
| Heterophils | 17.2 ± 2.4 | 24.6 ± 5.7 | 11(5/6) | 2.27 | 0.17 |
| Lymphocytes | 67 ± 5.6 | 50 ± 6.5 | 11(5/6) | 1.28 | 0.30 |
| Monocytes | 4.6 ± 2.2 | 5.2 ± 0.7 | 11(5/6) | 2.66 | 0.29 |
| Eosinophils | 11.2 ± 3.5 | 13.2 ± 3.2 | 11(5/6) | 0.00 | 0.98 |
| Nest success/attempt | 27.8% | 42.1% | 37(18/19) | 1.20 | 0.27 |
| Fledglings/successful brood | 3.4 ± 0.40 | 3.75 ± 0.16 | 16(5/8)* | 0.72 | 0.40 |
| Nestling body mass | 23.1 ± 0.55 | 22.4 ± 0.52 | 44 (17/27)<br>13 (5/8)[1] | 0.85 | 0.36 |
| Nestling agglutination (titre) | 2.0 ± 0.45 | 1.6 ± 0.36 | 40 (15/25)<br>13 (5/8)[1] | 1.17 | 0.28 |
| Nestling haptoglobin (mg/ml) | 0.23 ± 0.01 | 0.24 ± 0.02 |  | 0.24 | 0.63 |

* number of successful nests, not number of fledglings [1] number of nests

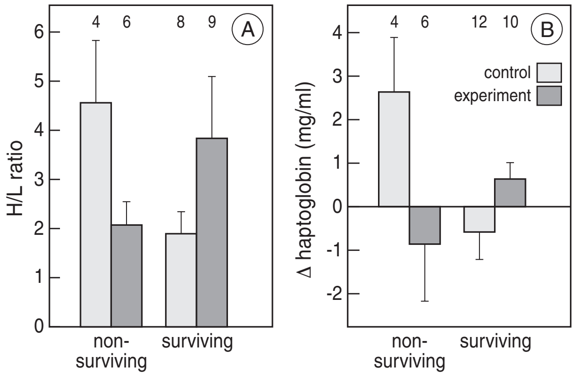

**Figure 9.3:** A) H/L-ratio at second brood and change in agglutination from first to second brood for experimental and control Skylarks that returned the year after and for birds that did not return. The interaction between survival and treatment was significant in both cases. Statistical analyses can be found in Results.

(62.5% versus 75%) but the difference was not significant ($\chi^2_{1,29} = 0.97$, $P = 0.33$). In the year following the experiment, surviving birds did not differ between treatment groups in reproductive parameters (Table 9.1). Moreover, the recaptured birds did not differ in any of the physiological parameters between treatment groups (Table 9.1). There was no relationship between the magnitude of change in any physiological parameter during the experiment and its value in the following year (always $P > 0.27$, $F < 1.38$), *e.g.* birds that lost more mass during the experiment were not necessarily the lightest ones in the following year.

### *Prediction of survival by immune function*

We explored for the two groups if the immune parameters measured at the end of the experiment differed between birds that returned in the next year and birds that did not return (interaction treatment × immune parameter). Separated by treatment, survivors and non-survivors differed in H/L-ratio and agglutination (Fig. 9.3), but not in any of the other immune parameters (always $\chi^2 < 1.25$, $P > 0.26$). Returning control Skylarks had lower H/L-ratios at the end of the experiment than non-returning birds. This pattern was reversed in experimental birds (treatment × H/L-ratio: $\chi^2_{1,23} = 6.58$, $P = 0.010$). This interaction occurred with both the proportion of heterophils ($\chi^2_{1,23} = 6.01$, $P = 0.014$) and lymphocytes ($\chi^2_{1,23} = 4.33$, $P = 0.037$). A similar trend occurred in agglutination titres at the end of the experiment (treatment × agglutination: $\chi^2_{1,27} = 3.67$, $P = 0.055$). The change in agglutination titre during the experiment from first to second brood predicted survival in control birds differently than in experimental birds (Fig. 9.3B). In control Skylarks, birds that returned had decreased their agglutination titres during the experiment, while birds that did not return showed an increase. In experimental birds survivors showed increased agglutination during the experiment, while non-survivors decreased agglutination (treatment × delta agglutination titre: $\chi^2_{1,27} = 6.55$, $P = 0.010$).

## Discussion

Skylarks handicapped by an extra weight modulated the trade-off between parental effort and investment into immune function differently at different time scales. Differences among years further influence this trade-off. While we found no effect on adult immune function on the short term, reproductive output of experimentally handicapped birds was decreased during first broods. However, later during the season after birds carried their extra weight for several weeks and had their second broods, adults exhibited differences in their immune function. Handicapped adults also changed their behavior and brought different food to their nestlings. While nestlings of control and experimental pairs did not differ in body mass, experimental nestlings from first broods showed altered immune parameters and had lower recruitment rates. Reduced survival of adults after removal of the handicap points to carry-over effects that were likely mediated by changes in the immune system. The H/L-ratio at the end of the experiment and the change in agglutination titres during the experiment predicted survival to the next year. In the breeding season one year after the experiment, treatment groups no longer differed in terms of immune parameters or reproductive performance.

After attachment of the extra weights, experimental birds faced higher nest failure rates, their fledglings expressed altered immune responses and these were less likely to be detected as recruits. However, we found no effects on the immune system or body mass in adults. This strongly indicates that during first broods, Skylarks shift the costs of increased work load onto the nestlings. Such a pattern has been described for several species (Mauck & Grubb 1995, Moreno *et al.* 1999, Alonso-Alvarez & Tella 2001, Navarro & Gonzalez-Solis 2007) but is generally associated with long-lived rather than short-lived species (Williams 1966, Mauck & Grubb 1995). By their second brood, handicapped adult Skylarks modulated several of their own immune indices, but their parental effort was not different from controls. This result suggests that the costs shifted back to the parents while parental effort was maintained. To our best knowledge, our study is the first to document a shift in the trade-off between reproduction and self-maintenance from first to second broods within a breeding season that is mediated by physiological changes.

Our study shows that the outcome of this trade-off differs over time and leads to carry-over effects for parents and their offspring, resulting in reduced survival rates and reduced recruitment rates, respectively. Nestlings had altered immune defences during first broods and adult Skylarks modulated their immune system after being handicapped for several weeks. We show that these immunological effects lead to reductions in survival of adults and recruitment of nestlings. Trade-offs between reproduction and immune investment are known (Deerenberg *et al.* 1997, Nordling *et al.* 1998, Hasselquist *et al.* 2001, Ardia *et al.* 2003, Knowles *et al.* 2009), but studies on the trade-offs between current reproduction and self-maintenance are often limited to one or two time points (*e.g.* Tinbergen 1987, Tieleman *et al.* 2008, Leclaire *et al.* 2010). Our results extend these findings by showing that a modulation of the immune system occurred only during second broods, when birds carried the extra weight for a few weeks, but not during the first brood, when they carried it for a couple of days.

One might ask, if reduced return rates indeed reflect survival rather than dispersal because birds with increased work load might anticipate the conditions as poor and emigrate. However, our study shows that Skylarks modulate parameters of the immune system and these predicted survival. Our findings build on the results of Daan *et al.* (1996), who demonstrated that kestrels indeed show increased mortality during winter rather than emigration. While their study lacked a mechanistic link, we provide evidence that changes in immune defences act as a mediator. In the year after the experiment, we did not find any immunological or reproductive effects of the experiment, while such carry-over effects are known for other species (Wernham & Bryant 1998).

Experimental manipulations of parental effort often have no effect on nestling body mass or structural size (Sanz *et al.* 2000, Tieleman *et al.* 2008, Leclaire *et al.* 2010). These negative results are typically interpreted as maintenance of current parental effort. In our study, feeding rates and body mass of fledglings did not differ between treatment groups. Despite this, nestlings did differ in terms of immunological indices, which suggest adjustments in parental effort. One mediator may be diet composition because developing the immune system requires energy and specific nutrients (Klasing 2004). Indeed, we found that parents brought a modified diet to their nestlings. Changes in foraging behavior have been described previously for handicapped birds (Tinbergen 1981, Wright & Cuthill 1990a, Wright & Cuthill 1990b, Wright *et al.* 1998, Beaulieu *et al.* 2010), but clear links to the physiology of the nestlings have reminded elusive. We shed light on these links by showing that dietary differences correlate with immunological effects and lowered recruitment rates.

In conclusion, we demonstrated that a temperate passerine bird species modulates the trade-off between current reproduction and survival differently over short-, medium- and long-time scales. Further we provided evidence that investment into the immune system is one physiological mechanism that mediates survival in adults and recruitment of their offspring.

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## Appendix 1: Details of statistical methods

We analysed data using R version 2.14.0 (R Development Core Team 2011). For within-brood and within-season measurements we used linear models with the difference between the two time points for every dependent variable. We always included treatment group, time since baseline measurement, sex and year. We also included baseline values as a fixed effect into the models to account for different starting values (e.g. birds with a high body mass might be able to lose more mass than initially light birds). The interaction between treatment and time since baseline measurement was included in within-brood analyses. Interactions between treatment and sex, year and time since baseline measurement were included in within-season analyses. Within-brood analyses included the interaction between treatment and time since initiation of the experiment. Julian day had no effect on baseline or within-season data and was therefore not included in analyses. Data collected on birds that we recaptured the following year were analysed with linear models using the measurement taken at recapture as response variable and treatment, sex, year, length of the experiment in days (from initiation until extra weights were removed from experimental birds), and the change in the explanatory variable during the experiment, as predictors. Because response variables were not calculated as a difference between two time points, but included as single time point measurements, we analyzed the proportion of each WBC type against the total WBC number minus the focal WBC type using a binomial approach.

Nest success, number of fledglings and number of recruits were analysed on the nest level with generalized linear models and the appropriate corresponding error structure (binomial, poisson, Gaussian or quasibinomial and quasipoisson when overdispersion occurred). Treatment, year, age of nestlings when the experiment started (or stopped, for second broods) and the two-way-interaction between treatment and year were always included. Feeding rates were tested with a generalized linear mixed model with poisson error distribution and including nest as random effect to avoid pseudoreplication. Models included treatment, age of nestlings, days since initiation of the experiment and the two-way-interactions with treatment. The variables from the dropping analyses were tested in generalized linear models with year, treatment and number of nestlings as fixed effects and a quasipoisson (number of animal prey), poisson (diversity of prey items) or Gaussian (length of prey items) error structure.

Individual nestling data were analysed with linear mixed models including sex, treatment, year, age, number of nestlings and age of nestlings when parents were captured as well as the two-way interactions of treatment with year and number of nestlings. Nest identity was always included as random factor to account for non-independence of siblings.

Survival and recruitment data were analysed with generalized linear models with binomial error structure and treatment, sex, year, length of the experiment and the two-way interactions involving treatment as fixed explanatory variables. We tested if survival could be predicted by any measurement at the end of the experiment by sequentially including the interaction of treatment with each measured immune parameter and body mass as well as their change during the experiment. As covariates we included the length of the experiment (in days), sex and year.

We always started with the full model and simplified it using backward elimination based on likelihood-ratio test and F-Statistics (Chisq-Statistics for generalized linear models with binomial or poisson error structure) and with $P < 0.05$ as the selection criterion ("drop1"-function of R) until reaching the minimal adequate model. Assumptions of all models were checked on the residuals of the final model. We report interactions only when significant.

## Appendix 2: Statistics and coefficients

**Table A.1:** (Right and next page) Statistics and coefficients of the linear models for within-brood analyses during a handicap experiment in Skylarks. Results are from linear models after removing all non-significant interactions ($P > 0.05$). *P*-values < 0.05 are bold. Beta values are only given for significant factors. A) Adults, B) Nest, C) Nestlings. Nest data were analysed with generalized linear models and the appropriate error structure. For nestlings and feeding rates, nest identity was included as random effect. For further details see statistic section in methods. t = number of days since baseline measurement; Baseline=measurement of y at the initial capture.

**A) Adults**

| y-variable | Source of variation | *df* | *F* | *P* | mean | beta | reference |
|---|---|---|---|---|---|---|---|
| Δ Body mass | ….. | | | | -2.34 | | |
| | Treatment | 1 | 0.45 | 0.521 | | | |
| | Year | 1 | 0.34 | 0.574 | | | |
| | Sex | 1 | 1.18 | 0.299 | | | |
| | t | 1 | 0.31 | 0.591 | | | |
| | Baseline | 1 | 2.03 | 0.182 | | | |
| Δ Lysis titre | ….. | | | | 0.75 | | |
| | Treatmentment | 1 | 0.16 | 0.699 | | | |
| | Year | 1 | 4.69 | 0.062 | | | |
| | Sex | 1 | 2.04 | 0.196 | | | |
| | t | 1 | 11.77 | **0.008** | | -0.78 | |
| | Baseline | 1 | 16.83 | **0.003** | | -0.90 | |
| Δ Agglutination titre | ….. | | | | -0.83 | | |
| | Treatment | 1 | 0.46 | 0.523 | | | |
| | Year | 1 | 14.89 | **0.004** | | 2.19 | 2008 |
| | Sex | 1 | 3.19 | 0.112 | | | |
| | t | 1 | 0.15 | 0.710 | | | |
| | Baseline | 1 | 64.75 | **<0.001** | | -0.95 | |
| Δ Haptoglobin | ….. | | | | -0.17 | | |
| | Treatment | 1 | 0.28 | 0.622 | | | |
| | Year | 1 | 0.11 | 0.750 | | | |
| | Sex | 1 | 1.36 | 0.278 | | | |
| | t | 1 | 0.08 | 0.781 | | | |
| | Baseline | 1 | 1.86 | **<0.001** | | -1.06 | |
| Δ Heterophils | ….. | | | | 8.73 | | |
| | Treatment | 1 | 0.01 | 0.946 | | | |
| | Year | 1 | 0.43 | 0.528 | | | |
| | Sex | 1 | 0.01 | 0.938 | | | |
| | t | 1 | 0.44 | 0.523 | | | |
| | Baseline | 1 | 0.23 | 0.642 | | | |
| Δ Lymphocytes | ….. | | | | 2.11 | | |
| | Treatment | 1 | 0.03 | 0.874 | | | |
| | Year | 1 | 0.39 | 0.405 | | | |
| | Sex | 1 | 0.01 | 0.932 | | | |
| | t | 1 | 1.25 | 0.292 | | | |
| | Baseline | 1 | 3.98 | 0.074 | | | |
| Δ Monocytes | ….. | | | | -1.39 | | |
| | Treatment | 1 | 2.06 | 0.175 | | | |
| | Year | 1 | 0.63 | 0.451 | | | |
| | Sex | 1 | 0.08 | 0.791 | | | |
| | t | 1 | 0.76 | 0.410 | | | |
| | Baseline | 1 | 35.27 | **<0.001** | | -0.97 | |
| Δ Eosinophils | ….. | | | | -9.15 | | |
| | Treatment | 1 | 0.01 | 0.921 | | | |
| | Year | 1 | 0.01 | 0.928 | | | |
| | Sex | 1 | 0.13 | 0.730 | | | |
| | t | 1 | 0.40 | 0.542 | | | |
| | Baseline | 1 | 93.26 | **<0.001** | | -0.76 | |
| Δ H/L-ratio | ….. | | | | 0.17 | | |
| | Treatment | 1 | 0.14 | 0.723 | | | |
| | Year | 1 | 0.65 | 0.444 | | | |
| | Sex | 1 | 0.01 | 0.938 | | | |
| | t | 1 | 1.13 | 0.311 | | | |
| | Baseline | 1 | 0.94 | 0.356 | | | |

**B) Nests**

| y-variable | Source of variation | *df* | $\chi^2/F$ | *P* | mean | beta | reference |
|---|---|---|---|---|---|---|---|
| Nest success | ….. | | | | 0.64 | | |
| | Treatment | 1 | 3.69 | 0.055 | | | |
| | Year | 1 | 2.23 | 0.135 | | | |
| Number fledglings (successful nests only) | ….. | | | | 1.69 | | |
| | Treatment | 1 | 0.02 | 0.889 | | | |
| | Year | 1 | 0.00 | 0.999 | | | |
| | Age when exp. started | 1 | 0.70 | 0.404 | | | |
| Recruits per fledgling (successful nests only) | ….. | | | | 0.2 | | |
| | Treatment | 1 | 0.82 | 0.366 | | | |
| | Year | 1 | 0.29 | 0.588 | | | |
| | Age when exp. started | 1 | 0.19 | 0.662 | | | |
| Feeding rate | ….. | | | | 10.75 | | |
| | Treatment | 1 | 0.497 | 0.481 | | | |
| | Age of Nestlings | 1 | 8.50 | **0.004** | | 0.46 | |
| | t | 1 | 0.24 | 0.627 | | | |
| Droppings (length animals) | ….. | | | | 10.3 | | |
| | Treatment | 1 | 0.51 | 0.492 | | | |
| | Year | 1 | 0.31 | 0.591 | | | |
| | Number nestlings | 1 | 8.46 | **0.016** | | -2.1 | |
| Droppings (no animals) | ….. | | | | 25.03 | | |
| | Treatment | 1 | 0.43 | 0.527 | | | |
| | Year | 1 | 0.00 | 0.960 | | | |
| | Number nestlings | 1 | 0.46 | 0.512 | | | |
| Droppings (prey diversity) | ….. | | | | 9.67 | | |
| | Treatment | 1 | 0.25 | 0.614 | | | |
| | Year | 1 | 1.87 | 0.172 | | | |
| | Number nestlings | 1 | 4.86 | **0.027** | | 0.32 | |

**C) Nestlings**

| y-variable | Source of variation | *df* | $\chi^2/F$ | *P* | mean | beta | reference |
|---|---|---|---|---|---|---|---|
| Body mass | …. | | | | 21.06 | | |
| | Treatment | 1 | 1.66 | 0.198 | | | |
| | Year | 1 | 0.46 | 0.498 | | | |
| | Sex | 1 | 36.95 | **<0.001** | | 3.25 | Male |
| | Number nestlings | 1 | 4.35 | **0.037** | | 1.30 | |
| | Age nestlings | 1 | 11.22 | **<0.001** | | 3.74 | |
| | Age at experimental initiation | 1 | 0.33 | 0.567 | | | |
| Agglutination titre | …. | | | | 2.22 | | |
| | Treatment | 1 | See text for interaction | | | | |
| | Year | 1 | See text for interaction | | | | |
| | Sex | 1 | 0.24 | 0.627 | | | |
| | Number nestlings | 1 | 0.39 | 0.533 | | | |
| | Age nestlings | 1 | 0.86 | 0.354 | | | |
| | Age at experimental initiation | 1 | 0.75 | 0.385 | | | |
| Haptoglobin | …. | | | | 0.27 | | |
| | Treatment | 1 | See text for interaction | | | | |
| | Year | 1 | See text for interaction | | | | |
| | Sex | 1 | 0.62 | 0.430 | | | |
| | Number nestlings | 1 | 3.16 | 0.076 | | | |
| | Age nestlings | 1 | 1.18 | 0.276 | | | |
| | Age at experimental initiation | 1 | 0.00 | 0.983 | | | |

**Table A.2:** (Next pages) Statistics and coefficients of the linear models for within-season (second brood) analyses during a handicap experiment in Skylarks. Results are from linear models after removing all non-significant interactions ($P > 0.05$). $P$-values $< 0.05$ are bold. A) Adults, B) Nest, C) Nestlings. Nest data were analysed with generalized linear models and the appropriate error structure. For nestlings nest identity was included as random effect. For further details see statistic section in methods. t = number of days since baseline measurement; Baseline=measurement of y at the initial capture.

A) **Adults**

| y-variable | Source of variation | *df* | *F* | *P* | mean | beta | reference |
|---|---|---|---|---|---|---|---|
| Δ Body mass | ….. | | | | -0.95 | | |
| | Treatment | 1 | 0.00 | 0.995 | | | |
| | Year | 1 | 1.26 | 0.271 | | | |
| | Sex | 1 | 5.30 | **0.029** | | 1.57 | Male |
| | t | 1 | 7.52 | **0.011** | | -0.07 | |
| | Baseline | 1 | 9.29 | **0.005** | | -0.38 | |
| Δ Lysis titre | ….. | | | | 0.66 | | |
| | Treatment | 1 | 4.79 | **0.037** | | -0.82 | handicapped |
| | Year | 1 | 5.15 | **0.031** | | -1.05 | 2008 |
| | Sex | 1 | 2.19 | 0.152 | | | |
| | t | 1 | 3.49 | **0.073** | | | |
| | Baseline | 1 | 4.78 | **0.038** | | -0.39 | |
| Δ Agglutination titre | ….. | | | | 0.15 | | |
| | Treatment | 1 | See text for interaction | | | | |
| | Year | 1 | See text for interaction | | | | |
| | Sex | 1 | 0.02 | 0.902 | | | |
| | t | 1 | 0.64 | 0.432 | | | |
| | Baseline | 1 | 18.28 | <**0.001** | | -0.71 | |
| Δ Haptoglobin | ….. | | | | -0.07 | | |
| | Treatment | 1 | See text for interaction | | | | |
| | Year | 1 | 12.59 | 0.002 | | -0.23 | 2008 |
| | Sex | 1 | See text for interaction | | | | |
| | t | 1 | 0.12 | 0.729 | | | |
| | Baseline | 1 | 43.49 | <**0.001** | | -0.29 | |
| Δ Heterophils | ….. | | | | 0.03 | | |
| | Treatment | 1 | 0.01 | 0.933 | | | |
| | Year | 1 | 1.47 | 0.237 | | | |
| | Sex | 1 | 1.01 | 0.325 | | | |
| | t | 1 | 0.04 | 0.837 | | | |
| | Baseline | 1 | 8.84 | **0.006** | | -0.61 | |
| Δ Lymphocytes | ….. | | | | 0.004 | | |
| | Treatment | 1 | See text for interaction | | | | |
| | Year | 1 | 2.86 | 0.105 | | | |
| | Sex | 1 | 1.74 | 0.201 | | | |
| | t | 1 | 0.22 | 0.644 | | | |
| | Baseline | 1 | See text for interaction | | | | |
| Δ Monocytes | ….. | | | | -0.0003 | | |
| | Treatment | 1 | 1.53 | 0.228 | | | |
| | Year | 1 | 0.58 | 0.453 | | | |
| | Sex | 1 | 0.16 | 0.696 | | | |
| | t | 1 | 1.09 | 0.307 | | | |
| | Baseline | 1 | 32.99 | <**0.001** | | -1.19 | |
| Δ Eosinophils | ….. | | | | 0.03 | | |
| | Treatment | 1 | See text for interaction | | | | |
| | Year | 1 | 1.32 | 0.264 | | | |
| | Sex | 1 | 0.69 | 0.416 | | | |
| | t | 1 | 0.27 | 0.612 | | | |
| | Baseline | 1 | See text for interaction | | | | |
| Δ H/L-ratio | ….. | | | | 0.04 | | |
| | Treatment | 1 | 0.05 | 0.823 | | | |
| | Year | 1 | 2.29 | 0.143 | | | |
| | Sex | 1 | 0.77 | 0.388 | | | |
| | t | 1 | 0.05 | 0.818 | | | |
| | Baseline | 1 | 20.10 | <**0.001** | | -0.74 | |

**B) Nests**

| y-variable | Source of variation | *df* | $\chi^2/F$ | $P$ | mean | beta | reference |
|---|---|---|---|---|---|---|---|
| Nest success | ….. | | | | 0.69 | | |
| | Treatment | 1 | See text for interaction | | | | |
| | Year | 1 | See text for interaction | | | | |
| Number fledglings (successful nests only) | ….. | | | | 2.28 | | |
| | Treatment | 1 | 0.11 | 0.735 | | | |
| | Year | 1 | 0.03 | 0.801 | | | |
| | Age of Nestlings | 1 | 0.58 | 0.446 | | | |
| | Age when parents recaptured | 1 | 0.12 | 0.728 | | | |
| Recruits per fledgling (successful nests only) | ….. | | | | 0.21 | | |
| | Treatment | 1 | 0.41 | 0.524 | | | |
| | Year | 1 | 0.01 | 0.926 | | | |
| | Age of Nestlings | 1 | 0.36 | 0.550 | | | |
| | Age when parents recaptured | 1 | 1.81 | 0.178 | | | |
| Droppings (length animals) | ….. | | | | 11.3 | | |
| | Treatment | 1 | 4.37 | **0.037** | | 2.02 | handicapped |
| | Year | 1 | 2.72 | 0.099 | | | |
| | Number nestlings | 1 | 0.39 | 0.532 | | | |
| Droppings (no animals) | ….. | | | | 33.0 | | |
| | Treatment | 1 | 1.81 | 0.201 | | | |
| | Year | 1 | 0.04 | 0.836 | | | |
| | Number nestlings | 1 | 0.62 | 0.448 | | | |
| Droppings (prey diversity) | ….. | | | | 11.1 | | |
| | Treatment | 1 | 2.28 | 0.131 | | | |
| | Year | 1 | 0.17 | 0.680 | | | |
| | Number nestlings | 1 | 0.71 | 0.400 | | | |

**C) Nestlings**

| y-variable | Source of variation | **df** | $\chi^2$ | $P$ | mean | beta | reference |
|---|---|---|---|---|---|---|---|
| Body mass | …. | | | | 21.62 | | |
| | Treatment | 1 | 0.89 | 0.344 | | | |
| | Year | 1 | 1.85 | 0.174 | | | |
| | Sex | 1 | 15.11 | **0.001** | | 2.65 | Male |
| | Number nestlings | 1 | 0.24 | 0.617 | | | |
| | Age nestlings | 1 | 0.03 | 0.859 | | | |
| Agglutination titre | …. | | | | 2.08 | | |
| | Treatment | 1 | 0.60 | 0.438 | | | |
| | Year | 1 | 3.78 | 0.052 | | | |
| | Sex | 1 | 10.63 | **0.001** | | 1.74 | Male |
| | Number nestlings | 1 | 0.37 | 0.534 | | | |
| | Age nestlings | 1 | 0.04 | 0.848 | | | |
| Haptoglobin | …. | | | | 0.28 | | |
| | Treatment | 1 | 0.05 | 0.821 | | | |
| | Year | 1 | 2.74 | 0.097 | | | |
| | Sex | 1 | 6.27 | **0.012** | | -0.09 | Male |
| | Number nestlings | 1 | 0.19 | 0.658 | | | |
| | Age nestlings | 1 | 5.14 | **0.023** | | 0.08 | |

**A) Adults**

| y-variable | Source of variation | *df* | *F* | *P* | mean | beta | reference |
|---|---|---|---|---|---|---|---|
| Body mass | ….. | | | | 33.65 | | |
| | Treatment | 1 | 2.54 | 0.150 | | | |
| | Year | 1 | 0.11 | 0.753 | | | |
| | Sex | 1 | 23.80 | **<0.001** | | 5.28 | Male |
| | Length experiment | 1 | 0.74 | 0.417 | | | |
| | Δ body mass | 1 | 0.02 | 0.882 | | | |
| Lysis titre | ….. | | | | 1.05 | | |
| | Treatment | 1 | 4.43 | 0.065 | | | |
| | Year | 1 | 0.11 | 0.753 | | | |
| | Sex | 1 | 1.60 | 0.241 | | | |
| | Length experiment | 1 | 0.02 | 0.889 | | | |
| | Δ lysis titre | 1 | 1.16 | 0.317 | | | |
| Agglutination titre | ….. | | | | 4.18 | | |
| | Treatment | 1 | 1.77 | 0.216 | | | |
| | Year | 1 | 0.458 | 0.520 | | | |
| | Sex | 1 | 0.11 | 0.747 | | | |
| | Length experiment | 1 | 0.07 | 0.800 | | | |
| | Δ agglutination titre | 1 | 1.00 | 0.346 | | | |
| Haptoglobin | ….. | | | | 0.36 | | |
| | Treatment | 1 | 0.658 | 0.444 | | | |
| | Year | 1 | 2.53 | 0.146 | | | |
| | Sex | 1 | 0.52 | 0.502 | | | |
| | Length experiment | 1 | 2.09 | 0.186 | | | |
| | Δ haptoglobin | 1 | 1.15 | 0.324 | | | |
| Heterophils | ….. | | | | 21.3 | | |
| | Treatment | 1 | 2.27 | 0.166 | | | |
| | Year | 1 | 0.07 | 0.794 | | | |
| | Sex | 1 | 0.26 | 0.624 | | | |
| | Length experiment | 1 | 0.01 | 0.915 | | | |
| | Δ heterophils | 1 | 0.02 | 0.904 | | | |
| Lymphocytes | ….. | | | | 57.7 | | |
| | Treatment | 1 | 1.27 | 0.296 | | | |
| | Year | 1 | 3.87 | 0.081 | | | |
| | Sex | 1 | 5.15 | 0.053 | | | |
| | Length experiment | 1 | 0.03 | 0.867 | | | |
| | Δ lymphocytes | 1 | 0.23 | 0.651 | | | |
| Monocytes | ….. | | | | 4.9 | | |
| | Treatment | 1 | 2.66 | 0.147 | | | |
| | Year | 1 | 0.03 | 0.878 | | | |
| | Sex | 1 | 7.08 | **0.026** | | 0.96 | Male |
| | Length experiment | 1 | 1.91 | 0.216 | | | |
| | Δ monocytes | 1 | 1.38 | 0.274 | | | |
| Eosinophils | ….. | | | | 12.3 | | |
| | Treatment | 1 | 0.00 | 0.975 | | | |
| | Year | 1 | 9.21 | **0.014** | | 1.32 | 2009 |
| | Sex | 1 | 3.96 | 0.082 | | | |
| | Length experiment | 1 | 0.01 | 0.910 | | | |
| | Δ eosinophils | 1 | 0.30 | 0.603 | | | |
| H/L-ratio | ….. | | | | 0.46 | | |
| | Treatment | 1 | 0.98 | 0.359 | | | |
| | Year | 1 | 1.57 | 0.250 | | | |
| | Sex | 1 | 0.52 | 0.503 | | | |
| | Length experiment | 1 | 0.02 | 0.896 | | | |
| | Δ H/L-ratio | 1 | 1.28 | 0.292 | | | |

**B) Nests**

| y-variable | Source of variation | *df* | $\chi^2$ | *P* | mean | beta | reference |
|---|---|---|---|---|---|---|---|
| Nest success | ….. | | | | 0.35 | | |
| | Treatment | 1 | 1.20 | 0.273 | | | |
| | Year | 1 | 2.93 | 0.087 | | | |
| Number fledglings (all nests) | ….. | | | | 1.3 | | |
| | Treatment | 1 | 0.72 | 0.395 | | | |
| | Year | 1 | 1.93 | 0.165 | | | |

**C) Nestlings**

| y-variable | Source of variation | *df* | $\chi^2$ | *P* | mean | beta | reference |
|---|---|---|---|---|---|---|---|
| Body mass | …. | | | | 22.7 | | |
| | Treatment | 1 | 0.85 | 0.358 | | | |
| | Year | 1 | 10.38 | **0.001** | | 2.32 | 2009 |
| | Sex | 1 | 41.79 | **<0.001** | | 3.22 | Male |
| | Number nestlings | 1 | 2.50 | 0.114 | | 1.83 | |
| | Age nestlings | 1 | 7.97 | **0.005** | | | |
| Agglutination titre | …. | | | | 1.8 | | |
| | Treatment | 1 | 1.17 | 0.280 | | | |
| | Year | 1 | 2.27 | 0.132 | | | |
| | Sex | 1 | 1.86 | 0.173 | | | |
| | Number nestlings | 1 | 2.18 | 0.139 | | | |
| | Age nestlings | 1 | 1.28 | 0.258 | | | |
| Haptoglobin | …. | | | | 0.24 | | |
| | Treatment | 1 | 0.24 | 0.626 | | | |
| | Year | 1 | 1.80 | 0.180 | | | |
| | Sex | 1 | 0.01 | 0.924 | | | |
| | Number nestlings | 1 | 0.18 | 0.670 | | | |
| | Age nestlings | 1 | 0.753 | 0.386 | | | |

**Table A.3:** Statistics and coefficients of the linear models for carry-over effects after a handicap experiment in Skylarks. Results are from linear models after removing all non-significant interactions ($P > 0.05$). *P*-values $< 0.05$ are bold. A) Adults, B) Nest, C) Nestlings. Nest data were analysed with generalized linear models and the appropriate error structure. For nestlings, nest identity was included as random effect. For further details see statistic section in methods. t = number of days since baseline measurement; Baseline=measurement of y at the initial capture.

# CHAPTER 10

# Partial migration has carry-over effects on immune function, body mass and return rates in a passerine bird

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**Unpublished manuscript**

## Abstract

Partial migration in birds occurs when some individuals of a breeding population migrate seasonally to non-breeding areas while other individuals of the same population remain resident year-around. Studying partial migrants offers the unique opportunity to investigate the causes and consequences of migration by comparing migrants and residents from the same population. Such studies can reveal if the wintering strategy, either migration or local wintering, has carry-over effects on the fitness of an individual bird. We analysed stable hydrogen isotopes in claw material to determine if individual Skylarks (*Alauda arvensis*) were migratory or resident the previous winter. We then compared migratory status to several immune parameters, body mass and reproductive performance in the subsequent breeding season as well as future return rates. We demonstrate that the wintering strategy has carry-over effects on immune function, body mass and future return rates. Resident Skylarks had higher haptoglobin concentrations in some years, showed a trend of having lower lysis titres, and had lower return rates over the following winter than migrants. Furthermore residents had higher body mass than migrants, but this difference was partly explained by a difference in size. Reproductive parameters, agglutination titres and wing length did not differ between groups. Future return rates showed roughly the reverse pattern of haptoglobin concentrations, which suggests links between immune function and survival. For 20 individuals we could assign a wintering strategy for multiple winters, and 45% of these birds switched strategies between winters. Thus, the decision to migrate is not genetically determined but flexible. We conclude that migrating or wintering locally has carry-over effects on immune function, which we hypothesise to increase mortality and consequently result in different return rates after the following winter. Differences in carry-over effects between years probably reflect different evolutionary costs and benefits of both strategies in different years, resulting in coexistence. That resident Skylarks have lower subsequent return rates could indicate deteriorated local wintering conditions and thus has important implications for conservation measures for this rapidly declining species.

## Introduction

Partial migration occurs when some individuals of a breeding population migrate seasonally to non-breeding areas while other individuals of the same population remain resident year-round (Gauthreaux 1982, Terrill & Able 1988, Alerstam 1990). Even though partial migration is hypothesized to be an evolutionary early form of full migration (Berthold 1996), most studies of avian migration and its causes and consequences focus on obligate long-distance migration (Alerstam 1990, Gwinner 1990, Berthold 2001, Berthold *et al.* 2003, Newton 2008). Studying partial migrants offers the unique opportunity to investigate causes and consequences of migration by comparing migrants and residents of the same population (Adriaensen & Dhondt 1990, Chapman *et al.* 2011a, Chapman *et al.* 2011b, Palacin *et al.* 2011). Because of our inability to track small migratory birds beyond a few kilometres, it has been historically challenging to quantify, but partial migration appears to be more widespread than previously thought and may even be considered the norm among migratory birds (Berthold 2001, Newton 2008). The underlying factor driving partial migration is hypothesized to be a combination of environmental and genetic factors (Schwabl & Silverin 1990, Newton 2008, Chapman *et al.* 2011a, Pulido 2011). Individual birds either can persist in one strategy throughout their life (Gillis *et al.* 2008) or can switch strategies between years (Ogonowski & Conway 2009, Palacin *et al.* 2011).

The decision to migrate to distinct and remote non-breeding (hereafter wintering) areas or to remain resident on breeding areas year-round is likely to affect physiology and fitness. Previous research on partial migrants shows that resident individuals have slower moult and lower basal metabolic rates (Nilsson *et al.* 2011), higher reproductive success (Harper 1985, Adriaensen & Dhondt 1990, Gillis *et al.* 2008) and lower survival rates (Gillis *et al.* 2008). However, these studies focus on either partial migrants with local rather than large-scale movements, compare migrants and residents that winter together and breed in different areas, or study birds with unknown breeding sites. No study has yet investigated the consequences of partial migration within a breeding population by comparing year-round residents with migrates that fly several hundreds or thousands of kilometres to distinct wintering grounds. Such a system is essential to quantify trade-offs, either within a season or as carry-over effect to a subsequent season. Carry-over effects are well established for obligate long-distance migratory birds. Variation in individual condition, date of departure from non-breeding areas, and date of arrival on breeding grounds are supposed causes of carry-over effects (Marra *et al.* 1998, Marra & Holmes 2001, Studds & Marra 2005, Reudink *et al.* 2009). However, the physiological mechanisms underlying carry-over effects are poorly understood (but see Tonra *et al.* 2011a,b). One potential candidate is immune function. The immune system promotes survival by reducing the probability of disease-related mortality (Roitt *et al.* 1998). However, the system incurs costs in terms of its production, maintenance and activation (Schmid-Hempel 2003, Klasing 2004) and is therefore supposed to be traded off against competing physiological and behavioural processes (Sheldon & Verhulst 1996, Lochmiller & Deerenberg 2000, Norris & Evans 2000). For example, migratory birds can delay migration when their immune system is challenged (van Gils *et al.* 2007), but delays likely affect subsequent reproductive output. Alternatively birds can modulate immune function during migration (Owen & Moore 2008, Hegemann *et al.* 2012b), but reductions might affect survival.

Determining the wintering strategy (i.e., migrant vs non-migrant) of individual birds requires knowing where they spent the winter. Stable isotope analyses provide a powerful non-invasive technique for assigning individuals to particular geographic regions. The methods is based on tissue samples collected at a single capture event and avoid potentially influencing the study object with attached tracking devices (Hobson & Wassenaar 2008). Growing animal tissues incorporate the isotopic signature of the local food web and this signature remains fixed in tissue like feathers and claws (Hobson 1999). Stable hydrogen (deuterium) isotopes are particularly useful to track migratory birds since their signature shows predictable large-scale patterns (Bowen *et al.* 2005). This method has been successfully applied to assign migratory birds to wintering or breeding grounds in North America (Greenberg *et al.* 2007, Mazerolle & Hobson 2007) and Europe (Hobson *et al.* 2004, Bearhop *et al.* 2005, Hobson *et al.* 2009).

The Skylark (*Alauda arvensis*) is a partial migrant in Europe. Breeding populations in northern and eastern Europe tend to be migrants, and birds from southern Europe tend to be residents (Glutz von Blotzheim & Bauer 1985). Skylarks from our study population in the northern Netherlands consist of both, residents that winter close to their breeding territory and migrants that winter in SW Europe (Hegemann *et al.* 2010). In addition, we know much about their physiology, especially their immune system and energetics, and about other fitness-related parameters (Tieleman *et al.* 2003, Tieleman *et al.* 2004, Hegemann *et al.* 2012b, chapter 6 and 9 this thesis, Hegemann *et al.* 2012c). The Skylark is, therefore, ideally suited to study the costs and benefits associated with different wintering strategies and to investigate carry-over effects of migration on physiology, reproductive success and subsequent return rates.

In this study, we determined the wintering strategy of individual Skylarks in the past winter based on the deuterium signature in their claws, which were collected during four breeding seasons. Repeated sampling of birds across years allowed us to determine whether a strategy was fixed or flexible. We compared fitness-related parameters among the two wintering strategies to test for carry-over effects related to the previous winter strategy (resident or migrant). We used three immune parameters and body mass, in addition to reproductive parameters (number of nestlings, fledglings and recruits) and return rates. We expected birds with different strategies to differ with respect to immune parameters because immune function reflects the outcome of trade-offs with other annual-cycle demands and environmental conditions (Sheldon & Verhulst 1996, Hegemann *et al.* 2012b). If the wintering strategy affects immune function, we expect future return rates to differ as well because patterns of immune function can predict survival (chapter 9 this thesis). We also expect that the cost-benefit balance between migration and residency differs between years, resulting in coexistence of both strategies. Resident and migrant Skylarks return before mid-March to their territories (Hegemann *et al.* 2010), and the majority of birds starts reproduction in early May (Hegemann unpubl.

Data). We thus expect no difference in breeding performance driven by different timing of territory establishment. We furthermore expect wing length to differ only between groups if the wintering strategy is genetically fixed and persistent across winters.

## Methods

### *Study subjects*

We caught adult Skylarks from our study population at the Aekingerzand in the northern Netherlands (N 52°55'; E 6°18') during the breeding seasons 2006-2009 (Hegemann *et al.* 2012b). Birds were caught at nests while feeding nestlings during 10 May – 20 July 2006 ($n = 30$), 21 April – 17 July 2007 ($n = 67$), 4 May – 18 July 2008 ($n = 49$) and 4 May – 24 July 2009 ($n = 40$). Individuals were sampled in one year ($n = 88$), and in some cases over multiple years ($n = 27$ in 2 years, $n = 12$ in 3 years and $n = 2$ in all 4 study years). Birds were sexed biometrically and in some doubtful cases molecularly (Hegemann *et al.* 2012a). We sampled 76 males and 110 females.

Skylarks undergo a complete postnuptial moult in adults and a complete post-juvenile moult in birds of the year on their breeding grounds (Glutz von Blotzheim & Bauer 1985). Therefore, instead of feathers we collected Skylark claws to obtain a tissue that incorporates deuterium during wintering periods. Bird claws grow somewhat continuously and stable-hydrogen isotope analyses from the distal part can provide information about the region the bird visited several months ago (Bearhop *et al.* 2003, Bearhop *et al.* 2005, Mazerolle & Hobson 2005). The length of time that can be traced back prior to sampling depends mainly on the length of the claw. Skylarks have a very long back toe nail which can reach a length of more than 20 mm (Hegemann unpublished data). As a result, Skylark claws collected during the breeding season have the potential to reveal wintering strategy in the previous winter.

### *Sampling protocol*

We collected blood samples (~150uL) into heparinised capillary tubes from the brachial vein immediately after capture (median: 5 min; range: 2.25–30 min) and thus before impacts of handling stress on immune parameters (Buehler *et al.* 2008). Structural measurements were taken after blood collection. We collected a claw sample by cutting the back toe nail with a pair of scissors about 10 mm distal from the skin. Afterwards birds were ringed with a single metal ring and a unique combination of 4 colour rings and released.

Claw samples were stored at room temperature until laboratory analyses (see below). Blood samples were stored on ice until returning to the lab later the same day and then centrifuged at 7000 rpm for 10 min. Plasma and red blood cells were separated and stored at -20°C. Blood sampling was performed under license D4743A and DEC5219B of the Institutional Animal Care and Use Committee of the University of Groningen.

### *Stable isotope analyses*

Claws were washed in a 2:1 chlorform:methanol solution and air-dried (fume hood) for 48 hours. Claws were transported to the Smithsonian Institution Museum Support Center in Suitland, Maryland, and equilibrated with the local atmosphere for 72 hours. Because claw samples were often up to >1 cm in length we clipped multiple samples starting from the distal end. Samples were 0.7 – 4.6 mm long and loaded into a silver capsule that was crushed, pyrolized at 1350°C in an elemental analyzer (Thermo TC/EA), and introduced to an isotope ratio mass spectrometer (Thermo Delta V Advantage) via a Conflo IV interface. Four standards were run for every ten unknowns. Isotope ratios are reported in delta notation relative to Vienna Standard Mean Ocean Water (VSMOW), where dD = ([2H/1Hsample / 2H/1Hstandard]–1) ´ 1000. Analytical error (± 1 SD) was better than 2‰ based on replicate analyses of the same claw (*n* = 18) and replicate analyses of standards. We ran hydrogen (H) standards provided by the International Atomic Energy Agency (IAEA-CH-7) to monitor machine stability and three keratin standards to correct for the combined exchangeable + non-exchangeable hydrogen values. The dD values reported include only non-exchangeable H, as determined by a correction using three isotopically different keratin standards (Wassenaar & Hobson 2003).

### *Assignment of migratory strategy*

Claw samples varied in length (average: 5.77 mm, range 1.6–14.3 mm). Long samples were cut into two or more pieces. For short samples, we analysed the complete sample (average length 3.02 mm, range: 1.6–4.1 mm; *n* = 45). For long samples, we separately analyzed the basal part and the tip (base: 1.57 mm, 0.7–2.9 mm, *n* = 143; tip: 2.89 mm, 1.45–4.6 mm, *n* = 143). For 9 individuals with long claws (*n* = 54), we also sampled one or more middle sections (*n* = 16). Thus, in total we analysed 349 claw pieces from 186 claw samples collected from 129 individual Skylarks over a 4-year period.

In Europe, stable isotope ratios of hydrogen vary with latitude and also over the annual cycle (Bowen *et al.* 2005). Based on models by Bowen *et al.* (2005) birds that are resident year round in north western Europe experience stable isotope values in December to February that are depleted by approximately 20 ppm compared to during the breeding season. In contrast, birds from the same population that migrate to winter in southwestern Europe will incorporate stable isotope values which are more enriched by approximately 5–15 ppm than values during breeding (Bowen *et al.* 2005, Bowen 2012). Consequently, individual birds from the same breeding population will have different stable-hydrogen isotopes depending on their wintering strategy: migrants have enriched deuterium values and residents more negative isotopic signatures compared with values from the breeding period.

Before assigning a wintering strategy to individual claw samples, we first separated claw pieces by year to account for variation between years in deuterium samples (Farmer *et al.* 2008, Gow *et al.* 2012, Hache *et al.* 2012). We then assigned for each year 20% with the most depleted parts as belonging to resident birds and the 20% with the most enriched pieces as belonging to migrants. Claw pieces with intermediate deuterium values were assigned to "unknown". Applying this rule, we assigned birds as resident and as migrants, respectively, with the following isotopic values: 2006 resident: -67.4 to -64.3 ‰, migrant: -51.0 to -48.1 ‰; 2007 resident: -69.9 to -60.2‰, migrant: -47.4 to -31.0 ‰; 2008 resident: -70.1 to -60.6‰, migrant: -56.6 to -27.0‰; 2009 resident: -83.7 to -69.1 ‰, migrant: -50.8 to -34.4‰. Thus the difference between the most enriched resident and the most depleted migrant and thus the unclassified range, was on average -14.6 ‰. This makes our assignment criteria conservative when compared with published data of variation in deuterium estimates from tissue of known origin (Wunder *et al.* 2005, Rocque *et al.* 2006, Langin *et al.* 2007) and on the previously used difference of 9‰ that was established to reflect differences in dispersal distances (Studds *et al.* 2008).

Based on the most extreme isotope values of all pieces of each claw, an individual was classified as "migrant", "resident" or "unknown". Using this assignment rule, in 3 of the 187 complete samples the bird was simultaneously classified as resident and migrant based on different pieces of a single claw. In these cases we used the strategy reflected by the basal part of the claw. Across all four study years, we were able to assign 107 individuals to a wintering strategy and 79 individuals remained unclassified (2006: 6 migrants, 6 residents, 18 unknown; 2007: 17, 19, 31; 2008: 16, 17, 16; 2009: 12, 14, 14).

To further validate our assignments we conducted two tests. First, we analyzed the claw sample of a single individual, which was proven by means of radio-telemetry to winter in the study area (Hegemann *et al.* 2010). Based on our assignment rules, this bird was indeed classified as a resident by the deuterium signature of -62.7‰ in a proximal piece of its claw. Second, to validate the deuterium estimates within individual birds we compared the tips of the right and the left back claw for 14 individuals. The average stable deuterium difference between right and left was -0.53 permil (SE=1.08, $n = 14$). Knowing that the analytical error can be up to 2 permil (Studds *et al.* 2008, Wassenaar 2008) and that the two pieces of an individual varied in length (mean difference: 0.6 mm, range: 0.1–1.9 mm), this repeatability is extremely high.

### *Immune assays*

We used a hemolysis-hemagglutination assay to quantify titres of complement-like lytic enzymes and non-specific natural antibodies from preserved plasma samples (Matson *et al.* 2005, Hegemann *et al.* 2012b). Scans of individual samples were randomized among all plates and scored blindly to year and migratory strategy (by AH). We used a commercially available colorimetric assay kit to quantify haptoglobin concentrations in plasma samples (Hegemann *et al.* 2012b, Matson *et al.* 2012).

### *Fitness components*

In each of the four study years the breeding population was intensively monitored. We ringed all nestlings with a metal ring and a unique combination of three colour rings before they fledged. Number of fledglings was determined when chicks were ringed at about 8 days old; after ringing nestlings leave the nest (Hegemann unpublished). Capture and ring-readings revealed the identity of the parents. We examined return rates of adults (survival) and young (recruitment) by ring readings and catching parents on nests. As detection probabilities of ringed birds during the breeding season were almost 100% during the study years (Hegemann unpublished) and as we have no indication that detection rate differed between strategies, we used the percentage of resighted birds per year as return rates.

### *Statistics*

We compared migratory and resident groups for each response variable using linear mixed models with the program R version 2.15.0 (R Development Core Team 2012). We included wintering strategy, year, sex, Julian day and the two-way interactions of migratory strategy with year and sex, respectively, as explanatory variables. Individual identity was included as random effect. We used generalized linear mixed models with a Poisson error structure to analyse the number of nestlings, number of fledglings and number of recruits. Return rates were analysed with the same model type and a binomial error structure.

We always started with the full model and simplified it using backwards elimination based on log likelihood ratio test with $P < 0.05$ as selection criterion ("drop1" in R) until reaching the minimal adequate model. Model assumptions were always checked on the residuals of the final model. Sample sizes differ slightly among response variable (*e.g.* due to insufficient plasma volume) and are shown in the graphs.

## Results

Among the 107 claw samples that we assigned to one of the two strategies based on their isotope signature, we identified 21 male and 30 female migrants, and 28 male and 28 female residents. Migrants and residents differed in immune function, body mass and probability of return in a subsequent year. Haptoglobin concentrations in migratory Skylarks differed from resident individuals depending on year (interaction strategy × year $\chi^2 = 16.49$ $P < 0.001$, Figure 10.1C). In all years, migrants had higher lysis titres than resident Skylarks (Fig. 10.1A), a non-significant trend ($\chi^2 = 3.01$, $P = 0.08$). Residents and migrants did not differ in terms of agglutination titres (Table 10.1, Fig. 10.1A,B). Resident Skylarks were on average 1.67 g (4.9%) heavier than their migratory conspecifics (Figure 10.1D). This effect was independent of sex (interaction strategy × sex $\chi^2 = 0.20$, $P = 0.651$) and borderline non-significant when taking into account tarsus length as measure of structural size ($\chi^2 = 6.66$, $P = 0.08$). Wing length did not differ between migrating and non-migrating birds (Table 10.1). Skylarks that migrated the previous winter had a significantly higher chance of returning after the following winter (migrants: 77%, residents: 60%, Table 10.1). The difference was particularly pronounced in 2006 and 2009 (100% vs 67% and 67% vs 21%); birds of both strategies had equal probabilities to return after the 2007 breeding season (69% vs 68%) and residents survived better after the 2008 breeding season (84% vs 91%, Figure 10.1G). However the interaction between strategy and year was not significant (df=3, 5.45, $P = 0.141$). Repro-

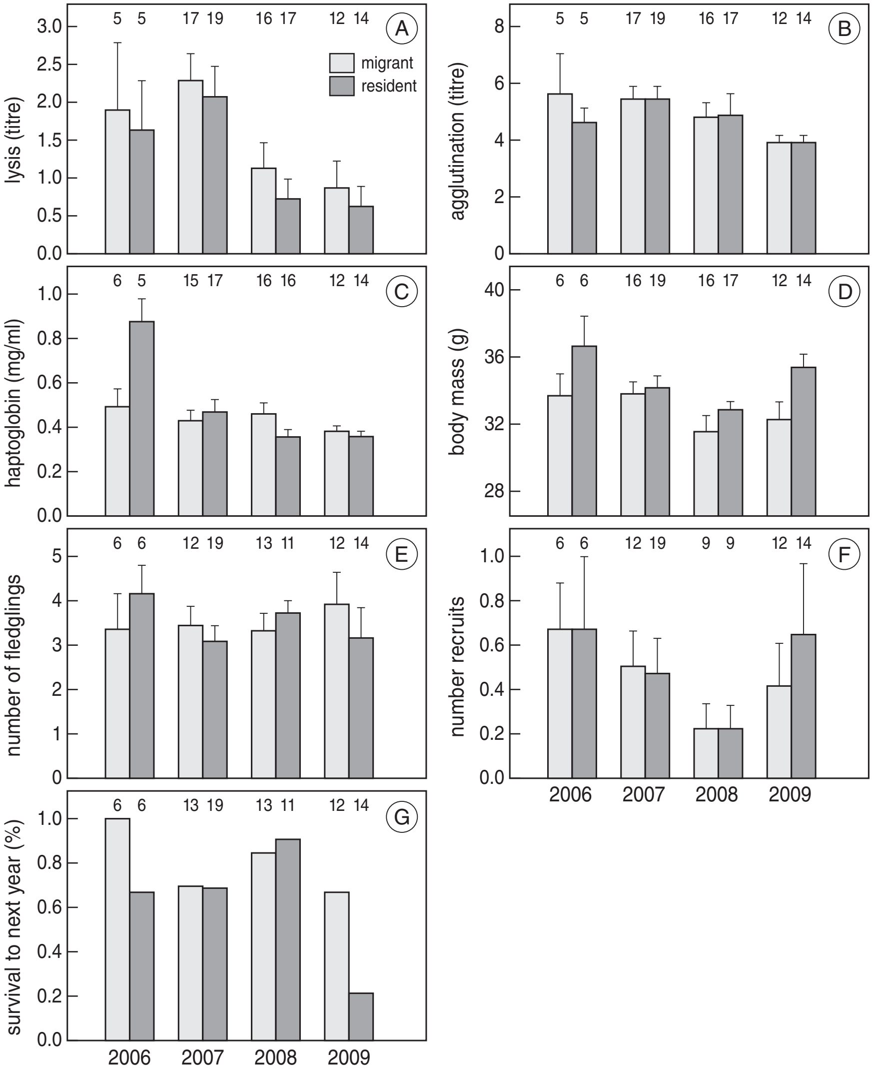

**Figure 10.1:** Patterns of A) lysis, B) agglutination, C) haptoglobin concentrations, D) body mass, E) number of fledglings, F) number of recruits and G) future return rates to the next breeding season of Skylarks in relation to their wintering strategy in the preceding winter. Data are collected during the breeding season. The wintering strategy is determined by means of stable deuterium analyses of claw samples. Bars depict means and standard errors. Numbers represent sample size of individual birds for which information of the corresponding response variable was available.

**Table 10.1:** Statistics and coefficients of the linear mixed models of measures of immune function, body mass and future return rates of Skylarks during the breeding season in relation to their wintering strategy in the previous winter. The wintering strategy is determined by means of stable deuterium analyses of claw samples. Individual bird identity was included as random effect to avoid pseudo replication. Statistics of significant interactions are given in the text. *P*-values < 0.05 are bold.

| Trait | Wintering strategy | | | | Year | | | Sex | | | | Julian day | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | df | $\chi^2$/F | β* | *P* | df | $\chi^2$/F | *P* | df | $\chi^2$/F | β# | *P* | df | $\chi^2$/F | β | *P* |
| Lysis titre | 1 | 3.01 | | 0.083 | 3 | 28.28 | **<0.001** | 1 | 0.16 | | 0.688 | 1 | 15.79 | 0.02 | **<0.001** |
| Agglutination titre | 1 | 0.08 | | 0.771 | 3 | 7.15 | 0.067 | 1 | 0.03 | | 0.087 | 1 | 0.05 | | 0.830 |
| Haptoglobin (mg/ml) | Interaction with year | | | | Interaction with strategy | | | 1 | 0.48 | | 0.483 | 1 | 0.18 | | 0.670 |
| Number of nestlings | 1 | 0.63 | | 0.426 | 3 | 3.03 | 0.388 | 1 | 0.29 | | 0.588 | 1 | 5.86 | | 0.016 |
| Number of flegdlings | 1 | 0.20 | | 0.658 | 3 | 0.65 | 0.886 | 1 | 0.00 | | 0.948 | 1 | 0.39 | | 0.534 |
| Number of recruits | 1 | 0.08 | | 0.370 | 3 | 3.49 | 0.322 | 1 | 0.36 | | 0.549 | 1 | 0.07 | | 0.797 |
| Return Rate | 1 | 4.28 | -1.11 | **0.039** | 3 | 10.82 | **0.013** | 1 | 0.00 | | 0.994 | 1 | 10.99 | 0.04 | **<0.001** |
| Body mass (g) | 1 | 5.66 | 1.10 | **0.017** | 3 | 6.29 | 0.098 | 1 | 41.22 | 3.90 | **<0.001** | 1 | 13.39 | -0.04 | |
| Wing length (mm) | 1 | 0.00 | | 0.968 | 3 | 6.87 | 0.076 | 1 | 121.19 | 9.11 | **<0.001** | 1 | 1.04 | | 0.309 |

# reference is Male, * reference is resident

ductive parameters (number of nestlings, fledglings and recruits) did not differ between the two wintering strategies (Table 10.1, Fig. 10.1E,F).

We could assign a wintering strategy to a total of 20 birds for more than one winter (Table 10.2). For 12 individuals we could assign their migration status across two years: six had the same strategy and six switched the strategy. For seven individuals we have a wintering strategy across three years: one bird wintered always locally and four birds always migrated while two switched the strategy across years (Table 10.2). We could assign a wintering strategy to one individual across the four years of this study. It was a migrant in the first three winters and a resident in the last winter.

## Discussion

We showed that the wintering strategy of partially migrating Skylarks had carry-over effects on the physiology and the probability to return. Determining the deuterium signature in the birds' claws as an indicator of the latitude where birds spent the previous winter allowed us to assign them to wintering strategies without tracking them throughout the complete annual-cycle. Skylarks that migrated to a southern wintering area in the previous winter had higher haptoglobin values in one of the four breeding seasons included in this study. Migrating Skylarks also tended to have higher lysis values, a pattern consistent in all years. Migrants were lighter during the breeding season than residents; this effect was partly driven by structural size. Skylarks that migrated the previous winter had a higher probability to return after the following winter. Reproductive parameters were not related to the preceding wintering strategy. We assigned a wintering strategy in multiple years to 20 individuals; 45% of these birds switched strategies between winters, suggesting that environmental factors rather than genetic effects determine the wintering strategy (Lundberg 1988, Ogonowski & Conway 2009, Palacin *et al.* 2011). A lack of difference in wing length between birds of the two strategies further supports the idea that the migratory tendency is not genetically fixed within our population of partially migrating Skylarks. Among Skylark populations, birds that breed at the northern range of the breeding distribution are obligate migrants and have longer wings than birds from partial migratory or resident populations (Glutz von Blotzheim & Bauer 1985, Hegemann *et al.* 2012a).

**Table 10.2:** Number of individual Skylarks for which we could assign a wintering strategy for multiple years based on the stable deuterium value in its claw. Top row gives different possible combinations of strategies in multiple winters. R=resident, M=migrant.

| N years | R,R | M,M | R,M | M,R | R,R,M | R,M,M | M,M,M,R |
|---|---|---|---|---|---|---|---|
| 2 | 2 | 4 | 3 | 3 | N/A | N/A | N/A |
| 3 | 1 | 4 | 0 | 0 | 1 | 1 | N/A |
| 4 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |

Wintering strategies had different carry-over effects on haptoglobin concentrations. Haptoglobin is an acute phase protein that is released from the liver during a pathogenic challenge. Normally in birds, concentrations of haptoglobin or iron-binding functional equivalents increase in association with inflammation (Thomas 2000). However, in Skylarks haptoglobin concentrations decreased 13 hours after an experimental immune challenge (chapter 6 this thesis). Thus, compared with other species, Skylarks appear to rely on relatively high constitutive concentrations of haptoglobin, rather than inducing its production when needed (Matson 2006, chapter 6 this thesis). In the 2006 breeding season, resident Skylarks had higher haptoglobin concentrations than their migratory counterparts and compared with both strategies in other years. This could indicate that resident birds needed to increase baseline haptoglobin concentrations in that winter (2005-2006) to resist a high pathogen pressure (Hegemann *et al.* 2012b). Lysis titres were consistently lower in resident individuals. In Skylarks, lysis titres increase following endotoxin challenge (chapter 6 this thesis). Baseline values also vary among annual-cycle stages and between years, probably reflecting environmental conditions like pathogen pressure and food supply (Hegemann *et al.* 2012b). Migrants are thought to encounter more pathogens than residents and thus face higher risks of infection (Piersma 1997, Møller & Erritzoe 1998). If migrants also experience increased food availability compared to residents, the combination could enforce and allow migrants to up-regulate lysis titres. Agglutination titres did not differ between Skylarks with different wintering strategies. Though agglutination titres in Skylarks also vary between annual-cycle stages and years (Hegemann *et al.* 2012b), titres do not change upon an immune challenge (chapter 6 this thesis). Furthermore agglutination is more genetically controlled than *e.g.* haptoglobin concentrations (Versteegh 2012), and this could contribute to the lack of difference between resident and migratory Skylarks.

Carry-over effects from wintering conditions on reproductive performance have been shown for long-distance (*e.g.* Marra *et al.* 1998, Marra & Holmes 2001, Studds & Marra 2005) and short-distance migrants (Dale & Leonard 2011). Circulating androgen and testosterone are thought to act as physiological mediators affecting preparation and condition of migration and the onset of reproduction (Tonra *et al.* 2011a,b). In our study, reproductive parameters did not differ between groups, but the wintering strategy had carry-over effects on the probability of future return. Across all years, birds that migrated in winter t had a 17% higher chance to return after winter *t* + 1. We did not detect a statistically significant effect of the interaction between wintering strategy and year on return rate, but apparently migrants did not survive better to the subsequent breeding than residents in all years. In one year there was no difference between the two strategies, and in another year residents had slightly higher chances to return. Interestingly return rates showed a roughly reversed pattern compared with haptoglobin concentrations. We have shown previously that immune function reacts rather slowly to increased workload and that immune patterns during the breeding season can predict mortality in the following winter (chapter 8 this thesis). We therefore hypothesize that if birds with one wintering strategy experience harsh conditions during non-breeding, the immune system might be affected during the following summer, which in turn can lead to increased mortality during the next winter. Thus, we present the first evidence for carry-over effects of wintering strategy on the physiology of birds during the subsequent breeding season that might influence future survival. Changes in immune function that lead to reduced resistance against diseases and parasites might be a mechanistic link between wintering conditions and fitness. Our results thus provide a possible mechanistic link to explain survival differences between migratory strategies.

That resident Skylarks, when compared with migratory conspecifics, have reduced future return rates has important implications for conservation plans. The Skylark, as many other farmland birds, is rapidly declining in many (western) European countries and especially in the Netherlands (SOVON 2002, BirdLife International 2004, van Dijk *et al.* 2008, PECBMS 2009). Earlier studies suggest that the general change in agricultural practice from summer to winter cereal and the subsequent loss of overwintering stubble fields reduces the food supply in winter (Donald *et al.* 2001, Newton 2004, Siriwardena *et al.* 2007, Siriwardena *et al.* 2008). Our study provides evidence that wintering in the Netherlands lowers future return rates in Skylarks and that this is potentially mediated by altered immune function. Increasing food supply during winter might help to solve this problem, since maintaining and activating the immune system requires energy and specific nutrients (Klasing 2004, Hegemann *et al.* 2012c). That resident Skylarks during the subsequent breeding season were heavier than migratory Skylarks does not necessarily contradict the hypothesis of poorer wintering conditions in the Netherlands. This effect was partly driven by structural size. That is, during a breeding season larger Skylarks were more likely to have been resident the previous winter. However, this does not suggest that larger and heavier birds are generally more likely to become resident. First, wing length did not differ between individuals exhibiting the two wintering strategies. Second, a high probability of switching strategies suggests flexibility. Instead, of the birds trying to winter locally, the ones with the heaviest bodies might be more likely to survive until the following breeding season. The “body-size hypothesis” suggests that individuals with the largest bodies are more likely to survive harsher wintering conditions (Ketterson & Nolan 1983). A further indication that wintering in the Netherlands is a less successful strategy comes from our finding that only 15% of the Skylarks for which we have repeated assignments to a wintering strategy were consistent residents, while 40% were consistent migrants. Overall, conservation plans should recognise the possible consequences of limited food supplies and include measures to increase habitat suitability during winter to help increase adult survival in Skylarks.

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Ring-Nr. V324647

**Skylark - Aekingerzand**

Date:

Time:

**C-rings:** OBG **LM / ML / [✓ BM] / MB / RM**

Field:

Nest-Nr.

**Recap** / no

**Adult** X Male Female X Unkown

**Fledgling** Age:

**Chick** Age:

No. chic

**Stage:** singing / Feeding / moulting / incuba / age / migratio / heath / et / unkown / No. / wintering

Claw

Feather yes no

Dropping yes no

Swabs Nick yes no

Blo

killing assay yes no

Blood slide yes no

Ketone: yes no

Glucose:

Duratio catching:

min after ca

Blood in freezer

Blood volume:

Body Mass (g):

Wing (mm): 106

Tongue spots:

Tarsus short (mm): 3,9

Tarsus long (mm): 27,4

Feathers mites: few

Bill (mm): 11,7

Head-Bill (mm): 34,7

Fat score:

Body Temp:

**Handicap experiment:** Control Exper.

Meas.: Baseline Short Medium Long

Radio transmitter X Added Removed X

EW: added Removed

Frequency

LPS experiment

st net with tape

lworm-baited trap

# CHAPTER 11

# From the field to the lab and back: physiology, ecology and conservation in the annual cycle of Skylarks – A synthesis

Arne Hegemann

In this thesis, my co-authors and I have studied aspects of ecology and physiology in the annual cycle of Skylarks (*Alauda arvensis*); a species that is rapidly declining in many countries. Specifically, we shed light on physiological mechanisms shaping the annual cycle. To accomplish this challenging task we used different approaches (e.g., observational and correlational studies, field and lab experiments), a variety of methods and techniques (from traditional behavioural observations to radio-telemetry and cutting edge stable isotope analyses, immunological tests, and molecular analyses) and exploited the power of a fine-tuned combination of field and lab work.

Our physiological studies focused on immune function because the immune system is one of the key factors preventing mortality. Maintaining and activating the immune system incurs costs and consequently trade-offs with other physiological and behavioural processes are expected. This gives immune function the potential to be a key parameter while optimising fitness and it might be a mediator behind processes that are fundamental in life-history theory.

In this final chapter I will discuss how the work presented in this thesis has shaped our ideas about how birds need to adjust their immune system during the different annual-cycle stages in order to survive and reproduce. I will discuss our findings not only in light of fundamental scientific questions but also in light of the urgent need to develop powerful conservation strategies for this rapidly declining species. In the context of discussing the different aspects of my thesis I will also point out open questions and present recommendations for future research.

## The pitfalls of sexing and aging Skylarks and their consequences for the EURING-Database

In chapter 3 (Hegemann *et al.* 2012a) we answered methodological questions regarding the aging and sexing of Skylarks. The results are not only relevant to (our own) scientific studies, but also provide significant benefit for the many (mainly) volunteer bird ringers across Europe. We show that Skylarks can be sexed reliably using their wing length as long as populations from different parts of Europe are not encountered in the same area. As soon as birds from different populations co-occur in the same area, *e.g.* during migration and winter, sexing based on wing length becomes more difficult. The wing of small males from Dutch Skylarks can be shorter than wings of the largest females from more northern European breeding grounds. Ringers are often not fully aware of such a pattern, even though it potentially applies to other short-distance or partial migrants that lack plumage dimorphism like Coot (*Fulica atra*) (see also Visser 1976, Fjeldsa 1977) and Rock Pipit (*Anthus spinoletta*). We also demonstrated that tongue spots cannot be used as an age characteristic in Skylarks. This result questiones the use of tongue spots as reliable ageing criteria in other species, as suggested *e.g.* for Warblers (Svensson 1992, B. van den Brink in van Tol 2012). Our results suggest that incorrect sexing and aging during ringing activities of Skylarks (and potentially other species as well) might be more frequent than previously thought. These pitfalls of sexing and ageing point out a shortcoming of the EURING-Database and its affiliated ringing centres around Europe. Currently, the database does not contain information on how birds were aged and sexed. Given our findings, we recommend that ringers should be obligated to report the criteria on which they based the sex and age determination. This would allow excluding sex or age determinations that are based on incorrect, unreliable or insufficient criteria and would make future analyses of ringing data less prone to bias by obviously wrong sex or age conclusions. It would further allow to clearly distinguish all molecularly, and thus highly reliable, sexed individuals from birds sexed on morphometric or plumage characteristics. The incorporation of this additional information would clearly improve the value of the EURING-database.

## Skylarks, Cuckoos and Meadow Pipits

Spending endless hours in the Aekingerzand, one cannot escape from finding additional nests of other species than Skylarks. Among the many ($n$ = 43) Meadow Pipit (*Anthus pratensis*) nests we found, 21% were parasitized by Common Cuckoos (*Cuculus canorus*) but only 1 of 348 Skylarks nests was so (Hegemann & Voesten 2011/chapter 7). It has been suggested that Skylarks build their nests too far away from trees to be a potential Cuckoo host and that this would explain the difference in parasitism rate between two species with similar breeding biology (Roskaft *et al.* 2002, Antonov *et al.* 2010). However, this hypothesis cannot explain the differences in the Aekingerzand, where both species share the same habitat and nests of both species are often found only meters apart from each other. Skylarks also lack egg recognition abilities and are unable to reject cuckoo eggs, which is the most common defence mechanism among potential host species (Antonov *et al.* 2010). Our observation (chapter 7) is anecdotal, but it raises the question if Skylarks evolved an alternative anti-parasite defence mechanism by discriminating parasite nestlings rather than parasite eggs. Clearly, experimental tests are needed to shed light on this interesting topic. How do Skylarks react towards experimentally added cuckoo nestlings? Are they able to discriminate and reject cuckoos? To further investigate the mechanism that triggers parasite nestling rejection I suggest adding Meadow Pipit nestlings and cross-fostering Skylark nestlings. Such studies will reveal if the behaviour we observed was an evolutionarily evolved response or just a coincidence.

## The difference between baseline immune function and induced immune responses, and the influence of environmental factors: a new concept

In part II of this thesis, my co-workers and I investigated patterns of immune function across the annual cycle of Skylarks and put several aspects of a central hypothesis of ecoimmunology to a test: Does immune function show seasonal patterns as result of trade-offs with other physiological and behavioural processes (Nelson & Demas 1996, Nelson *et al.* 2002, Buehler & Piersma 2008, Martin *et al.* 2008)?

We show that seasonal patterns of constitutive immune function indeed occur over the annual cycle (Hegemann *et al.* 2012b/chapter 4). However, these seasonal patterns varied between years. Body mass showed similar levels of variation as immune function, both within and between years. Within individuals (repeated measures) the observed seasonal and annual variation of immune function and body mass paralleled variation measured at the population level. Given our results, immune modulation does not seem to be a simply pre-programmed phenomenon. Instead, we conclude that fluctuating environmental conditions such as food availability, temperature or pathogen pressure that vary among years likely contribute to immunological variation in Skylarks. To my best knowledge, this idea has never been tested in free-living birds of any species before.

Knowing that Skylarks modulate innate and acquired immune defences among annual-cycle stages, and given the hypothesis that especially costly immune defences are traded off (Lee 2006, Buehler *et al.* 2010a), one would have expected seasonal modulation in the acute phase response, a particularly costly form of defence (reviewed by Owen-Ashley & Wingfield 2007). However, in Skylarks both the energetic (Hegemann *et al.* 2012c/chapter 5) and the immunological (chapter 6) consequences of an endotoxin challenge are constant throughout the year. The responses were independent of other annual-cycle demands and independent of baseline immune values. We conclude that an immune response after an endotoxin challenge is crucial for survival and cannot be compromised.

## A new concept of immune function in Skylarks

Combining the results of chapters 4-6 (Hegemann *et al.* 2012b, Hegemann *et al.* 2012c) highlights the importance of distinguishing between baseline (constitutive) values and induced responses when studying ecological immunology (Adamo 2004). To integrate variable baseline immune function and constant immune responses in a general concept, we suggest that baseline immune function and induced immune responses have different principles and independently regulated functions, and are traded off in different ways. Based on our integrated results, I introduce a new concept of immune function in free-living Skylarks (Fig. 11.1). This concept synthesizes our current thinking of how Skylarks modulate their immune system. Variable baseline immune function (Hegemann *et al.* 2012b/chapter 4 and chapter 6) is hypothesized to be the result of a trade-off between three variables: annual-cycle demands, food availability, and pathogen and/or parasite (hereafter disease) pressure. Disease pressure varies over space and time and high disease pressure selects for an upregulation of baseline immune function (Horrocks *et al.* 2011). Maintaining the immune system, however, is costly in terms of energy and nutrients (Klasing 2004). This also applies to annual-cycle activities like reproduction, moult or migration (Drent & Daan 1980, King 1980, Klaassen *et al.* 2000). Furthermore, the availability of food varies in space and time. The trade-offs between annual-cycle activities, food availability and disease pressure will determine the available resources and the selective pressure that shape baseline immune function. For example, a

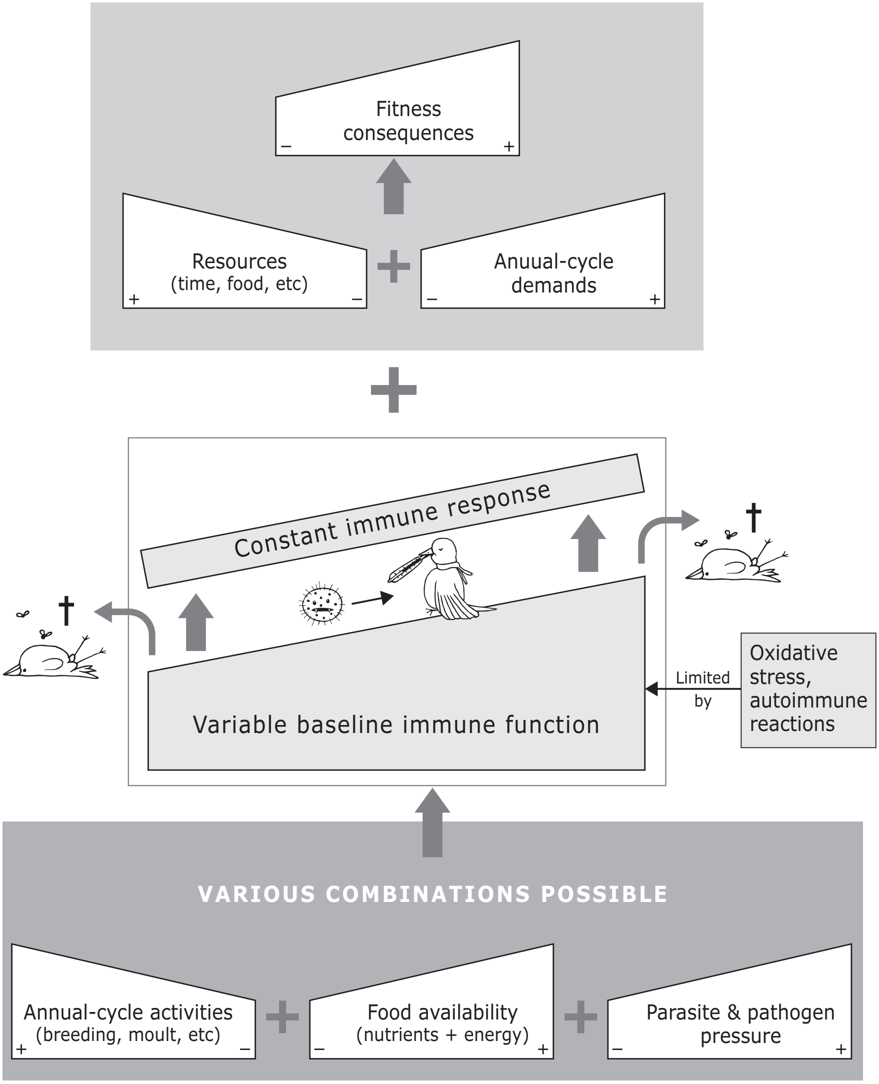

**Figure 11.1:** A concept of immune function in free-living Skylarks. Baseline (constitutive) immune function is variable but induced immune responses are constant. The trade-off between food availability, the demands of annual-cycle activities and the pathogen and parasite pressure (disease pressure) (dark grey shaded area) results in variable levels of baseline immune function. For example low demands by annual-cycle activities combined with high food availability and a high pathogen/parasite pressure results in high baseline immune function. High levels of baseline immune function are limited by negative effects of oxidative stress and autoimmune reactions. If baseline immune function cannot clear a pathogen and the pathogen establishes itself in the host body, an immune response (independent of baseline immune values) is required. If the bird fails to mount this response, death is a likely consequence. The immune responses cannot be modulated, but other annual-cycle demands will be compromised instead. The impact on fitness is probably depending on resource availability and annual-cycle demands (medium grey shaded area). For a more detailed description of the concept see main text.

combination of high food availability, high disease pressure and low demands for other annual-cycle activities will enable and force birds to upregulate baseline immune function. The upregulation is potentially limited by costs of oxidative stress and autoimmune function that are associated with high immune activity (Sorci & Faivre 2009, Hasselquist & Nilsson 2012). In a scenario with low disease pressure and low food availability, but high demands of annual-cycle activities, baseline immune function will be down-regulated. Which specific parts of the immune system (i.e., complement activity, antibody titres, cell type distributions) gets down- or up-regulated ultimately depends on the type of activity (*e.g.*, migrating or resting), available micro-nutrients and the specific pathogens that a bird encounters (see also Klasing 1998, Buehler *et al.* 2010a).

To verify the predicted causes of variable baseline immune function, experiments that manipulate the combination of annual-cycle activity, food availability and disease pressure are required. We conducted such an experiment (chapter 9). We manipulated the demands of an annual-cycle activity by handicapping adult Skylarks during the breeding season with extra weights. Our conceptual model predicts a shift in baseline immune function. Indeed, we observed adjusted baseline values of immune function as response to experimentally manipulated energy demands of breeding Skylarks. Thus, this experiment provides direct evidence that demands of annual-cycle activities are traded off against baseline immune function. The experiment further provided direct evidence that food availability plays an important role in shaping baseline immune function. Nestlings of handicapped adult Skylarks received a different diet compared to nestlings of un-handicapped control birds (chapter 9). This resulted in a change of nestling immune function. Consequently our experiment also provides evidence that difference in food availability impact levels of baseline immune function. We have no experimentally tested evidence that variable disease pressure indeed leads to variation in baseline immune function as predicted by our conceptional model. However, in a collaborative approach together with N. Horrocks we show that baseline immune function correlates with disease pressure in an inter-species comparison among larks from different environments (Horrocks *et al.* 2012). In summary our data provide direct and indirect evidence that trade-offs between food availability, annual-cycle activities and disease pressure cause variable baseline immune function in free-living Skylarks.

If baseline immune function is not able to clear a pathogen from the birds' body and the pathogen establishes itself in the host, a more costly induced immune response is required (see Hegemann *et al.* 2012c/chapter 5, chapter 6, and Buehler *et al.* 2010). In this scenario mounting an immune response is crucial for survival and cannot be compromised. The strength of this induced immune response is independent of baseline immune function (chapter 6). We hypothesize that failure to mount a (sufficiently high) response may result in mortality. Mounting an immune response is costly (Hegemann *et al.* 2012c/chapter 5) and thus we hypothesize that the consequences for an individuals' fitness depend on both annual-cycle demands and available resources. For example, mounting an immune response during a quiescent period of the year when food availability is high might have little impact on fitness. In contrast, mounting an immune response during the breeding season, when finding food for nestlings is time and energy

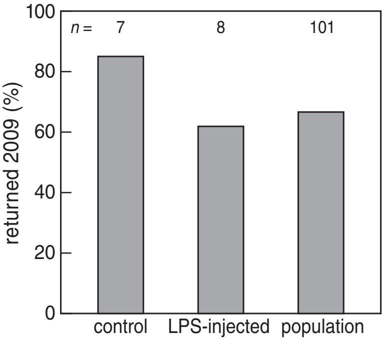

**Figure 11.2:** Return rates of adult breeding Skylarks to the Aekingerzand from 2008 to 2009. Control birds and LPS-injected birds were part of the experiment on induced immune responses described in chapter 5 and 6. Only LPS-injected birds faced an experimentally triggered immune response. The third bar refers to all adult Skylarks present in the Aekingerzand in the 2008 breeding season that were not part of any experimental manipulation. Numbers in bars represent samples size of initial birds.

demanding, mounting an immune response might force birds to abandon their young, resulting in a high fitness impact. Experimental studies in Skylarks that verify the consequences of an induced immune response are lacking so far. However, studies on other bird species support our predictions. For example, Bewick`s Swans (*Cygnus columbianus bewickii*) naturally infected with low-pathogenic avian influenza delayed spring migration for about one month compared with uninfected birds (van Gils *et al.* 2007). After an endotoxin challenge White-crowned Sparrows (*Zonotrichia leucophrys gambelii*) decreased song activity and territorial aggression (Owen-Ashley & Wingfield 2006, Owen-Ashley *et al.* 2006). An experimental challenge of the immune system in House sparrows (*Passer domesticus*) resulted in decreased feeding rate which lowered reproductive success (Bonneaud *et al.* 2003) and in reduced feather growth during moult (Martin 2005).

Minimising the probability of infections and thus avoiding subsequent negative consequences of mounting an immune response is probably the driving selective pressure forcing birds to increase baseline immune function when disease pressure (and thus the probability of getting sick) is high. It remains to be tested if mobilising acute immune responses has consequences for future survival *e.g.* via autoimmune reactions and oxidative stress. We found no indications that the experimentally triggered acute phase response during the breeding season (Hegemann *et al.* 2012c/chapter 5, chapter 6) had strong impacts on subsequent survival of Skylarks (Fig. 11.2), but sample sizes are too small to draw sound conclusions.

I hypothesize that the concept I developed for immune function in Skylarks holds true for comparisons between annual-cycle stages and between years (as described in Hegemann *et al.* 2012b/chapter 4, Hegemann *et al.* 2012c/chapter 5, chapter 6). Additionally, I suggest that it can be further extended to allow comparisons between individual birds living in different territories or different habitats. This would make the concept general to immune function and independent from spatial and temporal dimensions. However, studies on captive Red Knots (*Calidris canutus*) suggest that these long-distance migrating shorebirds evolved different strategies to stay healthy throughout the year. Thus, there may be limits to the global generality of our concept. Specifically, Red Knots modulate aspects of induced immune responses when resources are scarce (Buehler *et al.* 2009, Buehler *et al.* 2010a). Differences in the outcome of the trade-offs between annual-cycle demands, food availability and disease pressure of an arctic breeding long-distant migrating shorebird compared to a temperate breeding partial migrating passerine may explain the evolution of different immunity strategies. Hypothesized differences in pathogen pressure, specifically rather clean marine environments (Piersma 1997), could be one of the main reasons for different immune strategies in shorebirds. Clearly, mores studies on baseline immune function and induced immune responses in free-living birds throughout the annual cycle, and from different environments are needed to proof the generality of our concept of immune function.

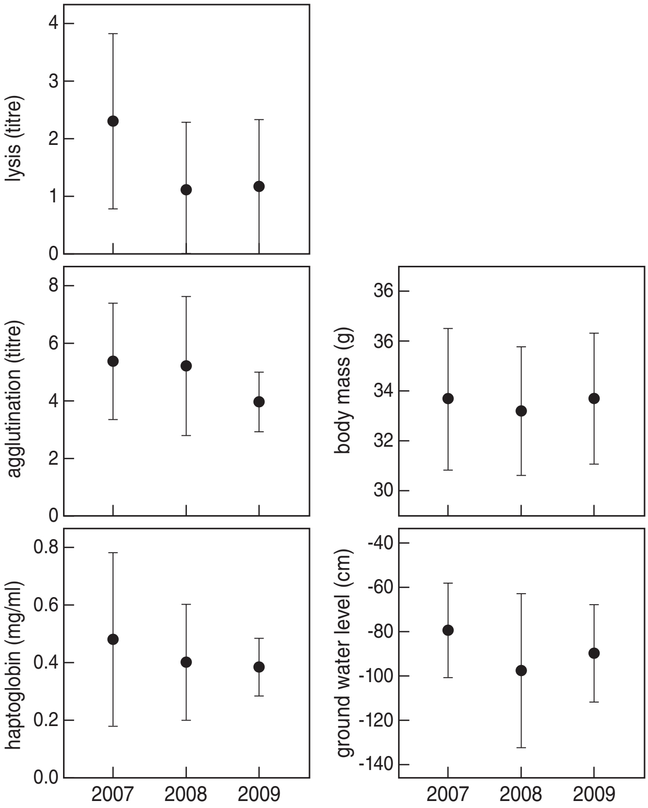

**Figure 11.3:** Patterns of average immune function and body mass of Skylarks and ground water levels in the Aekingerzand. Data on immune function and body mass are taken from Hegemann et al. 2012b/chapter 4. Measurements of ground water levels were done by AH using a ground water pipe of Staatsbosbeheer Drents-Friese Wold. Ground water levels are averaged over multiple measurements per months during 1 April- 31 July. Shown are average and SD.

### *Environmental factors*

Our finding that patterns of immune function vary among years (Hegemann *et al.* 2012b/chapter 4) and our concept of immune function (summarised in Fig. 11.1) highlight the need for incorporating measurements of environmental conditions in ecoimmunological studies. Including weather variables like temperature and precipitation can be a first step, as they are known to influence both food availability (*e.g.* Frampton *et al.* 2000, Ferguson & Joly 2002) and parasite abundance (*e.g.* Moyer *et al.* 2002). As a proxy for precipitation and humidity, I compiled average ground water levels in the Aekingerzand. I measured ground water levels on a ground water pipe of Staatsbosbeheer Drents-Friese Wold one to five times per months during the breeding seasons 2007-2009. I plotted average baseline immune function of Skylarks during the breeding season against these levels (Fig. 11.3). The highest average ground water level (in 2007) parallels the highest average lysis titres and haptoglobin concentrations in our Skylark population. The highest fluctuations in water levels in 2008 coincided with the highest spread in agglutination titres. Although suggestive, more detailed studies need to reveal if there is any functional relationship behind these patterns. Future studies should further investigate if higher humidity promotes the transmission of diseases (*e.g.* Moyer *et al.* 2002) and resulting in an increased need for high baseline immune function. Also, ground water levels (as included here) may not be an accurate measure of surface moisture. Furthermore, within breeding season patterns of ground water levels vary considerably between years (Fig. 11.4). Thus incorporating weather variables as a proxy for disease risk and food availability can only be a first step. Going forward, ecoimmunologists need to strive for direct measurements of food availability and abundance of parasites, bacteria and viruses to reveal links between available resources, the bird's defence mechanisms and its threats (see also Horrocks *et al.* 2011).

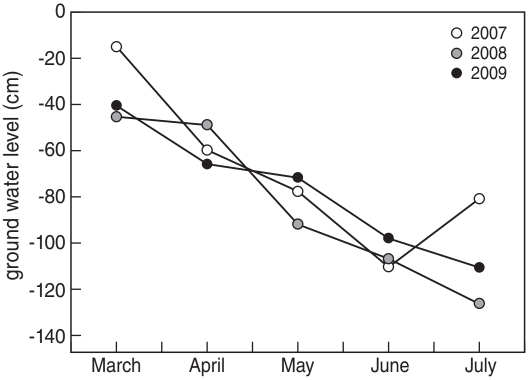

**Figure 11.4:** Average ground water levels in the Aekingerzand per months for the breeding seasons 2007-2009. Ground water levels are averaged over multiple measurements per month during 1 April- 31 July. Measurements of ground water levels were done by AH using a ground water pipe of Staatsbosbeheer Drents-Friese Wold.

In chapter 7 (Zehtindjiev *et al.* 2012) we made a first step into this direction by measuring blood parasite load by combining microscopic and molecular analyses. We found a striking difference in the prevalence of haemosporidian infections of Skylarks from the Aekingerzand and birds sampled in Italy, which suggests high spatial and temporal dynamics of infections. Future studies should focus on monitoring parasite (*e.g.* feather mites), bacterial and virus communities (*e.g.* cloacal and choanal communities) across all annual-cycle stages and over multiple years. This framework will allow addressing questions such as: How do population patterns of these parasites and pathogens look like? Is their variation linked to patterns of avian immune function? And if so, does this relationship also manifest itself at the individual bird level? Such questions and relationships were beyond the scope of this thesis, but our results clearly indicate that ecoimmunologists should strive to identify influential environmental factors and incorporate relevant measurements of these identified factors. Finally, experimental manipulations of environmental factors are needed to reveal causal relationships. For example, combining experimentally manipulated parasite loads and food availability with subsequent measurements of baseline immune function will gain further insights in the fine-tuning of trade-offs that modulate baseline immune function.

## Immune function, ecology and behaviour in the annual cycle of Skylarks: What do we learn for life-history theory and conservation?

In part I we looked at natural history, part II focused on physiology, and in part III we looked at parasite infections. In chapter 9 and 10 (part IV) we integrate all levels and connect annual-cycle events with immune function. First (chapter 9), we provide evidence that immune function might be a mechanistic link modulating the trade-off between current and future reproduction – one of the key concepts of life-history theory (Williams 1966, Stearns 1992). Then (chapter 10), we show that the wintering strategy of the partially migrating Skylark has consequences for immune function and future return rates. As immune function predicted survival after our handicap experiment (chapter 9), we hypothesize that the difference in immune function between the wintering strategies within a partially migratory population causes different survival rates of migrant and resident larks. Thus, both studies stress the importance of immune function as a physiological mediator for annual-cycle events.

The recent steep population decline in Skylarks has been strongly associated with agricultural intensification (Chamberlain *et al.* 2000, Newton 2004, Donald *et al.* 2006). Reductions in the number of successful breeding attempts per season and in the number of fledglings have been suggested to be the main causes for the population decline (Donald *et al.* 2002, Donald 2004). In this thesis, we provide evidence that several additional factors are likely to contribute to the Skylark population decline, and our data facilitate insights in the mechanistic links acting during different parts of the annual cycle.

### Wintering and migration

In this thesis we bridged one fundamental gap in our understanding of the annual cycle of Dutch Skylarks: Where do Dutch Skylarks winter? The combination of a ring-analysis with radio-telemetry (Hegemann *et al.* 2010/chapter 2) suggests a partial migration strategy, i.e. coexistence of migration and local wintering. The findings of our study are important for planning conservation efforts, because protection of this rapidly declining species can only be successful when we understand their behaviour, ecology and whereabouts throughout the entire annual cycle (Sutherland *et al.* 2004, van Beusekom 2006, Bos *et al.* 2009). By using stable deuterium analyses in claw samples as a proxy for wintering location, we further extended our discovery of Dutch partially migrating Skylarks and determined the wintering strategy for a larger number of individual Skylarks from our Aekingerzand study population (chapter 10). We found that Skylarks wintering close to the breeding grounds had higher mortality rates following the subsequent breeding season. Wintering in the Netherlands consequently lowers future return rates in Skylarks. Furthermore, only 15% of the Skylarks for which we have repeated assignments to a wintering strategy were consistent residents, while 40% were consistent migrants. The remainder of birds switched from migration to residency, or vice versa, between years.

The combination of reduced future return rates after local wintering with a low percentage of birds that are consistently resident suggests that wintering in the Netherlands might currently be a less competitive wintering strategy. Wintering close to the breeding grounds is generally seen as a profitable strategy and associated with higher breeding success (Newton 2008). If this holds true for Skylarks, our findings suggest that improving the wintering situation for Dutch Skylarks could significantly improve adult winter survival and should positively impact population trends. Our results were quickly picked up in discussions for a more powerful conservation strategy and conservation efforts now strive for an improvement of wintering habitats in the Netherlands (Kragten & Koks 2011). Given that our data call for a two-pronged wintering conservation strategy for the Dutch breeding population, improving the wintering situation for migrants in south western Europe needs to be incorporated as well.

Studying a bird population exhibiting a partial migration strategy offers the unique opportunity to investigate causes and consequences of migration by comparing migrants and residents of the same population. Yet, only few studies focus on partial migratory populations (Adriaensen & Dhondt 1990, Chapman *et al.* 2011a, Chapman *et al.* 2011b, Palacin *et al.* 2011). In chapter 10 we could demonstrate that the wintering strategy (local wintering or migration to the south) in Dutch Skylarks had carry-over effects on baseline immune function during the breeding season. Because baseline immune function predicted survival over the following winter in our handicap experiment (chapter 9), we hypothesize that different future return rates in residents and migrants are also caused by altered immune function. Consequently, changes in immune function, and thus reduced resistance against pathogens and parasites, might be a mechanistic link between wintering conditions and fitness. This mechanistic link might explain survival differences between migratory strategies within a partially migrating population.

This is, to my best knowledge, the first evidence for carry-over effects of wintering conditions on the physiology of birds during the subsequent breeding season that might influence future survival. We hypothesize that differences between years (chapter 10) reflect different evolutionary costs and benefits of either strategy in different years, resulting in maintaining the coexistence of migration and residency in Dutch Skylarks.

These results present an important first step in our understanding of costs and benefits of different migration strategies. Our Skylark study system and our knowledge about natural history and physiology of Skylarks (Tieleman *et al.* 2003, Tieleman *et al.* 2004, this thesis) provide an excellent model for future research questions on partial migration. Future studies should investigate the relationship between reproductive performance and immune function in the preceding breeding season to further enhance our understanding of migratory decisions. Furthermore, a refined approach using tracking devices with higher resolution and precision (compared to stable isotopes) should allow linking migratory distance and/or timing, rather than the wintering strategy per se, with fitness-related patterns. Is there an optimal migratory distance depending on the environmental conditions? Light level geo-locators (Stutchbury *et al.* 2009) are now small enough for deployment on Skylarks and have a resolution of less than 200 km (Fudickar *et al.* 2012). The ultimate goal should be to integrate data on physiology and reproduction not only of returning survivors, but also of birds that die on their wintering grounds or during migration. Satellite based tracking, for instance, that does not require the recapture of birds and provides information about when and where birds die (Wikelski *et al.* 2007) would clearly bring us another step closer to understanding causes, consequences and connections of annual-cycle events.

**Box 1: Advice for tracking studies**

The combination of the handicap experiment presented in chapter 9 with the radio-telemetry study presented in chapter 2 (Hegemann *et al.* 2010) contains an interesting result for those working with tracking devices. A rule of thumb is that tracking devices should weigh not more than 5% of an animal's body mass to avoid significant impacts on the individual. While being widely accepted, there seems to be no empirical evidence for the '5%-rule' (Barron *et al.* 2010). The few studies using heavier devices do not provide any evidence for increased negative effects compared to lighter devices (Barron *et al.* 2010). Skylarks carrying a transmitter equalling no more than 5% of their body mass for several months seemed unaffected by this handicap (Hegemann *et al.* 2010/chapter 2). However, Skylarks carrying an extra 10% of their body mass for just a few weeks experienced severe consequences for reproduction and survival (chapter 9). This comparison provides strong indications that devices heavier than 5% can have significant impacts on behaviour and fitness of the studied individual. Consequently, tracking studies that want to avoid strong negative impacts on their study objects should continue to strive for light devices.

### *Breeding*

During the breeding season, handicapping adult Skylarks with an extra weight resulted in lower breeding success, reduced immune function and lower survival rates. We assume that the extra weight increased their locomotion costs and this caused the effects (chapter 9). The results from this experiment are relevant for other situations that alter locomotion costs of Skylarks as well. Specifically, locomotion costs can be especially relevant for birds in poor quality habitats due to increased flight distances and time spent on foraging trips (Lovvorn & Gillingham 1996, Hinsley 2000, Bruun & Smith 2003, Stauss *et al.* 2005). Skylarks living in modern agricultural landscapes experience low food availability (Jenny 1990a). For the Netherlands there is indeed empirical evidence that Skylarks in intensive agricultural areas have to spend more time and carry out longer flights to collect food for their offspring than their conspecifics in extensive grasslands (Teunissen *et al.* 2007). As a consequence, these birds might face similar trade-offs as the Skylarks in our handicap experiment. We hypothesize that Skylarks breeding in agricultural areas are likely to suffer from reduced reproductive output and lower adult survival, mediated by reduced immune function. This mechanistic link can at least partly explain the observed population declines. We suggest that conservation measures (in agricultural areas) should not only strive for a general increase in profitable habitat patches (Henderson *et al.* 2012). They also need to consider the spatial design and resulting locomotory costs of birds.

Another striking discovery of the handicap experiment was the link between nestling diet, immune function and survival (chapter 9). Our data suggest that differences in nestling diet have consequences for the development of their immune function, which subsequently affected their survival probabilities. This link does not only provide evidence for our hypothesis that environmental conditions like food supply are important in shaping immune function (see above the introduced concept on immune function), but may further help understanding the rapid population declines of Skylarks. A reduction in number of successful breeding attempts and the number of fledglings raised has been suggested to be the major cause for the population decline (Donald *et al.* 2002, Donald 2004). We hypothesise that the less diverse diet in agricultural areas (*e.g.* Wilson *et al.* 1999, Benton *et al.* 2002) negatively impacts nestling immune function resulting in reduced future survival of fledglings. This mechanistic link might also contribute to the rapid population decline. A thorough comparison of Skylark nestling diet and immune function between areas (natural areas like the Aekingerzand, different types of intensive and extensive agricultural areas) will help shedding light on differences in nestling diet between different habitat types. This should then help to develop management schemes that carefully incorporate the nutritional needs for successful Skylark reproduction.

### *The annual-cycle stages combined*

Taken together, our results provide strong evidence how different phases of the annual cycle are connected. We furthermore demonstrate that immune function is a likely mediator of carry-over effects. It is beyond the scope of this thesis to provide a clear answer to how we can successfully conserve Skylark populations breeding in agricultural areas. Still, we provide evidence that some of the mechanistic links we revealed in our natural high-density study population may also apply for Skylarks breeding in agricultural areas. We also point out how our results may be used for conservation purposes. We hypothesise that Skylark conservation plans in the Netherlands (and beyond) may be more successful and sustainable, if conservation policies incorporate results from fundamental ecological and evolutionary studies as presented in this thesis.

## Concluding remarks

Like any innovative research, this thesis raises more questions than it answers. Nevertheless, the work presented in this thesis clearly illustrates a number of key points for a better understanding of bird immunology, ecology and behaviour in an annual-cycle perspective:

- Dutch Skylarks are partial migrants, and the wintering strategy has carry-over effects on immune function, body mass and future return rates (Hegemann *et al.* 2010/chapter 2 & chapter 10).
- Baseline immune function is variable (Hegemann *et al.* 2012b/chapter 4), but costly immune responses are constant throughout the annual cycle (Hegemann *et al.* 2012c/chapter 5 & chapter 6).
- We introduce a new concept of immune function (this chapter) and point out the importance of environmental factors like food availability and disease pressure (Hegemann *et al.* 2012b/chapter 4, chapter 9, this synthesis).
- Immune function is a mediator for carry-over effects between current and future reproduction (chapter 9).
- Our studies provide several mechanistic links that might contribute to the population decline in agricultural areas. Conservation efforts may be improved by incorporating results from fundamental ecological and evolutionary studies (this synthesis).

Furthermore, the thesis highlights two points:

1) The power of combining many different approaches and tools (*e.g.* intensive descriptive field work, lab and field experiments, radio-telemetry, stable isotope analyses, immunological tests, microscopy, genetic tools). This integrative and multifaceted approach allowed us to combine aspects of physiology with the natural history of our study species and to reveal mechanistic links between annual-cycle stages.
2) The importance of taking an annual-cycle perspective. Only considering all annual-cycle stages of our study species and linking events in different parts and on different levels of the annual cycle with each other allowed us to draw conclusions on how birds optimise their fitness by trading off year-round survival and reproduction.

In conclusion, my thesis highlights that integrating not only different approaches and techniques (1), but also different phases during the annual cycle (2) is one of the most promising approaches to gain insight in fundamental evolutionary and ecological questions. In turn, this knowledge can also be used to develop powerful management plans for specifically tailored conservation strategies.

**Box 2: Additional recommendations for future research**

Studying Skylarks for several years and spending thousands of hours throughout the year in the field makes you think of many interesting questions which are worth to be studied in detail. Despite the different approaches and angles presented in this thesis I could only touch upon a few aspects of my ideas. I have pointed out several promising future research topics throughout chapter 11 when discussing results of our current work. Here, I want to outline two more of the numerous ideas and thoughts that crossed my mind. The first, about extra-pair paternity, could add crucial information for our understanding of bird behavioural ecology. The second, about sex ratios, may provide important information for evolutionary biology and conservation purposes. Our Aekingerzand Skylark study population seems ideal to address both topics.

***(a) Extra-pair paternity***

The existence of extra-pair copulations and young has been proven for many bird species (reviewed by Petrie & Kempenaers 1998, Griffith *et al.* 2002). However, most studies on extra pair paternity have been conducted in species where the majority of individuals produce only a single brood per year. Furthermore in many of the species, most individuals reproduce only once in their life (*e.g.* many tits). The Skylark as ecological model offers two unique characteristics that could increase our understanding of behavioural consequences of extra pair copulations: Skylarks have multiple breeding attempts per season, and adults often stay paired with the same partner for multiple breeding seasons (Delius 1964, Jenny 1990, and own unpublished data). This study system offers the unique opportunity to test whether the rate of extra pair copulations relates to the rate of pair separations and to investigate the rate of extra-pair paternity over the course of the breeding season (and in relation to the success of previous breeding attempts). Intense mate guarding and high aggression between males throughout the breeding season indicate a high risk of extra pair copulation. Indeed, a recent study in Skylarks demonstrates that extra-pair paternity exists (Hutchinson & Griffith 2008), but this study did not explore rates of extra-pair paternity in the course of a breeding season, or in relation to pair bond changes. Our colour-ringed breeding population in the Aekingerzand would offer an ideal study system to investigate these aspects in detail.

### *(b) Sex ratio*

There is some evidence that in agricultural landscapes adult sex ratio of Skylarks is skewed towards males (Donald 2011). A skewed adult sex ratio can occur for different reasons. One reason is a skewed secondary (fledgling) sex ratio. Different costs and benefits of sons and daughters are the main reason (Komdeur 2012), and two mechanisms can lead to an uneven secondary sex ratio. Either the female manipulates the primary (egg) sex ratio, which might be controlled by a number of different physiological mechanisms or environmental cues (West *et al.* 2002, Pike & Petrie 2003, Alonso-Alvarez 2006), or differential egg or chick mortality occurs between laying and fledging (Szekely *et al.* 2006).

In Skylarks, the dimorphism in size between the sexes is already established when the nestlings leave the nest (own unpublished data). Hence, rearing sons and daughters might incur different costs for the parents. If these costs and benefits change throughout the long breeding season with multiple breeding attempts, seasonal trends in the secondary sex ratio might occur. Such a pattern has been documented in Kestrels (*Falco tinnunculus*) (Dijkstra *et al.* 1990). The only study investigating sex ratio of Skylark nestlings also shows a seasonal trend in Skylark nestling sex ratio (Eraud *et al.* 2006). Early in the season more males fledged, whereas later in the season more females fledged, resulting in an overall equal proportion of male and female offspring. This study, however, investigated a small number of nests during only one year. If this is indeed a more general trend, this would have important implications for Skylark conservation. The fast growing vegetation in agricultural areas has been hypothesized to reduce the chance for Skylarks to produce a successful second or third brood (Donald *et al.* 2002, Donald 2004). If this trend of more male offspring produced early in the breeding season proves to be consistent, a male-skewed adult Skylark population could result from the fact that Skylarks in modern agricultural areas gain their reproductive output mainly from first broods. Our study population in the Aekingerzand with its natural habitat and multiple breeding attempts per year would provide an ideal opportunity to investigate seasonal trends in offspring sex ratio.

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**Onderzoek Vogeltrek**
(niets aanraken a.u.b.)

**Onderzoek naar de winter verplaatsingen van veldleeuweriken, die in de zomer in het Aekingerzand broeden.**

Voor meer informatie kunt u contact opnemen met:

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Nederlandse samenvatting

# Streven naar overleving: ecologie en fysiologie van veldleeuweriken in hun jaarcyclus

## Veranderingen door het jaar

Vogels en veel andere dieren moeten hun leven aan de verschillende tijden van het jaar aanpassen. Jongen groot brengen, ruien, trekken naar broed- en overwinteringsgebieden en veel andere activiteiten moeten niet alleen aan elkaar aangepast worden maar ook aan de veranderingen in de omgeving tijdens verschillende perioden van het jaar. Om dit te bereiken moeten vogels hun gedrag en fysiologie aanpassen. Beslissingen en omstandigheden tijdens één periode van het jaar kunnen consequenties hebben voor latere perioden. Slecht weer tijdens de voorjaarstrek kan bijvoorbeeld voor een latere aankomst in het broedgebied zorgen. Een latere aankomst in het broedgebied betekent meestal dat vogels later beginnen met broeden met het gevolg dat broedsucces verlaagd is. Hoewel het bestaan van zulke connecties tussen de verschillende perioden van het jaar al lange tijd bekend zijn, zijn studies die de gehele jaarcyclus van een vogelsoort onderzoeken (en niet slechts één bepaalde tijd van het jaar) zeer schaars. Daarbovenop zijn de meeste studies beperkt tot langeafstandstrekkers, of bestuderen ze ecologische aspecten zoals timing. Studies die zich op fysiologische oorzaken en consequenties van connecties tussen de verschillende perioden van het jaar concentreren zijn nog steeds een uitzondering.

## Het belang van het immuunsysteem in de jaarcyclus van vogels

Het immuunsysteem beschermt het lichaam tegen ziektes en bevordert zo de overleving. Als het immuunsysteem niet werkt loopt ieder levend wezen de kans om ziek te worden en mogelijk te overlijden. Daarom is het van groot belang een goed werkend immuunsysteem te hebben. Het handhaven van het immunsysteem brengt echter ook kosten met zich mee. Bij mensen is het bijvoorbeeld bekend dat een gezond en gevarieerd dieet een goed immuunsysteem mogelijk maakt. De „kosten“ van het immuunsysteem hebben mogelijk een bepalende invloed op de keuzes die een vogel maakt gedurende zijn leven. Als bijvoorbeeld de hoeveelheid voedsel beperkt is, moet een vogel een keuze maken tussen het investeren van energie in het immuunsysteem (en dus de langdurige overleving), of in het groot brengen van jongen. Het immuunsysteem is echter complex: verschillende onderdelen hebben verschillende kosten en reageren op verschillende ziekteverwekkers (virussen, bacteriën, parasieten etc.). Bovendien veranderen de aantallen en soorten ziekteverwekkers gedurende het jaar. Het immuunsysteem moet zich altijd aanpassen. Een centrale wetenschappelijke hypothese over het immuunsysteem van wilde dieren (ook wel eco-immunologie genoemd) gaat ervan uit, dat investering in (verschillende delen van) het immuunsysteem moet worden afgewogen tegen andere fysiologische processen en gedag. Het belang van het functioneren van het immuunsysteem staat dus tegenover de kosten die met investeringen in andere activiteiten (zoals broeden, ruien, trekken) moeten worden afgewogen. Deze kosten-baten-afweging maakt dat het immuunsysteem mogelijk een belangrijke rol speelt bij de keuzes die een dier maakt gedurende zijn leven. Echter, ons begrip van het immuunsysteem van wilde vogels tijdens de jaarcyclus, de reactie van het immuunsysteem op veranderingen in de omgeving en de consequenties hiervan zijn nog zeer beperkt.

## Doel van dit proefschrift: integratie van fysiologie, ecologie en gedrag van veldleeuweriken gedurende de jaarcyclus

Dit proefschrift integreert aspecten van gedrag en ecologie van veldleeuweriken met onderzoek naar hun immuunsysteem gedurende de complete jaarcyclus. Om deze integratie te bereiken heb ik in samenwerking met verschillende collega's diverse methoden gebruikt om vogels door het jaar te volgen (zenders, ringen, analyse stabiele isotopen). Verder heb ik verschillende technieken en methoden gebruikt om data over de fysiologie in het algemeen, en het immuunsysteem in het bijzonder, te verzamelen (immuuntests, genetische vingerafdruk, stofwisselingsmetingen). Bovendien heb ik samen met collega's verschillende factoren experimenteel gemanipuleerd om te testen hoe vogels zich aanpassen. Deze innovatieve, integrale en diverse benadering geeft mij de mogelijkheid om een combinatie van fysiologie, gedrag en ecologie te onderzoeken. Zo zijn we in staat te onderzoeken hoe vogels in het algemeen, en veldleeuweriken in het bijzonder, zich tijdens hun jaarcyclus aan veranderingen in de omgeving aanpassen en hoe ze de uitdaging van gezond blijven met de uitdaging van broeden, ruien, trekken en overwinteren kunnen combineren.

Dit proefschrift is in 4 delen onderverdeeld. Na een algemene inleiding (hoofdstuk 1) worden in deel 2 (hoofdstuk 2 en 3) veldbiologische aspecten bestudeerd die de basis zijn voor de volgende hoofdstukken. Deel 2 (hoofdstuk 4, 5 en 6) focust op het immuunsysteem. Deel 3 (hoofdstuk 7 en 8) kijkt naar bloedparasieten en broedparasieten. Deel 4 (hoofdstuk 9, 10 en 11) combineert de ecologische en fysiologische onderzoeksbenaderingen en identificeert mechanische verbanden en interacties. Ik eindig dit proefschrift met een synthese waar ik alle resultaten van de voorafgaande hoofdstukken in een breed verband bediscussieer. Bovendien laat ik zien hoe de moderne bescherming van veldleeuweriken in het agrarisch gebied van dit wetenschappelijk fundamenteel onderzoek kan profiteren.

## De veldleeuwerik

De veldleeuwerik is een algemene zangvogel in Europa die in open gebieden met niet te dichte vegetatie voorkomt. Oorspronglijk leefden veldleeuweriken in natuurlijke open landschappen, maar nu broeden de meeste veldleeuweriken in cultuurlandschappen. In Nederland wordt de jaarcyclus van de veldleeuwerik door verschillende perioden gekarakteriseerd. De vogels komen vanaf half februari in hun territoria aan en de mannetjes beginnen te zingen. Vanaf eind april tot en met eind juli bouwen ze nesten, goed verstopt in het gras. Veldleeuweriken broeden tot vijf keer per jaar en elk legsel bevat 3-5 eieren. Een groot deel van de legsels is echter niet succesvol; ze worden door predatoren opgegeten. In augustus en september ruien de jonge en adulte veldleeuweriken in hun broedgebied. In oktober en begin november trekken veldleeuweriken naar wintergebieden in Zuid-West Europa, maar er overwinteren echter ook veldleeuweriken in Nederland. In februari en maart trekken ze terug in hun broedgebieden. Tijdens de zomer zijn veldleeuweriken territoriaal en agressief tegen soortgenoten. Buiten het broedseizoen leven ze echter in groepen. Granen, andere zaden en groene delen van planten vormen het voedsel in de winter. In de zomer worden ook veel insecten en hun larven gegeten. Jonge leeuweriken worden zelfs uitsluitend met insecten(larven) gevoerd. Zoals veel andere soorten vogels van agrarisch cultuurlandschap loopt de veldleeuwerik in Midden-Europa sterk terug in aantal. In Nederland is het aantal sinds de jaren zeventig zelfs met 95% afgenomen. Deze populatieafname is sterk gekoppeld aan de intensivering van de landbouw. Vooral de overstap van zomergranen naar wintergranen heeft negatieve gevolgen gehad. Wintergranen groeien sneller en in hogere dichtheid dan zomergranen met als gevolg een gebrek aan nestmogelijkheden in de tweede helft van het broedseizoen. Bovendien zijn er in de winter minder voedselrijke stoppelakkers.

## Het studiegebied: het Aekingerzand

Het onderzoek voor dit proefschrift is grotendeels uitgevoerd aan een populatie broedende veldleeuweriken in het Aekingerzand. Het Aekingerzand is onderdeel van het Nationale park Drents-Friese Wold op de grens tussen Drenthe en Friesland. In dit gebied zijn behalve begrazing met schapen gedurende het hele jaar geen agrarisch activiteiten. Het Aekingerzand bestaat uit een mozaïek van open stuifduinen, gras- en heidevelden en kleine boomgroepen op zandige ondergrond. De korte open vegetatie en kleine heuvels komen overeen met de natuurlijke habitat van de veldleeuwerik en maken een hoge dichtheid aan broedparen mogelijk. Deze leefomgeving maakt het ook mogelijk gekleurde pootringen (kleurringen) vrij gemakkelijk te herkennen. Met behulp van deze kleurringen, die door een verrekijker of telescoop te herkennen zijn, is het mogelijk vogels individueel te herkennen en te volgen. Op deze manier kunnen verschillende aspecten van de individuele levensgeschiedenis bestudeerd worden (bijvoorbeeld welke ouders bij een nest horen, het aantal legsels per jaar, en of een vogel in het volgende jaar terugkeert naar het broedgebied). De combinatie van individuele identificatie en de hoge dichtheid van broedvogels maakt onderzoek in het Aekingerzand mogelijk wat in moderne akkerbouwgebieden onmogelijk zou zijn.

## De overwinteringstrategie

In hoofdstuk 2 heb ik samen met mijn medeauteurs de overwinteringsstrategie van Nederlandse veldleeuweriken bestudeerd. Hoewel de veldleeuwerik nog steeds een algemene vogel is, was het niet bekend waar de Nederlandse broedvogels overwinteren. Er werd gedacht dat Nederlandse veldleeuweriken vooral in eigen land overwinteren en gezelschap van vogels uit Noord en Oost-Europa krijgen. Alternatieve hypothesen gingen ervan uit dat de meeste of alle in Nederland broedende leeuweriken trekvogels zijn en in Frankrijk overwinteren. Overwinteraars zouden uitsluitend vogels uit noordelijke populaties zijn. Data die een van de twee hypothesen zouden kunnen ondersteunen zijn er tot nu niet geweest. Om deze vraag te beantwoorden hebben wij ervoor gekozen het onderzoek van twee kanten te benaderen. Aan de ene kant hebben wij de terugmeldingen van het vogeltrekstation van de laatste 100 jaar geanalyseerd, aan de ander kant hebben wij enkele vogels uit onze eigen studiepopulatie met kleine radiozenders uitgerust. Zowel de analyse van terugmeldingen als het zenderonderzoek bewijst duidelijk dat er zowel trekvogels als standvogels zijn. Een deel van de populatie trekt in de winter naar Zuid-West Europa, een ander deel overwintert vlakbij het broedgebied.

## Leeftijds- en geslachtsbepaling bij veldleeuweriken

Mannelijke en vrouwelijke veldleeuweriken zijn niet te onderscheiden aan de hand van hun verenkleed. Vogelringers gebruiken daarom meestal de vleugellengte voor een geslachtsbepaling. Leeftijdsbepaling is niet mogelijk bij veldleeuweriken, maar er wordt wel gespeculeerd dat tongvlekken een onderscheiding van jonge en adulte velleeuweriken in het najaar en in de winter mogelijk maken. In hoofdstuk 3 testen wij deze twee criteria. Wij maken onder ander aan de hand van moleculaire methoden duidelijk dat bij veldleeuweriken een geslachtsbepaling aan de hand van de vleugellengte bijna zonder fouten mogelijk is, mits uitsluitend vogels van één populatie bekeken worden. Echter, wanneer veldleeuweriken van verschillende populaties tegelijk in een locatie voorkomen (bijvoorbeeld tijdens te trek of in winter) is een geslachtsbepaling van veel individuen niet meer mogelijk. Grote vrouwtjes uit noordelijke populaties hebben namelijk langere vleugels dan kleine mannetjes uit Midden-Europa. Een leeftijdsbepaling door naar tongvlekken te kijken blijkt niet mogelijk. Er werd gedacht dat jonge vogels tongvlekken hebben en deze in de loop van de eerste zomer compleet verdwijnen, maar tongvlekken blijken geen goed kenmerk. Ten eerste hebben sommige nestjonge vogels geen tongvlekken. Ten tweede hebben veel vogels zelfs na meerdere jaren nog tongvlekken. Onze resultaten geven ringers betrouwbare criteria voor de geslachtsbepaling van veldleeuweriken en geven aan dat tongvlekken niet gebruikt kunnen worden om de leeftijd te bepalen. Om in de toekomst fouten in de databanken van de Europese vogeltrekstations te voorkomen, moet worden bijgehouden op welke basis de leeftijd en het geslacht van een vogel is bepaald.

## Het immuunsysteem van veldleeuweriken in de jaarcyclus

In de hoofdstukken 4-6 heb ik samen met mijn medeauteurs verschillende aspecten van het immuunsysteem van de veldleeuwerik gedurende de jaarcyclus onderzocht. In hoofdstuk 4 testen wij een centrale hypothese van de eco-immunologie: verandert het immuunsysteem in de loop van het jaar omdat de investeringen in het immuunsysteem worden afgewogen tegen investeringen in andere fysiologische en gedragsbiologische processen? Tot nu zijn er slechts een handvol studies over dit onderwerp bij wilde vogels. Bovendien is er geen enkele studie die onderzoekt of de variatie gedurende het jaar in het immuunsysteem elke jaar hetzelfde is, of dat deze variatie tussen jaren verschilt. Wij hebben veldleeuweriken in twee complete jaarcycli en in vier verschillende broedperioden gevangen en kleine bloedmonsters afgenomen. Aan de hand van deze bloedmonsters hebben wij in het laboratorium verschillende parameters van het immuunsysteem gemeten. Omdat veldleeuweriken door het jaar karakteristieke en jaarlijks herhalende patronen in dieet, activiteit en sociaal gedrag hebben, zou het goed kunnen dat het immuunsysteem verglijkbare herhalende patronen tijdens de jaarcyclus laten zien. Echter, wanneer omstandigheden in het milieu, zoals de beschikbaarheid van voedsel of weersinvloeden, invloed op het immuunsysteem hebben, zouden veranderingen door het jaar in het immuunsysteem tussen jaren kunnen verschillen. In hoofdstuk 4 laten wij zien dat het immuunsysteem van veldleeuweriken inderdaad sterke veranderingen door het jaar vertoont. We vonden ook dat het immuunsysteem zelfs binnen het broedseizoen verandert. Deze patronen waren echter niet elk jaar hetzelfde, maar verschilden tussen jaren. Dit hebben wij zowel binnen individuen als tussen individuen kunnen vaststellen. Mannetjes en vrouwtjes verschilden echter nauwelijks. Wij concluderen uit dit onderzoek dat aanpassingen in het immuunsysteem tijdens het jaar niet alleen genetisch bepaald worden, maar ook door omgevingsomstandigheden gestuurd zijn.

De resultaten in hoofdstuk 4 verwijzen naar de "baseline" immuunfunctie zonder dat een vogel ziek is. In hoofdstuk 5 en 6 testen wij of de immuunreactie, dus de reactie van een vogel op een ziekteverwekker, gedurende het jaar ook aanpassingen vertoont. Omdat een immuunreactie veel energie vergt en vermoeiend is (denk aan een koortsachtige griep bij mensen) zijn er hypothesen dat vogels, door bijvoorbeeld veranderingen in de voedselbeschikbaarheid, in sommige perioden van het jaar gedwongen zijn hun immuunreactie aan te passen. Toch is deze hypothese nooit bij wilde vogels getest. Wij hebben de immuunreactie van veldleeuweriken tijdens verschillende perioden van het jaar gemeten. Om dit te verwezenlijken hebben wij vogels gevangen en ze voor één nacht naar het laboratorium gebracht. Wij hebben ze een injectie gegeven die een lichte immuunreactie veroorzaakt; dit is verglijkbaar met de reactie na een vaccinatie. Na de „vaccinatie" hebben wij in de aansluitende nacht de stofwisseling van de vogels gemeten en de volgende ochtend de lichaamstemperatuur en bloedsuikerwaarden. Hoewel het lichaamsgewicht en de stofwisseling van gezonde veldleeuweriken gedurende het jaar variëren (wat erop wijst dat ze tijdens sommige perioden energie moeten besparen), was de immuunreactie het hele jaar constant. De „gevaccineerde" vogels hadden altijd een hogere temperatuur, een hogere stofwisseling en hebben gedurende de nacht meer gewicht verloren dan hun soortgenoten die wij niet "gevaccineerd" hadden. Deze resultaten bewijzen dat een immuunreactie veel energie kost, maar onze resultaten weerleggen ook de hypothese van een aangepaste immuunreactie in bepaalde perioden van het jaar. In plaats daarvan suggeren wij dat de immuunreactie zo belangrijk voor de overleving is, dat deze ondanks de hoge kosten niet aangepast kan worden.

In hoofdstuk 4 hebben mijn collega's en ik gedemonstreerd dat de "baseline" van het immuunsysteem zonder dat een vogel ziek is, gedurende het jaar verandert. Als het een ziekteverwekker toch lukt om zich in het lichaam van een vogel te vermeerderen, dan wordt de vogel ziek en een dure immuunreactie is nodig om de ziekteverwekker onschadelijk te maken. De energetische kosten van een immuunreactie als gevolg van een ziekte zijn echter altijd gelijk, onafhankelijk van de periode van het jaar (hoofdstuk 5). In hoofdstuk 6 onderzoeken wij wat er binnen het immuunsysteem gebeurt tijdens een immuunreactie. In de ochtend na de „vaccinatie“ hebben wij van elke vogel ("gevaccineerd" en "ongevaccineerd") een bloedmonster genomen en aan de hand van dit monster tien verschillende parameters van het immuunsysteem gemeten. Zes van de tien parameters waren in de ochtend na de „vaccinatie" veranderd. Sommige waarden waren hoger, terwijl andere waarden lager waren in de "gevaccineerde" vogels vergeleken met de "ongevaccineerde" vogels. Deze variatie toont aan hoe complex het immuunsysteem is. Ondanks deze complexiteit is de samenstelling van de immuunreactie in elke periode van het jaar gelijk en onafhankelijk van de "baseline" van het immuunsysteem. We kunnen hieruit concluderen dat de veranderingen binnen het immuunsysteem tijdens een immuunreactie constant zijn (zoals de energetische kosten) en niet flexibel zoals de "baseline" van het immuunsysteem van een gezonde vogel.

## Bloedparasieten en broedparasieten

In hoofdstuk 7 veranderen wij het perspectief en bestudeer ik een tegenspeler van het immuunsysteem, namelijk bloedparasieten. In samenwerking met Pavel Zehtindjiev en anderen vergelijk ik infecties door bloedparasieten tussen veldleeuweriken uit het Aekingerzand en veldleeuweriken uit Italië. Interessant genoeg hebben veldleeuweriken uit Italië een hoger infectiepercentage dan veldleeuweriken uit het Aekingerzand.

In hoofdstuk 8 staat een andere parasiet in het middelpunt, ook al daagt hij niet het immuunsysteem uit: de koekoek. Veldleeuweriken zijn slechts zelden als waardvogels voor koekoeken omschreven. Ook in het Aekingerzand bleek dit te gelden. Gedurende vier jaar hebben wij 348 veldleeuweriknesten gevonden, terwijl er slechts één (0.3%) was geparasiteerd door een koekoek. Tegelijkertijd werden echter negen van de 43 (21%) nesten van graspiepers door de koekoek geparasiteerd. Bijzonder interessant was dat de veldleeuwerik-ouders schijnbaar de jonge koekoek uit het nest hebben gegooid om hun eigen jongen groot te brengen. Dit gedrag is nog nooit voor een potentiele waardsoort van de koekoek beschreven.

## Overleving en reproductie van veldleeuweriken in relatie met het immuunsysteem

Gedragsbiologische theorieën voorspellen dat individuen een afweging moeten maken tussen de eigen overleving en het grootbrengen van jongen, omdat er energie en tijd slechts in beperkte mate beschikbaar is. Het voeren van jongen vermindert de hoeveelheid tijd en energie die in het eigen lichaam geinvesteerd kan worden. Eerder onderzoek toonde bijvoorbeeld aan dat vogels die je extra jongen in hun nest plaatst een hogere kans hebben om dood te gaan gedurende de volgende winter. De fysiologische mechanismen die hieraan ten grondslag liggen, en wanneer ze werkzaam zijn, zijn echter nauwelijks bekend. Het immuunsysteem zou een sleutelfactor kunnen zijn, omdat oudervogels die extra jongen moeten grootbrengen vaak meer parasieten hebben. Hoofdstuk 9 omvat de resultaten van een veldexperiment, waarin ik dit verband onderzoek. Wij hebben enkele veldleeuweriken-ouders tijdens het voeren van het eerste broedsel van het jaar een klein rugzakje opgebonden zodat ze meer energie voor lopen en vliegen verbruikten. Andere vogels zijn ter vergelijking niet met een rugzakje uitgerust. Vervolgens hebben wij zowel tijdens het eerste als ook tweede legsel het broedsucces (aantal en gewicht jongen) en verschillende parameters van het immuunsysteem gemeten. Uit de metingen bleek dat tijdens het eerste broedsel de oudervogels de extra kosten, veroorzaakt door het gewicht van het rugzakje, afschuiven op hun jongen. Het broedsucces van de vogels met rugzakje is lager en de jongen die wel in leven blijven hebben een minder goed immuunsysteem. Het immuunsysteem van deze jongen verandert omdat ouders met het rugzakje (en dus meer kosten voor lopen en vliegen) ander voer voor hun jongen verzamelen als hun soortgenoten zonder rugzakje. Tijdens het tweede legsel dragen de oudervogels de extra kosten van het rugzakje zelf. Hun immuunsysteem verandert, maar het gewicht en de overleving van de jongen uit het tweede legsel verandert niet door het experiment. Hoewel ik de rugzakjes aan het eind van de broedperiode weer heb verwijderd, hebben deze oudervogels een hogere sterftekans in de winter, waarschijnlijk veroorzaakt door de veranderingen in hun immuunsysteem. Samengevat laten wij voor de eerste keer zien dat het immuunsysteem een sleutelfactor is bij de afweging tussen overleving en voortplanting.

## Trekken of niet trekken: wat zijn de consequenties?

Als binnen een populatie sommige individuen in het broedgebied overwinteren en andere individuen wegtrekken geeft dit de unieke kans om de voor- en nadelen van verschillende overwinteringsstrategieën te bestuderen. In hoofdstuk 2 hebben wij al laten zien dat zo een tweeledige overwinteringsstrategie bij onze veldleeuweriken bestaat. In hoofdstuk 10 bestuderen mijn collega's en ik de consequenties van deze twee verschillende strategieën. Bovendien onderzoeken wij of een vogel altijd dezelfde strategie volgt of dat een vogel tussen verschillende winters van strategie kan wisselen. Om te achterhalen welke strategie een vogel in de voorafgaande winter had, hebben wij tijdens het broedseizoen een stuk van de teennagel geknipt. Uit dit stuk nagel hebben wij de zogenaamde stabiele isotopen geanalyseerd. Dit zijn varianten van natuurlijk voorkomende elementen, die afhankelijk van de geografische plek op aarde in licht verschillende hoeveelheden voorkomen. Deze informatie wordt in groeiend weefsel van organismen (bv. nagels van vogels) opgeslagen en verandert dan niet meer. Dus de punt van een veldleeuwerik-nagel in de zomer bevat informatie over waar de vogel in de voorafgaande winter is geweest (je kunt dit vergelijken met een blauwe vingernagel: als je jezelf met een hamer op je vinger hebt geslagen ontstaat er een blauwe stip in de nagel die in de loop van weken/maanden langzaam naar de punt groeit). Met hulp van de analyse van stabiele isotopen hebben wij voor veel vogels uit het Aekingerzand kunnen vaststellen of ze in de voorafgaande winter trekvogel of standvogel zijn geweest. Wij ontdekten dat de overwinteringsstrategie effect had op het immuunsysteem, het lichaamsgewicht en de toekomstige overleving, maar niet op het broedsucces. De effecten verschilden tussen de jaren, wat er op wijst dat wegtrekken in de ene winter meer voordelen heeft, terwijl in een andere winter in het broedgebied blijven juist voordeliger is. Dit kan ook verklaren waarom beide strategieën naast elkaar kun blijven bestaan. Voor 20 veldleeuweriken zijn wij in staat geweest om een overwinteringsstrategie in verschillende winters te bepalen. Van deze vogels is 45% tussen verschillende winters van strategie gewisseld. Dit wijst erop dat de beslissing om te trekken of lokaal te overwinteren niet genetisch is bepaald, maar door nog onbekende prikkel(s) uit het milieu veroorzaakt wordt.

## Van het veld naar het laboratorium en weer terug: Fysiologie, ecologie en soortbescherming in de jaarcyclus van de veldleeuwerik - een synthese

In het afsluitende hoofdstuk van dit proefschrift vat ik de resultaten van de verschillende detailstudies samen en bediscussieer ze in een grotere samenhang. Ik laat zien hoe gedurende het hele jaar vogels zich aanpassen aan veranderingen in de omgeving. In het bijzonder ga ik in op het belang van het immuunsysteem en ik ontwerp een nieuw concept dat verklaart hoe vogels het hele jaar door gezond blijven en de eisen van het immuunsysteem met andere activiteiten afstemmen. De „baseline“ functie van het immuunsysteem is afhankelijk van de beschikbaarheid van voedsel, het aantal ziekteverwekkers en de om energie concurrerende activiteiten zoals jongen groot brengen of migratie. Als het een ziekteverwekker toch lukt om zich in het lichaam van een vogel te vermeerderen, dan wordt de vogel ziek en een dure immuunreactie is nodig. Deze immuunreactie is onafhankelijk van de „baseline” immuun functie en van andere activiteiten. Om deze immuunreactie op te bouwen moet zo nodig op andere activiteiten bezuinigd worden. Dit kan in het ergste geval tot het verlaten van het nest met eieren of jongen leiden.

In dit afsluitende hoofdstuk laat ik ook zien, hoe de hier gepresenteerde resultaten van fundamenteel wetenschappelijk onderzoek relevant kunnen zijn voor soortbescherming. Zo hebben wij bijvoorbeeld laten zien dat een deel van de Nederlandse veldleeuweriken in de winter naar Zuidwest-Europa trekt, en dat een ander deel in Nederland overwintert. Deze kennis is van groot belang voor de manier waarop men aan soortbescherming wil doen. Beschermingsmaatregelen in de winter moeten tweeledig zijn en de situatie zowel in Nederland als ook in Zuidwest-Europa verbeteren. Bovendien hebben wij aangetoond dat lokale overwintering momenteel een nadelige strategie is. Vogels die lokaal overwinteren hebben een veranderd immuunsysteem en in de volgende winter een lagere kans om te overleven. Het lijkt erop dat het voedselaanbod tijdens de winter vooral in Nederland verbeterd moet worden. Verder laten wij in een experiment zien dat adulte veldleeuweriken minder broedsucces en een hogere sterftekans hebben als de kosten voor lopen en vliegen tijdens het voeren van jongen omhoog gaan. In moderne akkerbouwgebieden moeten veldleeuweriken vaker grotere afstanden vliegen en hebben ze meer tijd nodig om voedsel voor hun jongen te zoeken. Dit zou een vergelijkbaar effect op broedsucces en overleving van deze vogels kunnen hebben als in ons experiment. Verder wijst ons onderzoek erop dat de voedselsamenstelling bij jonge veldleeuweriken invloed heeft op het immuunsysteem en de latere overleving. Het is bekend dat in akkerbouwgebieden minder insectensoorten leven, dus lijkt het goed mogelijk dat jonge veldleeuweriken in het agrarisch gebied vergelijkbare consequenties ondergaan van dit voedselaanbod als in ons onderzoek. Dergelijke resultaten van fundamenteel onderzoek kunnen gebruikt worden om effectievere beschermingsmaatregelen te ontwikkelen.

Verder laat ik op verschillende plekken in dit hoofdstuk zien wat de meest kansrijke stappen zijn voor toekomstig onderzoek om onze kennis over het samenspel tussen ecologische, fysiologische en gedragsbiologische aspecten verder te verdiepen.

## Conclusie

Mijn proefschrift illustreert meerdere sleutelfactoren die bijdragen aan een beter begrip hoe vogels hun immuunsysteem, hun ecologie en hun gedrag aanpassen aan de veranderingen gedurende het jaar om te overleven. Bovendien onderstreept mijn onderzoek de mogelijkheden die ontstaan als veel verschillende onderzoeksmethoden en invalshoeken gecombineerd worden om de complete jaarcyclus van een soort te onderzoeken. Alleen door de integrale en ruime aanpak van dit proefschrift was het mij mogelijk aspecten van fysiologie en ecologie over het hele jaar van een vogel te combineren en mechanistische combinaties te ontrafelen. Het onderzoek naar de hele jaarcyclus maakte het mogelijk om verbanden te ontdekken tussen gebeurtenissen die tijdelijk en ruimtelijk gescheiden zijn en blijkt een zeer sterke en kansrijke aanpak. Op deze manier kunnen wij beter begrijpen hoe vogels overleven en zich voortplanten. Ook kunnen de resultaten gebruikt worden voor het ontwikkelen van beschermingsmaatregelen.

# Deutsche Zusammenfassung

## Streben nach Überleben: Ökologie und Physiologie von Feldlerchen im Jahresverlauf

## Veränderungen im Jahresverlauf

Vögel müssen, wie viele andere Tiere ihr Leben auf die verschiedenen Phasen des Jahres abstimmen. Aufzucht der Jungen, Wechsel des Gefieders (Mauser), Zug in Brut- oder Überwinterungsgebiete und viele andere Aktivitäten müssen nicht nur aufeinander, sondern auch auf jahreszeitliche Veränderungen der Umwelt abgestimmt sein. Dazu müssen Vögel ihr Verhalten und ihre Physiologie anpassen. Entscheidungen oder (Umwelt-) Bedingungen in einer Phase des Jahres können Auswirkungen auf spätere Phasen haben. Verzögert sich zum Beispiel der Heimzug eines Zugvogels aufgrund schlechten Wetters, hat dies eine spätere Ankunft im Brutgebiet zur Folge. Das verzögert wiederum den Brutbeginn und verringert damit möglicherweise den Bruterfolg. Obwohl es schon früh erste Hinweise für einen Zusammenhang zwischen den verschiedenen Phasen des Jahreszyklus gab, blieben Studien, welche eine Vogelart über das gesamte Jahr (und nicht nur zu einer bestimmten Jahreszeit) untersuchen, lange Zeit eine Ausnahme. Vorhandene Studien beschränken sich zudem überwiegend auf ökologische Aspekte, z.B. zeitliche Abläufe. Studien, die sich auf physiologische Ursachen und Folgen von Zusammenhängen zwischen den verschiedenen Phasen des Jahreszyklus konzentrieren, sind bisher kaum vorhanden.

## Die Bedeutung des Immunsystems im Jahreszyklus von Vögeln

Das Immunsystem schützt den Körper gegen Krankheiten und sichert damit das Überleben. Funktioniert das Immunsystem nicht, läuft ein Lebewesen Gefahr, krank zu werden und womöglich zu sterben. Daher ist es für jedes Individuum sehr wichtig, ein gut funktionierendes Immunsystem zu haben. Dies verursacht jedoch auch Kosten. Zum Beispiel wissen wir von uns Menschen, dass nur eine gesunde und ausreichende Ernährung ein gutes Immunsystem ermöglicht. Diese “Kosten” des Immunsystems haben möglicherweise einen entscheidenden Einfluss auf die genaue Lebensgeschichte eines Tieres. Wenn zum Beispiel die Nahrung knapp ist, muss sich ein Vogel „entscheiden“ ob die Energie in das Immunsystem (und damit das langfristige Überleben) oder aber in die Aufzucht von Jungen investiert wird. Das Immunsystem ist jedoch sehr komplex: Verschiedene Bereiche des Immunsystems haben unterschiedliche Kosten und reagieren auf unterschiedliche Krankheitserreger (z.B. Viren, Bakterien, Parasiten). Hinzu kommt, dass sich die Anzahl und Art von Krankheitserregern im Jahresverlauf verändert. Daran muss sich das Immunsystem jedes Mal aufs Neue anpassen. Eine zentrale Hypothese in der Erforschung des Immunsystems von freilebenden Tieren (Ökoimmunologie) geht daher davon aus, dass Investitionen in (verschiedene Teile des) Immunsystems mit Investitionen in anderen physiologische Prozesse und Verhaltensweisen abgewägt werden. Somit steht auf der einen Seite die wichtige Funktion des Immunsystems für das Überleben, und auf der anderen Seite die Kosten, die mit Investitionen in andere Aktivitäten (z.B. Brut, Mauer, Zug) abgewägt werden müssen. Diese Kosten-Nutzen-Abwägung gibt dem Immunsystem das Potential, entscheidend bei der Ausgestaltung eines Vogellebens beteiligt zu sein. Inwiefern sich das Immunsystems wilder Vögel innerhalb eines Jahres verändert und welche Konsequenzen sich aus dieser Veränderung ergeben, ist jedoch weitgehend unbekannt.

## Ziel dieser Arbeit: Integration von Physiologie, Ökologie und Verhalten von Feldlerchen im gesamten Jahresverlauf

Diese Doktorarbeit integriert Aspekte des Verhaltens und der Ökologie von Feldlerchen mit Untersuchungen zu deren Immunsystem im gesamten Jahreszyklus der Feldlerche. Dafür habe ich in Zusammenarbeit mit verschiedenen Kollegen diverse Methoden angewendet, um individuelle Feldlerchen zu verfolgen (Besenderung, Beringung, Analyse stabiler Isotopen). Weiterhin habe ich zahlreiche Techniken und Methoden verwendet, um Daten über die Physiologie im Allgemeinen und das Immunsystem im Besonderen zu gewinnen (z.B. Immuntests, genetischer Fingerabdruck, Stoffwechselmessungen). Außerdem habe ich zusammen mit Kollegen in kleinen Experimenten verschiedene Faktoren manipuliert, um zu sehen, wie sich die Vögel daran anpassen. Dieser innovative, integrative und vielfältige Untersuchungsansatz erlaubt es mir, die Physiologie, die Ökologie und das Verhalten zu kombinieren. Dadurch lernen wir, besser zu verstehen, wie sich Vögel im Allgemeinen und Feldlerchen im Besondern an Umweltveränderungen innerhalb eines Jahres anpassen, und wie sie die Herausforderung, gesund zu bleiben, mit den Herausforderungen von Jungenaufzucht, Überwinterung, Mauser und Zug vereinen können. Die Arbeit ist in 4 Teile gegliedert. Nach einer allgemeinen Einleitung (Kapitel 1) werden in Teil 1 (Kapitel 2,3) feldbiologische Aspekte untersucht, welche die Grundlage für die weiteren Kapitel liefern. Teil 2 (Kapitel 4-6) konzentriert sich auf das Immunsystem. Teil 3 (Kapitel 7,8) beleuchtet Blutparasiten und Brutparasiten. Teil 4 (Kapitel 9-11) kombiniert die ökologischen und physiologischen Forschungsansätze und identifiziert mechanistische Zusammenhänge und Wechselwirkungen. Ich beende meine Doktorarbeit mit einer Synthese, in der ich die Ergebnisse der einzelnen Kapitel in einem weiteren Zusammenhang diskutiere. Dabei zeige ich auch auf, wie moderner Artenschutz von Feldlerchen in der Agrarlandschaft von der vorliegenden wissenschaftlichen Grundlagenarbeit profitieren kann.

## Die Feldlerche

Die Feldlerche ist ein weitverbreiteter Singvogel Europas und lebt in offenen Landschaften mit nicht zu dichter Bodenvegetation. Ursprünglich in natürlichen Offenlandschaften beheimatet, brüten die meisten Feldlerchen heutzutage in der Kulturlandschaft. In Holland (und Deutschland) ist der Jahresverlauf von Feldlerchen durch bestimmte Phasen charakterisiert. Die Vögel kommen Mitte bis Ende Februar in ihren Revieren an, und die Männchen beginnen zu singen. Von Ende April bis Ende Juli werden Nester gut versteckt am Boden im Gras gebaut. Feldlerchen produzieren Gelege mit je 3-5 Eiern.

Pro Jahr können bis zu fünf Gelege produziert werden. Ein Großteil der Bruten ist jedoch nicht erfolgreich, weil sie von Prädatoren gefressen werden. Im August und September wechseln Jung- und Altvögel ihr Gefieder (Mauser). Im Oktober und Anfang November ziehen Feldlerchen nach Südwesteuropa zum Überwintern. Jedoch überwintern auch Feldlerchen in den Niederlanden (und Deutschland). Im Februar und März ziehen Feldlerchen dann zurück in ihre Brutgebiete. Während des Sommers sind Feldlerchen sehr territorial und aggressiv gegen Reviernachbarn. Außerhalb der Brutzeit leben sie jedoch in Gruppen. Körner und andere Pflanzenteile sind die Nahrungsgrundlage im Winter. Im Sommer werden viele Insekten bzw. Insektenlarven gefressen; Jungvögel werden sogar ausschließlich mit Insekten(larven) gefüttert. Wie viele andere Arten des Offenlandes nehmen die Bestände der Feldlerche in Mitteleuropa rasant ab, in den Niederlanden seit den 1970iger Jahren um 95%. Der Rückgang ist eng mit der Intensivierung der Landwirtschaft verbunden. Besonders der Wechsel von Sommer- auf Wintergetreide hat sich negativ ausgewirkt: Wintergetreide wächst schneller und dichter, weshalb sich Nestgelegenheiten verringern. Außerdem fehlen nahrungsbietende Stoppelfelder im Winter.

## Das Untersuchungsgebiet: Der Aekingerzand

Die Untersuchungen zu dieser Arbeit wurden überwiegend an einer Feldlerchenpopulation im Aekingerzand durchgeführt. Der Aekingerzand ist ein Teil des Nationalparks Drents-Friese Wold in den nördlichen Niederlanden, wo außer ganzjähriger Schafbeweidung keine landwirtschaftliche Nutzung stattfindet. Der Aekingerzand besteht aus einem Mosaik aus offenen Sanddünen, Gras- und Heideflächen, sowie einzelnen Baumgruppen auf sandigen Böden. Die kurze, lückige Vegetation, viele offene Bodenstellen und kleine Hügel entsprechen dem natürlichen Habitat der Art und ermöglichen eine hohe Dichte an Brutpaaren. Die Lebensraumeigenschaften erlauben auch ein relativ einfaches Erkennen von farbigen Fußringen. Durch solche Farbringe, die mit Hilfe eines Fernglases oder Fernrohrs erkennbar sind, können Vögel individuell markiert werden. So können verschiedene Aspekte individueller Lebensgeschichten studiert werden (z.B. welche Eltern zu einem Nest gehören, die Anzahl der Bruten, ob der Vogel im nächsten Jahr zurückkommt). Die Kombination aus individueller Identifizierung und hoher Brutpaardichte erlaubt im Aekingerzand Studien, die an Populationen in der modernen Landwirtschaft unmöglich wären.

## Die Überwinterungsstrategie

In Kapitel 2 habe ich zusammen mit Kollegen die Überwinterungsstrategie von holländischen Feldlerchen untersucht. Obwohl die Feldlerche noch immer ein häufiger Vogel ist, war bisher unbekannt, ob holländische Feldlerchen Zugvögel sind oder im Brutgebiet überwintern. Es wurde spekuliert, dass holländische Feldlerchen zumindest überwiegend in den Niederlanden überwintern und dabei Gesellschaft von Feldlerchen aus Nord- und Osteuropa bekommen. Eine andere Hypothese ist, dass die meisten oder alle Brutvögel Zugvögel sind und vermutlich in Frankreich überwintern. Hiesige Überwinterer wären somit ausschließlich Vögel, die weiter im Norden gebrütet haben. Daten, die eine der beiden Hypothesen belegen könnten, gab es bisher nicht. Um dies zu untersuchen, haben wir einen zweigleisigen Untersuchungsansatz gewählt. Einerseits haben wir die Ringfunde der holländischen Vogelwarte der letzten 100 Jahre ausgewertet und andererseits einige Vögel unserer Untersuchungspopulation mit kleinen Radiosendern ausgestattet. Sowohl die Ringfundanalyse als auch die Telemetrie liefert eindeutige Beweise, dass es unter den holländischen Feldlerchen sowohl Zug- als auch Standvögel gibt. Einige Vögel ziehen nach Südwesteuropa während andere in der Nähe ihrer Reviere überwintern.

## Alters- und Geschlechtsbestimmung von Feldlerchen

Da sich männliche und weibliche Feldlerchen nicht an Gefiedermerkmalen unterscheiden, benutzen Vogelberinger üblicherweise die Flügellänge als Geschlechtsmerkmal. Eine Altersbestimmung kann meist nicht durchgeführt werden. Gelegentlich wurde jedoch vermutet, dass sich Jungvögel im Herbst und Winter an Flecken auf der Zunge von Altvögeln unterscheiden lassen. In Kapitel 3 teste ich zusammen mit einigen Kollegen diese beiden Kriterien. Wir zeigen unter Zuhilfenahme molekularer Methoden, dass Feldlerchen nahezu fehlerfrei anhand der Flügellänge geschlechtsbestimmt werden können, wenn man Vögel innerhalb einer Population betrachtet. Wenn Feldlerchen verschiedener Herkunftsgebiete gleichzeitig an einem Ort vorkommen (z.B. auf dem Zug oder im Winter), können viele Individuen nicht mehr einwandfrei geschlechtsbestimmt werden, denn die größten Weibchen nördlicher Populationen haben längere Flügel als die kleinsten Männchen mitteleuropäischer Populationen. Eine Altersbestimmung anhand der Zungenflecken ist nicht möglich. Es wurde vermutet, dass junge Feldlerchen Zungenflecken haben und diese im Lauf des ersten Sommers verschwinden. Dies ist jedoch nicht der Fall. Einerseits haben bereits einzelne Nestlinge keine Zungenflecken, andererseits behalten andere Vögel diese für mehrere Jahre. Unsere Ergebnisse geben Beringern verlässliche Kriterien zur Alters- und Geschlechtsbestimmung. Um dennoch mögliche Fehlbestimmungen zu identifizieren, sollten die Datenbanken der europäischen Vogelwarten zukünftig registrieren, an welchen Kennzeichen Beringer Geschlechts- und Altersbestimmungen vorgenommen haben.

## Das Immunsystem der Feldlerche im Jahresverlauf

In den Kapiteln 4-6 habe ich zusammen mit meinen Mitautoren verschiedene Aspekte des Immunsystems der Feldlerche im Jahresverlauf untersucht. In Kapitel 4 testen wir eine zentrale Frage der Ökoimmunologie: Verändert sich die Grundabwehr des Immunsystems (eines gesundes Vogels) im Laufe des Jahres, da die Investitionen in die Immunabwehr gegen Investitionen in andere physiologische und verhaltensbiologische Prozesses abgewägt werden? Bisher gibt es hierzu nur sehr wenige Studien an Wildvögeln. Darüber hinaus hat keine einzige Studie überprüft, ob die jahreszeitlichen Schwankungen jedes Jahr gleich sind. Wir haben Feldlerchen über zwei komplette Jahreszyklen sowie in vier verschiedenen Brutperioden gefangen und ihnen eine kleine Blutprobe entnommen. Anhand der Blutprobe habe ich später im Labor verschiedene Parameter des Immunsystems gemessen. Da Feldlerchen über das Jahr charakteristische und jährlich wiederholende Veränderungen in der Nahrungszusammensetzung, Aktivität und im sozialen Leben zeigen, sind ebenso regelmässige, sich wiederholende jahreszeitliche Veränderungen im Immunsystem denkbar. Wenn jedoch äußere Umstände wie Nahrungsverfügbarkeit oder Witterung einen Einfluss auf das Immunsystem haben, könnten jahreszeitliche Veränderungen im Immunsystem in verschiedenen Jahren unterschiedlich ausgeprägt sein. Hier zeigen wir, dass das Immunsystem von Feldlerchen tatsächlich Veränderungen im Jahresverlauf unterworfen ist. Manche Immunfunktionen sind zu bestimmten Zeiten stärker ausgeprägt als andere. Selbst innerhalb der Brutsaison verändert sich das Immunsystem. Die Schwankungen sind jedoch nicht jedes Jahr konstant, sondern können sich von Jahr zu Jahr ändern. Dies konnten wir sowohl innerhalb von Individuen als auch im Vergleich zwischen verschiedenen Individuen feststellen. Männchen und Weibchen unterscheiden sich dagegen nur sehr wenig. Wir schließen aus unseren Untersuchungen, dass Veränderungen des Immunsystems im Jahresverlauf nicht starr genetisch programmiert sind, sondern zusätzlich von Umwelteinflüssen gesteuert werden.

Die in Kapitel 4 gezeigten Veränderungen im Immunsystem beziehen sind auf die Grundabwehr von gesunden Vögeln. In Kapitel 5 und 6 teste ich mit Hilfe einiger Kollegen, ob die Immunreaktion, d.h. die Reaktion eines Vogels in dessen Körper sich ein Krankheitserreger etablieren konnte, auch Veränderungen im Jahresverlauf unterliegt. Da eine Immunreaktion eine sehr energieaufwändige und anstrengende Situation ist (man denke an eine fiebrige Grippe beim Menschen), gibt es Hypothesen, dass Vögel z.B. aufgrund von Veränderungen im Nahrungsangebot zu bestimmten Jahreszeiten dazu gezwungen sind, unterschiedliche Krankheitsreaktionen zu zeigen. Diese Hypothese wurde bisher jedoch noch nicht bei Wildvögeln getestet. In Kapitel 5 haben wir haben die energetischen Kosten einer Immunreaktion von Feldlerchen zu verschiedenen Zeiten des Jahres gemessen. Dazu haben wir Vögel gefangen und für eine Nacht ins Labor gebracht. Ihnen wurde eine Substanz gespritzt, die eine leichte Immunreaktion hervorruft, die vergleichbar mit Symptomen ist, die nach einer Impfung auftreten. Nach der "Impfung" haben wir in der darauf folgenden Nacht die Stoffwechselrate, sowie am nächsten Morgen die Körpertemperatur und Blutzuckerwerte gemessen. Obwohl gesunde Feldlerchen das Körpergewicht und die Stoffwechselrate im Jahresverlauf variieren (was dafür spricht, dass sie zu bestimmten Zeiten sparsam mit ihrer Energie umgehen müssen), war die Immunreaktion das gesamte Jahr über konstant. Die "geimpften" Vögel hatten immer eine etwas erhöhte Temperatur, einen höheren Stoffwechsel und verloren mehr Gewicht über Nacht als ihre Artgenossen ohne eine experimentell ausgelöste Immunreaktion. Die Daten zeigen, dass eine Immunreaktion viel Energie verbraucht. Unsere Ergebnisse widerlegen jedoch die Hypothese von angepasster Immunreaktion im Jahresverlauf. Stattdessen postulieren wir, dass eine Immunantwort für das Überleben so bedeutsam ist, dass sie trotz ihrer energetischen Kosten nicht eingeschränkt werden kann.

In Kapitel 4 haben wir gezeigt, dass sich das Immunsystem eines gesunden Vogels über den Jahresverlauf verändert. Schafft es jedoch ein Krankheitserreger sich im Körper des Vogels zu etablieren und der Vogel wird krank, muss eine energetisch kostspielige Immunreaktion aufgebaut werden. Die energetischen Kosten einer solchen Immunreaktion sind immer konstant, egal zu welcher Jahreszeit ein Vogel krank wird (Kapitel 5). In Kapitel 6 untersuchen wir, was innerhalb des Immunsystems während der Immunreaktion passiert. Am Morgen nach der „Impfung“ haben wir sowohl den „geimpften“ als auch von „ungeimpften“ Vergleichsvögeln eine Blutprobe genommen und anhand dieser 10 verschiedene Parameter des Immunsystems gemessen. Sechs der Parameter zeigten am Morgen nach der “Impfung” veränderte Werte. Einige Werte stiegen, andere Werte sanken in den „geimpften“ Vögeln im Vergleich zu nicht „geimpften“ Vögeln. Dies zeigt, wie komplex eine Immunreaktion ist. Trotz dieser Komplexität war die Zusammensetzung der Immunreaktion zu allen Phasen des Jahreszyklus gleich. Die einzelnen Parameter des Immunsystems während einer Immunantwort reagieren zu jeder Zeit des Jahres gleich, unabhängig wie die Grundabwehr des Immunsystems augenblicklich ist. Dies bedeutet, dass die Veränderungen innerhalb des Immunsystems konstant sind und nicht flexibel wie die Basiswerte des Immunsystems vor einer Erkrankung. Die Zusammenstellung der Immunreaktion bleibt ebenso wie deren energetischen Kosten das gesamte Jahr über gleich.

## Blutparasiten und Brutparasiten

In Kapitel 7 wechseln wir die Perspektive und studieren einen Gegenspieler des Immunsystems: Blutparasiten. Zusammen mit Pavel Zehtindjiev und anderen vergleichen wir Infektionen durch Blutparasiten in Feldlerchen aus dem Aekingerzand und von Feldlerchen, die in Italien gefangen wurden. Interessanterweise zeigen Feldlerchen aus Italien eine viel höhere Infektionsrate als Feldlerchen aus dem Aekingerzand. In Kapitel 8 steht ein anderer Parasit im Fokus, auch wenn er das Immunsystem nicht herausfordert: Der Kuckuck. Feldlerchen wurden bisher nur sehr selten als Wirtsvögel für Kuckucke beschrieben. Dies gilt auch für unser Untersuchungsgebiet. Im Laufe von 4 Jahren fanden wir 348 Feldlerchennester, aber nur eines wurde von einem Kuckuck parasitiert. Im gleichen Zeitraum enthielten jedoch 21% der 43 Wiesenpiepernester, die wir fanden, ein Kuckucksei oder -junges. Besonders interessant war jedoch, dass der junge Kuckuck offenbar von den Feldlercheneltern aus dem Nest geworfen wurde und die Feldlerchen dann den eigenen Nachwuchs grosszogen. Ein solches Verhalten wurde bisher noch für keine potentielle Wirtsart des Kuckucks beschrieben.

## Der Zusammenhang zwischen Überleben, Reproduktion und dem Immunsystem von Feldlerchen

Verhaltensbiologische Theorien sagen voraus, dass Individuen zwischen dem eigenem Überleben und der Aufzucht von Jungen abwägen müssen, da ihnen nur begrenzt Energie zur Verfügung steht. Die Jungenaufzucht verringert oft die Energie und Zeit, welche in den eigenen Körper investiert werden kann. Es wurde z.B. gezeigt, dass Vögel, denen man zusätzliche Jungtiere ins Nest setzt, im darauf folgenden Winter eine erhöhte Sterblichkeitsrate haben. Die physiologischen Mechanismen, und wann sie genau wirken, sind jedoch weitgehend unbekannt. Dem Immunsystem könnte eine Schlüsselfunktion zukommen, da Altvögel, die zusätzliche Jungvögel aufziehen müssen, erwiesenermaßen meist einen stärkeren Parasitenbefall haben. Kapitel 9 beinhaltet die Ergebnisse eines Feldexperimentes, das diese Zusammenhänge untersucht. Ich habe einigen adulten Feldlerchen während der ersten Brut des Jahres einen kleinen Rucksack aufgesetzt, wodurch sich ihre energetischen Kosten für Laufen und Fliegen erhöht haben. Zum Vergleich haben wir andere Vögel ohne dieses Extragewicht gelassen. Anschließend haben wir sowohl während der ersten als auch während der zweiten Brut den Bruterfolg (Anzahl und Gewicht der Jungen) und verschiedene Parameter des Immunsystems von Alt- und Jungvögeln gemessen. Offenbar wälzen die Altvögel während der ersten Brut die Nachteile des zusätzlichen Gewichtes auf die Jungen ab. Der Bruterfolg ist geringer und die ausfliegenden Jungen haben ein schlechteres Immunsystem. Das Immunsystem ist offenbar schlechter, weil die Altvögel mit Extragewichten aufgrund der zusätzlichen Kosten für Laufen und Fliegen anderes Futter für ihre Jungvögel sammeln als ihre Artgenossen ohne Rucksack. Während der zweiten Brut tragen dann die Altvögel die zusätzlichen Kosten und ihr Immunsystem verändert sich. Die Jungen bleiben jedoch von Auswirkungen verschont. Obwohl ich am Ende der Brutsaison den Vögeln die Rucksäcke wieder abgenommen habe, hatten Altvögel eine höhere Sterblichkeit im darauf folgenden Winter, vermutlich bedingt durch die Veränderungen im Immunsystem. Damit zeigen wir erstmals, dass dem Immunsystem eine Schlüsselrolle in der Abwägung zwischen Selbsterhaltung und Reproduktion zukommt.

## Ziehen oder nicht Ziehen: was sind die Konsequenzen?

Wenn innerhalb einer Population einige Individuen im Brutgebiet überwintern und andere wegziehen, bietet sich die einmalige Chance, Vor- und Nachteile unterschiedlicher Überwinterungsstrategien zu studieren. In Kapitel 2 haben wir bereits gezeigt, dass eine solche zweigleisige Überwinterungsstrategie bei unseren Feldlerchen vorkommt. In Kapitel 10 untersuchen meine Kollegen und ich die Konsequenzen dieser unterschiedlichen Strategien. Auch untersuchen wir, ob ein Vogel jeden Winter derselben Strategie folgt oder zwischen Zug und lokaler Überwinterung wechseln kann. Um herauszufinden, welcher Strategie Vögel im vorangegangenen Winter folgten, haben wir während der Brutperiode ein Stück der Kralle abgeschnitten und daraus dann sogenannte stabile

Isotope analysiert. Dabei handelt es sich um Variationen natürlich vorkommender Elemente, die sich je nach geographischer Herkunft geringfügig unterscheiden. Diese Information wird in wachsendem Gewebe eingebaut und verändert sich danach nicht mehr. Daher enthält die Spitze der Kralle im Sommer Informationen darüber, wo der Vogel im Winter war (man kann dies z.B. mit einem blauen Fingernagel vergleichen: hat man sich im Winter mit einem Hammer auf den Finger gehauen, wächst die blaue Stelle langsam über Wochen oder Monate zur Spitze heraus). Mit Hilfe der Analysen stabiler Isotope konnten wir für viele Feldlerchen feststellen, ob sie im vorangegangenen Winter Stand- oder Zugvogel waren. Wir fanden heraus, dass die Überwinterungsstrategie Auswirklungen auf das Immunsystem, das Körpergewicht und die zukünftigen Überlebenschancen hat, nicht jedoch auf den Bruterfolg. Die Effekte unterschieden sich zwischen den Jahren, was darauf hindeutet, dass eine Strategie in einem Winter mehr Vorteile bringt und die andere Strategie in einem anderen Winter. Dies kann auch erklärten, warum beide Strategien nebeneinander vorkommen. Für 20 Feldlerchen konnten wir die Überwinterungsstrategie in mehreren Wintern bestimmen. Fast die Hälfte der untersuchten Feldlerchen (45%) wechselte die Strategie zwischen verschiedenen Wintern. Dies bedeutet, dass die Strategie nicht genetisch vererbt wird, sondern die Entscheidung zu ziehen oder lokal zu überwintern durch äußere Umstände beeinflusst wird.

## Aus dem Gelände ins Labor und wieder zurück: Physiologie, Ökologie und Artenschutz im Jahresverlauf der Feldlerche - eine Synthese

Im abschließenden Kapitel dieser Arbeit fasse ich die Ergebnisse der verschiedenen Einzelstudien zusammen und diskutiere sie in einem größeren Zusammenhang. Ich zeige, wie die Ergebnisse meiner Arbeiten unser Verständnis darüber vertiefen, wie Vögel sich an Umweltveränderungen im Jahresverlauf anpassen. Insbesondere gehe ich auf die Bedeutung des Immunsystems ein und entwickle ein neues Konzept. Dieses Konzept erklärt, wie Vögel das gesamte Jahr über gesund bleiben und die Anforderungen des Immunsystems mit anderen Aktivitäten und Umweltbedingungen abstimmen. Es beinhaltet, dass die Grundabwehr des Immunsystems abhängig von der zur Verfügung stehenden Nahrung, der Anzahl Krankheitserreger und den um Ressourcen konkurrierenden Aktivitäten wie Jungenaufzucht oder Zug ist. Wenn es ein Krankheitserreger trotzdem schafft, sich im Körper eines Vogels zu etablieren und der Vogel krank wird, muss eine kostspielige Immunreaktion aufgebaut werden. Diese Immunantwort ist unabhängig von den Basiseigenschaften des Immunsystems im gesunden Zustand und von anderen Aktivitäten. Um eine adäquate Immunreaktion zu gewährleisten und das eigene Überleben zu sichern, müssen ggf. andere Aktivitäten eingeschränkt werden. Dies kann im schlimmsten Falle das Verlassen der eigenen Jungen bedeuten.

In diesem abschließenden Kapitel zeige ich aber auch auf, wie die hier präsentierten Ergebnisse wissenschaftlicher Grundlagenforschung für den Artenschutz relevant sein können. Zum Beispiel haben wir gezeigt, dass ein Teil der holländischen Feldlerchen im

Winter nach Südwesteuropa zieht und der andere Teil im Brutgebiet überwintert. Dieses Wissen ist sehr wichtig bei der Entwicklung von Schutzstrategien. Schutzmaßnahmen im Winter müssen zweigleisig sein und die Situation sowohl in den Niederlanden als auch in Südwesteuropa verbessern. Darüber hinaus haben wir gezeigt, dass lokale Überwinterung momentan eine nachteilhafte Strategie ist. Vögel, die lokal überwintern, hatten ein verändertes Immunsystem und haben im folgenden Winter eine geringere Überlebenswahrscheinlichkeit. Dies spricht dafür, dass das Nahrungsangebot für Feldlerchen im Winter, vor allem in den Niederlanden, verbessert werden muss. Außerdem zeigen wir, dass adulte Feldlerchen einen geringeren Bruterfolg und eine höhere Sterblichkeit haben, wenn die Kosten für Laufen und Fliegen während der Jungenaufzucht experimentell erhöht wurden. In der modernen Agrarlandschaft müssen Feldlerchen oft längere Strecken fliegen und benötigen mehr Zeit, um Nahrung für die Jungen zu sammeln. Dies könnte ähnliche Folgen haben wie unser Experiment mit den kleinen Rucksäcken. Weiterhin zeigen wir auf, dass eine veränderte Nahrungszusammensetzung bei jungen Feldlerchen ein verändertes Immunsystem zur Folge hat, was wiederum mit einer geringeren Rückkehrrate dieser Vögel im folgenden Jahr korreliert. Es ist bekannt, dass in der modernen Agrarlandschaft weniger Insektenarten leben. Daher ist es möglich, dass junge Feldlerchen in der Agrarlandschaft ähnlichen Konsequenzen unterliegen. Wenn Artenschutz solche Ergebnisse von Grundlagenforschung berücksichtigt, besteht die Chance, effektivere Schutzmaßnahmen zu entwickeln. Außerdem erläutere ich die vielversprechendsten Schritte zukünftiger Forschung, die das Wissen über das Zusammenspiel von ökologischen, physiologischen und verhaltensbiologischen Aspekten während des gesamten Jahreszyklus einer Vogelart vergrößern werden.

## Fazit

Meine Dissertation weist mehrere Schlüsselfaktoren nach, wie Vögel ihr Immunsystem, ihre Ökologie und ihr Verhalten anpassen, um die sich ändernden Bedingungen innerhalb eines Jahres zu überleben. Außerdem verdeutlicht meine Arbeit die Möglichkeiten, die entstehen wenn man einen Forschungsansatz wählt, der viele Methoden und Techniken sowie eine Betrachtung des gesamten Jahreszyklus einer Art kombiniert. Nur durch diesen integrativen und vielzeitigen Ansatz, war es mir möglich, Aspekte von Physiologie und Ökologie über den gesamten Jahreszyklus zu vereinen und mechanistische Verbindungen aufzuzeigen. Die Betrachtung des gesamten Jahreszyklus erlaubte es mir, Verbindungen zwischen Ereignissen zu finden, die zeitlich und räumlich getrennt sind. Letztendlich ist die Kombination aus vielen Methoden und die Betrachtung des gesamten Jahreszyklus eine der vielversprechendsten und stärksten Forschungsansätze um Grundlagenforschung zu betreiben. Das daraus gewonnen Wissen kann dann auch in Artenschutz umgesetzt werden.


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Accomplishing this intensive study and learning so much over the years would have been impossible without receiving support in many different ways. Numerous friends and colleagues helped me with various things over the past years. Many of them have already been mentioned in the acknowledgments of the different chapters for specific contributions to each sub-study of my project. So please read these acknowledgments as well. This section tries to summaries the help and support I received since my arrival in Groningen.

Right at my application interview in early 2006 Irene Tieleman trusted me with the challenging and fascinating project about Skylarks in the Aekingerzand. She then gave me all the freedom to develop my own ideas and lines of research while supporting me with her sharp sense for science. I much enjoyed developing my own project rather than being just a tiny part of a long-term project, even though the former comes with some difficulties every now and then. I'm also very glad that Irene now gives me the unique opportunity to continue for another year with the work we started. Hopefully we can give the Aekingerzand work a glory future. Kevin Matson taught me most of my knowledge about lab work and immune assays and was for all the things that happened in the lab and office a great daily supervisor. Your door was always open (often literally) and I could just drop by and bother you with any question at almost any time.

Especially in the first years, when I developed the research lines of my project, meetings with Theunis Piersma were very stimulating. Christiaan Both helped me out in the years that Irene was sick and unavailable. Thanks to both of you. Christiaan, Niels Dingemanse and the late Rudi Drent provided useful discussions and feedback in the early stages of my project. I also thank the members of my reading committee (Dennis Hasselquist, Marcel Klaassen and Jan Komdeur) for taking the time to read and evaluate my thesis. Collaborations with Pete Marra, Heiner Flinks, Auxi Villegas and Pavel Zehtindjiev were very fruitful and brought me many new insights. I very much hope we can continue collaborations in the future. I also want to thank Barbara Helm for the help with compiling a strong application and supporting me while searching for a PhD-position. I am not sure if I would made the step into science without the experience at the Max Planck Institute in Andechs.

Irene's little lab group provided helpful feedback on immunological problems. Especially Debbie Buehler, being a senior PhD student when I started, taught me a lot about lab work and administrative life at the University. Debbie also made me one of the nicest compliments I ever got ("Seeing Arne doing field work is like observing a fish in water; a perfect match"). The collaboration with Maaike Versteegh and Kevin (and others) for the LPS-project was very fruitful and stimulating. I am very grateful for your great input into every component of this project. It was great fun doing it together and I enjoyed it very much. Maaike also helped me out with R-problems so many times, especially when it came to graphs. Sometimes it was enough for her to just stand next to my computer (but that was crucial at that moment) and R then finally did the things I wanted R to do. The weekly lunch meetings of Animal Ecology are infamous. Some people are even afraid of them. I learned much from these critical discussions. There I learned to be critical, to be precise in what you tell and how to ask questions. This stimulating envi-

ronment is one of the main advantages of working in Groningen. I am grateful to everyone who regularly contributed to lively lunch meetings. Our secretaries Suus Bakker and Joyce Rietveld helped with many administrative problems and tasks. Dick Visser did an excellent job and turned the different chapters into this nice little book. Maaike, Martijn Hammers and Marco van der Velde improved my Dutch summary, Silke Steiger and Chris Trierweiler commented on my German summary. Also many thanks for these last minute tasks.

Catching Skylarks during moult and winter was only possible with helpers. I very much appreciated the help during the night catching in the Aekingerzand or on (sometimes very muddy) fields in cold conditions somewhere at the border between Drenthe and Friesland. Thanks to Martin Keiser, Rob Voesten, Stephi Michler, Richard Ubels, Chris Trierweiler, Flavia Geiger, Merlijn de Graaf, Lotte Oostebrink, Martijn Hammers, Rose Kentie, Pedro Lourenco, Oscar Vedder, Liz Kleynhans, Jorna Ariz, Elske Schut, Aafke van Erk, Janne Ouwehand, Jelmer Poelstra, Tim Tausenfreund, Sophie Jaquier, Claudia Burger, Luc te Marvelde, Kristina Pfannkuche, Freek Mandema, Kobus Boeke, Maaike Versteegh, Sebastian Prinz and the ones I might have forgotten. Janco Mulder counted as a volunteer Skylarks for us in Winter 07/08. Many thanks. In Winter 08/09 Flavia Geiger from University of Wageningen joint our winter field work. We developed great plans how to use the locally wintering radio-tagged birds to combine their spatial use with Flavia's excellent knowledge on food availability. Unfortunately all except one skylark decided to migrate that winter, so plans needed to be adjusted. Still, Flavia and her coworkers Maurits Gleichman and Sebastian Prinz collected very interesting data and I much enjoyed working with you. I look forward to get the data published.

A very special thanks goes to Kees van Eerde and Rob Voesten who were both so important for my thesis. I still remember the first encounters with both of them, even though I did not realize at that moment that such fruitful collaborations would emerge. Kees had (very sadly he hasn't anymore) a fascinating system of big clap nets at the Dwingelerveld (you can find several photos in this thesis). I needed some Skylarks during migration and Kees wrote me that he sometimes catches Skylarks during autumn migration. Without knowing how his system works, I had no special expectation when arriving completely sleep deprived at his catching place on an early October morning. I had spent the entire night trying to catch migrants with the "French method" in the Aekingerzand. I hadn't slept at all that night, neither caught a single Skylark. But what was happening then blew all my tiredness and letdowns entirely away. Kees easily caught the first larks for me, then more and more. So many that I couldn't handle them. In between we caught Meadow Pipits, Redwings, Fieldfares, Wagtails, Starlings (a species I like, but Kees hates) and many more just "pulling" them out of the sky. His little "keet" (construction trailer) was perfect to sample the birds. I was so full of adrenaline that day that I completely forgot my tiredness. Many more successful catching days followed in that, and in other seasons including spring. Sometimes we modified his "keet" to a "mobile lab". The days catching (and eating cookies) with Kees certainly belong to the nicest of my whole project and the data contributed to several chapters!

Rob arrived in 2007 as a volunteer field assistant to help for two weeks of the breeding season. At that time, he spoke hardly any English, I hardly any Dutch. But still there weren't any communication problems, because he is as much a fanatic field worker as I am. I was so impressed by his enthusiasm and his field work abilities that we hired him as a field assistant for each breeding season ever since. He had very important contributions to much of the field working that was going on in the Aekingerzand, especially the handicap experiment. Working with Rob was not only of high quality but also involved a lot of fun. Rob also took over "my" population work in the Aekingerzand while I was writing up this thesis. I hope you will be involved in the work for many more years and we keep the good connections!

Exchanging experiences with Henk Jan Ottens during breeding seasons was always of great help and fun. As well as our "competitions", with daily sms-updates, who will find most nests. Sorry, Henk Jan that I always beat you. Stef Wasdorp was a good (even though partly chaotic) coworker in the field. I am impressed by your easy-doing style of field work while still being able to accomplish so much. Hope we will stay in though. I much enjoyed the two short (and very spontaneous!) trips to France to catch Skylarks with Moana Grysan. Thanks to you and your family for your hospitality.

During the breeding seasons several volunteer field assistants from all over the world joint our team. I especially want to mention Nicole Schneider, Mauro(ne Mafia Boss) Varaschin, Andreas Bange and Liz Morgan. Staatsbosbeheer Drents-Friese Wold generously allowed us working in the Aekingerzand and were open to Stef's and my suggestions on how to fix some problems in the Aekingerzand. Thanks.

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During the project I also supervised several students (which was highly "illegal", because bursal PhD students are officially not allowed to teach; still supervising students is commonly expected, but we are not allowed to mention it in official reports...). Anyhow, I learned a lot from the interactions with the Master students Merlijn de Graaf, Joppe Rijpstra, Lotte Oostebrink as well as with the course students Maite Punter, Nicky Lustenhouwer, Koen Meirmans and Sander Wallert. Thanks for this learning experience and the fun we had. I hope you also learned some things from me and remember more then that I was a slave driver (I recently heard this was my reputation under some of you).

At the old BC in Haren I shared my office with Nick Horrocks and having a native English speaker sitting right (actually left) next to you had many advantages. After the move to the new building in Groningen Steffi Nolte, Freek Mandema and Roel van Klink from COCON were very nice roommates. I also appreciated coffee breaks and chats with other COCON members (Wimke, bedankt voor de lekkere appeltaart; hoop ik mag nog een keer mee voor het ganzenvangen).

Beside all the work-related interactions, social events have been important too. I still remember all the great and funny social events we had in Animal Ecology, mostly at the old BC in Haren, sometimes at other places: Coffee breaks, borrels, table tennis, football matches, preparing and playing stukjes, poker nights and much more. Many students, PhD students, PostDocs, guests and other people joint these events during the years. I especially want to thank Cas, Karen, Martin v/d P., Lyanne, Elske, Eelke, Peter D., Götz, Martin, Stephi, Luc, Popko, Janske, Janne, Berber, Michael M., Oscar, Roos, Richard, Marco, Martijn H., Jildou and Reinder. My year-around field work left not much time for a social life outside the University, but maybe that is why these activities and contacts were so important, too. Evenings in the Pintelier with Ulrike & Carlos and Romy & Reinhard, skyping and mailing with Miriam and Silke, playing football with "GVVC1" and bird counts with the counting groups in the Lauwersmeer, on Schiermonnikoog and in the Dollard; all were welcome distractions from work. Likewise were the dinners with Elske. Thanks also for trusting me to be your paranimf. Hope you enjoy your role as a paranimf on 23 November with Aniek. Chris certainly deserves a hearty thanks for accompanying me for a great part of this project and for helping me in many ways.

Coming to the end (both of this acknowledgements, but also of my time as a PhD-student), I am very flattered that Janne and Marco have agreed to be my paranimfen. Marco, I enjoyed many birding trips with you (wacht nog steeds op mijn patat voor de Noordse Pijl) and the football evening (GER – NED 3: 0 and pizza). Not to forget the fun we had (mostly together with Richard) with quips during breaks; the content of these were usually not suitable to be made public (remember this when preparing my stukje!). And Janne, what would be a day at the university without some spiteful comments by you? So far you always managed to make up with your concernment of how things are or with some charming smiles. I am looking forward to have you and Marco on my side at my promotion day.

Finally I thank my family for letting me go my way and giving me the opportunity to develop the way I did.

Thanks to everyone for everything, Arne

PS: do not forget to read the acknowledgment of the different chapters.

# List of publications

***International peer-reviewed journals***

1. Anthes N, Bergmann HH, **Hegemann A**, Jaquier S, Kriegs JO, Pyzhjanov SW & Schielzeth H (2004): Waterbird phenology and opportunistic acceptance of a low quality staging site at Lake Baikal, eastern Siberia. Wader Study Group Bulletin 105: 75-83.
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### *Non-international peer-reviewed journals (all in German with English summaries)*

1. **Hegemann A**, Leferink J & Bergmann H-H (2002): Dichteänderungen in Trupps überwinternder Dunkelbäuchiger Ringelgänse *Branta b. bernicla* - Methode und erste Ergebnisse. [Density changes in groups of winter staging Dark-bellied brent geese: methods and first results]. SEEVÖGEL 23: 56-59.
2. **Hegemann A** (2004): Illegale Greifvogelverfolgungen im Kreis Soest von 1992 bis 2003 – eine Auswertung mit Hinweisen zur Erkennung von Greifvogelverfolgungen. [Illegal Raptor persecution in the county of Soest from 1992 to 2003 – An analysis with notes on how to recognise persecution]. Charadrius 40: 13-27.
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11. **Hegemann A** (2008): Vorkommen und Phänologie von Trauerente *Melanitta nigra* und Samtente *M. fusca* sowie Bemerkungen zum Auftreten weiterer Meeresenten auf dem Möhnesee zwischen Herbst 1999 und Frühjahr 2003. [Occurrence and phenology of Common Scoter *Melanitta nigra* and Velvet Scoter *M. fusca* and other seaducks at lake Möhnesee from autumn 1999 to spring 2003]. Charadrius 44: 32-35.
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13. Sudmann SR, Grüneberg C, **Hegemann A**, Herhaus F, Mölle J, Nottmeyer-Linden K, Schubert W, von Dewitz W, Jöbges M & Weiss J (2008): Rote Liste der gefährdeten Brutvogelarten Nordrhein-Westfalens (5. Fassung). [Red List of threatened breeding birds of Northrhine-Westphalia (5th edition)]. Charadrius 44: 137-230.
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