university of groningen

University Medical Center Groningen

University of Groningen

The role of disease risk and life history in the immune function of larks in different environments

Horrocks, Nicholas Piers Christopher

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2012

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Horrocks, N. P. C. (2012). *The role of disease risk and life history in the immune function of larks in different environments*. s.n.

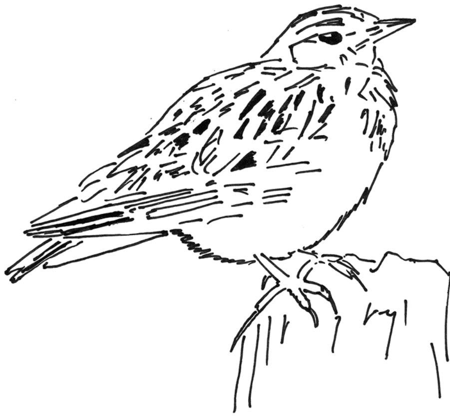
Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Download date: 17-04-2024



CHAPTER 5

Immune defences are associated with microbial pressure rather than life history in larks from contrasting environments

Nicholas P.C. Horrocks, Arne Hegemann, Kevin D. Matson, Kathryn Hine, Sophie Jaquier, Mohammed Shobrak, Joseph B. Williams, Joost M. Tinbergen & B. Irene Tieleman

Unpublished manuscript

Abstract

Variation in immune defence is hypothesised to arise either through parallel variation in disease risk or due to trade-offs with other life history traits encompassed in the 'pace-of-life' concept. We studied lark species (Alaudidae) in the Arabian Desert and temperate Netherlands to test contrasting predictions from these two hypotheses. Based on expected lower exposure to disease risk, desert species are predicted to have relatively weaker immune defences, while based on their slower pace-of-life, desert-living larks are predicted to have relatively stronger immune defences than temperate larks. We developed and applied a novel technique to quantify host-independent and host-dependent measures of disease risk by assaying the abundance of culturable microbes in ambient air and on the surface of birds. We also measured four indices of constitutive innate immunity. Desert-living larks were exposed to significantly lower concentrations of airborne microbes than temperate larks. Densities of some classes of bird-associated microbes were also lower in desert species, although compared with airborne microbes, differences between environments in bird-associated densities were less pronounced. Compared to their temperate relatives, desert-living larks exhibited significantly lower levels in two of four immune indices. Variation in microbial abundance and variation in immune defence among species was less in the desert than in the temperate zone. Because immune system levels broadly matched with microbial exposure but not with pace-of-life, we concluded that, in our study system, disease risk is a more important modulator of immune defences than life history. The use of both host-independent and host-dependent measures of disease risk, including assessment of microbial assemblages, provides novel insight into the mechanisms underlying immunological variation.

Introduction

Investment in immune defences is hypothesised to be shaped by disease risk and by trade-offs associated with life history. If immune defences are related to the threats posed by disease risk, for example the abundance of pathogens and/or parasites (e.g. Piersma 1997; Møller 1998; Blount *et al.* 2003; Guernier, Hochberg and Guegan 2004; Mendes *et al.* 2005; Matson 2006; Spottiswoode 2008), then environments with numerous pathogens should favour selection for robust immune systems. Furthermore, if as predicted, immune systems consume limited resources (e.g. energy; Sheldon and Verhulst 1996; Lochmiller and Deerenberg 2000; Norris and Evans 2000), then environments with fewer pathogens should favour selection for relatively weaker immune systems (Lindström *et al.* 2004; Tschirren and Richner 2006). Another hypothesis, also based on consumption of limited resources by the immune system, predicts trade-offs between immune function and other resource-demanding activities such as reproduction (Ilmonen, Taarna and Hasselquist 2000). Life history theory (Roff 1992; Stearns 1992) predicts that species with low reproductive rates and long life spans (slow 'pace-of-life') should invest more in (certain) immune defences than species with high reproductive rates and short life spans (fast 'pace-of-life'; Ricklefs and Wikelski 2002; Tieleman *et al.* 2005; Lee 2006). Interestingly, when applying these hypotheses to species with a slow pace-of-life that live in environments with predicted low disease risk (e.g. deserts), opposing predictions about their immune defences arise (Horrocks, Matson and Tieleman 2011).

Microbes represent a component of disease risk encountered by all animals. Vectors are typically not required for transmission (Kulkarni and Heeb 2007), and the presence of specific microbes may vary among environments (Corby-Harris *et al.* 2007). Variation in microbial pressure has physiological implications: higher concentrations of airborne microbes produce greater inflammation and have higher toxicity *in vitro* (Huttunen *et al.* 2010); increased microbial diversity and abundance correlates with greater immunological investment *in vivo* (Alcaide *et al.* 2010).

We introduced a novel technique to quantify two measures of broad, non-specific microbial pressures: host-independent microbial concentrations present in ambient air and host-dependent densities of microbes on the surface of birds. Host-dependent measures (e.g. load or prevalence of specific parasites) are more typically used, but such measures are affected by host immune defences and may not reflect the disease risk associated with an environment (Horrocks, Matson and Tieleman 2011).

General microbial challenges are first countered by the constitutive innate arm of the immune system, which provides a non-specific defence against infection (Janeway *et al.* 2004). We measured three aspects of constitutive innate immunity. The ability of whole blood to limit bacterial growth integrates humoural and cell-

mediated aspects of constitutive innate immunity (Tieleman *et al.* 2005; Millet *et al.* 2007). The ability of plasma to agglutinate and lyse foreign cells (Matson, Ricklefs and Klasing 2005) involves natural antibodies and the complement system (Ochsenbein and Zinkernagel 2000). Lastly, concentrations of haptoglobin, an acute phase protein, increase in concentration in response to inflammation or infection (Dobryszycka 1997; van de Crommenacker *et al.* 2010).

To investigate how disease risk and life history may be associated with immunity, we compared microbial exposure and immune defence in seven species of larks (Alaudidae) from the Arabian Desert and the mesic temperate Netherlands. Well-characterised life histories, behaviours and physiologies (reviewed by Tieleman 2005) illustrate that the pace-of-life is slower in desert-living larks than temperate larks (Tieleman, Williams and Visser 2004). A slow pace-of-life may result in higher immune investment by larks in the desert (Tieleman *et al.* 2005; Lee 2006). However, relative to other environments, deserts may pose a lower risk of infection by endo- (Little and Earlé 1995; Valera *et al.* 2003; Jex *et al.* 2007; Froeschke *et al.* 2010) and ecto-parasites, (Moyer, Drown and Clayton 2002; but see Carrillo *et al.* 2007). The combined effects of low primary productivity, high ambient air temperature, minimal precipitation and humidity and high solar radiation likely limit growth rates, abundance and diversity of microbial assemblages in xeric environments (Tong and Lighthart 1997; Burrows *et al.* 2009; Tang 2009; Bachar *et al.* 2010). Thus, compared with temperate larks, desert larks might encounter fewer microbes and a relatively lower microbial pressure, a circumstance that may lead to lower investment in immune defence. In this study we examined differences between desert and temperate environments in concentrations of microbes in ambient air, densities of bird-associated microbes and immune indices. Because little is known of other sources of variation that may affect our interpretation of differences between environments, we also explored year-to-year variation in immune indices within each environment. In addition, we investigated variation among species, also within environments, in densities of bird-associated microbes and immune indices.

Methods

Study species, study locations and bird handling

We studied seven lark species during the breeding season. Arid-zone larks (hoopoe lark *Alaemon alaudipes*, $n = 37$; Dunn's lark *Eremalauda dunni*, $n = 29$; bar-tailed desert lark *Ammomanes cincture*, $n = 11$; black-crowned finchlark *Eremopterix nigriceps*, $n = 27$; crested lark *Galerida cristata*, $n = 13$) were captured in May and June 2006 and 2007 at two locations in central Saudi Arabia. In 2006 all five species were captured at Mahazat as-Sayd (henceforth Mahazat), a reserve in the Arabian Desert (N 22°15' E 41°50'). In 2007 the nomadic species –

black-crowned finchlark and crested lark – were absent from Mahazat and were instead captured about 170 km away at the National Wildlife Research Center, Taif (N 21°15’ E 40°42’). Mahazat is characterised by gravel plains, sparse vegetation, and annual mean rainfall of 96 ± 71 mm (± SD). Spring conditions are hot and dry, with mean air temperatures of at least 30°C (Tieleman and Williams 2002). Throughout the year, environmental conditions at Taif are wetter and cooler than at Mahazat (Tieleman, Williams and Bloomer 2003).

Temperate larks (woodlark *Lullula arborea*, n = 58; skylark *Alauda arvensis*, n = 123) were captured in the Aekingerzand, northern Netherlands (N 52°56’ E 06°18’) between April and July in 2006–2008. The Aekingerzand consists of heath and grazed meadowland, surrounded by agricultural fields and mixed woodland. Mean annual rainfall is 853 ± 160 mm (SD). Mean daily air temperature during the breeding season is 13.8°C (Koninklijk Nederlands Meteorologisch Instituut).

Birds were captured using mist nets and clap traps, sometimes with the addition of playback calls or bait (bird seed and mealworms). Upon capture, we sampled microbial loads from birds (desert n = 36, 2007 only; temperate n = 36, 2008 only) using an air-sampling method (bird air-sampling; see below). We then bled (45-60 minutes after capture), weighed (± 0.1 g) and measured (wing ± 0.1 cm; tarsus ± 0.01 cm) all birds. A further 226 birds (desert n = 81, temperate n = 145) were bled within 10 minutes of capture and measured but were not sampled for microbes. Between 200–300 μl of blood were collected from the brachial vein and stored on ice until processing later the same day. Samples were centrifuged to separate plasma from red blood cells. Plasma was frozen and stored at –20°C until use in immune assays. All birds were sexed by body measurements and behavioural observations. Permission to work with wild birds in Saudi Arabia was obtained from the National Wildlife Research Centre. Procedures in the Netherlands were conducted under licence from the Animal Experimentation Committee of the University of Groningen (DEC 5219, 5219A).

Air sampling

We used a battery-powered portable air sampler (Burkard, Rickmansworth, UK) to determine concentrations of microbes in ambient air and densities of microbes on birds. The air sampler drew air at a constant rate through a perforated metal plate, filtering microbial particles that collect onto an agar-filled Petri dish below. Following incubation of the agar plate, the number of colony-forming units (CFUs) was counted to obtain an index of the concentration of culturable microbes. Culture-dependent methods remain useful for measuring microbial exposure (e.g. Haas *et al.* 2010) despite the unculturability of many microorganisms (Rappé and Giovannoni 2003). For both environmental air-sampling and bird air-sampling we cultured three different microbial groups. Generalist aerobic bacteria were cultured on non-selective Tryptic soy agar; gram-negative bacteria were cultured on MacConkey agar with crystal violet sodium, chloride and 0.15%

bile salts (Sigma-Aldrich, St Louis, MO, USA); fungi were cultured on Sabouraud 4% Glucose agar (Sigma-Aldrich), with 50 mg $litre^{-1}$ Gentamicin antibiotic (Invitrogen, Breda, The Netherlands) to inhibit bacterial growth. Plates were incubated at 30°C and numbers of CFUs were counted after 24 hours (generalist bacteria,), 72 hours (fungi) and 96 hours (gram-negative bacteria), due to different growth rates. The collection efficiency of impaction air samplers varies with particle size: smaller particles are collected less efficiently than larger ones. If particles are cross-contaminated, for example fungal spores with bacteria on their surface, then CFU counts might be correlated among agar types. To account for the possibility that more than one culturable particle might be collected through a sampling hole but produce only a single countable colony we applied a correction to all CFU counts (Andersen 1958).

Environmental air-sampling

Sampling of ambient air for microbes took place over 20 days in the desert in 2007 and over 37 days in the Netherlands in 2008. We sampled at 16 sites in the desert and at 12 sites in the temperate zone, all of which were micro-habitats within our study areas where birds commonly occurred. Environmental air samples do not correspond with specific bird air-sampling events, however. Samples were collected throughout daylight hours to account for potential diurnal variation in microbial aerosol loads (Tong and Lighthart 1999). Sampling duration was optimised for both environments to avoid the situation where colonies were too numerous to count: air was sampled for 15–30 minutes per agar plate at the desert sites and for five minutes per agar plate at the temperate site. Sampling effort was standardised by multiplying the duration of sampling by the air flow rate (20 litres $minute^{-1}$) and expressing the data as CFU m^{-3} of air.

Bird air-sampling

We sampled bird-associated microbes by using the air sampler to collect the microbes shed from the surface of a bird as air passed over it. Birds were restrained inside a mesh tube and were placed, always facing the same way, inside a plastic box (33 × 22 × 16 cm) with fitted lid. The head of the air sampler was fitted through a hole in the side of the box so that air re-circulated back into the box. The order of agar types varied among birds, but duplicate plates were run for each agar type and for 5 minutes per agar plate. Handling of birds prior to being air-sampled was kept to a minimum. We cleaned our hands with antibacterial hand wash and used single-use bird bags to avoid cross-contamination among birds. All equipment was sterilised with ethanol before and after use. Since we sampled species of varying body size, we determined body surface area (Walsberg and King 1978) and expressed counts per bird as CFU cm^{-2} of body surface area. Bird air-sampling took place in the desert in 2007 and in the temperate zone in 2008.

Immune assays

We measured haptoglobin concentrations (mg ml^{-1}) with an assay that quantifies the haem-binding capacity of plasma (TP801; Tri-Delta Diagnostics, NJ, USA). We quantified natural antibody-mediated agglutination titres and complement-mediated lysis titres by incubating red blood cells from rabbits (B-0009D, Harlan, UK) with serially diluted plasma samples from birds, according to Matson, Ricklefs and Klasing (2005). We performed the microbial-killing assay under sterile conditions following Tieleman *et al.* (2005) and Matson, Tieleman and Klasing (2006). Fresh blood samples, taken immediately upon capture (from non-air sampled birds only) were transported directly to the laboratory, always arriving within 30 minutes. Blood was diluted with CO_2-independent media (Invitrogen) and subdivided for incubation at 41°C (bird body temperature) with different microbial strains. Microbicidal ability of blood was tested against three organisms, chosen to minimise effects of exposure histories of birds: *Escherichia coli* (ATCC 8739), *Candida albicans* (ATCC 10231), and *Staphylococcus aureus* (ATCC 6538; MicroBioLogics, St. Cloud, MN, USA). Lyophilised microbial pellets were reconstituted according to manufacturer's instructions and then incubated with the blood-media mixture under strain-specific conditions: *E. coli* was diluted 1:10 with blood-media mixture and incubated for 30 minutes; *C. albicans* and *S. aureus* were diluted 1:9 with blood-media mixture and incubated for 180 minutes. After incubation 75 μl of the diluted blood-media-bacteria mixture were plated in duplicate on agar plates. Control plates were also inoculated with 75 μl of microorganisms, diluted with media to the same final concentration as the blood-media-bacteria mixes, but not incubated at 41°C. These control plates reflected the number of microorganisms that the bird blood first encountered (i.e. the inoculating dose). All plates were incubated at 30°C and the number of CFUs was counted 24 hours later. We calculated killing ability as one minus the quotient of CFUs on the blood plates divided by the number of CFUs on control plates.

Statistical analyses

We used Mann-Whitney U tests and Fisher's F tests to examine environmental variation in mean values and variance of airborne microbial concentrations. We used generalised and linear mixed models to examine environmental variation in bird-associated microbes and immune indices. For each response variable we analysed data from all years and a restricted dataset containing only values collected from both environments in the same year(s). Results were qualitatively similar whichever dataset was used; we only report results obtained using the larger dataset. Full models contained the categorical factors environment (desert or temperate), sex, year and the interaction environment*sex. We controlled for species differences and repeated measures by including species and individual nested within species as random effects. To examine species differences within and among environments, we refitted models with species as a main effect and

without the term environment and its interaction. When the term species was significant, we used Tukey post-hoc tests to identify significant species differences. Full starting models to examine inter-annual variation in immune indices within environments contained the terms species, sex, year and the interaction species*year. We simplified models using stepwise backward elimination, based on log-likelihood ratio tests and $P > 0.05$. Residuals of models were checked for normality and homogeneity of variance by graphical examination. Analyses were performed using R version 2.10.01 (R Development Core Team 2009).

We included time of day as a covariate in analyses of bird-associated microbes to account for potential temporal fluctuations in concentrations of airborne microbes (Tong and Lighthart 1999). We grouped bird-derived data for our two desert sites since immune indices and bird-associated microbial densities did not differ between these two sites during the breeding season (Horrocks *et al.* unpublished data). We also combined data on haptoglobin concentration and agglutination and lysis titres for air-sampled and non-air sampled birds. There were no differences between these groups (all $P > 0.08$), despite differences in time of blood collection after capture (45–60 minutes vs. <10 minutes).

For the microbicidal assay, incubation with blood from some individuals promoted growth of bacteria, rather than killing, resulting in negative values (*E. coli* 21/90 individuals; *C. albicans* 14/79; *S. aureus* 65/83). Therefore, we followed the approach of Buehler *et al.* (2008) and present the results of analyses using a normal distribution to describe the data. Microbicidal ability might depend on the inoculation concentration, so we always included the mean control plate colony count as a covariate.

To investigate the effect of general microbial pressure on immunity, we examined correlations between immune indices – haptoglobin concentrations, agglutination and lysis titres – and total microbial density, which was the sum of all bacterial and fungal densities per bird. We calculated species means of total microbial density and individual deviations from these means to allow us to distinguish the contributions of within- and among-species variation (van de Pol and Wright 2009).

Results

Concentrations of environmental airborne microbes

Mean airborne concentrations of all three microbial groups were significantly lower in desert locations than in the temperate environment (Figs 5.1, 5.4A). Comparing the two desert locations, concentrations of generalist bacteria and fungi were significantly lower at Mahazat than at Taif (Fig. 5.1). Variance in the concentration of microbes within an environment was also lower in the desert than in the temperate environment (Figs 5.1, 5.4A). When combining data for

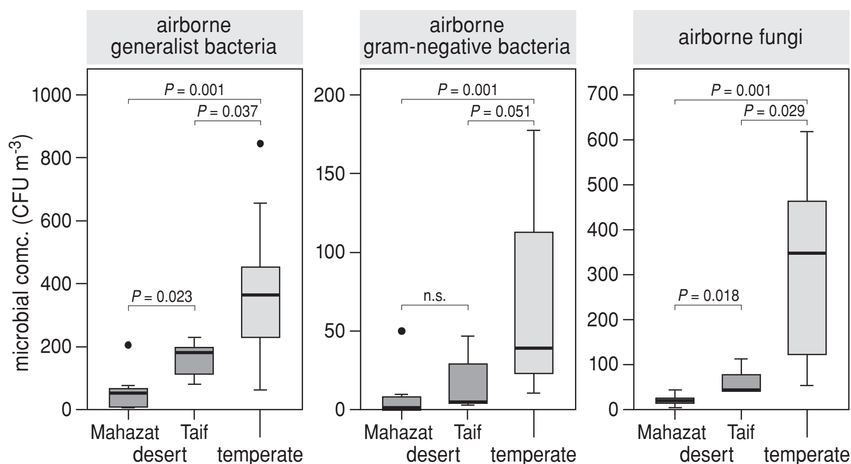


Figure 5.1. Concentrations of airborne microbes (generalist bacteria, gram-negative bacteria and fungi) measured at two sites in the Arabian Desert (Mahazat as-Sayd and Taif) and in the temperature Netherlands, during spring. Boxes encompass all data points between the 25th and 75th percentiles (interquartile range, IQR). Thick bars inside boxes indicate the median data value. 'Whiskers' indicate either the minimum or maximum data value, or 1.5 times the IQR (approximately two standard deviations), whichever is smaller. Data points outside this range ('outliers') are plotted individually as black dots.

Mahazat and Taif, the difference in variance was significant for all three microbial groups: generalist bacteria ($F_{11, 12} = 7.66$, $P = 0.001$); gram-negative bacteria ($F_{11, 13} = 11.34$, $P < 0.001$); fungi ($F_{10, 10} = 109.40$, $P < 0.001$). Gram-negative bacteria were the least numerous microbial group in both environments (Fig. 5.1).

Densities of bird-associated microbes

Densities of generalist bacteria ($z = 0.97$, $P = 0.334$) and gram-negative bacteria ($z = -1.42$, $P = 0.156$) associated with birds did not differ significantly between locations (Figs 5.2, 5.4B), but significantly lower densities of fungi were shed from larks living in the desert compared with temperate larks ($z = -3.74$, $P < 0.001$; Figs 5.2, 5.4B). Excluding three outliers (> two standard deviations from the mean) from the fungi data did not change the result. There was a significant effect of time of day on densities of bird-associated generalist bacteria ($z = 2.11$, $P = 0.035$) and fungi ($z = 2.35$, $P = 0.019$): birds sampled later in the day shed significantly more microbes, although the size of this effect was exceedingly small (an increase of approximately one CFU cm^{-2} per 12 hours, in each case). Female larks shed more of all microbe types than males, but this difference was significant only for fungi ($z = -2.01$, $P = 0.045$; generalist bacteria, $z = -1.91$, $P = 0.056$; gram-negative microbes, $z = -0.25$, $P = 0.799$).

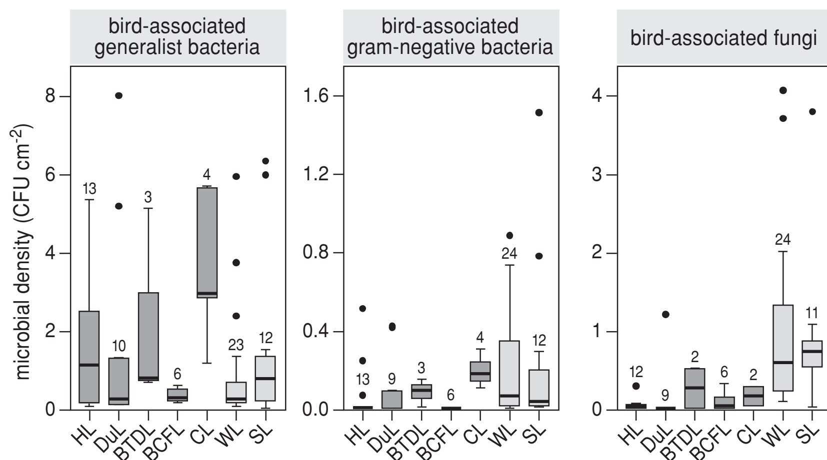


Figure 5.2. Densities of bird-associated microbes (generalist bacteria, gram-negative bacteria and fungi) air-sampled from seven lark species in the Arabian Desert (n = 5 species) and the temperate Netherlands (n = 2 species, light grey bars) in spring. Numbers above bars are sample size. HL = hoopoe lark; DuL = Dunn's lark; BTDL = bar-tailed desert lark; BCFL = black-crowned finchlark; CL = crested lark; WL = woodlark; SL = skylark. Boxes encompass all data points between the 25th and 75th percentiles (interquartile range, IQR). Thick bars inside boxes indicate the median data value. 'Whiskers' indicate either the minimum or maximum data value, or 1.5 times the IQR (approximately two standard deviations), whichever is smaller. Data points outside this range ('outliers') are plotted individually as black dots.

Excluding environment, and comparing among species, we found a significant effect of species on densities of bird-associated fungi ($F_{6,\,61}$ = 3.16, P = 0.009) and a non-significant trend in generalist bacteria ($F_{6,\,64}$ = 1.99, P = 0.080). For fungi, a post-hoc test could not resolve the source of the significance, while for generalist bacteria the near-significant species effect was driven by a single species, crested lark, which was significantly different from all other species (Fig. 5.2). There was no effect of species on densities of gram-negative bacteria ($F_{6,\,64}$ = 1.58, P = 0.167).

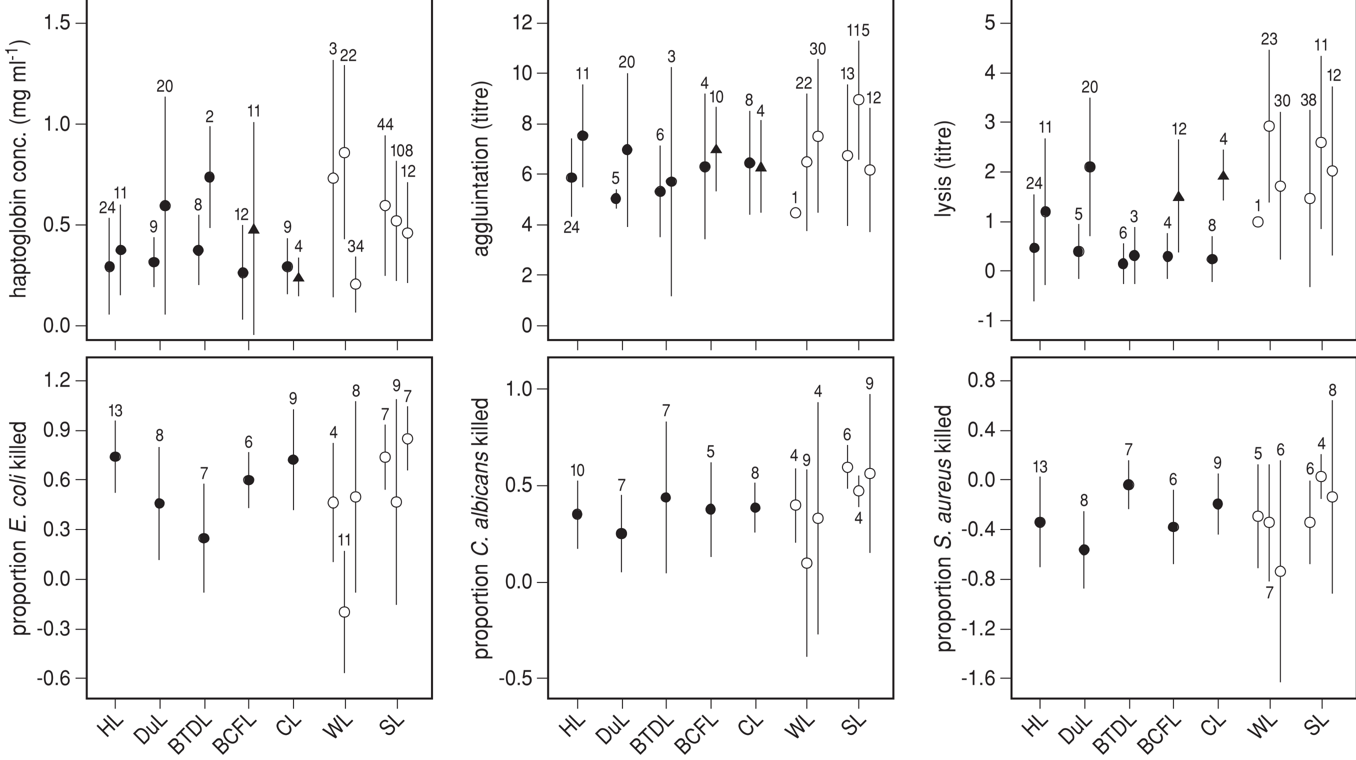
Immune indices

Taking into account species and variation among years, concentrations of haptoglobin ($F_{1,\,5}$ = 8.45, P = 0.034) and titres of lysis ($F_{1,\,5}$ = 30.25, P = 0.003) were significantly lower in arid-zone larks than in the two lark species measured at the temperate site (Table 5.1A; Figs 5.3, 5.4C). Agglutination titres ($F_{1,\,5}$ = 3.98, P = 0.102) and microbicidal ability against *E. coli* ($F_{1,\,5}$ = 0.03, P = 0.865), *C. albicans* ($F_{1,\,5}$ = 0.06, P = 0.816) and *S. aureus* ($F_{1,\,5}$ = 0.66, P = 0.452) all showed no significant differences between environments (Table 5.1a; Figs 5.3, 5.4B, 5.4C).

Table 5.1. Mean values (intercept), estimate sizes (difference) and 95% confidence intervals around the difference (95% C.I.) for environmental and inter-annual variation in indices of immunity measured in larks in the Arabian Desert (n = 5 species) and temperate Netherlands (n = 2) in spring. Desert larks were measured in 2006 and 2007 and temperate larks were measured in 2006-2008. Intercepts and differences are taken from final statistical models examining the role of environmental and inter-annual effects on each parameter. For estimates of environmental variation 'desert' is the reference category and differences are given relative to this. Estimates of inter-annual variation are given for each consecutive year (i.e. 2006 to 2007 and 2007 to 2008).

source of variance	immune index	mean (intercept)	difference	95% C.I.
(A) environmental				
desert vs. temperate	haptoglobin (mg ml^{-1})	0.37	0.21	0.02 – 0.40
	agglutination (titre)	5.58	1.03	-0.30 – 2.36
	lysis (titre)	0.15	1.15	0.61 – 1.67
	E. coli (% killed)	0.73	-0.04	-0.58 – 0.51
	C. albicans (% killed)	0.33	0.03	-0.28 – 0.34
	S. aureus (% killed)	-0.13	0.13	-0.27 – 0.53
(B) inter-annual desert				
2006 vs. 2007	haptoglobin (mg ml^{-1})	0.30	0.19	0.07 – 0.32
	agglutination (titre)	5.86	1.13	0.24 – 2.02
	lysis (titre)	0.37	1.27	0.82 – 1.73
(C) inter-annual temperate				
2006 vs. 2007	haptoglobin (mg ml^{-1})	0.60	-0.04	-0.15 – 0.07
2007 vs. 2008		0.55	-0.39	-0.52 – -0.26
2006 vs. 2007	agglutination (titre)	6.73	2.05	1.07 – 3.03
2007 vs. 2008		8.78	-0.72	-1.82 – 0.37
2006 vs. 2007	lysis (titre)	1.25	1.23	0.64 – 1.82
2007 vs. 2008		2.48	-0.94	-1.52 – -0.36
2006 vs. 2007	*E. coli* (% killed)	0.95	-0.35	-0.70 – -0.01
2007 vs. 2008		0.60	0.55	0.25 – 0.84
2006 vs. 2007	*C. albicans* (% killed)	0.61	-0.22	-0.60 – 0.17
2007 vs. 2008		0.39	0.18	-0.14 – 0.51
2006 vs. 2007	*S. aureus* (% killed)	-0.06	0.05	-0.40 – 0.50
2007 vs. 2008		-0.10	0.34	-0.31 – 1.00

Figure 5.3. (right). Patterns of species variation, inter-annual variation and environmental variation in indices of constitutive innate immunity (haptoglobin concentration, agglutination and lysis titres, and microbicidal ability (proportion killed) of whole blood against *E. coli*, *C. albicans* and *S. aureus*) measured in seven lark species in the Arabian Desert (n = 5 species; black symbols) and the temperate Netherlands (n = 2 species; white symbols) during spring. Desert larks were measured in two consecutive years in either Mahazat as-Sayd (black circles) or Taif (black triangles). Temperate larks were measured over three consecutive years. Data are plotted as mean values ± S.D. Numbers above bars are sample size. HL = hoopoe lark; DuL = Dunn's lark; BTDL = bar-tailed desert lark; BCFL = black-crowned finchlark; CL = crested lark; WL = woodlark; SL = skylark.


haptoglobin conc. (mg ml-1)
agglutination (titre)
lysis (titre)
proportion E. coli killed
proportion C. albicans killed
proportion S. aureus killed
HL
DuL
BTDL
BCFL
CL
WL
SL

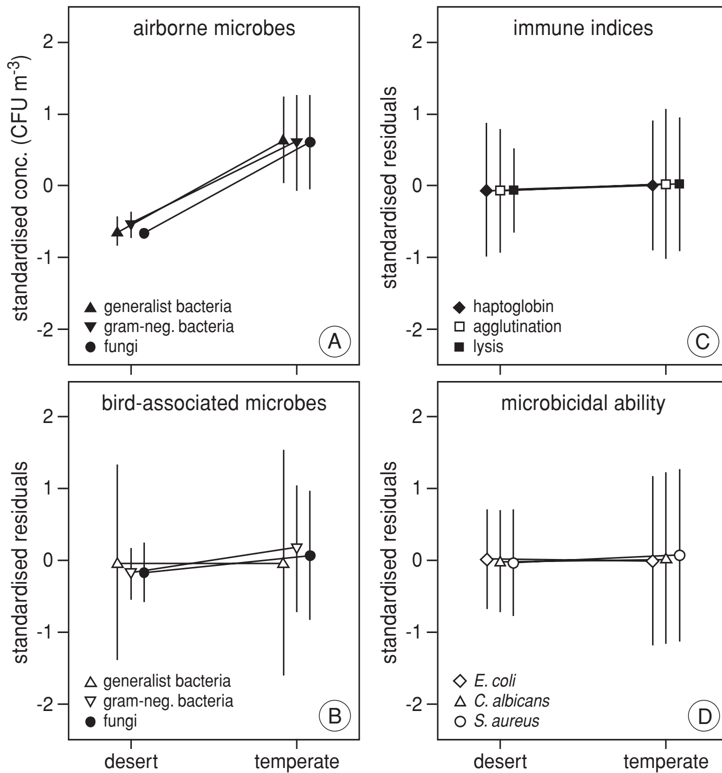


Figure 5.4. Differences in (A) concentrations of culturable airborne environmental microbes sampled from the Arabian Desert and temperate Netherlands, and (B) densities of bird-associated culturable microbes, (C) indices of constitutive immunity (haptoglobin concentration, agglutination and lysis titres) and (D) microbicidal ability of blood sampled from larks living in these environments. Data are mean values and error bars indicate standard deviations. In (B)-(D), data are plotted as standard residuals extracted from the final model for each variable. Non-significant differences between environments are represented by open symbols.

Excluding environment as a factor and focusing on inter-specific differences, all measures of immunity exhibited variation at the species level that was significant or close to significant at the $P = 0.05$ level. Post-hoc tests revealed that for some measures the effect of species was driven by members of a single species pair that were significantly different from each other (Table A5.1). Desert-living lark species were never significantly different from each other but the two temperate lark species sometimes were (Table A5.1).

Correlations between immune indices and total microbial load

Lysis titre was the only immune index that showed any relationship with total

microbial load (Table A5.2). Among species, there was no correlation between lysis titre and total microbial load ($F_{1, 5}$ =0.34, P = 0.587). Within species, birds with higher total microbial loads had greater lysis ability, but this finding did not reach significance ($F_{1, 57}$ = 3.21, P = 0.079). Analysing data for each environment separately revealed that the within-species relationship was strongly driven by desert birds ($F_{1, 27}$ = 5.61, P = 0.025) and was much weaker in temperate larks ($F_{1, 29}$ = 0.09, P = 0.761).

Inter-annual variation in immune function within environments

In desert-living larks, haptoglobin concentration ($F_{1, 108}$ = 9.05, P = 0.003), agglutination titre ($F_{1, 93}$ = 6.40, P = 0.013) and lysis titre ($F_{1, 95}$ = 31.13, P < 0.001) were all significantly higher in 2007 than in 2006 (Fig. 5.3, Table 5.1B). The interaction species*year was never significant, suggesting that all five desert lark species responded to the effect of year in a similar manner.

In the two temperate species, the interaction between species and year was not significant for lysis, but years differed ($F_{2, 60}$ = 11.85, P < 0.001; Fig. 5.3, Table 5.1C). The interaction between species and year was significant for agglutination titre ($F_{2, 57}$ = 6.46, P = 0.003) and for haptoglobin concentration ($F_{2, 59}$ = 10.81, P < 0.001) suggesting that woodlarks and skylarks differed in how these immune indices varied over years (Fig. 5.3). Analysing these variables separately for each species revealed that woodlarks demonstrated significant inter-annual variation in haptoglobin concentration ($F_{2, 9}$ = 32.89, P < 0.001) but not agglutination titre ($F_{2, 9}$ = 1.17, P = 0.355; Fig. 5.3). In skylarks the pattern was reversed; inter-annual variation in haptoglobin concentration was not significant ($F_{2, 50}$ = 1.34, P = 0.270) whereas inter-annual variation in agglutination titre was significant ($F_{2, 48}$ = 15.99, P < 0.001; Fig. 5.3). The two temperate species exhibited similar significant inter-annual differences in microbicidal ability against *E. coli* ($F_{2, 41}$ = 7.30, P = 0.002) and both exhibited no inter-annual differences in microbicidal ability against *C. albicans* ($F_{2, 31}$ = 0.96, P = 0.393) or *S. aureus* ($F_{2, 32}$ = 1.85, P = 0.174; Fig. 5.4, Table 5.1C). After accounting for species differences, inter-annual variation in immune indices within an environment could be of greater magnitude than variation in immune indices between desert and temperate environments (Fig. 5.3, Table 5.1A *vs.* Table 5.1B, C).

Discussion

Explanations for variation in immune defence focus on the role of either disease risk or life history but these two hypotheses can lead to opposing predictions (Horrocks, Matson and Tieleman 2011). We explored these opposing predictions simultaneously by contrasting the differences in disease risk and life history between desert and temperate environments. We found that desert-living larks –

with a slow pace-of-life (Tieleman, Williams and Visser 2004) – exhibited similar or significantly lower values of constitutive innate immunity than 'fast-living' temperate larks, Moreover, we found that host-independent and some-host-dependent measures of microbial pressure, a proxy for disease risk, were significantly lower for desert-living larks than for temperate larks. Thus, our data are consistent with the ideas that disease risks differ among environments and that increased disease risk is associated with stronger immune defences. In contrast, our data provide limited support for a relationship between pace-of-life and immune investment. Desert-living larks exhibited the same or reduced levels of immune indices compared with temperate-living larks. These results offer limited support to existing hypotheses regarding which types of immune defence might be limited by slow-living species (Lee 2006). However, we found no evidence that a slow pace-of-life is associated with overall increases in immune defence.

Our novel air-sampling technique allowed us to quantify host-independent and host-dependent measures of disease risk, providing greater insight into how host defences – both behavioural and immune – and disease risk may interact. The use of non-specific microbial assemblages provides an easily accessible and widely applicable index of disease risk. The emerging links between immune defence and microbial abundance (Alcaide *et al.* 2010; this study; Horrocks *et al.* unpublished data) suggest that microbial assemblages will be a fruitful avenue of further ecoimmunology research. Culture-independent techniques offer further possibilities to enhance and increase the amount of information about microbial assemblages that can be gained, including identification of pathogenic and other functions (Horrocks, Matson and Tieleman 2011).

We identified macro-scale variation in microbial abundance, since desert ambient air contained lower concentrations of microbes than air from the temperate zone. Low concentrations of airborne microbes in the desert likely relate to the fact that deserts are areas of low primary productivity (Field *et al.* 1998). This ecological characteristic and the associated low rainfall and humidity, but greatly elevated temperatures and solar radiation likely make deserts inhospitable for microbial growth and could reduce microbial abundance (Tong and Lighthart 1997; Burrows *et al.* 2009; Tang 2009). In fact, soil microbial abundance decreases with increasing aridity (Bachar *et al.* 2010) and fungal abundance increases with increasing precipitation (Talley, Coley and Kursar 2002). Within the desert, microbial concentrations were highest at Taif, which receives more annual rainfall and experiences lower average temperatures than does Mahazat (Tieleman, Williams and Bloomer 2003), further implicating a role for climatic conditions in influencing airborne microbial loads. Previous studies have examined environmental and habitat-related differences in antigens and pathogen pressure (e.g. Figuerola 1999; Mendes *et al.* 2005; Salkeld, Trivedi and Schwarzkopf 2008) but these studies have relied on host-dependent measures of disease risk, which as we have shown may differ from host-independent measures.

Compared with concentrations of airborne microbes, environmental differences in densities of microbes sampled from birds were small. Airborne and bird-associated microbes displayed similar relative abundances (generalist bacteria > fungi > gram-negative bacteria) in desert and temperate areas, but fungi were the only bird-associated microbial group that was significantly lower on desert-living larks compared with temperate larks. Gram-negative bacteria showed a similar but non-significant trend. Densities of microbes shed from birds are likely impacted by feather maintenance activities such as preening (Kulkarni and Heeb 2007). These activities, while perhaps limited in all larks during reproduction (Tieleman, Williams and Visser 2003; Tieleman, van Noordwijk and Williams 2008) might be particularly restricted in desert-living larks due to additional constraints on activity budgets imposed by high temperatures (Tieleman and Williams 2002). Habitat-specific behaviours might also influence densities of bird-associated microbes. For example, the use of burrows and soil scrapes as thermal refuges by larks in the desert (Williams, Tieleman and Shobrak 1999) might increase exposure to soil microbes (Shawkey *et al.* 2005). Thus, desert-living larks might carry higher densities of microbes than expected based on airborne concentrations. Sex-differentiated roles during the breeding season may be responsible for higher fungal and generalist bacterial densities on female birds than males. By spending more time incubating eggs than males, female larks increase their exposure to nest material and perhaps also to associated microbes (Berger, Disko and Gwinner 2003; Goodenough and Stallwood 2010). Females might also be constrained in the time they have available for feather care compared with males.

We found no evidence that the slow pace-of-life in desert-living larks was associated with increased investments in immune function. However, we measured only constitutive innate immune indices which we thought were particularly relevant in the context of microbial pressure. A more refined hypothesis of the relationship between pace-of-life and immune function states that slow-living species should rely more on developmentally-expensive adaptive immunity and less on energetically-costly constitutive innate immunity compared with fast-living species (Lee 2006). Two of four constitutive innate immune indices were higher in fast-living temperate larks, but information on adaptive immunity in desert-living and temperate lark species is currently unavailable. Interestingly, the acute phase protein haptoglobin, and complement-triggered cell lysis, the two indices that were greater in temperate larks, both relate to inflammatory responses. In general a greater reliance on inflammation might be associated with fast-living species (Lee 2006). The lack of correlation that we found between microbicidal ability of whole blood and life history in larks contrasts with other studies. Slow-living bird species exhibited relatively higher microbicidal ability in a study that focused on life history variation in a single (tropical) environment (Tieleman *et al.* 2005; Matson, Tieleman and Klasing 2006). However, comparing across

environments, Sparkman and Palacios (2009) found fast-living ecotypes of garter snakes *Thamnophis elegans* exhibited greater microbicidal ability than their slow-living counterparts. Comparisons among studies are complicated when packages of co-varying life history traits and environmental variation differ. Furthermore, the microbicidal ability assay is highly integrative, suggesting that similar end-points might be reached by employing strategies that differ in terms of cost of efficiency. Overall, relationships between innate immunity and life history remain unclear.

An important additional finding of our study was that the variation in immune indices between consecutive breeding seasons could sometimes be of similar or greater magnitude to the immunological variation explained by environmental differences. The extent of inter-annual variation also differed between environments and among species for temperate larks, but not for desert-living larks. Although we do not exclude the possibility that small sample sizes for some desert species meant that significant species*year interactions could not be detected, it is noteworthy that the five desert lark species (almost without exception) showed similar patterns of inter-annual variation in haptoglobin concentration and agglutination and lysis titres. We also detected no significant species differences among desert-living larks in any immune indices when accounting for the effect of year. In contrast, the two temperate lark species showed little consistency in immune indices over years, suggesting that different factors may be driving patterns of inter-annual variation for these species, despite them inhabiting the same mesic environment. This lack of consistency might be related to species-specific characteristics (e.g. greater phenotypic flexibility; Tieleman *et al.* 2003) or could indicate greater variability of conditions within the temperate environment, as suggested by the data on airborne microbial concentrations. Inter-annual variation in immune function within an environment or season can be expected if local conditions (e.g. resource availability, temperature, exposure to pathogens) are not fixed from year to year, and if these conditions influence the optimal investment in immune defence versus other fitness-enhancing traits (Hegemann *et al.* unpublished data). The importance of local conditions is illustrated by the clear seasonal patterns in airborne and bird-associated microbes and immune indices that we identified in desert-living larks sampled in spring and winter (Horrocks *et al.* unpublished data). These findings illustrate the relevance of changes in local conditions to immune function and highlight the importance of considering such factors when trying to understand patterns of immunological variation.

In conclusion, weaker immune defences in desert-living larks compared with temperate larks were associated with microbial pressures and not life history. The use of both host-independent and host-dependent measures of disease risk, including assessment of microbial assemblages, offers a promising approach to understanding underlying causes in patterns of immunological variation.

Acknowledgements

We are grateful to HH Prince Bandar bin Saud, Secretary General of the Saudi Wildlife Commission, and Mr Ahmad Al Bouq, Director of the National Wildlife Research Center (NWRC) for their support during this study. We also thank the staff at the NWRC Taif and Mahazat as-Sayd, for logistical support. Staatsbosbeheer gave permission to work in the Aekingerzand. Several volunteers contributed greatly to the Aekingerzand Lark Project, particularly Rob Voesten. Maaike Versteegh provided advice on statistical analyses. Financial support for this study was provided to N.P.C.H. by the Schure-Beijerinck-Poppings Fonds, to A.H. and B.I.T. by BirdLife Netherlands, and to K.D.M. and B.I.T. by VENI grants (863.08.026 and 863.04.023, respectively) from the Netherlands Organisation for Scientific Research (NWO). B.I.T. also received a Rosalind Franklin Fellowship from the University of Groningen.

Appendix 5.1

Table A5.1. Results (F tests and *P* values) of general linear models examining species variation in immune indices of seven species of desert (n = 5 species) and temperate (n = 2 species) larks. The final column shows those species that differed significantly from each other (at the $P = 0.05$ level) in pair-wise post-hoc Tukey tests. HL = hoopoe lark; DuL = Dunn's lark; BTDL = bar-tailed desert lark; BCFL = black-crowned finchlark; CL = crested lark; WL = woodlark; SL = skylark.

immune measure	F	*P*	species pairs
haptoglobin (mg ml^{-1})	$F_{6, 263} = 4.56$	< 0.001	WL - HL WL - BCFL WL - CL SL - HL SL - CL
agglutination(titre)	$F_{6, 245} = 3.83$	0.001	SL - DuL
lysis (titre)	$F_{6, 247} = 0.70$	< 0.001	WL - HL WL - BTDL WL - BCFL SL - HL SL - BTDL SL - BCFL
% *E. coli* killed	$F_{6, 80} = 4.39$	< 0.001	WL - HL WL - CL WL - SL SL - BTDL
% *C. albicans* killed	$F_{6, 65} = 2.05$	0.071	WL - SL
% *S. aureus* killed	$F_{6, 71} = 2.19$	0.054	SL - DuL

Table A5.2. Correlations at the among-species and within-species level between total microbial density shed from birds and immune indices measured in five arid-zone and two temperate lark species in Saudi Arabia and the Netherlands. Within-species centreing was used to partition the contribution of among- and within-species variation to any relationship.

	among-species			within-species		
immune measure	estimate ± SE	F	*P*	estimate ± SE	F	*P*
haptoglobin (mg ml^{-1})	-0.07 ± 0.06	$F_{1, 5} = 1.22$	0.320	0.01 ± 0.02	$F_{1, 59} = 0.15$	0.702
agglutination (titre)	-0.31 ± 0.40	$F_{1, 5} = 0.58$	0.480	0.11 ± 0.17	$F_{1, 55} = 0.40$	0.530
lysis (titre)	0.11 ± 0.19	$F_{1, 5} = 0.34$	0.587	0.13 ± 0.08	$F_{1, 57} = 3.21$	0.079