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

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CHAPTER 3

# Environmental disease risk proxies explain variation in immune investment better than indices of pace-of-life

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**Abstract**

Investment in immune defences is often predicted to co-vary with a variety of ecologically and evolutionarily relevant axes, with physiological pace-of-life and environmental disease risk being two notable examples. These axes may themselves co-vary directly or inversely, and such relationships can sometimes lead to conflicting predictions regarding immune defences. We investigated the relative influence of variation in pace-of-life and environmental disease risk on immune investment using comparable species of larks (Alaudidae) with different life histories and risks of infection. We used number of eggs per clutch and number of clutches per year as indicators of pace-of-life, and we used climatic variables, including aridity, as correlates of environmental disease risk. We quantified immune investment by measuring concentrations of haptoglobin and ovotransferrin and titres of agglutination and lysis, all indices of innate immunity. If pace-of-life shapes immune investment then we expected slow-living arid-zone lark species to invest more in immune defence than fast-living temperate and tropical species. Alternatively, if disease risk drives immune investment, then we expected larks in high-risk temperate and tropical environments to exhibit higher immune indices than larks from low-risk arid locations. We found that pace-of-life indices explained little of the variation in immune investment: only agglutination titre showed a significant correlation, although not in the predicted direction. Conversely, environmental disease risk proxies were highly predictive of immune function, and larks in high-risk environments had higher immune indices than those living in arid, low-risk locations. Overall, our study suggests that environmental variables that have strong ties to disease risk are more powerful drivers of immunological variation than reproductive indices related to the pace-of-life.

## Introduction

Variation in immune investment is widely observed within and among species. Understanding the causes of this variation is one general goal of ecological immunology (Sadd and Schmid-Hempel 2009). Two particular explanations of immunological variation are often looked to in order to make sense of patterns observed in nature. One explanation takes a life history perspective (Roff 1992; Stearns 1992) and focuses on the role of pace-of-life, the costs associated with immunity, and the presence of trade-offs between key physiological processes (Sheldon and Verhulst 1996; Norris and Evans 2000; Schmid-Hempel 2003). The other explanation takes an environmental disease threat perspective (e.g. Mendes *et al.* 2006) and focuses more on the benefits of immune defence. In fact, pace-of-life, disease risk and immune defences might all co-vary. Consequently, forming clear predictions about immune investment can be difficult.

Life history theory states that because resources are finite, the time and energy invested in, for example, reproduction, are not available to other essential functions such as growth or self-maintenance (Soler *et al.* 2003; Ardia 2005a; Ardia 2005b). Comparative studies of birds and mammals reveal that a single axis dominates life history variation (Saether 1988; Promislow and Harvey 1990; Ricklefs 2000). Combinations of life history traits lie along this slow-fast axis, known as the pace-of-life: at the slow end low extrinsic mortality combines with low annual reproductive output; at the fast end high extrinsic mortality associates with high annual reproductive output. Since the immune system is integral to self-maintenance and hence to survival and opportunities for future reproduction, ecological immunologists have invoked trade-offs between current and future reproduction and exploited differences in pace-of-life to explain variation in immune defence (Sheldon and Verhulst 1996; Norris and Evans 2000; Schmid-Hempel 2003). Fundamental to this explanation is the idea that the costs of development, maintenance and usage differ among immune defence components and the immune systems they constitute (Klasing and Leshchinsky 1999; Tieleman *et al.* 2005; Lee 2006).

Environmental disease risk represents another axis that might explain immune investment. Immune systems provide clear benefits in terms of protection against an array of endogenously and exogenously originating threats, including fitness-reducing infections by micro- and macro-parasites. Thus, immune investment might be greater in situations where the risk of infection is higher (Tschirren and Richner 2006; Horrocks, Matson and Tieleman 2011), which could be associated with environment, time, and other ecological factors (e.g. Piersma 1997; Møller 1998; Guernier, Hochberg and Guegan 2004; Guerra *et al.* 2010). For example, environmental moisture conditions appear to be important in shaping parasitic and microbial assemblages. Parasites tend to have low prevalence in arid environments (Little and Earlé 1995; Moyer, Drown and Clayton 2002; Valera *et al.*

2003; Jex *et al.* 2007; Froeschke *et al.* 2010), and microbial assemblages exhibit reduced abundance and diversity as environmental aridity increases (Tong and Lighthart 1997; Guernier, Hochberg and Guegan 2004; Burrows *et al.* 2009; Tang 2009; Bachar *et al.* 2010). If aridity is considered as a proxy for disease risk then immunological investment should be greater in environments that are cooler, wetter and more humid, suggesting a negative correlation between aridity and immune function.

Disentangling the relative contributions of pace-of-life and disease risk to observed variation in immune investment is difficult, particularly since pace-of-life and disease risk may themselves co-vary directly or inversely, depending on the study system (Horrocks, Matson and Tieleman 2011). Where pace-of-life and disease risk co-vary directly, predictions about immune investment should coincide, even if the causal factor responsible for immunological variation is not clear. For example, relative to temperate birds, those in the tropics might invest relatively more in immunity due to their slower pace-of-life (Martin II, Hasselquist and Wikelski 2006; Wiersma *et al.* 2007), because of increased exposure to disease risk (Møller 1998; Guernier, Hochberg and Guegan 2004), or perhaps as a result of both factors. Where pace-of-life and disease risk co-vary inversely, conflicting predictions can arise. For example, in addition to tropical birds, birds living in deserts are also predicted to invest strongly in immunity due to their slow pace-of-life (Tieleman, Williams and Visser 2004), even though the tropics and deserts may pose very different disease risks (Horrocks, Matson and Tieleman 2011). Investigating the drivers and correlates of immunological variation in diverse environments therefore requires careful consideration of study system characteristics. If the goal is to separate the contributions of pace-of-life and disease risk to immunological variation, then these two factors must be as un-confounded as possible.

An ideal study system to investigate the relative contributions of pace-of-life and disease risk to immune investment is a large-scale aridity gradient inhabited by a group of closely-related larks (Alaudidae). Despite large geographical and environmental differences, lark species inhabiting this gradient exhibit similar ecological traits: larks are ground-nesting passerines that consume similar diets, display similar behaviours and inhabit open grassland habitats (del Hoyo, Elliott and Christie 2004). Moreover, physiological, demographic and life history traits of several lark species are well-documented (reviewed in Tieleman 2005). Along the gradient from hyper-arid to mesic, pace-of-life co-varies with environmental aridity, a finding unaffected by phylogeny (Tieleman, Williams and Bloomer 2003; Tieleman, Williams and Visser 2004). Slower-living arid-zone larks lay smaller and fewer clutches per year and have slower nestling growth rates than faster-living larks from more mesic environments (Tieleman, Williams and Visser 2004). As defined, the environmental gradient varies predominantly in terms of environmental moisture levels, but locations along the gradient differ in terms of

related abiotic characteristics (e.g. temperature) as well. The net result of this variation in abiotic conditions is that the environments most associated with species exhibiting a slow pace-of-life (which may select *for* immune investment) are the same environments with the lowest presumed risks of disease (which may select *against* immune investment). This contrast is useful for comparing alternative hypotheses to explain variation in immune defences.

We studied larks from arid, semi-arid and mesic locations, which have been studied previously (Tieleman 2005). We also incorporated species from cold-desert and tropical locations that could further help us to tease apart the roles of life history and environmental disease risk in shaping immune investment. Cold-desert larks have clutch sizes more typical of a fast pace-of-life yet live in a predicted low disease risk environment. Tropical larks display life history traits consistent with a slow pace-of-life yet live in potentially high disease risk settings. We used clutch size and total number of eggs per year as indicators of pace-of-life (Saether 1988; Ricklefs 2000), and we used aridity, and the climatic variables that influence aridity as proxies for environmental disease risk. To assess immune investment we focused on four indices of innate immunity, which represent the initial circulating defences encountered by pathogens that have breached physical defensive barriers (Janeway *et al.* 2004). The acute phase proteins haptoglobin and ovotransferrin respond to inflammation or infection by increasing in concentration (van de Crommenacker *et al.* 2010; Horrocks, Tieleman and Matson 2011) in order to limit microbial growth (Cray, Zaias and Altman 2009). Natural antibodies (measured as agglutination titres) opsonize invading microorganisms to facilitate phagocytosis and activate the complement system, which leads to cell lysis (measured as lysis titres; Ochsenbein & Zinkernagel 2000).

We predicted that if immunological investment is driven by pace-of-life and life history trade-offs, then slow-living, arid-zone and tropical larks should invest relatively more in immune defence than fast-living species from temperate and cold-arid environments. However, if disease risk is more important for determining investment in the immune system, then we predicted that immune indices should be lowest in lark populations from arid locations and be higher in temperate and tropical larks living in environments with greater risk of disease.

## Methods

### *Birds*

We captured individuals of 12 species of larks in 23 locations during the breeding season and in winter from 2006 to 2009. Details of species, sample sizes and geographic locations are provided in Table 3.1. Upon capture of birds, we collected 200-300 $\mu$l of blood from the brachial vein and stored it on ice until processing by centrifugation to separate plasma and cellular fractions. Plasma was then frozen

**Table 3.1.** Sample size (*n*), sampling period (breeding (B), non-breeding (NB), or sampled in both periods (both)), geographic origin and climatic variables for twelve species of lark. The climatic variables are mean annual values for precipitation (P), temperature (T), potential evapotranspiration (PET), and two indices of aridity: $A_{UNEP}$ (P / PET) and $A_M$ (P / T + 10).

| # | species | *n* | sampling | latitude | longitude | P (mm) | T (°C) | PET (mm) | $A_{UNEP}$ | $A_M$ |
|---|---|---|---|---|---|---|---|---|---|---|
| a | hoopoe lark *Alaemon alaudipes* | 4 | B | 19° 53' N | 16° 18' W | 49.90 | 24.55 | 2081.12 | 0.02 | 1.44 |
| b | | 61 | both | 22° 20' N | 41° 44' E | 82.01 | 25.38 | 2427.54 | 0.03 | 2.32 |
| c | bar-tailed desert lark *Ammomanes cincturus* | 56 | both | 22° 20' N | 41° 44' E | 82.01 | 25.38 | 2427.54 | 0.03 | 2.32 |
| d | black-crowned finchlark *Eremopterix nigriceps* | 19 | both | 22° 20' N | 41° 44' E | 82.01 | 25.38 | 2427.54 | 0.03 | 2.32 |
| e | | 14 | B | 21° 15' N | 40° 42' E | 201.26 | 21.12 | 2165.66 | 0.09 | 6.47 |
| f | crested lark *Galerida cristata* | 18 | both | 22° 20' N | 41° 44' E | 82.01 | 25.38 | 2427.54 | 0.03 | 2.32 |
| g | | 4 | B | 21° 15' N | 40° 42' E | 201.26 | 21.12 | 2165.66 | 0.09 | 6.47 |
| h | | 2 | NB | 34° 22' N | 41° 44' E | 208.2 | 16.14 | 1571.63 | 0.13 | 7.96 |
| i | Dunn's lark *Eremalauda dunni* | 35 | both | 22° 20' N | 41° 44' E | 82.01 | 25.38 | 2427.54 | 0.03 | 2.32 |
| j | short-toed lark *Calandrella brachydactyla* | 2 | NB | 22° 15' N | 41° 45' E | 82.01 | 25.38 | 2427.54 | 0.03 | 2.32 |
| k | bimaculated lark *Melanocorypha bimaculata* | 7 | NB | 34° 22' N | 41° 44' E | 208.2 | 16.14 | 1571.63 | 0.13 | 7.96 |
| l | | 6 | NB | 36° 54' N | 67° 11' E | 199.88 | 16.98 | 1409.99 | 0.14 | 7.41 |
| m | | 6 | B | 36° 42' N | 67° 06' E | 225.92 | 15.61 | 1369.28 | 0.16 | 8.82 |
| n | | 14 | NB | 34° 54' N | 66° 53'E | 359.48 | 4.48 | 1159.61 | 0.31 | 24.83 |
| o | calandra lark *Melanocorypha calandra* | 11 | NB | 34° 22' N | 41° 44' E | 208.2 | 16.14 | 1571.63 | 0.13 | 7.96 |
| p | | 3 | NB | 36° 54' N | 67° 11' E | 199.88 | 16.98 | 1409.99 | 0.14 | 7.41 |
| q | | 6 | NB | 34° 54' N | 66° 53'E | 359.48 | 4.48 | 1159.61 | 0.31 | 24.83 |
| r | red-capped lark *Calandrella cinerea* | 5 | B | 0° 52' S | 36° 23' E | 570.48 | 20.25 | 1463.95 | 0.39 | 18.86 |
| s | | 8 | B | 0° 34' S | 36° 28' E | 806.77 | 15.39 | 1043.3 | 0.77 | 31.78 |
| t | rufous-naped lark *Mirafra africana* | 4 | B | 0° 52' S | 36° 23' E | 570.48 | 20.25 | 1463.95 | 0.39 | 18.86 |
| u | | 2 | B | 0° 34' S | 36° 28' E | 806.77 | 15.39 | 1043.3 | 0.77 | 31.78 |
| v | skylark *Alauda arvensis* | 144 | both | 52° 56' N | 6° 18' E | 770.11 | 9.19 | 557.82 | 1.38 | 40.13 |
| w | woodlark *Lullula arborea* | 61 | both | 52° 56' N | 6° 18' E | 770.11 | 9.19 | 557.82 | 1.38 | 40.13 |

and stored at -20°C until use in immune assays. We gathered data on pace-of-life indicators (mean clutch size and number of clutches per year; Table 3.2) directly from our own study populations and from Tieleman, Williams and Visser (2004), Cramp (1988) and del Hoyo, Elliott and Christie (2004).

### *Climatic variables and aridity indices*

We obtained high-resolution (0.5 × 0.5 degree) gridded data on climatic variables for the period 1901–2009 from http://badc.nerc.ac.uk/view/badc.nerc.ac.uk_ATOM_dataent_1256223773328276. This dataset is described in detail by

**Table 3.2.** Mean clutch size and number (year$^{-1}$), concentrations of acute phase proteins haptoglobin and ovotransferrin, and agglutination and lysis titres for 23 populations of 12 lark species. Values for life history variables are from this study and from the literature (data source column).

| # | species | clutch size | clutches year$^{-1}$ | hapto-globin (mg ml$^{-1}$) | ovotrans-ferrin (mg ml$^{-1}$) | agglu-tination (titres) | lysis (titres) | data source* |
|---|---|---|---|---|---|---|---|---|
| a | hoopoe lark | 2.88 | 1 | 0.25 | 7.41 | 4.50 | 1.63 | 3 |
| b | | 2.99 | 1 | 0.28 | 7.47 | 5.85 | 0.86 | 2 |
| c | bar-tailed desert lark | 3.24 | 1 | 0.29 | 9.08 | 6.13 | 0.38 | 2 |
| d | black-crowned finchlark | 2.00 | 1 | 0.27 | 9.11 | 5.83 | 0.58 | 3, 4 |
| e | | 2.57 | 1 | 0.49 | 5.43 | 7.03 | 1.52 | 3, 4 |
| f | crested lark | 4.15 | 2 | 0.25 | 5.18 | 6.24 | 0.53 | 2 |
| g | | 4.15 | 2 | 0.25 | 15.28 | 6.31 | 1.94 | 2 |
| h | | 4.75 | 2 | 0.07 | 11.20 | 5.25 | 0.00 | 2, 3 |
| i | Dunn's lark | 2.88 | 1 | 0.49 | 9.76 | 6.65 | 1.63 | 2 |
| j | short-toed lark | 3.50 | 2 | 0.37 | 9.18 | 11.00 | 1.00 | 2 |
| k | bimaculated lark | 3.96 | 1.5 | 0.11 | 12.72 | 4.21 | 0.21 | 3 |
| l | | 3.96 | 1.5 | 0.19 | - | 5.17 | 2.08 | 3 |
| m | | 3.96 | 1.5 | 0.17 | - | 7.63 | 4.88 | 3 |
| n | | 3.96 | 1.5 | 0.33 | 14.53 | 4.90 | 2.63 | 3 |
| o | calandra lark | 4.20 | 2 | 0.07 | 9.78 | 5.90 | 1.25 | 2 |
| p | | 4.20 | 2 | 0.08 | - | 7.25 | 3.58 | 2 |
| q | | 4.20 | 2 | 0.06 | 6.01 | 6.46 | 1.75 | 2 |
| r | red-capped lark | 1.83 | 2 | 0.15 | 9.10 | 4.50 | 0.13 | 1 |
| s | | 1.89 | 2 | 0.57 | 7.25 | 5.17 | 2.42 | 1 |
| t | rufous-naped lark | 2.11 | 1 | 0.74 | 9.08 | 6.31 | 3.69 | 1, 4 |
| u | | 2.00 | 1 | 0.19 | 10.20 | 5.63 | 3.63 | 1, 4 |
| v | skylark | 3.56 | 3.5 | 0.48 | - | 7.82 | 2.26 | 1 |
| w | woodlark | 4.02 | 2.5 | 0.46 | 9.41 | 7.20 | 2.21 | 1 |

* 1 Own data; 2 Tieleman, Williams and Visser (2004); 3 Cramp (1988); 4 del Hoyo, Elliott and Christie (2004).

Mitchell and Jones (2005). For each bird-sampling location we extracted mean annual values for precipitation (P; mm), temperature (T; °C), and potential evapotranspiration (PET; mm). PET is a derived reference measurement of the amount of water lost to the atmosphere through the combined processes of evaporation and plant transpiration. PET is dependent on a range of climatic and environmental factors but tends to be low in cool and humid environments and high in arid locations such as deserts. We used the three climatic variables to calculate two alternative indices of aridity: the United Nations Environment Programme aridity index ($A_{UNEP}$: P / PET; UNEP 1992) and de Martonne's aridity index ($A_M$: P / T + 10; de Martonne 1926). Climatic variables and aridity indices for each lark population are shown in Table 3.1.

*Immune assays*

We determined haptoglobin concentrations (mg $ml^{-1}$) using a functional assay that measures the haem-binding capacity of plasma (TP801; Tri-Delta Diagnostics, NJ, USA), following the 'manual method' instructions provided by the manufacturer and with incubation at 30°C for 5 minutes. We measured ovotransferrin concentrations (mg $ml^{-1}$) according to Horrocks, Tieleman and Matson (2011), but not all populations were measured due to blood volume limitations and logistical reasons (Table 3.2). We quantified natural antibody-mediated agglutination titres and complement-mediated lysis titres against rabbit red blood cells (B-0009D, Harlan, UK), according to the assay of Matson, Ricklefs & Klasing (2005).

*Statistical analyses*

Our dataset consisted of values at the individual level (immune measures) and values at the population- or species-level (all other variables). We took a conservative approach and calculated mean values per population (i.e. per species per location) of each dependent variable for which we had measurements on individuals. We treated species and geographically distinct populations as independent points. First, we tested whether populations differed in their immune responses: haptoglobin $F_{22, 444} = 2.89$, $P < 0.001$; ovotransferrin $F_{18, 106} = 0.89$, $P = 0.594$; agglutination $F_{22, 409} = 2.21$, $P = 0.001$; lysis $F_{22, 412} = 5.30$, $P < 0.001$. Then we used linear models to investigate relationships between immune indices and climatic and life history variables. Because sample size varied among species and populations (Table 3.1), we weighted regression models by the square root of the number of individuals sampled in each population (Sokal and Rohlf 1995). Since some species or populations were only sampled during one period (breeding or non-breeding; Table 3.1) we ran analyses using restricted datasets containing values per period, as well as with the entire dataset of all values. The results of these analyses were qualitatively similar and so we only present results based on the entire dataset. We performed all analyses using R 2.13.0 (R Development Core Team 2009).

## Results

To disentangle the roles of pace-of-life and environmental disease risk in directing immune investment, these factors should not be positively correlated. We confirmed that this was the case in our dataset by examining correlations between pace-of-life indicators (clutch size and number of eggs per year) and our primary proxies of environmental disease risk (aridity indices $A_{UNEP}$ and $A_M$). When restricting analyses to previously-studied species by excluding tropical and cold-desert larks (where pace-of-life and environmental disease risk do not correlate), clutch size and number of eggs per year correlated negatively with environmental disease risk (Table 3.3), as previously shown (Tieleman, Williams and Visser 2004). These correlations were not significant for clutch size but were significant for total number of eggs per year (Table 3.3). When including tropical and cold-desert species, correlations generally remained negative overall, but were much weaker and were all non-significant (Table 3.3).

Relationships between immune indices and pace-of-life parameters did not display consistent patterns, and with the exception of agglutination titres, were always non-significant and weak (excluding agglutination, all $r^2 \leq 0.12$; Table 3.4). Haptoglobin and ovotransferrin concentrations showed weak but opposite trends with respect to clutch size (Figs 3.1A, 3.1C) but not with number of eggs per year. (Figs 3.1B, 3.1D). Agglutination titres were positively associated with both life history variables (Figs 3.1E–F), and significantly associated with number of eggs per year (Fig. 3.1F; Table 3.4). Lysis titre showed no relationship with mean clutch size (Fig. 3.1G) and a very weak but positive relationship with number of eggs per year (Fig. 3.1H; Table 3.4).

Lark populations consistently exhibited lower indices of immunity in environments with reduced proxies of disease risk (Fig. 3.2), regardless of which aridity index was used. These negative correlations were significant for haptoglobin concentrations and agglutination and lysis titres (Fig. 3.2; Table 3.4). In line with

**Table 3.3.** Correlations between pace-of-life indicators (clutch size and number of eggs laid per year) and proxies of environmental disease risk (aridity indices $A_{UNEP}$ and $A_M$ – see Methods for details) for 23 populations of 12 lark species.

| correlation | Excluding tropical and cold-desert species | | | All species | | |
|---|---|---|---|---|---|---|
| | t | *P* | *r* | t | *P* | *r* |
| clutch size vs. $A_{UNEP}$ | -1.24 | 0.245 | -0.38 | 0.53 | 0.603 | 0.11 |
| total eggs year$^{-1}$ vs. $A_{UNEP}$ | -3.51 | 0.007 | -0.76 | -1.89 | 0.073 | -0.38 |
| clutch size vs. $A_M$ | -1.28 | 0.238 | -0.39 | 0.60 | 0.554 | 0.13 |
| total eggs year$^{-1}$ vs. $A_M$ | -3.59 | 0.006 | -0.77 | -1.50 | 0.149 | -0.31 |

this, haptoglobin concentration and agglutination and lysis titres were also positively and significantly correlated with mean annual precipitation (Fig. 3.3; Table 3.4). Ovotransferrin concentration showed no relationship with aridity or precipitation (Figs 3.2B and 3.3D). Mean annual temperature correlated negatively with immune indices (Fig. 3.3), but only lysis showed a significant relationship with this climatic variable (Fig. 3.3K; Table 3.4). Mean annual PET also showed negative relationships with immune indices (Fig. 3.3): these relationships were only

**Table 3.4.** Results (F tests and *P* values) of linear models examining relationships between immune indices of 23 populations of 12 lark species in relation to pace-of-life indicators (mean clutch size and total number of eggs laid per year) and to climatic proxies of environmental disease risk. Significant *P* values are shown in bold.

| response variable | explanatory variable | $r^2$ | F | *P* |
|---|---|---|---|---|
| haptoglobin (mg $ml^{-1}$) | mean clutch size | 0.12 | $F_{1, 21} = 2.86$ | 0.105 |
| | total eggs $year^{-1}$ | 0.01 | 0.20 | 0.657 |
| | aridity index $A_{UNEP}$ | 0.27 | 7.55 | **0.001** |
| | aridity index $A_M$ | 0.21 | 5.48 | **0.029** |
| | mean annual precipitation (mm) | 0.24 | 6.49 | **0.019** |
| | mean annual temperature (°C) | 0.02 | 0.34 | 0.569 |
| | mean annual PET (mm) | 0.07 | 1.61 | 0.218 |
| ovotransferrin (mg $ml^{-1}$) | mean clutch size | 0.06 | $F_{1, 17} = 1.02$ | 0.327 |
| | total eggs $year^{-1}$ | 0.02 | 0.41 | 0.530 |
| | aridity index $A_{UNEP}$ | 0.02 | 0.38 | 0.545 |
| | aridity index $A_M$ | 0.05 | 0.97 | 0.339 |
| | mean annual precipitation (mm) | 0.03 | 0.59 | 0.450 |
| | mean annual temperature (°C) | 0.15 | 3.10 | 0.096 |
| | mean annual PET (mm) | 0.10 | 1.92 | 0.184 |
| agglutination (titre) | mean clutch size | 0.05 | $F_{1, 21} = 1.17$ | 0.292 |
| | total eggs $year^{-1}$ | 0.34 | 10.58 | **0.004** |
| | aridity index $A_{UNEP}$ | 0.37 | 12.39 | **0.002** |
| | aridity index $A_M$ | 0.25 | 6.96 | **0.015** |
| | mean annual precipitation (mm) | 0.20 | 5.39 | **0.030** |
| | mean annual temperature (°C) | 0.12 | 2.73 | 0.113 |
| | mean annual PET (mm) | 0.19 | 4.87 | **0.039** |
| lysis (titre) | mean clutch size | 0.01 | $F_{1, 21} = 0.23$ | 0.637 |
| | total eggs $year^{-1}$ | 0.07 | 1.59 | 0.222 |
| | aridity index $A_{UNEP}$ | 0.17 | 4.15 | **0.055** |
| | aridity index $A_M$ | 0.23 | 6.30 | **0.020** |
| | mean annual precipitation (mm) | 0.24 | 6.68 | **0.017** |
| | mean annual temperature (°C) | 0.28 | 8.32 | **0.009** |
| | mean annual PET (mm) | 0.34 | 10.62 | **0.004** |

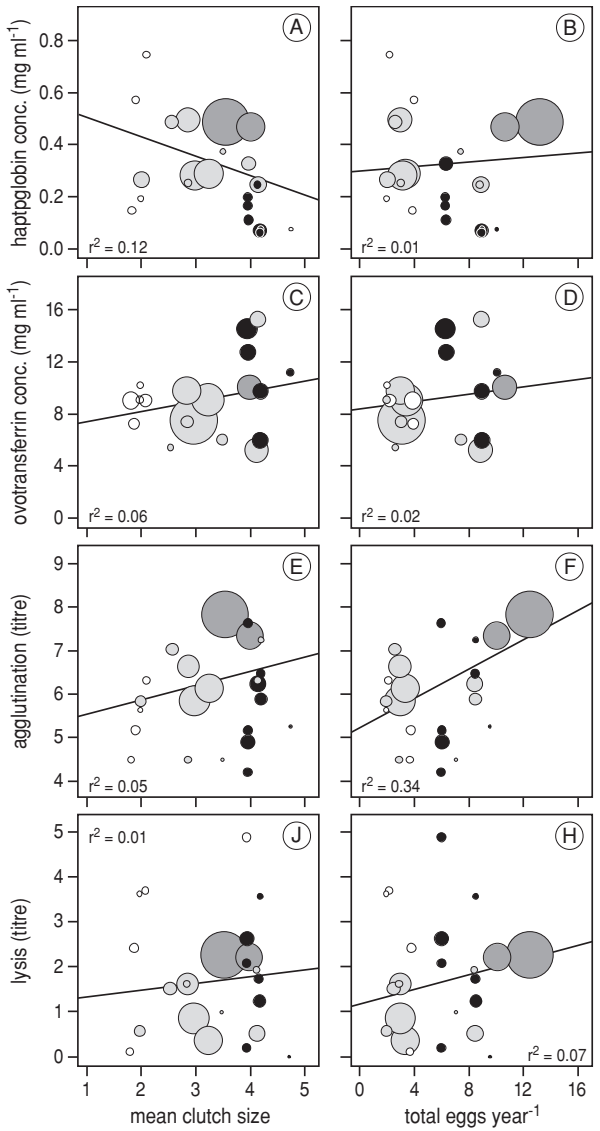


**Figure 3.1.** Haptoglobin (A-B) and ovotransferrin (C-D) concentrations and agglutination (E-F) and lysis (G-H) titres as a function of mean clutch size and mean total number of eggs year-1 in 23 populations of 12 lark species. The size of each data point is proportional to the number of records (number of individuals) contributing to the value. Hot-desert species are in light grey, cold-desert species in black, temperate species in dark grey and tropical lark species are in white.

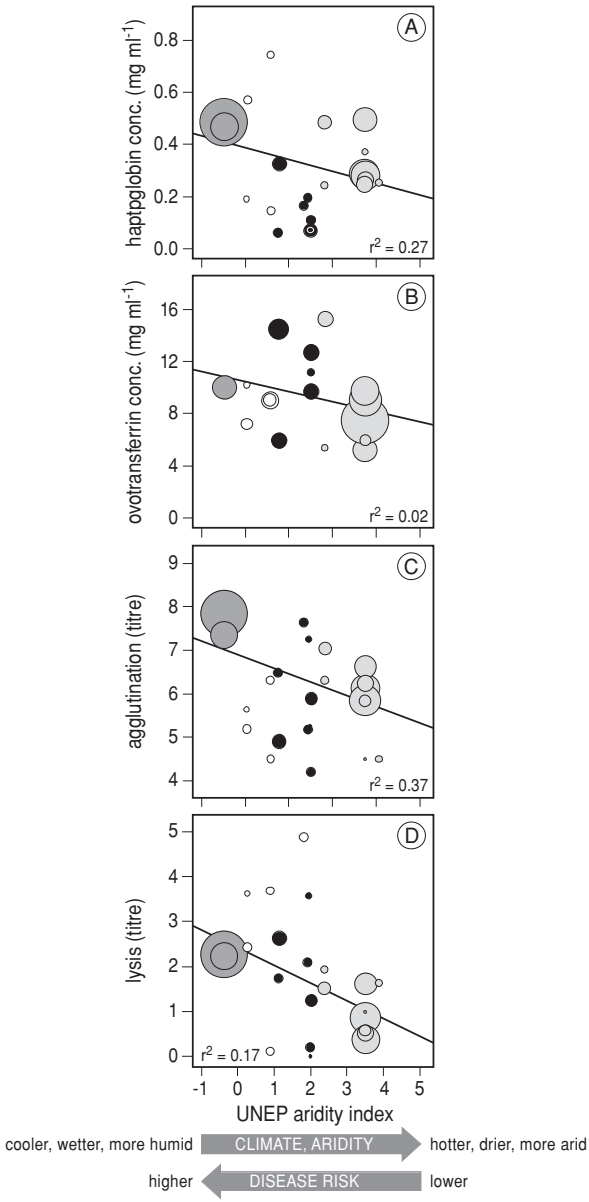


**Figure 3.2.** Haptoglobin (A) and ovotransferrin (B) concentrations and agglutination (C) and lysis (D) titres as a function of environmental aridity in 23 populations of 12 lark species. Aridity is plotted on a natural log scale and so that the aridity index increases with increasing aridity of the environment. The size of each data point is proportional to the number of records (number of individuals) contributing to the value. Hot-desert species are in light grey, cold-desert species in black, temperate species in dark grey and tropical lark species are in white.

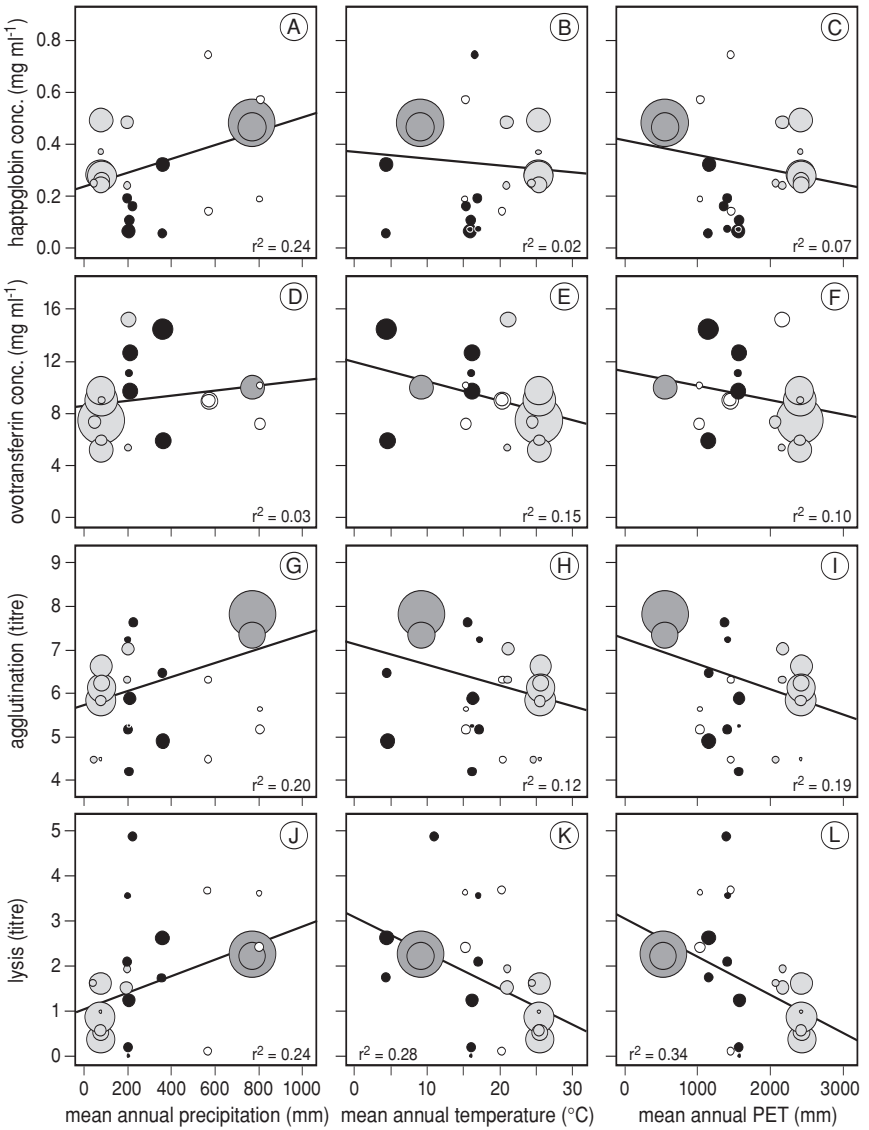


**Figure 3.3.** Haptoglobin (A-C) and ovotransferrin (D-F) concentrations and agglutination (G-I) and lysis (J-L) titres as a function of mean annual precipitation, mean annual temperature and mean annual potential evapotranspiration (PET) in 23 populations of 12 lark species measured along an environmental aridity gradient. The size of each data point is proportional to the number of records (number of individuals) contributing to the value. Hot-desert species are in light grey, cold-desert species in black, temperate species in dark grey and tropical lark species are in white.

significant for agglutination (Fig. 3.3I) and lysis (Fig. 3.3L) titres (Table 3.4). Thus, patterns of immune variation with precipitation, temperature and PET all supported the trend for decreasing immune indices with increasing predicted disease risk.

## Discussion

Pace-of-life and environmental disease risk represent two ecological axes with which immune investment may co-vary. Disentangling the relative contributions of these two factors can be complicated in some study systems (Horrocks, Matson and Tieleman 2011), particularly when pace-of-life and environmental disease risk are positively correlated. We studied lark populations living in diverse environments where pace-of-life and disease risk were not positively correlated. We found that pace-of-life explained very little of the variation in immune indices. In contrast, environmental correlates of disease risk were much better at explaining variation in immune investment. Our data provide an interesting counterpoint to studies suggesting a role for pace-of-life in shaping immune defences (e.g. Tieleman *et al.* 2005; Lee 2006; Sparkman and Palacios 2009) and underscore the value of incorporating indices of disease risk into ecological immunology studies.

In agreement with our second hypothesis, immune indices matched predictions based on disease risk. Measures of innate immunity were highest in populations of larks from humid locations where disease risk is expected to be higher, and these immune indices decreased with increasing aridity. In line with this, we also found significant positive associations between immune indices and precipitation and negative correlations between some immune indices and temperature and PET. Thus, with aridity as our *a priori* proxy for environmental disease risk, investment in innate immunity can be seen as increasing in tandem with this risk. Previous authors have linked disease risk and abiotic environmental variables (e.g. salinity exposure; Piersma 1997; Figuerola 1999; Mendes *et al.* 2005) and particularly disease prevalence and climatic factors (Guernier, Hochberg and Guegan 2004; Gage *et al.* 2008). These more general links and the specific links between aridity and parasitic (e.g. Jex *et al.* 2007; Froeschke *et al.* 2010) and microbial assemblages (e.g. Burrows *et al.* 2009; Tang 2009; Bachar *et al.* 2010; chapter 5) suggest that biotic disease risk variation is reflected by abiotic environmental variation. Directly quantifying and understanding biotic disease risk parameters is challenging, particularly among species and across environments, since parasitic and microbial assemblages may not be directly comparable, and universal measurement methods may not exist (Horrocks, Matson and Tieleman 2011). Abiotic environmental proxies can therefore provide a useful alternative while conceptual and methodological issues are resolved. Nevertheless, future work involving direct measurements of disease risk will no doubt shed additional

light on the strong associations we have identified between disease risk and immune defence.

In contrast to environmental disease risk, correlations between immune indices and two pace-of-life parameters were generally mixed and very weak ($r^2 \leq 0.12$, with one exception). Only one relationship, between agglutination and total eggs per year, was significant. Importantly, this significant correlation was positive, suggesting that lark species with a faster pace-of-life had higher agglutination ability. Thus, this finding contradicts our first hypothesis that larks with a slow pace-of-life should invest more in immunity than fast-living species. It also contradicts earlier work conducted on a range of tropical bird species that found higher natural antibody levels (i.e. greater agglutination ability) in birds with longer development times, indicative of a slower pace-of-life (Lee *et al.* 2008). In this context, populations in our dataset that are exceptions to the predominant inverse relationship between pace-of-life and environmental disease risk deserve further consideration. These exceptions can provide particular insight into the drivers of immune investment. For example, both tropical larks in our dataset (red-capped lark and rufous-naped lark; white spheres in figures) have small clutches and lay few clutches per year (Table 3.2) indicative of a slow pace-of-life. While their pace-of-life is similar to arid-zone larks (light grey spheres in figures), the disease risk experienced by tropical larks (based on aridity values; Table 3.1) is high and more similar to the temperate populations (dark grey spheres in figures) in our dataset. If pace-of-life strongly influenced immune investment, values of immune indices for red-capped larks and rufous-naped larks should cluster with values for arid-zone larks. In contrast, lysis titres in these two tropical larks were more similar to values for temperate larks (Fig. 3.1), which reasserts that immune investment is more likely related to disease risk. No obvious groupings were apparent with the other three immune indices. Conversely, cold-desert larks (bimaculated lark and calandra lark; black spheres in figures), which have clutch sizes indicative of a fast pace-of-life, inhabit low disease risk environments. In this case, if pace-of-life strongly influenced immune investment, values of immune indices for these larks should cluster with values for temperate lark populations. This was not the case, particularly for agglutination titres and haptoglobin concentrations (Fig. 3.1), which grouped more closely with arid-zone (hot-desert) species that are predicted to experience similar disease risks to cold-desert larks. Thus, this result also reinforces the idea that immune investment relates to disease risk rather than pace-of-life.

Natural antibody level (measured as agglutination titres) was the notable exception where there was a significant correlation with one measure of pace-of-life. This positive relationship between natural antibodies and pace-of-life might signify preferential investment in non-specific antibodies by short-lived species. Despite the otherwise weak relationships between pace-of-life and immune indices, this single correlation hints at a link between immune defence and life

history strategy (Lee 2006). Fast-living species are predicted to favour innate over adaptive immune defences, since adaptive immunity requires longer developmental times that are incompatible with the fast growth associated with a fast pace-of-life (Lee 2006; Lee *et al.* 2008). While this prediction is supported by the results of several studies (Martin II, Hasselquist and Wikelski 2006; Sparkman and Palacios 2009; although see Tieleman *et al.* 2005, conducted in a single environment), overall our data provide only limited support. Natural antibodies, although generally considered an innate immune defence, straddle the boundary between innate and adaptive immunity. For example, natural antibodies titres correlate positively with adaptive antibody responses (Parmentier *et al.* 2004) and provide links between the innate and adaptive arms of the immune system (Caroll and Prodeus 1998; Ochsenbein and Zinkernagel 2000). Nonetheless, measures of purely adaptive immune defences (e.g. induction of specific antibody responses) are necessary to draw conclusions about trade-offs within the immune system and overall protection.

Comparative studies, both within and among species and environments, represent a powerful approach for disentangling the roles of pace-of-life and environmental disease risk in shaping immune defences and for understanding immune defence variation in more general ecological and evolutionary terms. By employing a study system in which pace-of-life variation is uncoupled from environmental disease risk variation, we demonstrated that investment in innate immune defences is related more to environmental disease risk than to pace-of-life. An obvious next step is a comparative study that integrates indices of host-dependent and host-independent disease pressures. Adding, in essence, an axis that represents biotic environmental disease risk opens up an exciting frontier that will stimulate research and advance our understanding of immunological variation.

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