Environmental proxies of antigen exposure explain variation in immune investment better than indices of pace of life

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Abstract Investment in immune defences is predicted to covary with a variety of ecologically and evolutionarily relevant axes, with pace of life and environmental antigen exposure being two examples. These axes may themselves covary directly or inversely, and such relationships can lead to conflicting predictions regarding immune investment. If pace of life shapes immune investment then, following life history theory, slow-living, arid zone and tropical species should invest more in immunity than fast-living temperate species. Alternatively, if antigen exposure drives immune investment, then species in antigen-rich tropical and temperate environments are predicted to exhibit higher immune indices than species from antigen-poor arid locations. To test these contrasting predictions we investigated how variation in pace of life and antigen exposure influence immune investment in related lark species (Alaudidae) with differing life histories and predicted risks of exposure to environmental microbes and parasites. We used clutch size and total number of eggs laid per year as indicators of pace of life, and aridity, and the climatic variables that influence aridity, as correlates of antigen abundance. We quantified immune investment by measuring four indices of innate immunity. Pace of life explained little of the variation in immune investment, and only one immune measure correlated significantly with pace of life, but not in the predicted direction. Conversely, aridity, our proxy for environmental antigen exposure, was predictive of immune investment, and larks in more mesic environments had higher immune indices than those living in arid, low-risk locations. Our study suggests that abiotic environmental variables with strong ties to environmental antigen exposure can be important correlates of immunological variation.

Keywords Alaudidae · Aridity · Ecological immunology · Lark · Life history

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Introduction

Explanations for variation in immune investment have often focused on the identification of evolutionary and ecological axes along which immune defences might covary. For example, ecological immunologists have exploited differences in pace of life to explain variation in immune defences. Limited resources and the costs associated with immunity suggest that immune investment must be

counterbalanced against investment in other costly physiological processes such as growth and reproduction (Sheldon and Verhulst 1996; Ilmonen et al. 2000; Lochmiller and Deerenberg 2000; Norris and Evans 2000; Hegemann et al. 2013). Species at the fast end of the pace-of-life axis, with short lifespans and high reproductive rates, may allocate more of their limited resources to reproduction, and fewer to self-maintenance activities such as immune defence. Conversely, 'slow-living' species that develop slowly, have low extrinsic mortality, and low reproductive rates, can prioritise self-maintenance activities and invest more heavily in immunity (Roff 1992; Stearns 1992; Ricklefs and Wikelski 2002).

Variation in exposure to environmental antigens represents another axis that might explain immune investment. Immune systems provide clear benefits in terms of protection against exogenous threats, including fitness-reducing micro- and macro-parasites. Immune investment might be greater when the risk of infection is higher (Tschirren and Richner 2006; Horrocks et al. 2011a), which could be associated with environment, time, and other ecological factors (Piersma 1997; Møller 1998; Guernier et al. 2004; Hegemann et al. 2012, 2013; Horrocks et al. 2012a, b). For example, levels of environmental moisture shape endo- and ecto-parasitic communities, which show decreased prevalence, abundance and diversity in more arid environments (Little and Earlé 1995; Moyer et al. 2002; Valera et al. 2003; Guernier et al. 2004; Jex et al. 2007; Guerra et al. 2010; Froeschke et al. 2010; Pullan and Brooker 2012). Combined with reduced moisture, the increased solar radiation and temperature extremes associated with arid environments also act to limit microbial assemblages (Tong and Lighthart 1997; Saranathan and Burtt 2007; Burrows et al. 2009; Tang 2009; Bachar et al. 2010). If aridity is considered as a proxy for the level of antigenic exposure (Horrocks et al. 2014) then the requirement for immune investment should be greatest in cool, wet and humid environments where parasites and microbes are more likely to be encountered. This suggests a negative correlation between aridity and immune function.

Disentangling the relative contributions of pace of life and antigen exposure to immune variation is difficult because both axes may themselves covary (Horrocks et al. 2011a). Where pace of life and antigen exposure covary positively, predictions about immune investment 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 more in immunity due to their slower pace of life (Martin et al. 2006; Wiersma et al. 2007), because of increased exposure to environmental antigens such as parasites (Møller 1998; Guernier et al. 2004), or perhaps as a result of both factors. Where

pace of life and antigen exposure covary negatively, conflicting predictions can arise. For example, the slow pace of life of desert-living birds (Tieleman et al. 2004) predicts strong investment in immune defences, similar to birds in the tropics, even though deserts and the tropics may pose contrasting risks in terms of exposure to antigens (Horrocks et al. 2011a). Investigating the drivers and correlates of immune variation in diverse environments requires careful consideration of study system characteristics. If the goal is to separate the contributions of pace of life and antigen exposure, then these two factors must be as unconfounded as possible.

We investigated how immune investment is influenced by pace of life and antigen exposure—expressed as environmental aridity—by studying related species of songbirds (larks; Alaudidae) that inhabit environments differing in aridity. Larks are ideally suited to this study because of their ecological similarities in different environments (del Hoyo et al. 2004), and because environmental moisture is already known to influence exposure to antigens in birds. Bacterial loads in nests, and infestation of nestlings by parasitic fly larvae correlate negatively with precipitation (Berger et al. 2003; Antoniazzi et al. 2011), while haematozoan infections and prevalence of lice and bacteria on feathers are reduced under more arid conditions (Little and Earlé 1995; Moyer et al. 2002; Valera et al. 2003; Saranathan and Burtt 2007; Bush et al. 2009; Malenke et al. 2011; Horrocks et al. 2012b). Soil microbial abundance also correlates negatively with precipitation (Bachar et al. 2010; Drenovsky et al. 2010; Blankinship et al. 2011; Pasternak et al. 2013; Serna-Chavez et al. 2013) and soil microbes contaminate birds and their nests (Shawkey et al. 2005; Ruiz-de-Castañeda et al 2011; Potter et al. 2013).

We measured lark species living in hot, hyper-arid deserts and cooler, wetter, mesic locations, as well as those from cold desert and tropical locations. Cold desert larks have clutch sizes typical of a fast pace of life yet live in an environment predicted to pose a low risk of antigen exposure. Tropical larks display life history traits consistent with a slow pace of life yet live in potentially high-antigen-exposure settings (Tables 1, 2). For the remaining lark species in our study pace of life increases with decreasing aridity, a finding that is unaffected by phylogeny (Tieleman et al. 2003, 2004). This means that the environments most associated with species exhibiting a slow pace of life (which may select for immune investment) are the same environments that present the lowest exposure to environmental antigens (which may select against immune investment). These contrasts make larks particularly suited for teasing apart the roles of life history and environmental antigen exposure in shaping investment in immune defences.

Table 1 Sample size (n), sampling period [breeding (B), non-breeding (NB), or sampled in both periods (*both*)], geographic origin and habitat description, and climatic variables for 12 species of lark

	Species	n	Sampling	Latitude	Longitude	Country	Habitat	A_M	P (mm)	T (°C)
a	Hoopoe lark *Alaemon alaudipes*	61	Both	22°20′N	41°44′E	Saudi Arabia	Hot desert	2.29	81.19	25.38
b		4	B	19°53′N	16°18′W	Mauritania	Hot desert	2.01	69.33	24.55
c	Bar-tailed desert lark *Ammomanes cincturus*	56	Both	22°20′N	41°44′E	Saudi Arabia	Hot desert	2.29	81.19	25.38
d	Black-crowned finchlark *Eremopterix nigriceps*	14	B	21°15′N	40°42′E	Saudi Arabia	Hot desert	6.43	200.20	21.12
e		19	Both	22°20′N	41°44′E	Saudi Arabia	Hot desert	2.29	81.19	25.38
f	Crested lark *Galerida cristata*	4	B	21°15′N	40°42′E	Saudi Arabia	Hot desert	6.43	200.20	21.12
g		18	Both	22°20′N	41°44′E	Saudi Arabia	Hot desert	2.29	81.19	25.38
h		2	NB	34°22′N	62°11′E	Afghanistan	Hot desert	8.66	226.44	16.14
i	Dunn's lark *Eremalauda dunni*	35	Both	22°20′N	41°44′E	Saudi Arabia	Hot desert	2.29	81.19	25.38
j	Short-toed lark *Calandrella brachydactyla*	2	NB	22°16′N	41°45′E	Saudi Arabia	Hot desert	2.29	81.19	25.38
k	Bimaculated lark *Melanocorypha bimaculata*	6	NB	36°54′N	66°53′E	Afghanistan	Cold desert	7.96	214.88	16.98
l		14	NB	34°54′N	67°11′E	Afghanistan	Cold desert	26.92	389.80	4.48
m		7	NB	34°22′N	62°11′E	Afghanistan	Cold desert	8.66	226.44	16.14
n		6	B	36°43′N	67°06′E	Afghanistan	Cold desert	9.50	243.17	15.61
o	Calandra lark *Melanocorypha calandra*	3	NB	36°54′N	66°53′E	Afghanistan	Cold desert	7.96	214.88	16.98
p		6	NB	34°54′N	67°11′E	Afghanistan	Cold desert	26.92	389.80	4.48
q		11	NB	34°22′N	62°11′E	Afghanistan	Cold desert	8.66	226.44	16.14
r	Red-capped lark *Calandrella cinerea*	5	B	0°51′S	36°25′E	Kenya	Tropical	19.64	593.98	20.25
s		8	B	0°34′S	36°29′E	Kenya	Tropical	33.05	839.22	15.39
t	Rufous-naped lark *Mirafra africana*	4	B	0°48′S	36°32′E	Kenya	Tropical	19.64	593.98	20.25
u		2	B	0°34′S	36°29′E	Kenya	Tropical	33.05	839.22	15.39
v	Skylark *Alauda arvensis*	144	Both	52°56′N	6°18′E	Netherlands	Temperate	40.50	777.01	9.19
w	Woodlark *Lullula arborea*	60	Both	52°56′N	6°18′E	Netherlands	Temperate	40.50	777.01	9.19

The climatic variables are the aridity index (A_M; $P/T + 10$, where P is precipitation and T is temperature), and mean annual values for P and T. A lower value of A_M indicates a more arid environment

We assessed immune investment by measuring circulating levels of four non-specific immune indices that any environmental antigen that has breached defensive barriers such as the skin or mucosa might encounter (Janeway et al. 2004). Haptoglobin and ovotransferrin are acute phase proteins with immunomodulatory properties that counter microbial challenges and limit microbial growth by directly sequestering iron (Xie et al. 2002; Arredouani et al. 2003). Natural antibodies opsonize invading microorganisms to facilitate phagocytosis and activate the complement system, which leads to cell lysis (Ochsenbein and Zinkernagel 2000). We used clutch size and number of eggs laid per year as indicators of pace of life (Saether 1988; Ricklefs 2000), and aridity, precipitation and mean ambient temperature as proxies for environmental antigen exposure. We predicted that if immune investment is driven by pace of life, then slow-living, arid zone and tropical larks should invest relatively more in immune defences than fast-living species from temperate and cold-arid environments. If antigen exposure is more important for determining investment in immune defences, 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 abundance of microbes and macro-parasites.

Materials and methods

Study populations, sampling, and indicators of pace of life

We captured larks of 12 species in 11 climatically distinct locations during breeding and non-breeding periods from 2006 to 2009 (23 populations in total; Table 1). We collected <300 µl blood from the brachial vein of each bird and stored it on ice until processing by centrifugation to separate plasma and cellular fractions. The plasma was then frozen and stored at −20 °C until it was used in immune assays. We gathered data on mean clutch size and number of eggs laid per year (mean clutch size × mean number of clutches laid per year; Table 2) directly from our own study populations and from Tieleman et al. (2004), Cramp (1988), del Hoyo et al. (2004) and Hegemann et al. (2012, 2013). All birds were sampled under licence from the relevant authorities.

Table 2 Mean clutch size and number of clutches per year, concentrations of haptoglobin and ovotransferrin, and agglutination and lysis titres for 23 populations of 12 lark species

	Species	Clutch size	Clutches (no. year^{-1})	Haptoglobin (mg ml^{-1})	Ovotransferrin (mg ml^{-1})	Agglutination (titre)	Lysis (titre)	Data source[a]
a	Hoopoe lark	2.99	1	0.28	7.47	5.85	0.86	2
b		2.88	1	0.25	7.41	4.50	1.63	3
c	Bar-tailed desert lark	3.24	1	0.29	9.08	6.13	0.38	2
d	Black-crowned finchlark	2.57	1	0.49	5.43	7.03	1.52	3, 4
e		2.00	1	0.27	9.11	5.83	0.58	3, 4
f	Crested lark	4.15	2	0.25	15.28	6.31	1.94	2
g		4.15	2	0.25	5.18	6.24	0.53	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	0.41	9.18	11.00	1.00	2
k	Bimaculated lark	3.96	1.5	0.19	–	5.17	2.08	3
l		3.96	1.5	0.33	14.53	4.90	2.63	3
m		3.96	1.5	0.11	12.72	4.21	0.21	3
n		3.96	1.5	0.17	–	7.63	4.88	3
o	Calandra lark	4.20	2	0.08	–	7.25		
p		4.20	2	0.06	6.01	6.46		
q		4.20	2	0.07	9.78	5.90		
r	Red-capped lark	1.83	2	0.15	9.10	4.50		
s		1.89	2	0.57	7.25	5.17		
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		
v	Skylark	3.56	3.5	0.48	–	7.82		
w	Woodlark	4.02	2.5	0.46	9.41	7.20		

Values for life history variables are from this study and from the literature (*Data source*)

[a] Own data (*1*), Tieleman et al. (2004) (*2*), Cramp (1988) (*3*), del Hoyo et al. (2004) (*4*), Hegemann et al. (2012, 2013) (*5*)

Climatic variables and aridity index

We obtained high-resolution (0.5° × 0.5°—approximately 55 × 55 km) gridded data on climatic variables for the period 1901–2009 from the Climatic Research Unit time series data set [CRU TS 3.1 (Harris et al. 2013)]. For each bird-sampling location we extracted mean annual values for precipitation (*P*; mm) and temperature (*T*; °C). We used these climatic variables to calculate de Martonne's aridity index A_M [*P/T* + 10 (de Martonne 1926)]. A_M and climatic variables for each lark population are shown in Table 1. Low values of A_M indicate arid conditions, whereas higher values are associated with increasingly mesic environments.

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),

following the manual method instructions provided by the manufacturer and with incubation at 30 °C for 5 min (Matson et al. 2012). We measured ovotransferrin concentrations (mg ml^{-1}) according to Horrocks et al. (2011b). Three of the 23 populations were not measured due to blood volume limitations (Table 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 et al. (2005).

Statistical analyses

We calculated mean values per population (i.e. per species per location) for each immune index and used these values to conduct regression analyses to investigate relationships between immune indices and life history variables and aridity. Since our aridity index is a compound variable, we also ran separate models testing immune indices against each of the primary climatic variables precipitation and temperature. To account for potential non-independence among

species due to common ancestry we used a phylogenetic generalised least squares approach (Freckleton et al. 2002), simultaneously estimating maximum likelihood values of the parameter λ to test for phylogenetic signal in the model residuals [Pagel's λ (Pagel 1999)]. A value of $\lambda = 0$ indicates no phylogenetic signal, whereas $\lambda = 1$ suggests that trait evolution is consistent with a strong effect of phylogeny. We based our phylogeny on the phylogenetic tree of larks (Alström et al. 2013). For the seven species with multiple sampled populations we added branches to the tree for each population to create polytomies, with the branch length for each population within a species set to zero. We found no evidence for phylogenetic signal in any of our data sets: λ never differed significantly from zero and was always significantly different from one. Setting all branch lengths to one did not change this finding. Therefore, we present all results and figures based on simple linear regression models [ordinary least squares (Freckleton 2009; Revell 2010)], with species and geographically distinct populations treated as independent points. Because sample size varied among species and populations (Table 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 1) we ran analyses using restricted data sets containing values per period, as well as with the entire data set of all values. The results of these analyses were qualitatively similar and so we only present results based on the entire data set. To disentangle the roles of pace of life and environmental antigen exposure in shaping immune investment, these factors should not be correlated. This was the case in our data set: pace-of-life indicators and aridity were not significantly correlated (clutch size vs. A_M, $P = 0.57$, $r = -0.12$; number of eggs laid per year vs. A_M, $P = 0.16$, $r = 0.31$). All statistical analyses were conducted using R 2.15.2 (R Core Team 2012).

Results

Relationships between immune indices and pace-of-life parameters were non-significant and generally weak (Table 3; Fig. 1). The notable exception to this was the relationship between number of eggs laid per year and agglutination titres (Table 3; Fig. 1f).

Lark populations consistently exhibited lower immune defences in more arid locations (Fig. 2). These negative correlations with aridity were significant for haptoglobin concentrations and lysis titres, and approached significance for agglutination titres (Table 3; Fig. 2). Haptoglobin concentrations and lysis titres were also positively and significantly correlated with mean annual precipitation (Table 3; Fig. 2). Ovotransferrin concentrations showed no relationship with aridity or mean annual precipitation (Fig. 2d, e). Lysis titres correlated significantly and positively with mean annual temperature: larks from warmer locations had higher lysis titres (Table 3; Fig. 2l).

Table 3 Results of linear models examining relationships between immune indices of 23 populations of 12 lark species in relation to pace-of-life proxies and to climatic proxies of environmental antigen exposure

Response variable	Explanatory variable	F	P
Haptoglobin (mg ml^{-1})	Mean clutch size	$F_{1,21} = 2.86$	0.11
	Total eggs (no. year^{-1})	0.21	0.66
	Aridity index (A_M)	4.90	*0.038*
	Mean annual precipitation (mm)	5.79	*0.025*
	Mean annual temperature (°C)	0.31	0.59
Ovotransferrin (mg ml^{-1})	Mean clutch size	$F_{1,17} = 4.00$	0.06
	Total eggs (no. year^{-1})	1.74	0.21
	Aridity index (A_M)	0.53	0.72
	Mean annual precipitation (mm)	0.00	0.95
	Mean annual temperature (°C)	2.79	0.11
Agglutination (titre)	Mean clutch size	$F_{1,21} = 1.00$	0.33
	Total eggs (no. year^{-1})	8.34	*0.009*
	Aridity index (A_M)	3.14	0.09
	Mean annual precipitation (mm)	2.31	0.14
	Mean annual temperature (°C)	1.11	0.31
Lysis (titre)	Mean clutch size	$F_{1,21} = 0.23$	0.64
	Total eggs (no. year^{-1})	1.59	0.22
	Aridity index (A_M)	6.58	*0.018*
	Mean annual precipitation (mm)	7.03	*0.015*
	Mean annual temperature (°C)	8.32	*0.009*

$P < 0.05$ are shown in *italic*
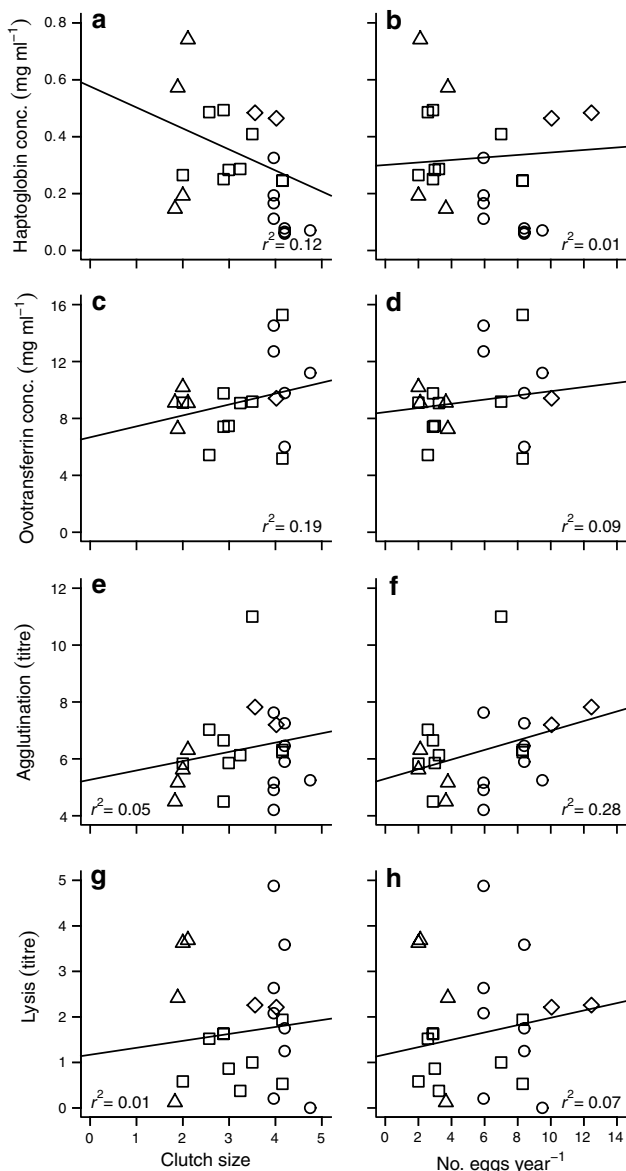
Fig. 1 Haptoglobin (**a**, **b**) and ovotransferrin (**c**, **d**) concentrations and agglutination (**e**, **f**) and lysis (**g**, **h**) titres as a function of mean clutch size and number of eggs per year in 23 populations of 12 lark species. *Squares* Hot desert larks, *circles* cold desert larks, *triangles* tropical larks, *diamonds* temperate larks

All other immune indices correlated negatively with mean annual temperature but these relationships were not significant (Table 3; Fig. 2).

Discussion

Contrary to the pace-of-life hypothesis, slow-living lark species did not invest more heavily in immune defences than their faster-living counterparts. Thus, our study adds to the list of publications that report no evidence for this

hypothesis (Cutrera et al. 2010; Horrocks et al. 2012a; Versteegh et al. 2012; Palacios et al. 2013), and contradicts those studies that provide at least some support for an association between pace of life and immune investment (Tieleman et al. 2005; Martin et al. 2006; Lee et al. 2008; Sparkman and Palacios 2009; Previtali et al. 2012). Taxonomic and methodological differences between studies, including measurement of different immune components and the use of different proxies for pace of life, may explain some of this variation. However, even within a single immune index and taxonomic group, interpretation of immune investment in relation to pace of life is not straightforward. The sole significant relationship in our study that might suggest a link between pace of life and immune investment was between agglutination titres and number of eggs laid per year. However, this correlation was positive, and contrary to the pace-of-life hypothesis this suggests that lark species with a faster pace of life have higher agglutination ability. This finding contradicts earlier work showing greater agglutination ability in tropical species with a slower pace of life (Lee et al. 2008). It also conflicts with Versteegh et al. (2012), who found no relationship between agglutination titres and pace of life when studying stonechat subspecies in a common garden set up. Natural antibody levels correlate positively with adaptive antibody responses (Parmentier et al. 2004) and these conflicting patterns might arise because natural antibodies, although usually considered an innate immune defence, straddle the boundary between innate and adaptive immunity (Caroll and Prodeus 1998; Ochsenbein and Zinkernagel 2000). Studies that measure purely adaptive immune defences, such as induction of specific antibody responses, are needed to test this possibility. More generally, the equivocal nature of the evidence linking immune investment and pace of life suggests that further exploration of this topic is required.

In agreement with the antigen exposure hypothesis (Horrocks et al. 2011a), haptoglobin concentrations and lysis titres decreased with increasing aridity and were highest in populations of larks from less arid locations where exposure to abundant immunological challenges is expected to be higher (Little and Earlé 1995; Moyer et al. 2002; Valera et al. 2003; Guernier et al. 2004; Jex et al. 2007; Tang 2009; Bachar et al. 2010; Guerra et al. 2010; Froeschke et al. 2010). Agglutination titres showed a similar, but non-significant trend and only ovotransferrin concentrations showed no relationship with aridity. This might relate to the iron binding and transport function of ovotransferrin, which, aside from its anti-microbial and immunomodulatory properties, make ovotransferrin important for vascularisation and nerve and muscle growth in the developing embryo (Giansanti et al. 2012).

In addition to links with aridity, we found significant positive associations between haptoglobin concentrations
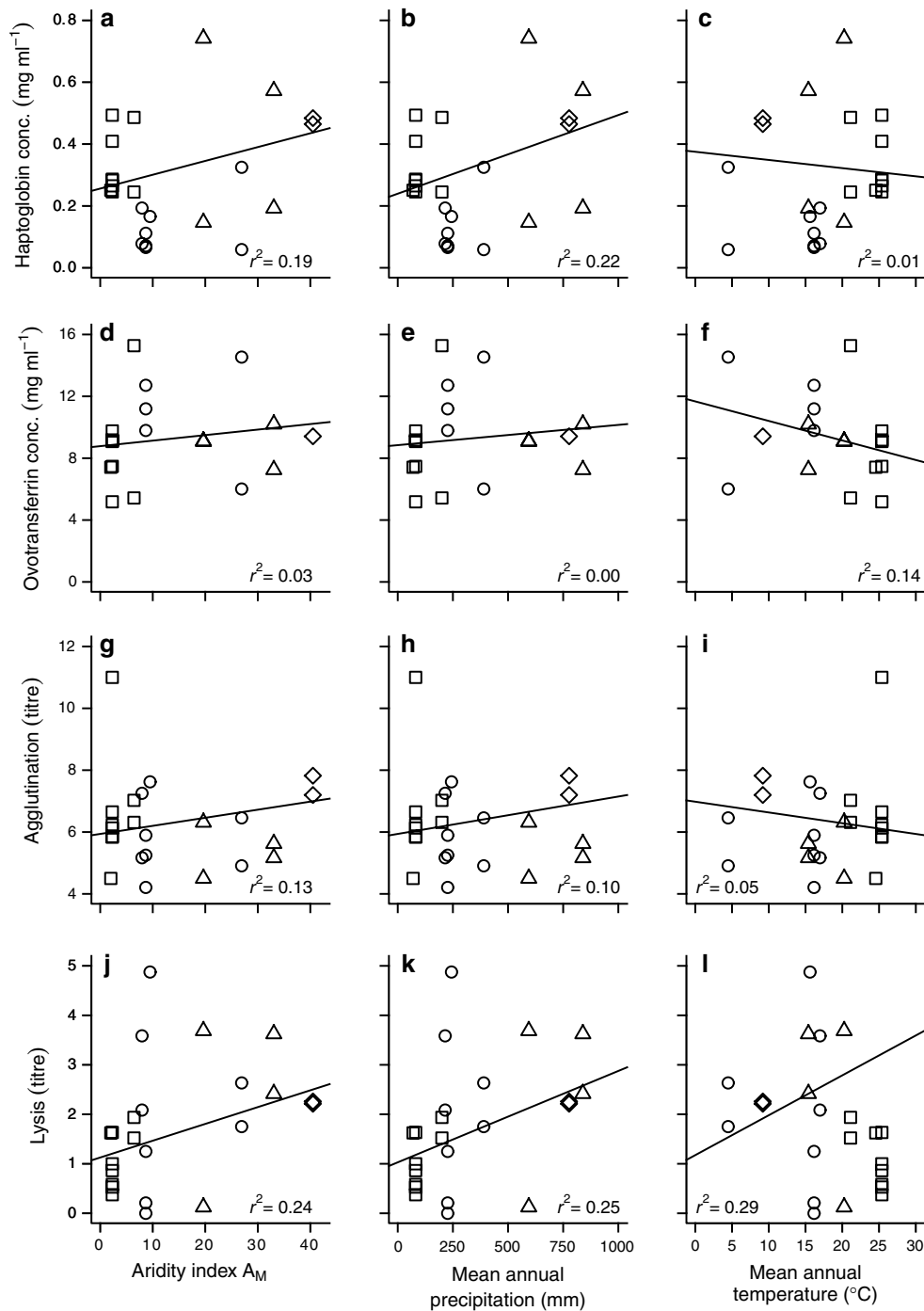
Fig. 2 Haptoglobin (**a**–**c**) and ovotransferrin (**d**–**f**) concentrations and agglutination (**g**–**i**) and lysis (**j**–**l**) titres as a function of de Martonne's aridity index (A_M), mean annual precipitation (mm) and mean annual temperature (°C) in 23 populations of 12 lark species measured along an environmental aridity gradient. A_M increases with decreasing aridity of the environment. *Squares* Hot desert larks, *circles* cold desert larks, *triangles* tropical larks, *diamonds* temperate larks

and lysis titres and precipitation, and between lysis titres and temperature. Previous authors have linked the risk of antigen exposure to climatic factors (Guernier et al. 2004; Gage et al. 2008; Guerra et al. 2010) and other abiotic

environmental variables [e.g. salinity (Figuerola 1999; Piersma 1997; Mendes et al. 2005)]. Our study extends this approach by showing that immune indices that are related to infection risk also correlate with abiotic environmental

variation. This demonstrates that abiotic measures can serve as useful proxies for antigenic exposure when direct biotic measurements are unavailable. Even so, it is important to recognise that other factors might influence immune investment, and may also covary with abiotic environmental variation. For example, physiological differences between birds along our aridity gradient, such as variation in metabolic rate (Tieleman et al. 2003), might influence immune responses, independent of any effect of antigen exposure. Temperature-constrained activity budgets (Tieleman and Williams 2002) and the reduced productivity associated with arid environments (Del Grosso et al. 2008; Serna-Chavez et al. 2013) could both restrict food availability in arid-living larks, leading to a resource trade-off that negatively impacts immune function. However, food availability might also be expected to influence clutch size and number of clutches per year (Tieleman et al. 2004; Lepage and Lloyd 2004), yet neither pace-of-life indicator correlated significantly with aridity. Earlier work showed that lark species in hyper-arid regions encountered lower microbial abundances than more mesic, temperate-living larks, and had correspondingly lower immune indices (Horrocks et al. 2012a). This supports our argument that aridity is a good proxy for risk of antigen exposure and consequently immune investment. Future work that takes a similar approach and focuses on direct measurement of relevant antigens will shed additional light on the associations we have identified between aridity, risk of antigen exposure, and immune defence. Combined with experimental studies, this work is also necessary to determine whether patterns of correlation between antigen exposure and immune responses reflect evolved responses to an antigen-rich environment or are simply a reflection of current exposure to antigens.

Comparative studies, both within and among species and environments, represent a powerful approach for disentangling the roles of pace of life and antigen exposure 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 variation in antigen exposure, we demonstrated that investment in innate immune defences might be related more to the likelihood of encountering an immunological challenge than to pace of life. Including relevant measures of environmental antigens and associated exposure risk in ecoimmunology studies will provide exciting opportunities for advancing our understanding of immunological variation.

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