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Density-dependent and geographical variation in bird immune response

A. P. Møller, M. Martín–Vivaldi, S. Merino and J. J. Soler

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Latitudinal gradients in parasitism are common, causing differences in the intensity of parasite-mediated natural selection. Such differences in selection pressures should affect optimal investment in anti-parasite defense, because defense levels should increase in response to increased intensity of parasite-induced selection. Likewise, latitudinal differences in population density may affect immune responses either by selecting for higher levels or defense, or by suppressing resources needed for mounting efficient immune responses. We tested these predictions in a study of T-cell mediated immune response in altricial bird species in subtropical Spain and temperate Denmark. There were highly consistent levels of T-cell mediated response between nestlings and adults in the two areas, with nestlings having stronger responses than adults. In addition, there were highly consistent levels of immune response in nestlings and adults between the two areas, with responses being consistently stronger in Denmark than in Spain, particularly in adults. Population density was much higher in Denmark than in Spain. We found evidence of density-dependent immune response in nestlings and adults, as shown by differences in T-cell response between study areas being positively related to differences in density. Given that the relationship between density and immune response was positive, we can reject the hypothesis that higher population densities suppressed immune response. Therefore, our results support the hypothesis that birds in areas with higher density allocate more resources to immune response, particularly in adults, suggesting that density-dependent effects of parasitism have selected for allocation strategies that minimize the risk of parasitism.

A. P. Møller (amoller@snv.jussieu.fr), Laboratoire de Parasitologie Evolutive, CNRS UMR 7103, Univ. Pierre et Marie Curie, 7 quai St Bernard, Case 237, FR-75252 Paris Cedex 05, France. – M. Martín-Vivaldi and J. J. Soler, Estación Experimental de Zonas Áridas, Consejo Superior de Investigaciones Científicas, Calle General Segura 1, ES-04001 Almería, Spain. – S. Merino, Museo Nacional de Ciencias Naturales, Consejo Superior de Investigaciones Científicas, José Gutiérrez Abascal 2, ES-28006 Madrid, Spain.

A key question in ecological and evolutionary studies concerns the factors that contribute to the uneven geographical distribution of parasite impact on hosts. An understanding of these patterns may not only have important health impacts for humans (Guégan et al. 2003, 2005, Guernier et al. 2004), but also affect the general understanding of geographical mosaic of coevolutionary interactions (Thompson 2005). Parasite abundance and diversity are consistently higher abundance at low latitudes (Rohde 1998, Guégan et al. 2003, 2005, Guernier et al. 2004, Yom-Tov and Geffen 2005, but see Scheuerlein and Ricklefs 2004). Furthermore, there is a higher impact of parasites on the fitness of their hosts at low latitudes (Janzen 1970, Connell 1971, Leigh 1994, Guégan et al. 2003, 2005, Guernier et al. 2004). In turn, this higher level of parasite-mediated natural selection at

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low latitudes has resulted in the evolution of high levels of host defense (Coley and Aide 1991, Hochberg and Van Baalen 1998, Møller 1998). However, such apparent latitudinal effects may be confounded by host density.

Parasites impose natural selection costs on their hosts by extracting resources from and reducing the fitness of hosts (Anderson and May 1991, Frank 1996). The level of virulence will depend on factors such as specificity of parasites, level of local adaptation and age-dependent resistance (Anderson and May 1991, Frank 1996). Parasite virulence and host resistance can coevolve to a coevolutionary stable strategy where no single party can benefit from changing its behavior (Van Baalen 1998). Levels of parasite offense and host defense may depend on availability of resources along productivity gradients with offense and defense levels increasing with greater amounts of resources (Hochberg and van Baalen 1998). Thus, level of parasite virulence will depend on level of host defense and vice versa, and local levels of resource availability will affect both. Consistent with this scenario, comparative studies of birds have shown that investment in immunity increases with parasite induced mortality (Martin et al. 2001, Møller et al. 2001, 2002). Thus, T- and B-cell mediated immune responses increased with increasing colony size in species of the bird family Hirundinidae, as did parasite-induced nestling mortality (Møller et al. 2001). Similarly, T-cell mediated immune responses of nestlings and adults increased with increasing parasite-induced mortality across bird species (Martin et al. 2001). Finally, relative size of the spleen (a lymphoid immune defense organ) increased with parasite-induced mortality across birds (Møller and Erritzøe 2002).

Current understanding of host–parasite interactions and their effects on populations of hosts derives from theoretical models that show that population regulation occurs when the growth rate of parasite populations exceeds that of hosts, causing the parasite to reduce the host population when the latter is large (Anderson and May 1978, May and Anderson 1978, Tompkins et al. 2001, Møller 2005). The Anderson and May (1978) model encapsulates the effects of aggregations of parasites on host population dynamics and the effects of parasites on survival and fecundity of hosts. Host–parasite interactions will tend to become stabilized by density-dependent effects on growth rate of the parasite population, for example, due to acquired immunity, and competition for space or resources within the host. Because density-dependent regulation is more likely to occur at early life stages of the host (i.e. when population sizes are the largest, Møller 2005), we can make the prediction that estimates of fitness impact of parasites on hosts during early life stages should be better predictors of host abundance than during later stages.

Population density of hosts can hypothetically have two different effects on host defense. First, immune defenses of hosts have a strongly condition-dependent component (Chandra and Newberne 1977, Glick et al. 1981, 1983, Willis and Baker 1981, Gershwin et al. 1985, Tsiagbe et al. 1987, Wan et al. 1989, Møller et al. 1998, Merino et al. 2000, Alonso-Alvarez and Tella 2001). Therefore, host population density can suppress levels of defense at high densities of hosts. Second, given that parasite transmission increases with host population density (Anderson and May 1978, May and Anderson 1978), hosts should allocate more resources to or evolve stronger anti-parasite defense at high population density (Wilson and Reeson 1998).

The objectives of this study were to (1) investigate geographical differences in anti-parasite defense, and (2) determine the relationship between population density and defense level. First, we compared the level of an important component of immunity in vertebrates, T-cell mediated immune response, in a number of the same free-living species of birds in a temperate study area in Denmark and a sub-tropical study area in Spain. Second, we assessed the level of T-cell response both in nestlings and adults because age-dependent selection pressures can be very important in host-parasite interactions due to immunologically naïve early life stages being particularly susceptible to parasitism. Theoretical models of parasitism and host life history have emphasized that the relative importance of parasite-induced juvenile and adult mortality is crucial in determining whether a larger or a smaller clutch size will be favored (Law 1979, Michod 1979, Hochberg et al. 1992). The factors contributing to early or late mortality during the life cycle of an organism will depend on factors such as exposure, susceptibility and natural selection against individuals with poor abilities of immune defense. Third, we used extensive information on population density of all species in the two study areas to test the relationship between difference in T-cell mediated immune response between the two areas and difference in population density. This intraspecific comparison of the hypothetical trade-off between resource competition and parasitism is particularly powerful because we are investigating the intraspecific difference in immune response in relation to intraspecific difference in population density and latitude.

Cell-mediated immunity constitutes an important arm of the immune system in vertebrates (Abbas et al. 1994). Extensive studies of T-cell responses in different species of birds have shown considerably greater responses in nestlings than in adults (Møller et al. 2001, 2003, Tella et al. 2002). Analyses of individual variation in T-cell response have shown that it is a viability indicator, because individuals with strong responses have considerably higher survival prospects than individuals with weak responses in both nestlings (Saino et al. 1997, Christe et al. 1998, Hõrak et al. 1999, Soler et al. 1999b, reviewed by Møller and Saino 2004) and adults

(González et al. 1999, Soler et al. 1999a, reviewed by Møller and Saino 2004). These relationships were not confounded by body condition or other factors that traditionally have been considered to be indicators of survival prospects (Moreno et al. 2005, Cichon and Dubiec 2005). Species of birds with a high rate of parasite-induced mortality have the strongest T-cell mediated immune responses (Martin et al. 2001). Intraspecific variation in T-cell response is a reliable predictor of blood parasite infection in birds (González et al. 1999). Likewise, bird species with specialist fleas and a higher species diversity of fleas have stronger T-cell responses (Møller et al. 2005). Furthermore, diversity of chewing lice of a sub-order that interacts with living tissue of bird hosts, but not of another sub-order that only feeds on dead tissue, is positively related to host T-cell response (Møller and Rózsa 2005). Finally, bird species with strong T-cell responses have high prevalence of blood parasites (Palacios and Martin 2006). In summary, these extensive studies of T-cell mediated immune response of a wide range of bird species under natural conditions suggest that this immune response provides reliable information about an important component of host defense, its efficiency and the level of host–parasite interaction.

Material and methods

Study sites

We studied breeding birds in Spain around Granada, Sierra Nevada, Badajoz and Segovia and in Northern Denmark during April-June 2000–2004. The study areas in both countries were open farmland habitats, scrub and forest with scattered small lakes and ponds.

Sampling nestlings and adults for immune tests

In Spain and Denmark we made extensive systematic searches for nests in suitable habitats throughout the breeding season. We relied on extensive help from amateurs with a good knowledge of birds in locating nests, and we requested help in locating nests of all commonly breeding species. Adult birds carrying food in their beak were particularly used as a means of locating nests with nestlings. We deliberately attempted not to touch nests or disturb the surrounding vegetation to avoid increasing the risk of nest predation. If nests contained eggs, when found, they were re-visited following a period of half the incubation period, and then again, if still not lost to predators, when nestlings were ca two thirds through the nestling period. The number of nest checks was in this way minimized to reduce any unnecessary predation due to investigators.

Adult birds were captured with mist nets during extensive capture events during the breeding season April-June. Typically, we captured birds by placing mist nets in habitats described above, mainly during morning (sunrise until noon) and evening (18:00 until sunset) capture sessions. Mist net captures of adult birds were subsequently used for T-cell immune challenge tests.

T-cell mediated immune response

We estimated the response to phytohemagglutinin to obtain an in vivo response of T-cells because phytohemagglutinin stimulates T-lymphocyte proliferation, followed by local recruitment of inflammatory cells and increased expression of major histocompatibility complex molecules at the site of injection (Goto et al. 1978, Abbas et al. 1994, Parmentier et al. 1998). T-lymphocyte immune responsiveness was assessed using injection with phytohemagglutinin, because this is a standard method to assess cell-mediated immunity in poultry (Stadecker et al. 1977, Goto et al. 1978, McCorkle et al. 1980, Cheng and Lamont 1988). The thickness of the left and right wing webs (patagium) of birds was measured at pre-marked sites with a pressure-sensitive caliper (Alpa SpA, Milano, cod SM112), to the nearest 0.01 mm. In order to avoid damage to the skin, we removed the spring from the pressure-sensitive caliper and replaced that by a weight of 16 g on top of the instrument. The right wing web was injected with 0.2 mg of phytohemagglutinin (Sigma, L-8754) in 0.04 ml of phosphate buffered saline. The left wing web was injected with 0.04 ml phosphate buffered saline only. Twenty-four hours later we re-measured the thickness of both wing webs at the pre-marked injection sites in all nestlings. APM instructed JJS and MMV about measurements with the pressure-sensitive caliper, ensuring that all measurers recorded measurements in a consistent and similar way. The precision of measurements with different calipers or different measurers with the same caliper was typically in the range 0.01 to 0.02 mm.

For adults we have shown in house sparrows *Passer domesticus* in captivity that the maximum response to challenge with phytohemagglutinin is already achieved after 6 h (Navarro et al. 2003). Thus, all adult birds were kept in a cloth bag in a dry place at ambient temperature for six hours before recording the response to phytohemagglutinin injection. This method has been cross-validated against data obtained from tests based on a 12 h and a 24 h internal between injection and measurement for a sample of different species, showing a high degree of consistency (Møller et al. 2003).

The estimate of immune response is the difference in wing web thickness between day 2 and day 1 in nestlings (and between six h post-injection and pre-injection for adults) for the phytohemagglutinin inoculated wing minus the difference in wing web thickness between day 2 and day 1 for the phosphate buffered saline inoculated wing (see Saino et al. 1997 for details of the methods used here). Measurements of T-cell response during repeat measurements of the same individuals were highly repeatable with repeatability values exceeding 0.68 (nestlings in Denmark 0.75 ($F = 24.45$, $DF = 52, 415$, $p < 0.0001$), adults in Denmark 0.68 ($F = 8.27$, $DF = 47, 167$, $p < 0.0001$), nestlings in Spain 0.74 ($F = 22.06$, $DF = 27, 204$, $p < 0.0001$), adults in Spain 0.70 ($F = 4.88$, $DF = 29, 62$, $p < 0.0001$)). Thus, our measurements are reliable.

All nestlings from the recorded broods were injected at a standard relative age during ontogeny (when they were two thirds through their normal nestling period) rather than at a similar absolute age. This procedure ensured that nestlings were tested at a similar developmental stage, which excludes the possibility that the recorded responses are dependent on developmental age. Preliminary studies of age-dependent cell-mediated immunity in barn swallow *Hirundo rustica* nestlings have shown little variation in intensity of response during the period 10–16 days (in a species with a 20 days nestling period, Møller unpubl.). A thorough characterization of immunocompetence (the ability to raise an efficient response to a parasite attack) requires that both T- and B-cell-mediated and innate immunity are quantified (National Research Council 1992). We note that at the interspecific level T-cell response is positively correlated with antibody production to a challenge with sheep red blood cells (Møller et al. 2001). Thus, two components of immunity are positively correlated across species of birds.

We only had information on T-cell response of five species in more than a single year. The year effect for these species did not reach statistical significance. A previous study of among-year variation in nestling T-cell response in barn swallows has shown significant variation among years (Møller 2002). However, we note that we did find highly consistent species-specific variation in T-cell response, despite potential differences among years. If among-year variation had been important, this would have increased the variance among individuals within species and hence reduced the difference among species. Any statistical tests of interspecific patterns would hence be conservative.

Estimating population density

We used population size of all species as reported by Martí and del Moral (2003) and Grell (1998). These estimates are consensus estimates derived from estimates of population density of suitable habitats and the range of such suitable habitats (Grell 1998, Martí and del Moral 2003). Because estimates were obtained during a period of several years, this should have prevented estimates to be influenced by short-term fluctuations in abundance. Likewise, we obtained an estimate of the breeding distribution of all species from the number of squares occupied, as reported by Martí and del Moral (2003) and Grell (1998). We estimated population density in Denmark and Spain by dividing population size with the area of occupied squares.

The estimates are reported in Appendix 1.

Statistical and comparative analyses

Species with an initially thick patagium may also be able to transport more immune cells and immune defense products to the site of injection. The linear regression between cell-mediated immune response and body mass also controlled for any additional effects of initial skin thickness on cell-mediated immune response, because a multiple regression with T-cell mediated immunity as the dependent variable and body mass and skin thickness before injection as independent variables did not show a significant partial regression coefficient for skin thickness (partial $F < 1.12$, $p > 0.29$ in tests of all species).

We used mean brood responses in the analyses to avoid dependence of data based on individual nestlings. Mean values of brood means are reported in Appendix 1.

Species or populations of birds cannot be considered statistically independent observations because some share a common ancestor more recently than others. We controlled for this effect of common ancestry in our analyses by calculating standardized linear contrasts between observations, using a phylogenetic hypothesis of the relationship among populations or species (Felsenstein 1985, Harvey and Pagel 1991). We used the computer program CAIC for these analyses (Purvis and Rambaut 1995). The resulting standardized linear contrasts represent a standardized estimate of the difference in phenotype between taxa, while analyses of species- or population-specific data rely on statistically dependent values with an inflated number of degrees of freedom.

The phylogenies that we used were based on that reported by Sibley and Ahlquist (1990) combined with more detailed information about phylogenetic relationships of species in Badyaev (1997), Barker et al. (2001), Blondel et al. (1996), Leisler et al. (1997), Martin and Clobert (1996) and Sheldon and Winkler (1993). The phylogeny is shown in Fig. 1.

T-cell response, body mass and population density were $\log_{10}$-transformed before analyses to allow use of parametric statistics. All regressions of contrasts were forced through the origin because the dependent variable is not supposed to have shown an evolutionary change, if the independent variable has not changed (Felsenstein 1985). Standardization of contrasts was checked by

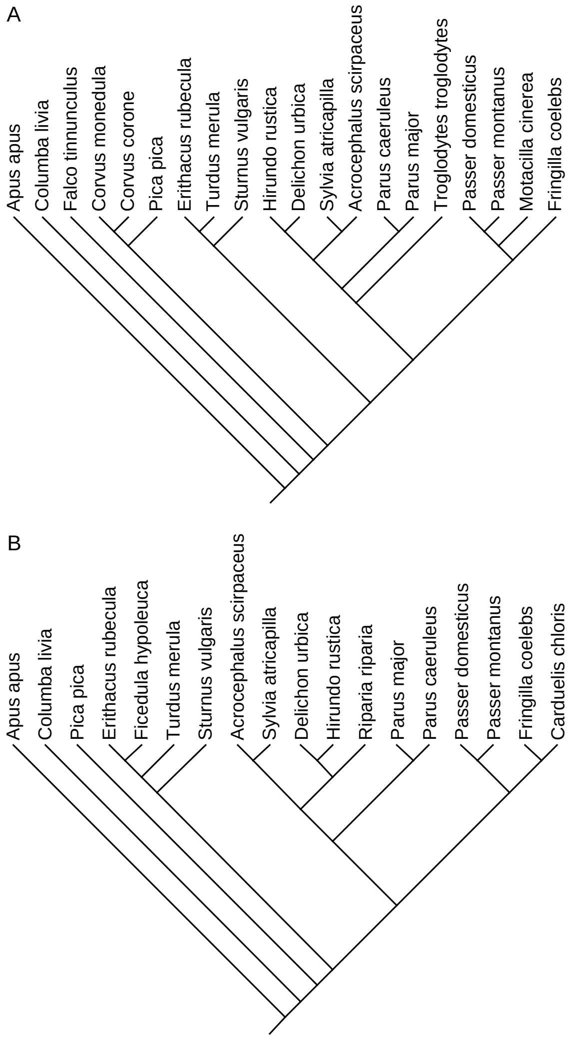

Fig. 1. Phylogeny of the species included in the study of (A) nestling and (B) adult T-cell mediated immunity. See Material and methods for sources.

examination of absolute values of standardized contrasts versus their standard deviations (Garland 1992, Garland et al. 1992).

Values reported are means (SE).

Results

Consistency in T-cell mediated immunity

We quantitatively assessed the consistency in T-cell mediated immune response for each age class and area in order to determine the relative degree of variability within and among species. The repeatability of T-cell response for nestlings and adults in both Denmark and Spain was significant with values ranging from 0.68 to 0.75, showing that most variation occurred among species (Table 1).

Consistency in T-cell response and age

Nestlings had significantly stronger mean T-cell responses than adults in Denmark (nestlings: 1.27 (0.13), $n = 42$, adults: 0.32 (0.05), $n = 42$, paired t-test, $t = 11.73$, $DF = 41$, $p < 0.0001$) and in Spain (nestlings: 0.95 (0.11), $n = 26$, adults: 0.24 (0.05), $n = 26$, paired t-test, $t = 10.62$, $DF = 25$, $p < 0.0001$). The effect size was approximately of the same magnitude in the two countries.

Mean nestling and adult responses were positively related in both countries (Denmark: $F = 8.98$, $DF = 1{,}39$, $r^2 = 0.19$, $p = 0.0047$, slope (SE) $= 0.48$ (0.16), Spain: $F = 3.40$, $DF = 1{,}28$, $r^2 = 0.11$, $p = 0.076$, slope (SE) $= 0.24$ (0.13)). That was not longer the case after adjusting immune responses for differences in body mass (Denmark: $F = 0.54$, $DF = 1{,}38$, $r^2 = 0.01$, $p = 0.47$, slope (SE) $= 0.16$ (0.22), Spain: $F = 0.09$, $DF = 1{,}27$, $r^2 = 0.00$, $p = 0.76$, slope (SE) $= 0.05$ (0.17)). The latter conclusion was confirmed in an analysis of contrasts (Denmark: $F = 0.02$, $DF = 1{,}37$, $r^2 = 0.00$, $p = 0.90$, slope (SE) $= -0.02$ (0.16), Spain: $F = 0.26$, $DF = 1{,}27$, $r^2 = 0.01$, $p = 0.62$, slope (SE) $= 0.08$ (0.16)).

Consistency in T-cell response between areas

T-cell mediated immune response of both nestlings and adults were significantly stronger in Denmark than in Spain (Table 2). Nestlings in Denmark had a 40% stronger response than in the same species in Spain, while adults had a 77% stronger response in Denmark than in the same species in Spain.

There was consistency in mean T-cell responses of species between areas both in nestlings and adults as shown by one-way analyses of variance with two mean values of response for each species, one for Denmark and the other for Spain (nestlings: $F = 3.14$, $DF = 19{,}20$, $p = 0.007$, adults: $F = 4.18$, $DF = 16, 17$, $p = 0.003$). This effect was independent of body mass (nestlings: $F = 3.37$, $DF = 19{,}20$, $p = 0.005$, adults: $F = 5.82$, $DF = 16, 17$, $p = 0.0004$).

Nestling T-cell responses were positively correlated between Spain and Denmark (Fig. 2A, $F = 24.32$, $DF = 1{,}18$, $r^2 = 0.57$, $p = 0.0001$, slope (SE) $= 0.63$ (0.13)). That was also the case for adult responses in the two areas (Fig. 2B, $F = 52.91$, $DF = 1{,}18$, $r^2 = 0.75$, $p < 0.0001$, slope (SE) $= 0.97$ (0.13)). The slope for adults was significantly steeper than that for nestlings (Fig. 2, test for difference in slopes: $t = 2.62$, $DF = 35$, $p < 0.05$). These relationships remained unchanged after adjusting for body mass (nestlings: $F = 14.55$, $DF = 1{,}17$, $r^2 = 0.36$, $p = 0.0014$, slope (SE) $= 0.69$ (0.18), adults: $F = 18.70$, $DF = 1{,}17$, $r^2 = 0.27$, $p = 0.0005$, slope (SE) $= 1.09$ (0.25)). Analyses of contrasts revealed similar conclusions (nestlings: $F = 7.36$, $DF = 1{,}17$, $r^2 = 0.20$, $p = 0.015$, slope (SE) $= 0.50$ (0.18), adults: $F = 16.95$, $DF = 1{,}17$, $r^2 = 0.41$, $p = 0.0007$, slope (SE) $= 0.98$ (0.24)).

Table 1. Repeatability (R) of T-cell response of nestlings and adults of bird species in Denmark and Spain.

	Denmark		Spain	
	Nestlings	Adults	Nestlings	Adults
F	24.45	8.27	22.06	4.88
DF	52,415	47,167	27,204	29,62
p	<0.0001	<0.0001	<0.0001	<0.0001
MS (within)	0.857	0.258	4.497	0.165
MS (among)	0.035	0.031	0.204	0.034
R (SE)	0.75 (0.06)	0.68 (0.09)	0.74 (0.08)	0.70 (0.12)

Density-dependent T-cell mediated immune response

Population density differed significantly between Denmark and Spain (paired t-test, t =4.22, DF =50, p < 0.0001). Mean density (SE) in Spain was only 0.61 pairs km^{-2} (0.10), n =51 species for which we had information on T-cell response in either Denmark or Spain, but in Denmark it was 5.34 pairs km^{-2} (1.19) for the same 51 species. For the 20 species for which there was information on T-cell response of nestlings in both Denmark and Spain, mean density was significantly higher in Denmark (paired t-test, t =6.07, DF =19, p < 0.0001); mean density (SE) in Spain: 1.11 pairs km^{-2} (0.22), Denmark: 9.03 (2.50), n =20 species). Likewise, for the 20 species for which there was information on T-cell response of adults in both Denmark and Spain, mean density was significantly higher in Denmark (paired t-test, t =5.89, DF =19, p <0.0001); mean density (SE) in Spain: 1.40 pairs km^{-2} (0.29), Denmark: 9.33 (2.47), n =20 species).

Density-dependent effects on T-cell response were investigated by relating the difference in T-cell response between countries to the difference in population density between countries. While the difference in nestling T-cell response was not significantly related to the difference in population density (Fig. 3A, F =3.55, DF =1, 18, r^2 = 0.16, p =0.08, slope (SE) =0.15 (0.08)), the relationship for adults was statistically significant (Fig. 3B, F =11.93, DF =1, 18, r^2 =0.40, p =0.003, slope (SE) =0.21 (0.06)). These effects were not confounded by effects of body mass (regression with difference in nestling T-cell response as the dependent variable and difference in population density and body mass as independent variables: F =4.28, DF =2, 17, r^2 =0.33, p =0.03, partial regression for difference in density: F =3.12, DF =1,17, p =0.10, slope (SE) =0.13 (0.07), adults: F = 3.84, DF =2, 13, r^2 =0.37, p =0.049, partial regression for difference in density: F =11.13, d.f. =1,17, P =0.004, slope (SE) =0.21 (0.06)). Furthermore, a model that controlled for covariation between T-cell response of nestlings and adults only revealed a significant effect for adults (partial regression for nestlings: F =0.01,

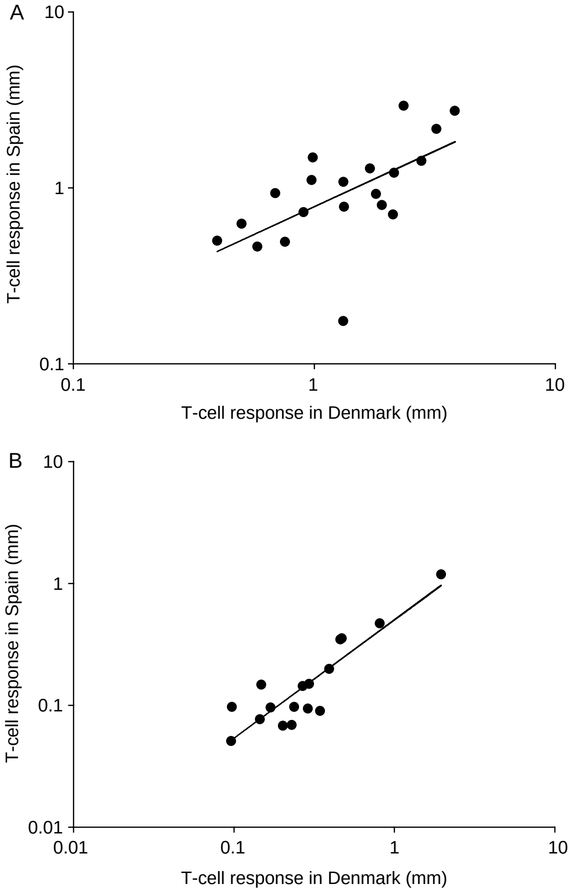

Fig. 2. Relationship between (A) mean nestling T-cell mediated immune response (mm) of different bird species in Denmark and Spain, and (B) mean adult T-cell mediated immune response (mm) of different bird species in Denmark and Spain. The lines are the linear regression lines.

Table 2. Mean (SE) T-cell response (mm) of nestling and adult bird species in Denmark and Spain and paired t-test for difference in response between countries.

	Nestlings	Adults
Denmark	1.58 (0.21)	0.39 (0.11)
Spain	1.13 (0.16)	0.22 (0.07)
No. species	20	17
t	2.72	6.87
p	0.014	<0.0001

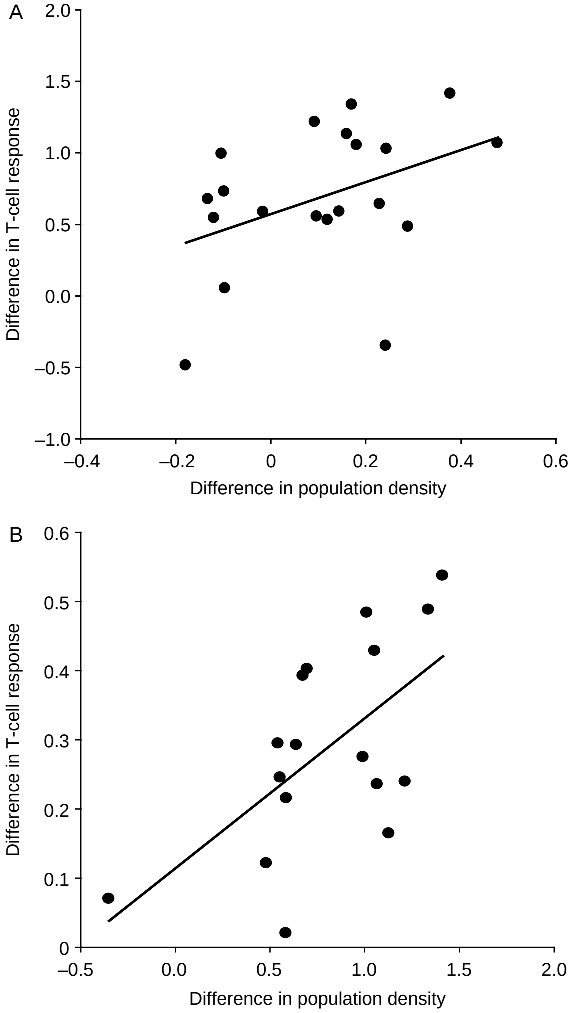

Fig. 3. Difference in mean T-cell mediated immune response between Denmark and Spain of (A) nestlings and (B) adults in relation to difference in mean population density between Denmark and Spain. The lines are the linear regression lines.

DF = 1,11, p = 0.91, slope (SE) = -0.01 (0.13), partial regression for adults: F = 8.39, DF = 1,11, p = 0.015, slope (SE) = 0.22 (0.08)). However, analyses of standardized linear contrasts revealed similar relationships for nestlings (F = 4.95, DF = 1, 18, $r^2 = 0.21$, p = 0.04, slope (SE) = 0.16 (0.07)) and adults as for the analyses based on species-specific data (F = 5.34, DF = 1, 15, $r^2 = 0.28$, p = 0.04, slope (SE) = 0.17 (0.08)).

Discussion

We investigated intraspecific and interspecific variation in an immune response measured in the field in both nestlings and adults of the same bird species in a temperate and a subtropical area. Repeatability of T-cell mediated immune responses among individuals was very high implying that estimates based on a few individuals are highly reliable. T-cell mediated immune responses were consistent between areas and age classes although nestlings generally showed much stronger responses than adults, and mean responses generally were much stronger in the temperate area than in the subtropical one. Population density was almost an order of magnitude larger in the temperate than the subtropical area, and differences in T-cell response between latitudes were positively related to differences in population density. We will briefly discuss each of these findings.

Parasites often have age-specific effects on their hosts because early life stages are immunologically naïve due to yet developing immune function, and due to a lacking ability to raise strong secondary responses arising from lack of encounters with different parasite antigens (Abbas et al. 1994, Wakelin 1996). However, nestlings also differ from adults by being forced to remain in the nest and hence fight parasites with their immune system rather than by physical escape. Nestlings had much stronger T-cell mediated immune responses than adults both in Denmark and Spain, and the effect of age was similar in the two countries. Furthermore, nestling and adult responses were strongly positively related in both Denmark and Spain, with similar effects in the two areas. These age-related differences are in accordance with previous results (Møller et al. 2001, Tella et al. 2002) and expectations given that nestlings are forced to remain in the nest until fledging, while adults at least have the option of leaving their nests and ultimately completely abandon their current reproductive event in case of severe parasite infestation (Emlen 1952). Nestling responses were quantified after 24 h, while adult responses were recorded after 6 hours, potentially resulting in a stronger response in nestlings. However, previous analyses of adult responses for the same species after 6 and 24 h revealed very similar results (Møller et al. 2003), suggesting that the difference in time interval for assessing immune response of nestlings and adults did not cause the difference in strength of immune response.

Parasite abundance and diversity generally increase towards the equator (Rohde 1998, Guégan et al. 2003, 2005, Guernier et al. 2004), and parasites also seem to have stronger fitness costs for their hosts at low latitudes (Janzen 1970, Guégan et al. 2003, 2005, Guernier et al. 2004). Latitudinal differences in productivity may provide a way of predicting latitudinal gradients in levels of anti-parasite defense. While we do not have any direct measure of productivity, clutch sizes are consistently larger in northern than southern Europe (Lack 1954, 1968). This implies that peak food abundance is likely to be greater with increasing latitude (Lack 1954), although productivity outside the short growing season at high latitudes may be much smaller than at low latitudes. This would suggest that the level of anti-parasite defense should increase with latitude (Hochberg and Van Baalen 1998). Therefore, we would predict stronger immune responses in our temperate study area than in our subtropical area. Consistently, we found that T-cell responses of the same bird species in Denmark were much stronger than in Spain, with the mean difference being 40% in nestlings and 77% in adults. Nestling and adult T-cell responses were positively related between Denmark and Spain, but the slope was much steeper for adults than for nestlings. This implies that responses in adults were relatively stronger in Spain for species that had strong responses in Denmark. In contrast, responses in nestlings were relatively weaker in Spain for species that had strong responses in Denmark. Resource availability is not the only factor responsible for gradients in level of host defense, because levels of defense will coevolve with levels of offense along productivity gradients (Hochberg and Van Baalen 1998). Hence, it is the greater fitness impact of parasites on hosts in productive environments that selects for greater investment in defense. If parasites did not play a key role in the evolution of strong host defenses, hosts would only pay the costs of strong defenses (such as auto-immune disease (Råberg et al. 1998, Lochmiller and Deerenberg 2000) or other negative impacts of defense such as direct pathological effects of immunity (Roitt et al. 1996)).

Density-dependence is an important aspect of host-parasite interactions, relating to the ability of parasites to control host populations (Anderson and May 1978, May and Anderson 1978, Tompkins et al. 2001, Møller 2005). Previous laboratory studies have found evidence of density-dependent prophylaxis in invertebrates, suggesting that hosts may allocate more resources to immune defense in the presence of more conspecifics (Reeson et al. 1998, Wilson and Reeson 1998, Barnes and Siva-Jothy 2000, Wilson et al. 2002, 2003, Cotter et al. 2004, Pie et al. 2005). Alternatively, species with high population densities may have shown a micro-evolutionary response to selection for strong immune responses. Here we provide a preliminary test of the association between immunity and density relying on our field data collected in two different areas. We hypothesized that density-dependence could affect host immune responses in two inherently different ways. Either high population density of hosts may suppress resource levels for hosts, giving rise to a negative relationship between density and immune response, or high density may select for greater investment in immunity due to density-dependent parasite transmission, producing a positive relationship between density and immune response. Population density was on average more than eight times higher in Denmark than in Spain, although there were considerable differences among species. The higher density in Denmark could be interpreted as implying a greater productivity compared to the situation in Spain. If that were the case, we should expect the level of parasite offense and host defense to be higher in Denmark than in Spain (Hochberg and Van Baalen 1998). Indeed, the difference in T-cell response between areas was positively correlated with the difference in population density in both nestlings and adults. This finding is consistent with the hypothesis that there has been greater allocation to immune function or selection for stronger immune responses in species and areas with higher population density, while the alternative hypothesis assuming depression of limiting resources at high densities clearly could be rejected. We can also conclude that parasites must have played an active role in this relationship between immunity and population density of hosts, because otherwise hosts would only pay the costs of immunity without achieving any benefits.

In conclusion, we have shown that a measure of cell mediated immune response varies consistently between Denmark and Spain, and with age and population density, as expected if the level of anti-parasite defense by hosts is determined by levels of productivity and density-dependent parasite-mediated natural selection.

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Appendix 1.

Nestling and adult T-cell response (mm) and population density (pairs km^{-2}) of birds in Denmark and Spain.

Species	Nestling T-cell response (mm)	SE	n (broods)	Adult T-cell response (mm)	SE	n	Population density (pairs km^{-2})
Denmark							
Accipiter gentilis	1.96	0.15	3				0.03
Accipiter nisus	1.69	0.17	2				0.09
Acrocephalus schoenobaenus				0.10		1	0.28
Acrocephalus palustris	0.44		1	0.20	0.02	6	0.91
Acrocephalus scirpaceus	0.40	0.01	2	0.10	0.01	9	1.49
Alauda arvensis	0.40	0.04	3				25.60
Anthus pratensis	0.61	0.07	3				1.17
Apus apus	1.80	0.01	3	0.46	0.16	3	3.98
Buteo buteo	2.03	0.37	3				0.11
Carduelis cannabina				0.15		1	5.54
Carduelis chloris	0.83	0.08	4	0.21	0.02	12	9.79
Carduelis flammea				0.06	0.01	7	0.60
Coccothraustes coccothraustes				0.25		1	0.68
Columba livia	3.83	0.30	4	1.95	0.45	4	5.58
Columba palumbus	2.26	0.32	3	0.31		1	5.56
Corvus corone	2.14	0.06	4				3.12
Corvus frugilegus	2.69	0.15	3				2.14
Corvus monedula	1.70	0.16	4				0.63
Cuculus canorus	1.13		1				0.43
Delichon urbica	2.78	0.14	5	0.47	0.06	3	0.37
Dendrocopos major				0.16		1	2.36
Emberiza citrinella	0.65	0.02	2	0.12	0.01	7	11.00
Emberiza schoeniclus	0.49	0.05	3	0.15	0.01	3	1.17
Erithacus rubecula	0.76	0.08	5	0.39	0.01	4	5.87
Falco tinnunculus	2.35	0.15	4				0.04
Ficedula hypoleuca				0.20		1	0.76
Fringilla coelebs	0.90	0.10	2	0.25	0.06	8	32.60
Garrulus glandarius				0.31		1	1.38
Hippolais icterina	0.27		1	0.21	0.01	5	1.50
Hirundo rustica	1.36	0.03	402	0.22	0.02	24	3.85
Lanius collurio	0.37	0.04	3	0.33	0.00	2	0.12
Luscinia luscinia				0.34		1	2.23
Milvus milvus	2.67	0.25	2				0.01
Motacilla alba	1.05	0.10	6	0.09	0.07	3	2.12
Motacilla cinerea	0.98	0.07	2				0.05
Motacilla flava	0.41	0.07	2	0.35		1	0.69
Muscicapa striata	0.41	0.02	3	0.10		1	0.62
Parus ater	1.10	0.13	8	0.15	0.00	2	4.59
Parus caeruleus	0.58	0.06	5	0.17	0.01	4	4.93
Parus cristatus	2.19		1	0.27	0.00	3	1.08
Parus major	0.75	0.08	5	0.26	0.02	3	14.30
Parus palustris	1.73	0.13	2	0.10	0.01	2	0.91
Passer domesticus	1.33	0.12	10	0.23	0.04	13	19.00
Passer montanus	1.83	0.34	5	0.29	0.04	6	9.84
Phoenicurus phoenicurus	1.13	0.06	2	0.13	0.01	3	1.10
Phylloscopus collybita				0.06	0.02	2	5.08
Phylloscopus trochilus	1.14	0.17	2	0.14	0.00	8	11.80
Pica pica	2.12	0.15	3	0.81	0.03	2	5.27
Prunella modularis	1.79		1	0.21	0.03	9	2.12
Pyrrhula pyrrhula				0.14	0.01	3	1.42
Regulus regulus	0.64		1	0.21	0.01	2	4.15
Riparia riparia	2.49	0.15	3	0.20	0.02	10	0.86
Saxicola rubetra	0.26	0.02	2	0.30		1	0.51
Sitta europaea	1.38		1	0.12		1	1.35
Streotopelia decaocto	2.46	0.12	3	1.07		1	1.17
Sturnus vulgaris	1.32	0.12	9	0.53	0.08	3	12.80
Sylvia atricapilla	0.69	0.09	2	0.19	0.02	7	5.92
Sylvia borin	0.17		1	0.26	0.04	3	4.55
Sylvia communis	0.51	0.04	2	0.15	0.01	6	6.93
Sylvia curruca				0.27	0.06	4	3.42
Troglodytes troglodytes	0.50	0.06	2	0.13		1	7.78
Turdus merula	1.91	0.04	4	0.31	0.03	16	43.10
Turdus philomelos	1.34		1	0.92		1	5.30
Turdus pilaris	1.61	0.16	3	0.31	0.01	3	0.21
Tyto alba	1.05	0.25	2				0.04

Appendix (*continued*)

Species	Nestling T-cell response (mm)	SE	n (broods)	Adult T-cell response (mm)	SE	n	Population density (pairs km^{-2})
Spain							
Acrocephalus scirpaceus	0.50	0.08	3	0.05	0.03	4	0.15
Apus apus	0.92	0.05	3	0.35	0.01	2	1.31
Athene noctua	1.72		1				0.15
Carduelis cannabina				0.15		1	1.27
Carduelis chloris				0.07	0.01	3	0.95
Columba livia	2.74	0.19	2	1.19	0.45	3	1.44
Coracias garrulus	1.45	0.07	4				0.10
Corvus corone	1.22	0.12	4				0.29
Corvus monedula	1.29	0.12	3				0.19
Cyanopica cyanus	0.93	0.05	10	0.27	0.05	29	2.12
Delichon urbica	1.59	0.06	11	0.40	0.07	28	0.83
Emberiza cirlus	0.38	0.03	2	0.16		1	0.35
Erithacus rubecula	1.00		1	0.20	0.03	7	1.67
Falco tinnunculus	2.93	0.14	4				0.04
Ficedula hypoleuca	0.52	0.04	25	0.20	0.04	8	5.24
Fringilla coelebs	0.73		1	0.14	0.03	5	1.99
Galerida theklae	0.79		1	0.32	0.08	2	0.74
Hippolais polyglotta				0.09	0.03	4	0.41
Hirundo daurica	0.94	0.05	23	0.11	0.03	3	0.11
Hirundo rustica	1.41	0.31	23	1.21	0.01	78	1.00
Lanius excubitor	1.30		1	0.18	0.10	2	0.08
Luscinia megarhynchos				0.15	0.03	3	0.53
Motacilla cinerea	1.49		1	0.17	0.06	2	0.15
Muscicapa striata				0.10	0.01	2	0.15
Oenanthe leucura	0.81	0.05	4	0.30	0.01	25	0.20
Oenanthe oenanthe				0.38		1	0.07
Otus scops	1.33	0.08	4				0.11
Parus caeruleus	0.46	0.13	16	0.10	0.02	5	1.37
Parus major	0.49	0.12	11	0.10	0.02	6	1.26
Passer domesticus	0.78	0.02	85	0.15	0.04	7	4.34
Passer montanus	2.16		1	0.09	0.01	4	0.45
Petronia petronia	1.74	0.09	3	0.24		1	0.56
Phoenicurus ochrurus	0.93	0.15	3	0.16	0.04	3	0.29
Phylloscopus bonelli	0.34	0.04	2	0.13	0.09	3	0.55
Pica pica	0.71	0.04	10	0.47	0.23	3	0.45
Regulus ignicapillus				0.09		1	0.63
Riparia riparia				0.08	0.03	6	0.17
Saxicola torquata	0.64	0.06	4				0.59
Serinus serinus	0.55	0.05	21	0.12	0.03	6	1.93
Sturnus unicolor	0.91	0.06	4	0.36	0.05	3	0.95
Sylvia atricapilla	0.93	0.05	2	0.08	0.02	3	1.25
Sylvia cantillans	0.60	0.12	3				0.38
Sylvia melanocephala	0.74	0.06	3	0.15	0.02	3	1.01
Sylvia undata	0.35		1	0.22	0.04	2	0.55
Troglodytes troglodytes	0.62		1				1.45
Turdus merula	0.80	0.03	5	0.09	0.04	4	1.67
Upupa epops	0.55	0.03	12	0.63	0.03	13	0.19