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Contemporary variations of immune responsiveness during range expansion of two invasive rodents in Senegal

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Biological invasions provide unique opportunities for studying life history trait changes over contemporary time scales. As spatial spread may be related to changes in parasite communities, several hypotheses (such as the evolution of increased competitive ability (EICA) or EICA-refined hypotheses) suggest immune changes in invasive species along invasion gradients. Although native hosts may be subject to similar changes in parasite selection pressures, their immune responses have been rarely investigated in invasion contexts. In this study, we evaluated immune variations for invasive house mice *Mus musculus domesticus*, invasive black rats *Rattus rattus* and native rodents *Mastomys erythroleucus* and *Mastomys natalensis* along well-characterised invasion gradients in Senegal. We focused on antibody-mediated (natural antibodies and complement) and inflammatory (haptoglobin) responses. One invasion route was considered for each invasive species, and environmental conditions were recorded. Natural-antibody mediated responses increased between sites of long-established invasion and recently invaded sites only in house mice. Both invasive species exhibited higher inflammatory responses at the invasion front than in sites of long-established invasion. The immune responses of native species did not change with the presence of invasive species. These patterns of immune variations do not support the EICA and EICA refined hypotheses, and they rather suggest a higher risk of exposure to parasites on the invasion front. Altogether, these results provide a first basis to further assess the role of immune changes in invasion success.

Biological invasions, i.e. the successful establishment and spread of species outside their native range, are increasingly frequent worldwide mostly due to human activities. They often have detrimental consequences for the communities invaded (Kolar and Lodge 2001). Despite the increasing burgeoning interest in invasion science these last decades, several gaps exist in our knowledge and understanding of the factors and forces driving invasion success of non-native species in new areas (Facon et al. 2006, Lowry et al. 2013).

Parasitism is one factor likely to promote invasion success by influencing a range of host community interactions (Dunn and Hatcher 2015). As such, invasive species may lose their natural enemies, including micro- and macroparasites, in their non-native ranges, with positive outcomes on invader fitness and demography (enemy release hypothesis, Colautti et al. 2004). Alternatively, invaders may introduce exotic parasites with detrimental effects on the survival, fecundity and/or regulation of indigenous host populations and provide opportunities for diseases to emerge (spill-over or novel weapon hypothesis, Strauss et al. 2012). They may also acquire local parasites from their new environment, amplifying the impact of some of them at the expense of native species, with effects at both the individual and population scales (spillback hypothesis, Kelly et al. 2009). As immunity is costly in terms of energy and immunopathology (Råberg et al. 2002, Klasing 2004), these changes in parasite pressure have led to some predictions regarding potential variations of invaders' immune defences in the course of invasion. With regards to the enemy release hypothesis, the invaders are expected to reallocate energetic resources from unnecessary defence mechanisms to life history traits favouring invasion success, such as dispersal or enhanced reproductive

output. This prediction, known as the evolution of increased competitive ability (EICA) hypothesis (Blossey and Notzold 1995), has been refined to take into account the widespread occurrence of parasitism (Lee and Klasing 2004). In addition to parasites that would not be lost during invasion, invaders could be infected by local parasites (including the possibility of spill-back of parasites carried by native hosts). A drastic reduction of immune responses would make invaders highly susceptible to generalist pathogens encountered in recently invaded host communities (Roberts and Janovy 2010). Then, successful invaders should be those that dampen the most expensive and/or least effective immune defences instead favouring less costly and more efficient immune strategies with respect to parasites that are kept from the source area or those newly encountered on the invasion front (EICA-refined hypothesis, Lee and Klasing 2004). Such tradeoff would allow promoting other life history traits without dampening invader defences.

The mechanisms potentially mediating these phenotypic variations include various processes: evolutionary ones as suggested in the name of this hypothesis, but also ecological changes. A major part of immune variation relies on differences in genetic background (reviewed by Ardia et al. 2011). Genetic drift and natural selection may therefore be key evolutionary processes by which invaders will evolve on invasion front after few generations (reviewed by Charbonnel and Cosson 2011). Recently, it was also assessed that epigenetic modifications – defined as changes in phenotypes that persist through mitosis and even meiosis, but occur independently of changes in underlying DNA sequence – may also play pivotal roles in immune changes related to invasion process (Brown et al. 2015, Na and Gijzen 2016). But alternatively, another important source of immune variation during invasion process could be related to phenotypic plasticity, which is the tendency/ability for phenotypes to change across different environments within generations (reviewed by Gervasi et al. 2015). This mechanism has been widely reported using experimental works modifying environmental conditions (resource quality and availability, cross-fostering) or individual surveys throughout their life, in both vertebrates and invertebrates (Schulenburg et al. 2009).

Another complexity resides in the immune system itself. In vertebrates, it is a highly diverse network of organs, cells and molecules that are generally classified into innate and adaptive compartments, both including cellular (e.g. macrophages, lymphocytes) and humoral components (e.g. antibodies, peptides), which interact together but may have different costs and benefits for the organism (Klasing 2004). It has been assessed that innate responses associated with local and/or systemic inflammation incur high-energy expenditure and major physiological, behavioural and pathological costs (Sorci and Faivre 2009) compared to responses mediated by other effectors, such as both natural (innate) and specific (acquired) antibodies (Råberg et al. 2002, Klasing 2004). This has led to suggest that invasion success would therefore be associated with dampened costly systemic inflammatory response and stronger less costly antibody-mediated immunity (Lee and Klasing 2004). Alternatively, because of potential high infection risks encountered on invasion fronts, the invaders' capacity to mount rapid, non-specific immune responses could be essential for invasion success. It would be therefore possible to make different predictions than those of Lee and Klasing (2004) for immune changes along invasion gradients, such as an increase of immune investment, or the absence of immune tradeoffs (Phillips et al. 2010).

The impacts that bioinvasions may have on immune responses in native species remain scarcely described and investigated. We can first consider that native species might be exposed to a lower infection risk as a result of dilution effects (the presence of a less competent host decreases the infection prevalence in the native host, Dunn 2009) or of density-dependent effects (the density of native hosts may decrease due to competition with invaders, and infection prevalence of parasites transmitted through contacts will consequently decreased too, Keesing et al. 2010). In this context, a decrease in immune investment could be expected (EICA hypothesis). Nevertheless, parasite spill-over from invasive to native species is frequent (Harris 2009) and can strongly affect native host communities, even leading to local extinction, which favors the invasive host (Daszak et al. 2000, Prenter et al. 2004). Such a strong impact of exotic infections on native species could result from the lack of efficient immune responses (either in terms of immune effectors or amounts of responsiveness). A dampened immune defense could result from direct competition with invaders through a lowered access to resources or from stress hormones such as glucocorticoids that may ultimately compromise immunity (Martin et al. 2010a). Strong immunopathologic effects affecting natives' fitness may also result from infections with exotic parasites (spill-over) or from amplified risk of infections with native parasites (spill-back) (Martin et al. 2010b).

Limited empirical data are currently available concerning the ecoimmunology of invasions (Pedersen and Babayan 2011, White and Perkins 2012). Indeed, few studies have investigated differences in immune phenotypes along invasion gradients for a given invasive species, and even fewer have considered several immune pathways simultaneously (Cornet et al. 2016, but see Llewellyn et al. 2012, Brown et al. 2015). Therefore, it remains important to improve our knowledge of such immune variations associated with invasion. It is a necessary pre-requisite before addressing the eco-evolutionary processes dictating invasion success. In this study, we focused on two invasive rodent species, the house mouse *Mus musculus domesticus* and the black rat *Rattus rattus*. We analyzed phenotypic variations in immune responsiveness occurring along their well-known invasion routes in Senegal. These murid rodents are exclusively commensal in the study area (i.e. living in/around human dwellings/man-made structures) and are worldwide significant invasive species (Global Invasive Species Database – <www.issg.org/database/>). Complete syntheses reporting description of their current invasion histories in Senegal (including data of historical inventories, molecular analyses and ecological longitudinal surveys) are provided elsewhere (Konecny et al. 2013, Dalecky et al. 2015). Briefly, both species originated from Asia and have expanded their distribution range worldwide, making use of human migration to colonize all the continents. They were first brought to west Africa by European explorers and settlers from the 15th century. Large and likely standing populations of rats and mice are reported

in coastal colonial cities from the middle of the 19th century. At the beginning of the 20th century, they began to expand eastwards with the development of inland commercial transport. Their distribution currently covers much of north and central Senegal for *M. m. domesticus* and south and central Senegal for *R. rattus*. Recent longitudinal surveys (Granjon and Duplantier 2009, Dalecky et al. 2015) have documented how the ongoing expansion of both species has resulted in the extirpation of native rodents (mostly *Mastomys erythroleucus* and *Mastomys natalensis*) from commensal habitats, these latter species being now found almost only in villages at the invasion front and in non-invaded areas.

Immune phenotypes were described using effectors involved in natural antibody (NAb)-mediated and inflammatory immune pathways. They are main components of innate constitutive immunity acting in the earliest phase of immune defence against general challenges and new parasites (Rossi et al. 2013). First, NAbs are humoral components of constitutive immunity, providing the first-line of non-cellular protection against antigens (Matson et al. 2005). They serve as recognition molecules capable of opsonising invading micro-organisms and initialising the Complement (Cp) enzyme cascade, which ends in cell lysis (Carroll and Prodeus 1998). They are unique among immunoglobulin molecules because their presence does not require prior exposure to exogenous antigens. The assessment of this immune pathway is particularly appealing because NAbs should be less sensitive than acquired antibody responses to short-term variations in environmental conditions, nutritional status, or stress levels (Baumgarth et al. 1999). Moreover, Nab levels in the serum could be positively correlated with the ability to produce antibodies after a challenge (Matson et al. 2005). Second, we used haptoglobin (Hp) concentration to assess the inflammatory state of the rodents, as previously performed in wild birds (Martin et al. 2010a). Hp is a multifunctional hepatic acute-phase protein highly released in the blood during inflammation, with strong anti-inflammatory and anti-oxidant properties (Huntoon et al. 2008). It circulates at low concentrations in the blood of a range of animal species and its concentration is dependent on health status (Dobryszycka 1997). Hp has been shown to be a distinctive trait of individuals (Matson et al. 2012). Both immune tests used here are simple, highly repeatable and non-specific. Moreover they do not require the recapture of animals or their maintenance in animal facilities. For these reasons, they enable to cope with the specific constraints of comparative immunological studies dealing with numerous samples of different non-model species caught in natural populations and sacrificed at the time of capture. All assays were performed at both long-established and recently invaded sites, for both invasive species, and for *Mastomys* species found either in sympatry with the invaders at the invasion front or alone at sites not yet invaded.

We specifically addressed the two following questions: 1) do the immune responsiveness patterns observed in rats and mice along invasion routes support the EICA or EICA refined hypotheses? We expected a decrease of immune responsiveness (Nabs and Hp) in recently invaded localities compared to long established ones under the EICA hypothesis, or a decrease of costly immune responsiveness (Hp) at the expense of less costly ones (NAbs) under the EICA-refined hypotheses; 2) do native species exhibit variations in immune responsiveness associated with the presence of invasive ones ? Up to now, no framework has been developed with regard to this question, what prevents us to make any predictions. Altogether, these results enabled to discuss some of the general predictions of Lee and Klasing's paper (2004), which is one of the reference work when studying the potential links between immunology and invasion. Nevertheless, this study is descriptive and as such, it was not designed to disentangle the ecological and evolutionary processes potentially underlying the immune patterns observed.

Material and methods

Ethical statement

Trapping campaigns within private properties were systematically realized with prior explicit agreement from relevant institutional (CBGP: 34 169 003) and local authorities. All animal-related procedures were carried out in accordance with relevant requirements of Senegalese and French legislation and following official ethical guidelines (Sikes et al. 2011).

Rodent sampling and blood collection

Field sampling was conducted separately along an invasion route for each invasive species (Fig. 1). It was carried out during the dry season, in March–April 2013 for the 'mouse' invasion route and from November 2013 to February 2014 for the 'rat' invasion route. The sampling sites belonged to one of three invasion status categories, defined on the basis of historical records and longitudinal surveys (Konecny et al. 2013, Dalecky et al. 2015): 1) sites of long-established invasion, in which invasive populations have been recorded for centuries; 2) sites at the invasion front (= sites recently invaded) in which invasive populations have been established for less than 30 years; and 3) non-invaded sites in which invasive species have never yet been recorded or trapped. For each category, we sampled three to four sites (Fig. 1). We used a standardised sampling protocol for all localities of the three categories of sites considered. It enabled to standardize the potential impacts of stress on immune responses due to animal capture and handling. The trapping procedures are described in detail elsewhere (Dalecky et al. 2015). At each site, we set at least 120 traps within houses during one to three successive nights, with the aim to capture 20 adult rodents per species. Traps were checked and baited once a day with peanut butter supplemented with fresh onions. Rodents captured at night were retrieved the following morning and then sacrificed by cervical dislocation within the following four hours. They were weighed to the nearest 0.5 g, sexed and dissected. Identification was based on morphometrics (head-body, tail, hind foot and ear lengths) and genetics (ZFY2 gene-based RFLP for identification to the subspecies level for *M. musculus*; cytochrome b gene-based RFLP for species characterisation in the genus *Mastomys*). As suggested by Granjon and Duplantier (2009), rodents were considered to be adults on the basis of body weight and reproductive

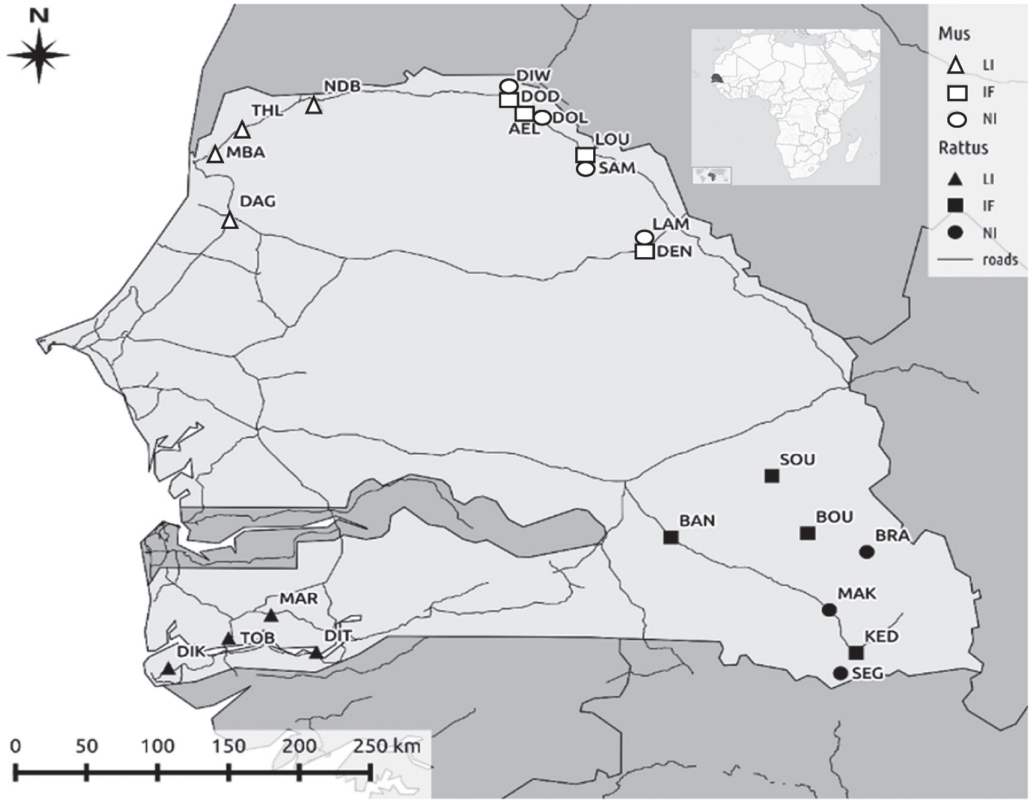

Figure 1. Sampling sites. Triangles, squares and circles correspond respectively to sites of long-established invasion (> 100 years), recently invaded sites (< 30 years) and non-invaded sites. Mouse invasion route (symbols in white): Dagathie (DAG), Mbakhana (MBA), Thilene (THL), Ndombo (NDB), Dodel (DOD), Aere Lao (AEL), Dendoudi (DEN), Lougue (LOU), Doumga Lao (DOL), Lambango (LAM), Saré Maoundé (SAM), Diomandou Walo (DIW). Rat invasion route (symbols in black): Diakene Wolof (DIK), Diattacounda (DIT), Marsassoum (MAR), Tobor (TOB), Badi Nieriko (BAN), Bountougoufara (BOU), Kedougou (KED), Soutouta (SOU), Bransan (BRA), Mako (MAK), Segou (SEG).

status. Blood samples were collected by cardiac puncture after the animals were euthanatized. They were kept on ice for 24 h and then centrifuged. The floating serum supernatant fraction was removed, frozen in liquid nitrogen, and then stored in a freezer (–20°C) in Dakar (Senegal). Samples were transferred in dry ice from Dakar to Montpellier (France) in accordance with the regulations enforced in Senegal and France.

Environmental data

Because environmental variations may drive differences in immune responses between rodent populations independently of the invasion status categories, we described relevant climatic and commensal habitat parameters for all sampling sites and included them in further statistical analyses. We focused on these factors and did not include vegetation information because house mice and black rats are strictly commensal in Senegal, and because both invasive species expand their range through human trade and transport rather than by individual dispersal in the wild (Dalecky et al. 2015). Means and standard deviations of climatic data collected between 1997 and 2012 were used (data available on <www.ncdc.noaa.gov/cdo-web/datasets> for temperatures, and <http://richardis.univ-paris1.fr/precip/rainser1.html> for rainfall with GPCP-1DD as the source of data). We considered rainfall data in mm (for each year: cumulated annual rainfall, cumulated rainfall during the rainy season, minimum and maximum monthly rainfall during the rainy season) and temperature data in degrees Celsius (for each year: monthly mean, monthly mean minimum and maximum, minimum of monthly mean minimum and maximum of monthly mean maximum). Commensal habitat characteristics were recorded during trapping sessions. In particular, we recorded for each sampled room the material used (sand, banco, cement, sheet metal, fibers) for each part of the building (floor, wall, ceiling), the type of room (dwelling house, shop, storehouse, kitchen) and for each site the inhabited area surface estimated using Google Earth Pro 7.1.

Hemagglutination-hemolysis (HA-HL) assay

The goal of this assay was to evaluate components of humoral innate immunity through the ability of plasma to agglutinate and lyse foreign cells through NAbs-Cp system. We characterised the serum agglutination of heterologous red blood cells (RBC) due to NAbs (HA assay) and RBC hemolysis due to NAbs-mediated Cp activation (HL assay), with a slightly modified version of the protocol described by Matson et al. (2005) for mammal species. Briefly, we used chicken RBCs as target cells, and serum samples from a rabbit immunised against chicken RBCs as positive controls. All the HA/HL

assays from an invasion route were carried out using the same RBC suspension. We diluted twofold serially (from 1/2 to 1/128) ten rodent serum samples per plate with 10 µl in every well. The plates were read blindly following Rossi et al. (2013) and scores were attributed for each sample (column) as the log2 of the last dilution exhibiting each phenomenon (HA, HL). To exclude potential observer effects, all images were scored the same day by the same trained observer; thus correction for observer effect was not necessary in the subsequent analyses. It was possible to assign a half-point score in cases of ambiguity. High scores of HA reflected high concentrations of NAbs in the blood. Levels of HL reflected both the NAbs and complement activities.

Haptoglobin (Hp) assay

Hp was quantified from 10µl of serum with a commercially available colorimetric immunoassay kit ('PHASE' Haptogloblin Assay, TP-801, Tridelta), according to the manufacturer's instructions. Absorbance at 650 nm was determined with a spectrophotometer, both before and after the addition of the final reagent triggering the colorimetric reaction. We used the pre-scan absorbance to correct for differences in plasma colour and cloudiness, including the initial redness of the serum (i.e. the initial serum hemolysis) that can hamper the assay if not taken into account (Matson et al. 2012). Serum Hp concentration (mg ml^{-1}) was estimated by comparing the difference in absorbance (final – pre-scan) to a calibration curve.

Statistical analyses

In order to assess whether environmental features differed between the three categories of sampled sites, we carried out a two-stage principal component analysis (PCA) with sites as observations, independently for climatic features (using 16-years mean values and their respective standard deviations) and commensal habitat characteristics (proportion data). As many indicators appeared highly correlated ($r > 0.75$), we kept only one variable in each set of highly correlated variables, based on both contributions to the axis construction and quality of representation. We then performed a final PCA with remaining variables. We tested statistically whether data were structured according to invasion status categories using a between/within-groups analysis (BWA). Monte Carlo tests of permutations (999 permutations) were applied to analyse the statistical significance of the groups graphically observed on the PCAs.

Variations in Hp concentration (log-transformed), HA and HL values were analysed independently, using linear models, in R ver. 3.1.0 (<www.r-project.org>). The 'mouse' and 'rat' invasion routes were investigated separately. The starting models included a factor combining the species and site status on the invasion route, hereafter referred to as 'specific invasion status' and classified into four categories: A) invasive species alone in sites of long-established invasion; B) invasive species at recently invaded sites; C) native species at recently invaded sites and D) native species in non-invaded sites. Combining the two factors 'species' and 'site category' into a single one (called 'specific invasion status' hereafter) enabled to avoid confounding effects between host species and invasion on immune responses. Individual factors (sex, body mass and age class) may greatly affect immune system parameters. We therefore included these factors and their two-ways interactions with specific invasion status as fixed effects. We also added the descriptors of environmental variations as potential predictors in the full models. Therefore, coordinates of each site on the two first PCA axes for both climatic and commensal habitat data were included. Finally, we had to include several additional factors specific to the different experimental protocols used: 1) a 'plate' factor in the HA model to take into account both the chronology in which the plates were analysed and the involvement of two different experimenters, and 2) HA scores in the HL model as HL directly depends on the presence of antibody–antigen complex revealed by HA. For both the HA and HL models, we also included the initial level of serum hemolysis as initial hemolysis is expected to interfere with the interpretation of HA and HL results. A value was then systematically given to the serum prior dilution, ranging from 1 (not red) to 8 (dark red) according to the intensity of the initial serum redness in order to correct for non-visibility and over-interpretation of the phenomena during the plate reading. The Akaike information criterion with correction for samples of finite size (AICc) was used for model selection. Models with all possible combinations of the terms included in the starting model were generated with the MuMIn ver. 1.10.5 R package (dredge function, Barton 2016). Models with a $\Delta AICc < 2$ with respect to the model with the lowest AICc were selected and the most parsimonious of these models was chosen. The significance of explanatory variables and their interactions was determined by deletion testing and log-likelihood ratio tests. The assumptions of each final model were checked graphically, by an analysis of their residuals. Post hoc tests for multiple comparisons were carried out with Tukey's test (95% family-wise confidence level).

Data deposition

Data available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.sv680> (Diagne et al. 2016).

Results

We analysed serum samples from 646 individuals belonging to four rodent species captured at 23 sites (Table 1, Fig. 1). The dominant species in the native rodent communities were *Maastomys erythroleucus* along the 'mouse' invasion route and *Ma. erythroleucus* or *Ma. natalensis* along the 'rat' invasion route. *Mastomys natalensis* was found specifically at the invasion front of *Rattus rattus*, coinciding geographically with the limited distribution area of this native species in Senegal. *Mastomys erythroleucus* was occasionally captured in sites of long-established invasion ($n = 15$) and in non-invaded sites beyond the invasion front in western Senegal ($n = 3$) along the 'rat' invasion route. These individuals were too few in number for more detailed analysis. No significant difference was detected in HA ($F_{1,49} = 0.18$, $p = 0.71$), HL ($F_{1,49} = 2.14$, $p = 0.28$) or Hp ($F_{1,53} = 1.27$, $p = 0.38$) levels between *Mastomys* species at sites recently invaded by *R. rattus*. *Mastomys natalensis* and *Ma. erythroleucus* were

Table 1. Sample size for each assay, by invasion status (LI = sites of long-established invasion; IF = invasion front; NI = non-invaded sites), sampled site (code in parentheses) and host species for a) the 'mouse' invasion route (166 *Mus musculus domesticus*, 145 *Mastomys erythroleucus*) and b) the 'rat' invasion route (196 *Rattus rattus*, 88 *Mastomys natalensis*, 50 *Mastomys erythroleucus*). Numbers in parentheses indicate sample size for males/females, respectively. '–' indicates that no rodent was trapped or analysed. Sample sizes differ between immune assays because of the limited volume of some serum samples prevented to perform both assays.

(a)

		Hemagglutination–hemolysis		Haptoglobin	
Invasion status	Sites	*M. m. domesticus*	*Ma. erythroleucus*	*M. m. domesticus*	*Ma. erythroleucus*
LI	Dagathie (DAG)	19 (10/9)	–	12 (6/6)	–
	Mbakhana (MBA)	23 (13/10)	–	12 (8/4)	–
	Thilene (THL)	20 (8/12)	–	13 (3/10)	–
	Ndombo (NDB)	21 (10/11)	–	15 (7/8)	–
IF	Dodel (DOD)	23 (9/14)	19 (11/8)	10 (3/7)	12 (9/3)
	Aere Lao (AEL)	21 (10/11)	19 (12/7)	17 (8/9)	11 (7/4)
	Dendoudi (DEN)	17 (12/5)	23 (16/7)	10 (6/4)	16 (12/4)
	Lougue (LOU)	22 (10/12)	22 (15/7)	10 (3/7)	14 (11/3)
NI	Doumga Lao (DOL)	–	20 (10/10)	–	14 (7/7)
	Lambango (LAM)	–	20 (9/11)	–	16 (7/9)
	Saré Maoundé (SAM)	–	9 (5/4)	–	5 (3/2)
	Diomandou Walo (DIW)	–	13 (9/4)	–	11 (7/4)

(b)

		Hemagglutination–hemolysis			Haptoglobin		
Invasion status	Sites	*R. rattus*	*Ma. erythroleucus*	*Ma. natalensis*	*R. rattus*	*Ma. erythroleucus*	*Ma. natalensis*
LI	Diakene Wolof (DIK)	24 (9/15)	–	–	24 (9/15)	–	–
	Diattacounda (DIT)	27 (13/14)	–	–	27 (13/14)	–	–
	Marsassoum (MAR)	26 (13/13)	–	–	26 (13/13)	–	–
	Tobor (TOB)	20 (6/14)	–	–	20 (6/14)	–	–
IF	Badi Nieriko (BAN)	21 (8/13)	11 (5/6)	–	23 (10/13)	12 (6/6)	–
	Bountougoufara (BOU)	31 (9/22)	12 (7/5)	–	31 (9/22)	13 (8/5)	–
	Kedougou (KED)	22 (9/13)	–	22 (9/13)	22 (9/13)	–	22 (9/13)
	Soutouta (SOU)	22 (11/11)	9 (5/4)	–	23 (12/11)	9 (5/4)	–
NI	Bransan (BRA)	–	–	23 (10/13)	–	–	23 (10/13)
	Mako (MAK)	–	–	23 (11/12)	–	–	26 (13/13)
	Segou (SEG)	–	–	20 (7/13)	–	–	21 (8/13)

therefore considered as a single taxon, native *Mastomys* sp., in further statistical analyses on the 'rat' invasion route.

Environmental data

We found significant climatic differences along 'mouse' and 'rat' invasion routes (Monte Carlo tests, $p < 0.05$). Along the 'mouse' invasion route, climate seemed cooler and drier in sites of long-established invasion compared to the other sites. Along the 'rat' invasion route, climate seemed cooler and wetter in sites of long-established invasion compared to the other sites. These results are well represented on the second axis of mouse and rat PCAs (Supplementary material Appendix 1 Fig. A1a, A2a). The between/within analysis on commensal habitat characteristics revealed a significant segregation between invaded and non-invaded sites along the 'mouse' invasion route (Supplementary material Appendix 1 Fig. A1b), suggesting more traditional buildings in non-invaded sites. Along the 'rat' invasion route, no significant difference in commensal habitat characteristics was detected (Monte Carlo test, $p > 0.05$) with regard to site invasion status (Supplementary material Appendix 1 Fig. A2b). However, these results must be taken cautiously as this study was not designed to investigate the influence of environmental characteristics on immune responsiveness.

Variation in HA/HL estimates

'Mouse' invasion route

The most parsimonious model explaining variation in HA included the climate (component 1: $F_{1,290} = 5.83$, $p < 0.0001$; component 2: $F_{1,290} = 16.81$, $p < 0.0001$), plate factor ($F_{1,290} = 11.27$, $p = 0.0163$), specific invasion status ($F_{3,290} = 12.48$, $p < 0.0001$, Fig. 2) and its interaction with sex ($F_{3,290} = 4.75$, $p = 0.0029$) (Supplementary material Appendix 1 Table A1). Sex accounted for differences in HA levels only at non-invaded sites, with higher values recorded for males than females. Mice from sites of long-established invasion had the lowest HA values. No significant difference in mean HA was detected in *Ma. erythroleucus* between recently invaded and non-invaded sites (post hoc Tukey's test, $p = 0.9999$), nor between *Ma. erythroleucus* and *M. m. domesticus* at the invasion front (post hoc Tukey's test, $p = 0.9999$).

The most parsimonious model explaining variation in HL included the initial degree of serum hemolysis ($F_{1,293} = 99.82$, $p < 0.0001$), HA score ($F_{1,293} = 50.72$, $p < 0.0001$), sex ($F_{1,293} = 9.87$, $p = 0.0018$) and specific invasion status ($F_{3,293} = 26.56$, $p < 0.0001$, Fig. 2, Supplementary material Appendix 1 Table A1). High values of HL were recorded for high values of HA and initial serum hemolysis, and for males

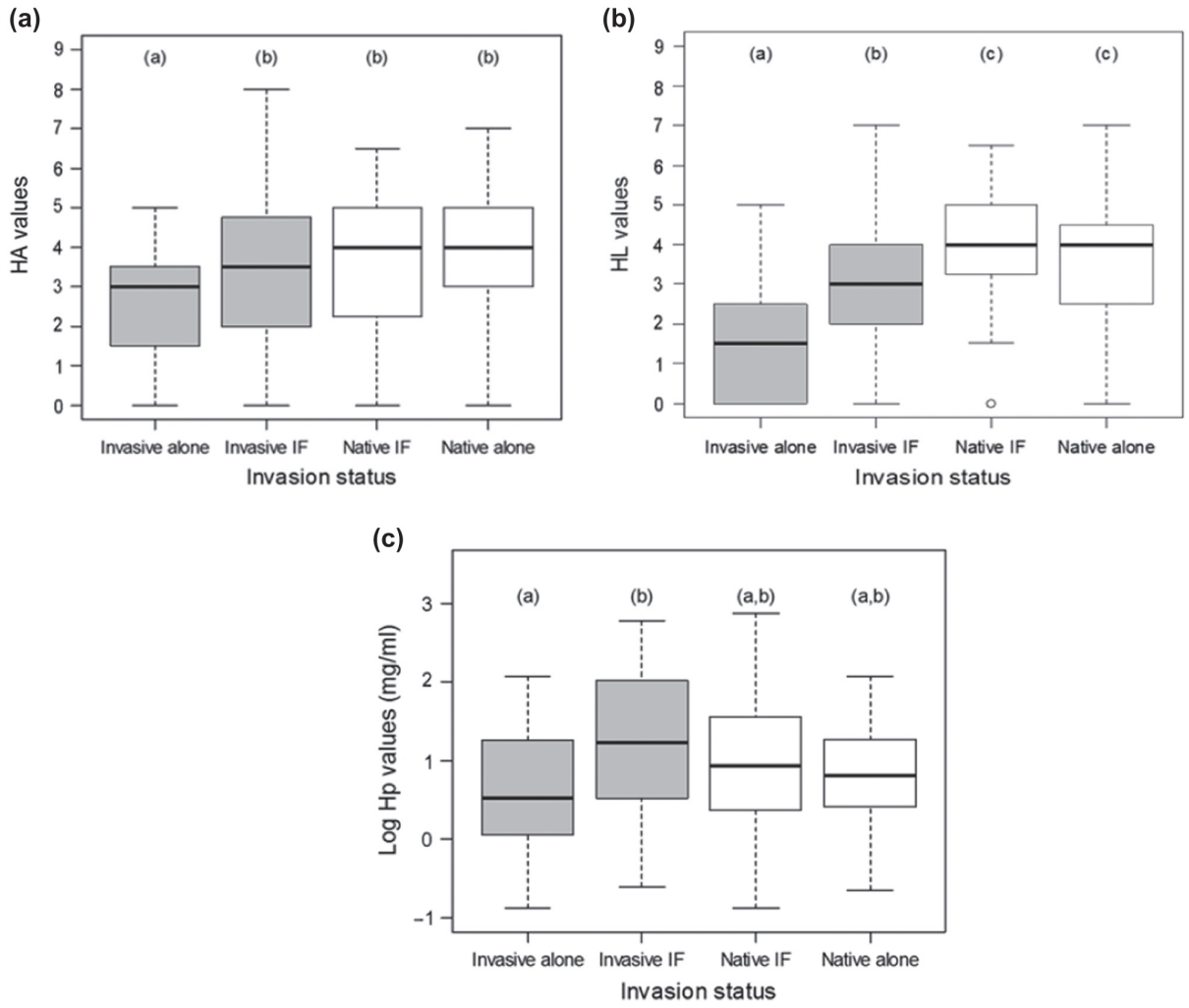

Figure 2. Effects of specific invasion status on the variation in (a) hemagglutination (HA), (b) hemolysis (HL) and (c) haptoglobin (Hp) levels in the serum of *Mus musculus domesticus* (boxplots in grey) and *Mastomys erythroleucus* (boxplots in white). The whiskers denote 1.5 inter-quartile range. Legend: Invasive alone = *M. m. domesticus* in sites of long-established invasion; Invasive IF = *M. m. domesticus* on invasion front (IF); Native IF = *Ma. erythroleucus* on IF; Native alone = *Ma. erythroleucus* in non-invaded sites. The letters above boxplots denote the significance of differences between specific invasion statuses: boxplots with the same letter above are no significantly different while boxplots with different letters above are significantly different.

compared to females. Mice from sites of long-established invasion had lower HL values compared with other invasion statuses. On invasion front, *M. m. domesticus* had lower values of HL than *Ma. erythroleucus* from recently invaded (post hoc Tukey's test, $p < 0.0001$) and non-invaded sites (post hoc Tukey's test, $p = 0.0006$). No significant difference in mean HL between recently invaded and non-invaded sites was detected in *Ma. erythroleucus* (post hoc Tukey's test, $p = 0.8723$).

'Rat' invasion route

The most parsimonious model explaining variation in HA included the initial degree of serum hemolysis ($F_{1,299} = 12.74$, $p = 0.0004$), age ($F_{1,299} = 9.40$, $p = 0.0024$), body mass ($F_{1,299} = 4.93$, $p = 0.0270$) and climate ($F_{1,299} = 12.91$, $p = 0.0004$) (Supplementary material Appendix 1 Table A2). Heavier and adult rodents had higher HA levels than lighter and juveniles, respectively. Serum hemolysis was negatively correlated with HA. No significant difference was found according to specific invasion status ($F_{3,299} = 1.41$, $p = 0.2399$).

The most parsimonious model explaining variations in HL included the initial degree of serum hemolysis ($F_{1,300} = 57.76$, $p < 0.0001$), HA score ($F_{1,300} = 355.92$, $p < 0.0001$) and commensal habitat characteristics (component 1: $F_{1,300} = 21.82$, $p < 0.0001$) (Supplementary material Appendix 1 Table A2). HL values were positively correlated with both HA scores and with initial serum hemolysis. Specific invasion status had no significant effect on HL values ($F_{3,297} = 0.82$, $p = 0.4862$).

Variation in Hp estimates

'Mouse' invasion route

The most parsimonious model explaining variation in Hp concentration included climate (component 1: $F_{1,183} = 13.31$, $p = 0.0003$; component 2: $F_{1,183} = 22.74$, $p < 0.0001$) and specific invasion status ($F_{3,183} = 5.22$, $p = 0.0017$) (Fig. 2,

Supplementary material Appendix 1 Table A1). Mice sampled from the invasion front had Hp levels twice those of mice from sites of long-established invasion (post hoc Tukey's test, p = 0.0039). No significant difference in mean Hp concentration was found between *Ma. erythroleucus* from recently invaded and non-invaded sites (post hoc Tukey's test, p = 0.9307). Furthermore, Hp levels were similar in *Ma. erythroleucus* and *M. m. domesticus* sampled in recently invaded sites (post hoc Tukey's test, p = 0.4349).

'Rat' invasion route

The most parsimonious model best explaining variation in Hp included climate ($F_{3,308} = 12.62$, p = 0.0004; Fig. 2, Supplementary material Appendix 1 Table A2) only. However, the specific invasion status was included in about half of the models within ΔAICc < 2 (in seven of the 16 selected) with *R. rattus* from sites of long-established invasion having lower Hp concentrations than rodents trapped in recently invaded sites (*R. rattus*: post hoc Tukey's test, p = 0.0362; *Mastomys* sp.: post hoc Tukey's test, p = 0.0058; Fig. 3). At the invasion front, Hp levels were similar in invasive and native species (post hoc Tukey's test, p = 0.74). No significant difference was detected in mean Hp between *Mastomys* sp. from recently invaded and non-invaded sites (post hoc Tukey's test, p = 0.2163).

Discussion

Most studies investigating the role of immunity in the context of biological invasions have focused on comparisons between sympatric invasive and native species or between phylogenetically related species with contrasted levels of invasion success (Lee et al. 2006, Martin et al. 2010a). Because differences in immune investment may be due to intrinsic species characteristics, sampling designs focusing on interspecific comparisons may not be entirely appropriate for evaluating immune defences – invasion issues. Comparing several invasive (areas in which invaders are well established versus newly colonised areas) and native (non-invaded versus recently invaded areas) populations along a well-defined invasion route appears to be a more relevant approach, as it allows overcoming specific differences and taking into account the invasion history as a spatio-temporal continuum. Moreover, potential immune changes in native communities should be considered as a full issue that may ultimately impact the invasion process. Several predictions have been proposed with regard to immunity and invasion (Lee and Klasing 2004, Phillips et al. 2010). Our results did not fully support the major one, which is the EICA-refined hypothesis. Antibody-mediated defences were found to increase along the invasion route, but for *Mus musculus domesticus* only (Fig. 2, Supplementary material Appendix 1 Table A1). Moreover, the inflammatory response was found to be stronger in invasive populations at the invasion front than in populations from sites of long-established invasion, for both invasive species (Fig. 2, 3, Supplementary material Appendix 1 Table A1, A2). Finally, we did not observe any difference in the immune effectors surveyed in native species between invaded and non-invaded localities.

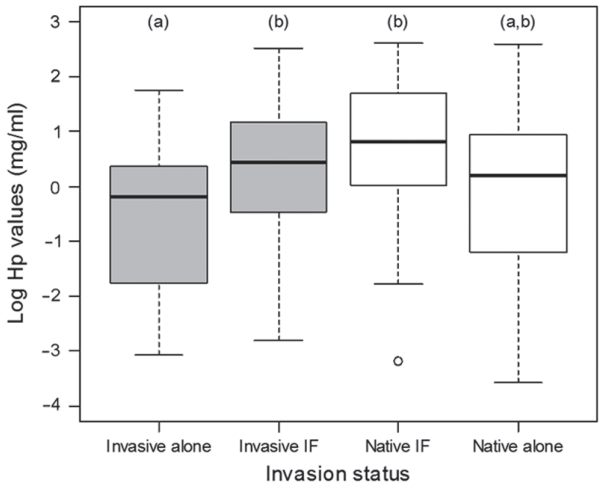

Figure 3. Effects of specific invasion status on the variation of serum haptoglobin (Hp) concentration in *Rattus rattus* (boxplots in grey) and *Mastomys* spp. (boxplots in white). The whiskers denote 1.5 inter-quartile range. Legend: Invasive alone = *R. rattus* at sites of long-established invasion; Invasive IF = *R. rattus* on invasion front (IF); Native IF = *Ma. erythroleucus* (n = 34) and *Ma. natalensis* (n = 22) on IF; Native alone = *Ma. natalensis* in non-invaded sites. The letters above boxplots denote the significance of differences between specific invasion statuses: boxplots with the same letter above are no significantly different while boxplots with different letters above are significantly different.

Effects of methodological biases and individual characteristics on immune variations

We carefully considered experimental factors in our statistical models as they could have biased our results. The initial levels of serum hemolysis and HA scores were found to significantly influence HA/HL results irrespective of the invasion route (Supplementary material Appendix 1 Table A1, A2). Higher values lead to higher HA/HL scores, regardless to the species and the invasion status of sites. These results were expected as Cp lysis is activated by the formation of antigen-NAb complexes (Matson et al. 2005). Furthermore, the initial serum hemolysis is part of the HL score and may represent the total final HL in extreme cases where NAb-mediated lysis did not occur in a serum. These results highlight the crucial importance to incorporate such 'experimental' factors as potential predictors in the explanatory models.

Immune responses could also be biased due to stress resulting from capture and handling procedures. However, the immune effectors studied here are not strongly affected by stressors. In particular, it has recently been shown that Nabs and Cp are insensitive to capture and handling stress (Buehler et al. 2008). Besides, Nab production seems to be largely independent of internal or external stimuli (Ochsenbein and Zinkernagel 2000). In addition, we minimized the potential biases due to different effects of stress between localities of colonization statuses by using a consistent standardized capture protocol among sampling sites. This design aimed at preventing any differences in methodology-related

stressors between rodent populations. Finally, a strong argument showing that capture and handling procedures had a very few impact on the immune patterns observed is that native rodents, which are also subject to these methodological stresses, exhibited no immune variation between non-invaded sites and invasion fronts. This result suggests that the invasion front itself is not an area generating specific stress reactions. Altogether, these elements argue in favour of a limited impact of stress on the immune variations observed here.

We found a significant influence of age, body mass and sex on some immune responses that were higher in adults, heavier individuals and males, respectively (Supplementary material Appendix 1 Table A1, A2). Age and body mass influenced significantly NAb levels for both native and invasive rodents on the 'rat' invasion route. These findings were congruent with other works (Rossi et al. 2013) and corroborated the common trend of an increased immune capacity with host growth and condition. Our result of male mice showing higher HL levels than females corroborates the life-history theory predicting that females should invest more in specific immune pathways than in constitutive immunity, which would thus be expected to be downregulated (Lee et al. 2006). Moreover, other factors including ecological, physiological, social and behavioural ones, probably interacting in a complex way, may contribute to the observed patterns of sexual differences observed in rodent immunity. For instance, it has been shown in voles that social environment and steroid hormones affect in a complex way sex differences in immune function (Klein et al. 1997). This question therefore remains a challenging area of research in evolutionary ecology.

Impact of environmental conditions on immune patterns

As *M. m. domesticus* and *Rattus rattus* are likely to encounter new environments along their respective invasion route, immune variations observed in these invasive species may be considered as responses to environmental factors (reviewed by Colautti and Lau 2015). It has been previously shown that environmental parameters can affect the immune functions of rodents (Beldomenico et al. 2008). Climate and commensal habitat structure may influence rodent immune responses, for instance through resource availability or community composition, and were thus considered in our statistical models. Nevertheless, these environmental features could not explain alone the patterns observed. Climatic data differed between sites of long- and recently-established invasion for both invasive mice and rats but no variation was observed in HA and HL levels for *R. rattus*, and climate was not a relevant predictor to explain variations of HL in *M. m. domesticus*. Likewise, habitat structure did not differ between sites of long- and recently-established invasion along the 'mouse' invasion route, although immune responses significantly varied. Conversely, habitat structure differed between non-invaded sites and those recently invaded by the house mouse, but no immune variation was observed in native populations along this invasion transect.

Absence of immune patterns supporting the EICA and EICA refined hypotheses

Variations in immune responses between long- and recently-established invasive populations did not support the predictions expected under the EICA and EICA refined hypotheses, which are respectively a general decrease of immune responsiveness or opposite changes between energetically costly immune pathways (decrease of responses) and less costly ones (increase of responses). Instead, this study revealed increased inflammatory and/or humoral responses in expanding invasive populations of mice and rats compared to long established populations (Fig. 2, 3).

These results questioned the relevance of the physiological and ecological interactions that are at the basis of EICA expectations, namely parasitism and immune costs. The risks of exposure to parasites and subsequent infections are supposed to be an important component of the novel environment encountered during invasion, which may explain these immune variations along invasion routes. Nevertheless, by contrast with the enemy release emphasized in the EICA hypothesis, invaders may experience novel parasite pressure as they may be exposed and/or infected with novel local parasites when pathobiome communities differ between native and invasive species (see Diagne et al. 2016, Galan et al. 2016 for examples). The overall infection risk in recently invaded areas could thus increase on invasion front. In particular, the ability to mount strong inflammatory responses may prevent 'naïve host syndrome' in invasive species or within the invasion front, a severe adverse effect of parasites on hosts with which they have no co-evolutionary history (Mastitsky et al. 2010). Besides, larger responses for Hp in invaders would be consistent with efforts to attenuate inflammation. Indeed, although the secretion of Hp is enhanced in inflammatory states, Hp has strong anti-inflammatory and anti-oxidative properties. During infection, Hp is best characterized as a protein that protects hosts against "all the dangers of the acute phase response" (Dobryszycka 1997). Within 24 h of infection, circulating Hp concentration increases considerably, to replenish hemoglobin stores and damp down inflammatory responses and thus, their detrimental effects. High Hp levels may therefore be of benefit to invaders due to a lessening of the negative impacts of immunity.

The extent to which the presumed costs of immune defences (high for inflammation, low for antibody-mediated responses, Lee and Klasing 2004) can be generalized to all biological models and ecological conditions must also be questioned. Immune strategies have been shown to differ significantly between species, or even between races of domesticated species (Mendes et al. 2006). Moreover, the estimation of these costs may be specifically dependent on the response examined for a given pathway, and/or the condition/energetics parameters chosen. However tradeoffs between inflammation and humoral responses involving immune effectors other than those investigated here cannot be excluded. We must keep in mind that the indicators studied here only reflect one part of the immune capacities to fight infections and may not be generalizable to other effectors. Although it is very difficult to measure immune costs in the wild, a full study dedicated to this issue should provide crucial information on immune tradeoffs during invasion process.

Stronger patterns of immune variations in mouse compared to rat invasive species

Although environmental parameters and interspecific differences might be obvious reasons to explain contrasted immune variations observed between rats and mice along their respective invasion routes, different abilities in direct competition with native communities may also be proposed. Direct competition is often put forward as an explanation for the replacement of native species by exotic rodents (Drake and Hunt 2009). The black rat and house mouse strongly differ in their competitive interactions with native rodents. *R. rattus* has been shown to be aggressive to intruders, physically eliminating experimentally introduced conspecific individuals from an insular population (Smith and Banks 2014). They are much larger than *Mastomys* species and would therefore be at an advantage in direct competition. On the contrary, *M. m. domesticus* is known to perform poorly in direct competition with several native rodents (Gomez Villafane et al. 2013). Variation in immune phenotype may therefore be a less important strategy for the invasion success of *R. rattus* than for *M. m. domesticus*. Invasion success mediated by immune variations in house mice would account for observations that this species is less parasitized than rats, for zoonoses for instance (Blackwell 1981, Meerburg et al. 2009). This hypothesis needs to be tested, and it would also be interesting to investigate whether it is observed in other cases of biological invasions.

No variation of immune response in native species

The questions of what happens in native species and whether immune responses change in invaded compared to non-invaded areas remain scarcely explored. In this work, native *Mastomys* species exhibited similar immune responses in non-invaded and recently invaded sites, along both the 'mouse' and 'rat' invasion routes (Fig. 2, 3). This pattern could result from the matching between environmental conditions (especially parasite pressure, resource availability, etc.) and the development of adequate immune responses by native rodents that have co-evolved with their natural environment. However, we found significant environmental differences between non-invaded sites and invasion fronts for both invasion routes. It is also likely that novel epidemiological conditions are established during invasion as the introduction of invaders directly affects host community composition and may also modify the infection risk of native species (Dunn and Hatcher 2015, Diagne et al. 2016, Galan et al. unpubl.). As such, the infection risk may be either amplified by spill-over and/or spill-back mechanisms, or decreased when invaders are not competent to maintain and transmit local parasites (dilution effect). Thus, the absence of immune variations in native species could reflect their inability to adapt their immune phenotypes.

Conclusion

Our data provide no support for 1) the predictions of the EICA-refined hypothesis that antibody-mediated immunity should be favoured over inflammation during invader range expansion, and 2) our expectations on immune changes experienced by native species when co-occurring with invaders. Whether immune changes could occur due to a higher risk of exposition to/infection with novel parasites at the invasion front and a greater ability of invasive species, such as *M. m. domesticus* in particular, to adjust their immune phenotypes during invasion are hypotheses that need to be confirmed experimentally in the future. Such studies could also enable to assess the respective roles of evolutionary and ecological processes in driving these phenotypic immune variations.

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Statement of authorship – CD performed the HAHL analyses and wrote the first draft of the paper. EGF participated in the interpretation and statistical analyses of HAHL data. SC, LH, SD performed the Hp analyses and SC carried out the statistical analyses. OFG participated in the multivariate analyses of environmental data. CB, AD and NC designed the sampling. NC designed the immunological experiments and formulated the hypotheses tested. AD and KB managed the extensive field work. KB, MK, YN, MD, AS, CB, NC and CD performed the field sampling. SP and EA were responsible for sample collection and the database.

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Supplementary material (available online as appendix oik-03470 at <www.oikosjournal.org/appendix/oik-03470>). Appendix 1.