Shifts in the immune response of an alien species along the invasion wave: testing the Evolution of Increased Competitive Ability hypothesis on invasive gray squirrels

Claudia Romeo[1,*], Joel Filipe[2], Lucas A. Wauters[3,4], Stefano Comazzi[2,5], Federica Riva[2], Nicola Ferrari[2,5]

[1] Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Via Bianchi 9, 25124 Brescia, Italy

[2] Università degli Studi di Milano, Department of Veterinary Medicine and Animal Science, Via dell'Università 6, 26900 Lodi, Italy

[3] Università degli Studi dell'Insubria, Department of Theoretical and Applied Sciences, Environment Analysis and Management Unit - Guido Tosi Research Group, Via Dunant 3, 21100 Varese, Italy

[4] University of Antwerp, Department of Biology - Evolutionary Ecology Group, Universiteitsplein 1, 2610 Wilrijk, Belgium

[5] Università degli Studi di Milano, WildlifeHealth Lab, Via dell'Università 6, 26900 Lodi, Italy

*corresponding author: Claudia Romeo (claudiarosa.romeo@izsler.it)

Abstract

Based on the Evolution of Increased Competitive Ability (EICA) hypothesis, a reduced investment in immunity, consequent to parasite loss, could partly explain the success of invasive alien species. We investigated the variation in the immune response of alien Eastern gray squirrels (*Sciurus carolinensis*) along the invasion wave of an expanding population, using multiple measures of immunity to investigate whether individuals at the invasion front, where parasite pressure is lower, 1) dampened their costly inflammatory response, and 2) increased their investment in less expensive acquired immunity compared to squirrels at the core of the invaded area. We first explored variation in hematological variables related either to the inflammatory or the acquired response. On a subset of individuals, we carried out *ex vivo* cell cultures to analyse the basal expression of MHC class II genes and the expression of TNF-α genes in response to an immune challenge. Platelet counts and TNF-α expression suggested higher inflammation in individuals living at the invasion core, whereas parameters associated with an acquired response (lymphocyte counts and MHC II expression by spleen cells) were conversely higher in squirrels at the front. Overall, our results suggest a shift between different immune strategies along the invasion wave, supporting a reduced investment in costly inflammatory responses and an increased investment in acquired immunity in individuals at the expanding edge of the range, which are subjected to high selective pressures for dispersal and reproduction.

Keywords

invasive species; biological invasions; immune strategy; expansion range; inflammatory response; Sciurus carolinensis

1. Introduction

Nowadays, biological invasions by alien species are widely recognised as one of the major threats to biodiversity and ecosystem functioning worldwide (Bellard et al., 2016; Diagne et al., 2021; Jeschke et al., 2014; Simberloff et al., 2013). Although, during the last decades, our knowledge in the field of invasion ecology has made relevant advances, the successful establishment and rapid spread of invasive species are likely the result of several complex interacting mechanisms whose exact interplay remains poorly understood (Enders et al., 2020).

Parasitism is considered one of the major selective forces leading to the evolution of diversity within natural populations (May and Anderson, 1983) and changes in host-parasite interactions occurring in introduced populations likely represent one of the key mechanisms governing invasions (Dunn and Perkins, 2012). One of the proposed hypotheses to explain the success of invasive species is the Evolution of Increased Competitive Ability (EICA), which postulates that a loss of natural enemies (including parasites, predators and grazers) should allow for a reduced investment in defense functions and a consequent reallocation of resources towards fitness and growth (Blossey and Notzold, 1995). In most cases, invasive plants and animals experience indeed a relevant loss of parasites during the introduction process, and will show an impoverished parasite community in the new range compared to the native one (Dunn et al., 2012; Torchin et al., 2003). However, although the EICA hypothesis has been verified in several invasive plant systems (reviewed in Callaway et al., 2022), evidence for its occurrence in animals is still scarce (reviewed in Cornet et al., 2016).

Considering the complexity of vertebrates' immune system, Lee and Klasing (2004) further refined the EICA, postulating that an overall reduction of the immune response might be risky as invading hosts will be likely exposed to new pathogens. They proposed therefore that successful vertebrate invaders should adopt a trade-off between different immune pathways (Lee, 2006), dampening their costly systemic inflammatory response in favor of less expensive forms of immunity. Systemic inflammation (or acute-phase inflammation) is a severe, innate immune response that ultimately induces in the host a series of physiological states and sickness behaviors (e.g. anorexia, lethargy, fever) aimed at subtracting resources or at creating an unfavorable environment for the pathogen (Ashley et al., 2012; Dantzer et al., 2008). This

diffuse inflammatory reaction can be triggered by a strong response to an external stimulus, through the release of pro-inflammatory signaling molecules (i.e., cytokines) (Ashley et al., 2012; Chaplin, 2010). Systemic inflammation represents an important defense mechanism against pathogens, but it is also considered one of the most expensive types of responses in terms of nutritional and metabolic costs (Lochmiller and Deerenberg, 2000). It will generally cause in the host decreased activity, reduced growth, decreased reproductive output (e.g. Aubert et al. 1997a, b; Bonneaud et al. 2003; Adelman and Martin 2009; Lopes et al. 2014), and, when deregulated or misdirected, may result in severe tissue damage, followed by morbidity and mortality (Ashley et al., 2012; Graham et al., 2005). Because of parasite loss, investing in inflammation and sustaining its high costs might therefore be unnecessary or even harmful to invading hosts, who would benefit by mounting less vigorous responses (Lee and Klasing, 2004; Phillips et al., 2010a). A reduced investment in inflammation may in turn allow for a reallocation of resources towards reproduction and growth and individuals which are able to down-regulate this type of response will thus be favored by natural selection and increase in frequency within the population. At the same time, a parallel enhancement of less expensive types of immunity might be advantageous, either an increased investment in some other forms of the innate response (Phillips et al., 2010a) or in antibody-mediated immunity (Lee and Klasing, 2004). This shift in the immune strategy from a costly inflammatory response to a less expensive one could at least partially explain why some invaders are more successful than others, and why alien species often perform better in the invaded area compared to their native range in terms of population growth rate (Lee and Klasing, 2004; White and Perkins, 2012).

Based on this framework of hypotheses, the strength and type of invaders' immune response can be expected to vary also among populations of the same alien species along its invasion wave (Phillips et al., 2010a; White and Perkins, 2012). Firstly, because moving towards the leading margin of range expansion a decrease in pathogen prevalence is usually observed, due to stochastic reasons and lower host densities that reduce transmission (Phillips et al., 2010b). Secondly, because selective pressures for high dispersal and reproduction at the front should favor trade-offs with other energetically expensive processes such as immunity (Burton et al., 2010; Phillips et al., 2010a; Phillips and Perkins, 2019; White and Perkins, 2012).

Here, we used the invasive North American Eastern gray squirrel (*Sciurus carolinensis*) as a model to test the EICA hypothesis, investigating individual variation in squirrels' immune response along the invasion wave of the main metapopulation established in Italy. Moving from the core towards the invasion front, we expected to observe: 1) a progressive decrease in hosts' inflammatory response and 2) a parallel increase in acquired immunity. To test these hypotheses, we made use of animals culled within an invasive species control plan and coupled observational data about immune-related blood parameters and MHC class II gene expression with experimental data on the expression of TNF-α genes following an *ex vivo* immune challenge. Data regarding parasite circulation in the invaded area were also collected to verify the assumption of a decreasing parasitic pressure towards the invasion front.

2. Material and Methods

2.1. Study area and sampling of squirrels

Gray squirrels used in the present study were culled during alien species control activities at nine different sites located within the main and oldest Italian metapopulation (Piedmont region, NW Italy, Figure 1), which originated from the first introduction of the species in the country in 1948 (Bertolino et al., 2014). The sites were characterized by homogeneous climate and similar vegetation (i.e., lowland broadleaf-conifer woodland patches surrounded by an agricultural matrix, see Supplementary File 1 for a detailed description). Depending on their position and time since gray squirrels' establishment, the areas were classified as core (i.e., gray squirrels established for > 15 years before sampling), intermediate (i.e., 5 – 15 years) or front sites (i.e., < 5 years), obtaining three independent replicates per type of area (Figure 1). At these nine sites, from 2017 to 2018 we sampled a total of 126 squirrels (54 males, 72 females): 43 from the core, 38 from intermediate areas and 45 from the front. Squirrels were trapped by using live-traps (model 202, Tomahawk Live Trap Co., Wisconsin, USA) baited with hazelnuts, and euthanized on the field through CO_2 overdose in compliance with EC and AVMA guidelines (Close et al., 1997, 1996; Leary et al., 2020) (see Ethics statement for details on trapping and handling methods). Each individual was sexed and weighed, and blood samples were collected immediately on the field through postmortem intracardiac puncture and stored at 4°C in ethylenediamine tetraacetic acid (EDTA) blood collection tubes. Samples and

carcasses for the *ex vivo* culture and experimental immune challenge were transported to the laboratory at 4°C and within 3 hours from culling, in order to collect fresh cells samples.

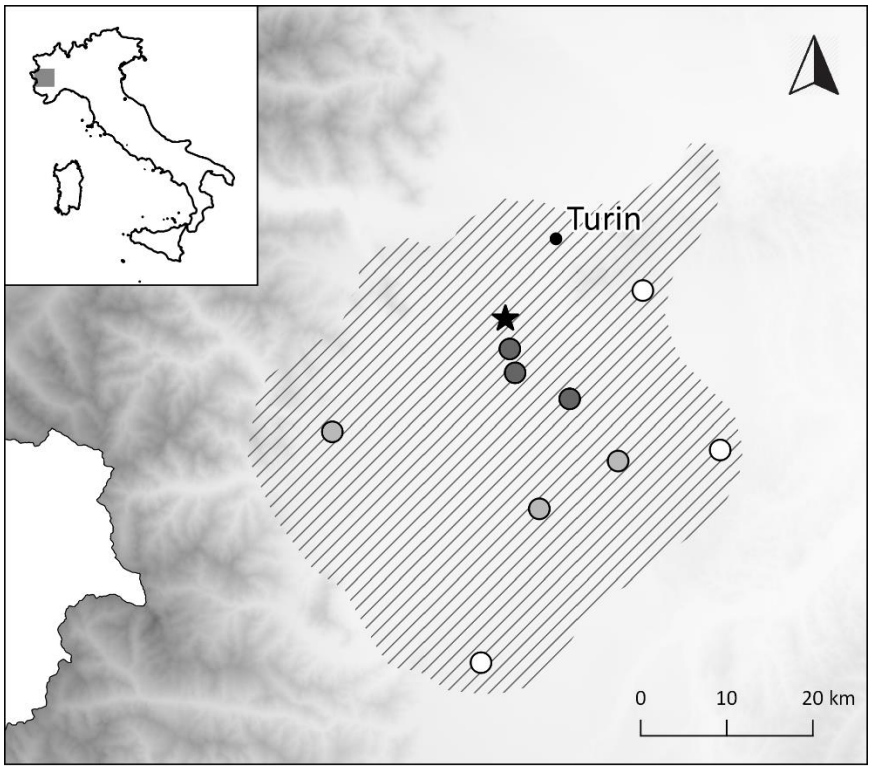


Figure 1. Location of sampling sites within the main Italian metapopulation of alien gray squirrels (*Sciurus carolinensis*) (shaded area, based on 2020 distribution data): core sites (dark gray circles, squirrels established for longer than 15 years), intermediate sites (light-gray circles, 5-15 years) and sites at the invasion front (white circles, <5 years). The star indicates the site of gray squirrels first release in 1948, from where they expanded mainly in a southwestern direction.

2.2 Study design and detailed hypotheses

Our set of hypotheses was based on the initial assumption of a progressive reduction in parasite prevalence from the core towards the front of the invasion range. To verify this condition, a preliminary parasitological analysis was carried out in the study area (see Section 2.3 for details on methods). Assuming such a decrease in parasitic pressure along the invasion wave, we expected to observe a parallel shift in the immune strategy of gray squirrels, with individuals at the invasion front showing 1) a dampened inflammatory response and 2) an enhanced acquired response. To test these two hypotheses, we used

multiple measures of immunity (Figure 2), combining hematological data and gene expression data and carrying out an *ex vivo* experimental immune challenge.

Firstly, we analyzed variation in selected hematological parameters (see "Parasitological and hematological analyses" for details on methods). Due to their involvement in the inflammatory response, hemoglobin concentration and platelet and neutrophil counts were considered proxies for inflammation (Figure 2, 1a, and 1b), while the lymphocyte count was considered a proxy for the acquired immune response since B and T cells are effectors of the response against specific pathogens (Figure 2, 2a). Finally, we predicted neutrophil-to-lymphocyte ratios (hereafter NLRs) to be lower in front animals, as they are expected to favor the acquired over the innate response (Figure 2, 2b).

Secondly, for the *ex vivo* experimental immune challenge and gene expression analysis, we isolated and cultured spleen and bone marrow cells from a subset of animals (see Section 2.4 for details on methods). Spleen and bone marrow tissues were chosen due to their different immunological roles, the first being involved mainly in lymphocyte development, while the latter is involved in the production of both lymphocytes and myeloid (neutrophils and monocytes) precursors. By means of quantitative PCR (qPCR), we therefore evaluated the basal expression of MHC Class II genes by spleen cells, and the expression of TNF-α genes by bone marrow cells before and after stimulation with bacterial lipopolysaccharide (LPS). LPS is a bacterial endotoxin that is found on the outer membrane of Gram-negative bacteria, and that can be used to simulate infection and elicit an immune response from vertebrate hosts, squirrels included (Prendergast et al., 2002; Previtali et al., 2012). The relative increase in TNF-α genes expression following LPS administration was considered a proxy for inflammation, as TNF-α is a pro-inflammatory cytokine produced by innate immune cells (monocyte/macrophages and neutrophils) after exposure to an inflammatory stimulus (Bradley, 2008; Falvo et al., 2010) (Figure 2, 1c). Conversely, MHC class II is a complex of molecules involved in the recognition of extracellular antigens and lymphocytes activation. MHC II is constitutively expressed by antigen-presenting cells (APCs), and induced in T lymphocytes (Holling et al., 2004; Neefjes et al., 2011). MHC II is therefore involved both in the innate and adaptive immune response pathways; however, we measured its expression limitedly to spleen cells. The spleen in mammals is the main secondary lymphoid organ, harboring mostly naïve lymphocytes which are locally primed by

APCs to initiate the adaptive immune response (Bronte and Pittet, 2013; Hensel et al., 2019). As such, MHC II basal expression by splenic cells was considered a proxy for the acquired immune response (Figure 2, 2c).

Main hypothesis		Proxy	Role	Prediction
1. Dampening of **inflammation** towards the invasion front 	**1a**	Platelet and neutrophil counts	participants to the inflammatory process	↓ front
	1b	Hemoglobin concentration	sequestered during inflammation	↑ front
	1c	TNF–α genes (expression after immune challenge)	encode for pro-inflammatory cytokines	↓ front
2. Enhancement of **acquired immunity** towards the invasion front 	**2a**	Lymphocyte counts	include cells involved in the acquired response	↑ front
	2b	Neutrophil to lymphocyte ratio	relative contribution of innate vs acquired response	↓ front
	2c	MHC class II genes (basal expression in spleen)	involved in T and B cells activation	↑ front

Figure 2. Main hypotheses and specific predictions regarding variation in the immune response of alien gray squirrels (*Sciurus carolinensis*) along the invasion wave.

2.3. Parasitological and hematological analyses

To investigate the spatial distribution of parasites along the invasion wave, faecal egg examinations were conducted on a total of 95 gray squirrels randomly sampled in the same study sites used for the immune survey. Briefly, faeces were collected at capture from the trap floor and stored dry at 4°C until examination, which was carried out within 3 days from collection to prevent eggs from hatching. Faecal samples were examined through faecal flotation with saturated NaCl solution (1200 g/l) as described in Romeo et al. (2014). Previous studies demonstrated that gray squirrels introduced in Italy experienced a relevant loss of pathogens compared to their native range: only a few viral and bacterial infections have been reported (Romeo et al., 2019, 2018, 2014a; Schilling et al., 2019; Schulze et al., 2020), and invaders show a particularly poor ecto- and endoparasite community (Hofmannová et al., 2016; Prediger et al., 2017;

Romeo et al., 2014c, 2014b; Santicchia et al., 2019). For this reason, we focussed only on gastro-intestinal helminths as a general proxy for infections.

Regarding hematological analyses, a complete blood count (CBC) and a leukocyte differential count (LDC) were run on the full sample of 126 squirrels. Whole blood stored in EDTA was processed within 48 hours from sampling by using a Sysmex XT-2000iV hematology laser analyzer (Sysmex Corporation, Kobe, Japan), adequately validated for the species (Romeo et al., 2021).

2.4. Ex vivo cultures, immune challenge, and gene expression analysis

The *ex vivo* experiment was carried out on a subset of 30 animals (15 males and 15 females; core: N=12; intermediate: N=9; front: N=9). From each animal, we collected the spleen for the analysis of MHC class II, and the bone marrow from the right femur for the LPS challenge and the analysis of TNF-α expression. Under a sterile hood, spleen and bone marrow cells were isolated, prepared for culture as described in Supplementary File 1, and plated in six wells plates at a concentration of $3x10^6$ cells/ml. Spleen cells were plated in duplicate and incubated for 4 hours at 37°C and 5% CO_2. Bone marrow cells were plated in duplicate for each condition: unstimulated and LPS 1 µg/ml (*E. coli* O111:B4 LPS, Sigma-Aldrich, St. Louis, MO, USA). Bone marrow cells were incubated at 37°C for 4 hours at 5% CO_2. After stimulation, cells of each well (both in suspension and in adhesion) were lysed with 1 ml of TRIreagent (Sigma-Aldrich, St. Louis, MO, USA) and stored at -20 °C until RNA extraction. Cells in suspension were centrifugated at 4 °C (5 minutes at 450 x g) and the supernatant was eliminated. Cell pellets were lysed with 1 ml of TRIreagent (Sigma-Aldrich, St. Louis, MO, USA) and the cells adhered to the corresponding well were also lysed and pooled together with the corresponding cell pellet. All lysed samples were stored at -20 °C until RNA extraction.

To investigate gene expression, total RNA was isolated following the manufacturer's instructions and quantified using a spectrophotometer (BioPhotometer, Eppendorf, Hamburg, Germany) at 260 nm wavelength. One µg of total RNA from each sample was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Foster City, CA, USA), accordingly to the manufacturer's instructions. The retro-transcribed cDNA obtained was used as a template for Real Time PCR in an optimized 25 µl volume reaction using Sybr Green chemicals, as previously described (Curone et al., 2018).

Due to the lack of species-specific primer pairs for target (TNF-α and MHC class II) and housekeeping (GAPDH) genes of *S. carolinensis,* primers were *de novo* designed based on available sequences from phylogenetically close species (red squirrel, *S. vulgaris,* for TNF-α and MHC class II, and marmot, *Marmota monax*, for GAPDH). Primers' design was carried out using the Primer Express Software (Applied Biosystem, Foster City, CA, USA) and their specificity assessed through qualitative PCR and sequencing of the amplicons. Quantitative Real-Time PCR was carried out in the 7000 Sequence Detection System (Applied Biosystem, Foster City, CA, USA) as previously described (Riva et al., 2010). Primers sequences and further details on cell culture, PCR and sequencing protocols are reported in Supplementary File 1 (Table S2 and S3).

2.5. Statistical analysis

Variation in blood parameters of gray squirrels was analyzed through a set of five Linear Models (LMs), using hemoglobin concentration, platelet counts, neutrophil counts, lymphocyte counts, and NLRs as response variables. Neutrophils, lymphocytes, and their ratios were log-transformed (ln(x+1)) to reduce skewness. On each of these parameters, we tested the effect of the study site's position (i.e., core, intermediate or front) and included as covariates a set of other variables that may potentially affect variation in blood parameters: squirrel sex, reproductive condition nested in sex (i.e., non-breeding or pregnant/ lactating for females, abdominal or scrotal testes for males), age class (i.e., subadult < 1yr old or adult), body mass nested in age class and season (i.e., spring, autumn or winter). The inclusion of study site ID as random intercept did not improve the fit of any of the models (likelihood ratio tests: all $p>0.05$), hence we started from full LMs and obtained minimal models through stepwise selection based on AICc. Interpretation of significant categorical variables with more than two levels was carried out through t-tests on differences of least square means, applying Tukey correction for multiple comparisons.

Variation in the expression of MHC class II and TNF-α by bone marrow and spleen cells was analyzed through two linear mixed models (LMMs), one for each gene, using efficiency-corrected RT-PCR Ct values as the response variable. The effect of site position on MHC class II and TNF-α basal expression, and the effect of treatment (control vs LPS) on TNF-α expression in each type of site were assessed through pre-planned

orthogonal contrasts. Normalization of target genes expression on the reference gene (GAPDH) was carried out within each model following the approach described in Steibel et al. (2009). Technical replicates and potential seasonal variability in gene expression were accounted for by including squirrel ID and season as random factors. Fold-changes and their 95% confidence limits were obtained through back-transformation of linear contrasts estimates. Normality of residuals was assessed visually.

All the analyses were carried out with PROC GLIMMIX and PROC MIXED, in SAS 9.4 software (Copyright © 2012 SAS Institute Inc., Cary, NC, USA). Gene expression data were analyzed by adapting to our data the code obtained through the SAS macro %QPCR_MIXED (Steibel et al., 2009).

3. Results

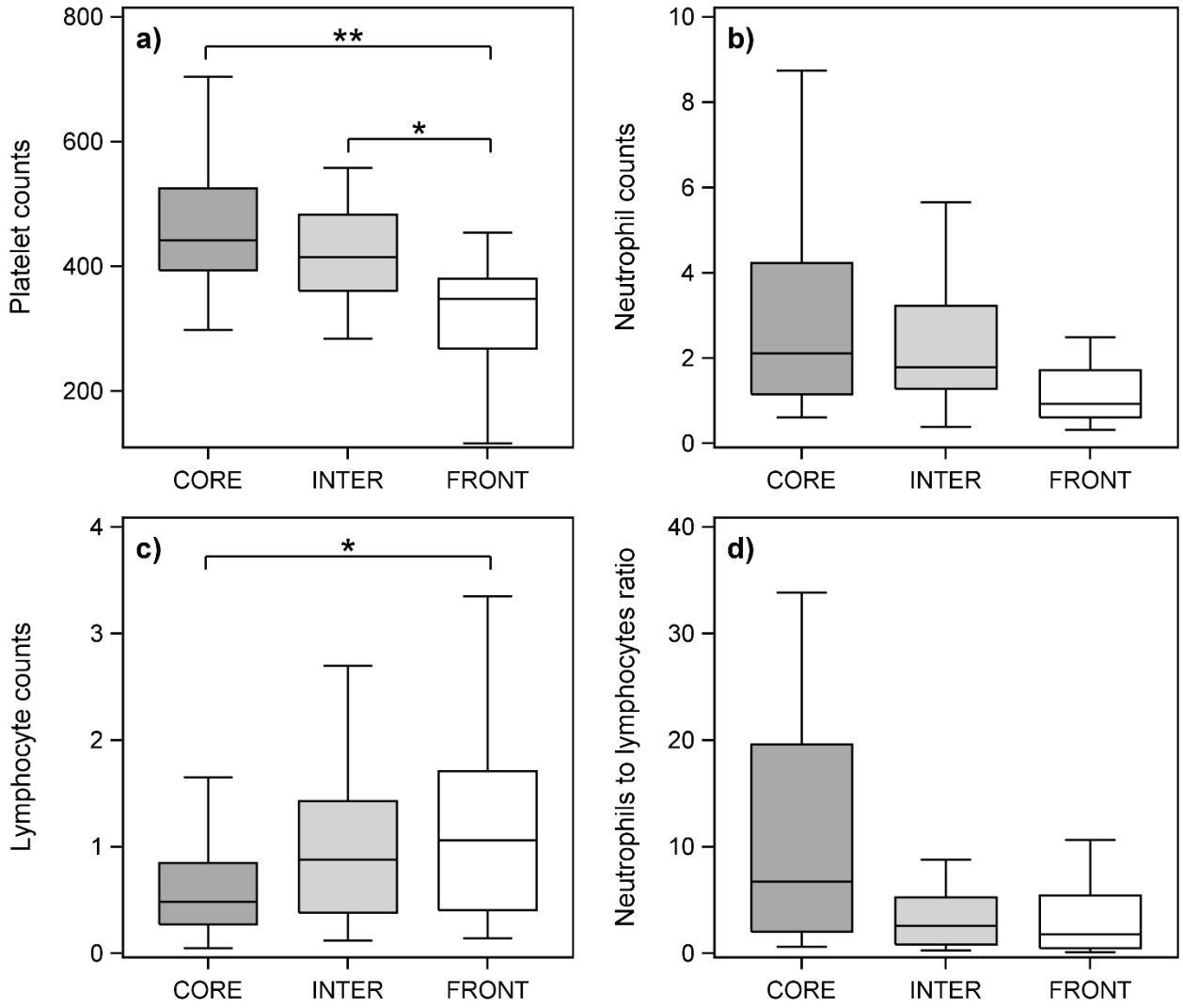
3.1. Parasitological analysis and post-mortem examination

Overall, 40% of faecal samples (38/95) were positive for helminth eggs: thirty-six individuals were infected only by the nematode *Strongyloides robustus*, in one animal we found only undetermined strongyle eggs, and another squirrel was infected by both. Endoparasites did not circulate uniformly in the study area: prevalence of infection gradually decreased moving from the core to the invasion front. While the prevalence of gastro-intestinal helminths at the invasion core was relatively high (i.e., 64%), moving outwards along the invasion wave it decreased markedly, with 37% prevalence in intermediate sites and 19% at the front (see Supplementary File 1, Table S1 for detailed data). Additionally, all the 126 squirrels used in this study were thoroughly inspected for pathological signs of other infections during post-mortem operations. All of them were in good condition and none showed any clinical sign of disease.

3.2. Variation in blood parameters

Mean values of selected hematological parameters in sampled gray squirrels and complete results of minimal models including all the variables affecting them are reported in Supplementary File 1 (Tables S4 and S5). Site position was retained in all selected models except for the one exploring variation in hemoglobin concentration. In particular, platelets ($F_{2,98}$=7.68; p=0.0008), lymphocyte counts ($F_{2,83}$=4.61; p=0.0126) and NLRs ($F_{2,79}$=3.42; p=0.0375) all varied significantly depending on a squirrels' position along

261 the invasion wave. In detail, the number of platelets in squirrels' blood was lower at the front than at the
262 core (parameter estimate ± SE=107.1 ± 27.5; t_{98}=3.90; p_{adj}=0.0005) and in intermediate sites (69.3 ± 29.5;
263 t_{98}=2.35; p_{adj}=0.04) (Figure 3a), while there was no difference in platelets counts between animals from
264 core and intermediate sites (p_{adj}=0.1). Squirrels living at the front had conversely a higher number of
265 lymphocytes than squirrels living at the invasion core (0.26 ± 0.10; t_{83}=2.57; p_{adj}=0.032) (Figure 3c). No
266 difference in lymphocyte counts between core animals and individuals from intermediate sites (p_{adj}=0.055),
267 nor between squirrels from intermediate sites and the invasion front (p_{adj}=0.7) was found. Overall, NLRs
268 varied significantly with site position as well, with higher ratios in animals living at the invasion core than in
269 intermediate or front sites (Figure 3d), however neither of the pair-wise comparisons was significant after p
270 value adjustment (p_{adj}=0.058 and p_{adj}=0.11, respectively). Finally, although neutrophil counts tended to
271 decrease from core to front (Figure 3b), the trend was not significant ($F_{2,79}$=2.57; p=0.082).
272
273 *3.3. Gene expression and immune challenge*
274 Both TNF-α ($F_{2,50}$=4.81; p=0.013) and MHC class II ($F_{2,25}$=5.11; p=0.014) basal expression by cultured cells
275 varied significantly depending on the position of the site inhabited by gray squirrels. Detailed results of pre-
276 planned contrasts are reported in Table S6. In particular, basal expression of MHC class II gene by spleen
277 cells was higher in animals from the invasion front than both core (parameter estimate=0.99 ± 0.32; t_{25}=3.1;
278 p=0.005) or intermediate sites (0.86 ± 0.36; t_{25}=2.43; p=0.023) (Figure 4a). Conversely, basal expression of
279 TNF-α gene by bone marrow cells was only higher in samples from core than intermediate sites (2.16 ±
280 0.63; t_{50}=3.45; p=0.012), while there was no difference with front sites (p>0.05). The response of bone
281 marrow cells to the immune challenge differed as well depending on the position of the site where
282 squirrels were sampled ($F_{5,50}$=9.29; p<0.0001). While the treatment had no effect on TNF-α expression in
283 samples from intermediate sites (p=0.2), LPS-stimulation induced a significant up-regulation in TNF-α
284 expression in samples from both core (3.29 ± 0.56; t_{50}=5.83; p<0.0001) and front sites (1.52 ± 0.68; t_{50}=2.26;
285 p=0.028) (Figure 4b). However, despite both responding to LPS stimulation, the increase in gene expression
286 was far greater in samples from core sites (Fold Change=9.8; 95% CI: 4.5 – 21.4) than in samples from the
287 invasion front (FC=2.9; 95% CI: 1.1 – 7.4).



288

289 **Figure 3.** Variation in hematological parameters of invasive gray squirrels (*Sciurus carolinensis*) along the

290 invasion wave: a) platelet counts (10^3/µl); b) neutrophil counts (10^3/µl); c) lymphocyte counts (10^3/µl); and

291 d) neutrophils to lymphocytes ratios.

292

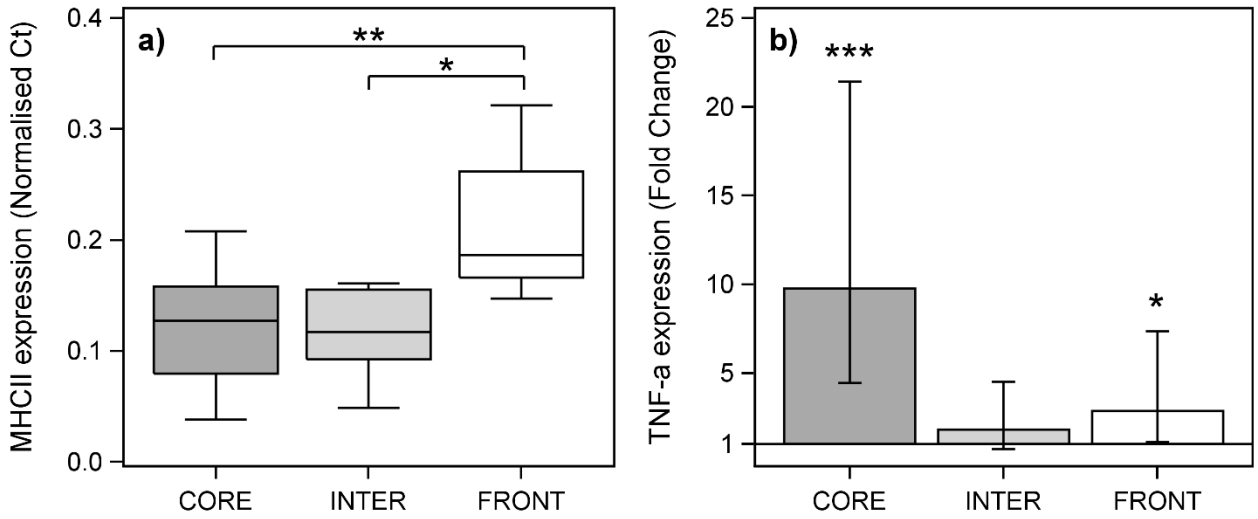


Figure 4. Expression of immune-related genes in invasive gray squirrels (*Sciurus carolinensis*) along the invasion wave: a) basal expression of MHC class II genes by spleen cells; and b) fold-change in TNF-α gene expression by bone marrow cells in response to LPS-stimulation. Error bars indicate 95% Confidence Interval.

4. Discussion

We investigated variation in the immune response of alien gray squirrels moving from the core of an expanding metapopulation, where gray squirrels had been established for 15 -30 years and parasite prevalence was high, to the invasion front, which was colonized less than 5 years before sampling and where parasites were nearly absent. Although the analyzed immune markers did not all concur to support the refined-EICA hypothesis (Lee and Klasing, 2004), overall our results suggest that populations living at the invasion front do favor a less costly acquired response over an inflammatory one. In detail, both platelet counts and experimental data on TNF-α gene expression following the immune challenge indicated a reduced inflammatory response in front populations compared to core populations, giving support to our first hypothesis (Figure 2, main hypothesis 1). Conversely, some of the markers associated with an acquired response (i.e., absolute lymphocyte counts and MHC class II basal expression) were significantly higher in squirrels living at the invasion front, supporting our second hypothesis (Figure 2, main hypothesis 2).

311 Platelets are important participants in the innate immune response, as they gather at the inflammation site
312 and release cytokines and chemokines to attract leukocytes and further enhance inflammation (Sonmez
313 and Sonmez, 2017). Hence, the linear decrease in the number of circulating platelets observed in gray
314 squirrels' blood when moving from core to front populations, may suggest a progressive dampening of the
315 inflammatory response. Among inflammation-related hematological variables however, only platelets fully
316 behaved as predicted: variation in hemoglobin concentration was not related to a squirrel's position along
317 the invasion wave, whereas the decrease in neutrophils, which are important indicators of systemic
318 inflammation, from core to front was not significant. On the other hand, NLRs, which were considered as a
319 surrogate marker of the shift between inflammatory and acquired response, did significantly vary with site
320 position, but the observed decrease from core to front sites was only marginally significant. A possible
321 reason for these unclear patterns might reside in the small sample size for neutrophil counts (and
322 consequently NLRs) in front sites (see Table S4), which likely hindered the detection of small-magnitude
323 effects.
324 Although observational data on inflammation-related hematological variables supported our prediction
325 only in part, experimental data on TNF-α gene expression confirmed the pattern suggested by platelet
326 counts and partially by NLRs, indicating a reduced inflammatory response in front populations. While TNF-α
327 basal expression by cultured bone marrow cells was similar in samples from the core and from the front,
328 they showed a differential response to the immune challenge: LPS-stimulation induced an almost 10-fold
329 increase in the expression of TNF-α in core samples, whereas front samples responded with just a 3-fold
330 increase (see Figure 3b). Since TNF-α is one of the main signaling proteins inducing systemic inflammation
331 (Bradley, 2008), this result strongly suggests that animals at the front respond to pathogens' threats (here
332 simulated by an LPS dose) with a reduced inflammation compared to animals living at the invasion core.
333 Interestingly, cultured cells from squirrels inhabiting intermediate sites showed an unexpected behavior as
334 their basal TNF-α expression was lower compared to the core and front animals, and they also did not
335 respond to LPS-stimulation. One possible explanation for this result could be that individuals living in
336 intermediate sites were maladapted to the parasitological conditions they were experiencing. Those areas

that were halfway between the core and the invasion front, until a few years earlier represented the leading margin of range expansion, where populations had presumably adapted to a reduced pathogen pressure. However, parasites that had lagged behind during host expansion progressively advance due to increased host densities, and conditions gradually become more similar to the core. It is thus possible that the lack of response to LPS-stimulation in squirrels at intermediate sites was due to a negative regulation of the inflammatory response pathway, consequent to a mismatch between their immunological phenotype and the actual parasitological conditions they were experiencing (Horrocks et al., 2011). Hematological variables of individuals living in intermediate sites showed indeed a mixed behavior as well, with platelet counts similar to those observed in core animals and lymphocyte counts conversely comparable to values from front squirrels. Similar results were obtained by Brown et al. (2015) on invasive cane toads (*Rhinella marina*) in Australia, where curvilinear relationships between some immune and physiological traits and time since population establishment were observed. In parallel to a reduction in the above-mentioned inflammation-related parameters, gray squirrels from the invasion front showed also higher MHC class II genes basal expression, higher lymphocyte counts and, as mentioned above, marginally lower NLRs compared to core individuals, suggesting a shift towards acquired immunity as predicted. In particular, since MHC class II molecules main functions lie in antigens binding and display, and in the subsequent activation of B and T cells (Chaplin, 2010; Neefjes et al., 2011), the higher MHC II gene expression observed in squirrels at the front could suggest a higher propensity for an acquired immune response when compared to core animals.

While there is a relevant body of literature concerning the EICA hypothesis in invasive plants (reviewed in Callaway et al., 2022), up to date, only a few studies investigated this hypothesis in invading vertebrates, with different approaches and contrasting results (reviewed in Cornet et al., 2016). For instance, Lee and colleagues (Lee et al., 2006, 2005) compared the immune response of two introduced passerine birds with different invasion success, finding that the most successful species (i.e., *Passer domesticus*) showed a weaker inflammatory reaction and a stronger antibody response compared to its less invasive congener (*P. montanus*), thus giving support to the EICA hypothesis. Similarly, at the biogeographical scale, Bailly and colleagues (Bailly et al., 2016) found that spectacled thrushes (*Turdus nudigensis*) showed a reduced

364 inflammatory response to an immune challenge in their introduction range compared to conspecifics in the
365 native range. However, in other vertebrate systems a pattern opposite to the EICA was found (i.e., Diagne
366 et al., 2017 on invasive rodents in Senegal), and other studies obtained mixed results, depending on the
367 different immune components that had been evaluated (i.e., Llewellyn et al., 2011; Brown et al., 2015;
368 Selechnik et al., 2017 on invasive cane toads in Australia). For example, a study comparing immunological
369 markers in native and invasive populations of the Egyptian goose (*Alopochen aegyptiacus*), reported higher
370 values of several energetically costly immune effectors in invasive geese, in contrast with EICA's predictions
371 (Prüter et al., 2020). However, the same invasive populations showed also higher variance for some of
372 these effectors, suggesting an increased plasticity in the immune response that would allow them to cope
373 better with new conditions. The authors suggest therefore that existing hypotheses, such as EICA, might
374 not fully cover the complex interplay between immunity and parasitism in biological invasions. The immune
375 response is indeed an extremely complex process, which involves many actors and is regulated by several
376 positive and negative feedback mechanisms of which we still have only a partial knowledge. The lack of
377 knowledge is even more challenging in the field of eco-immunology, as information about wild species
378 basic physiology, reference values and/or specific techniques to investigate their immune response is often
379 lacking (Boughton et al., 2011; Demas et al., 2011; Downs et al., 2014). For these reasons, to gain a clearer
380 picture of underlying immunological processes, it is advisable to measure as many immune traits as
381 possible, instead of focusing on a single component/indicator (Hawley and Altizer, 2011; Pedersen and
382 Babayan, 2011; Schoenle et al., 2018).
383 To test our hypotheses, we made use of different hematological and molecular variables coupled with an
384 experimental approach that allowed us to evaluate marginal variation in squirrels' immune responses
385 independently from their basal immune status. Although some parameters converged towards the same
386 results, partially supporting our predictions, we are aware that the range of immunological markers we
387 analyzed and the sample size for the *ex vivo* assay are still too limited to draw any robust conclusions on
388 the EICA hypothesis. This was due to both cost constraints and problems in the design of adequate primers
389 and set up of the experimental protocols. Nevertheless, despite these limitations, we believe that our work

adds valuable data to the still limited body of evidence regarding the role of immunological adaptations in the invasion process, contributing to shed more light on the potential mechanisms underlying invasiveness.

5. Conclusions

Our results suggest a shift in the immune response of invasive gray squirrels along the invasion wave, partially supporting the notion that individuals at the invasion front, where parasite prevalence is low and selective pressures for dispersal and reproduction are high, adopt a different immune strategy by reducing investment in costly inflammatory responses in favor of acquired immunity. Such flexibility in the immune strategy might be one of the mechanisms underlying the success of invasive alien species, as it may allow a reallocation of resources towards other processes, facilitating in turn population establishment, growth and spread. In a broader context, this kind of immunological adaptations along spatial gradients might indeed be common even in naturally expanding animal populations, assuming that infection gradients along the expansion wave occur. Although the complex immunological mechanisms underlying these shifts in defense strategies still need to be clarified, future research should also focus on quantifying the energetic costs and benefits of investing in different immune pathways in order to gain a better understanding of the ecological trade-offs adopted by individuals to cope with new parasitological conditions.

Ethics approval

This study did not require any ethics approval because gray squirrels were culled as part of a mandatory control program of Invasive Alien Species to adhere to the national and European guidelines (ITA Decree No. 230/2017; EU Regulation No. 1143/2014). Operators were trained by veterinary staff specialized in animal welfare and euthanasia methods followed EC and AVMA guidelines (Close et al., 1997, 1996; Leary et al., 2020). Trapping and handling of squirrels complied with the Italian national law on wildlife (L.N. 157 of 1992) and were carried out with permits of the authorities for wildlife research and management of Turin and Cuneo Provinces (D.D. n. 62-3025/2017 and Prot. n. 2016/98585 of 29/12/2016, respectively) based on the scientific guidelines and approval from the Italian Institute for Environmental Protection and Research (ISPRA).

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Claudia Romeo and Nicola Ferrari: conceptualization; Claudia Romeo, Joel Filipe, Federica Riva and Stefano Comazzi: methodology; Claudia Romeo, Joel Filipe, Federica Riva and Lucas A. Wauters: investigation; Federica Riva, Lucas A. Wauters and Stefano Comazzi: resources; Claudia Romeo and Joel Filipe; data curation; Claudia Romeo: project administration, formal analysis, visualization and writing – original draft; Joel Filipe, Federica Riva, Stefano Comazzi, Lucas A. Wauters and Nicola Ferrari: writing – review & editing; Nicola Ferrari: supervision and funding acquisition.

Acknowledgments

This research was part of the project "Immune response of invasive species as a driver of parasite dynamics" funded by the Department of Veterinary Medicine, Università degli Studi di Milano (Piano di Sostegno alla Ricerca, Linea 2). We would like to thank all the private estate owners for allowing sampling and the collection of field data, and all the students who helped with trapping and laboratory analyses.

Data availability

All data generated or analysed during this study are included in this published article as supplementary material (Supplementary File 2).

References

Adelman, J.S., Martin, L.B., 2009. Vertebrate sickness behaviors: adaptive and integrated neuroendocrine immune responses. Integr. Comp. Biol. icp028. https://doi.org/10.1093/icb/icp028

Ashley, N.T., Weil, Z.M., Nelson, R.J., 2012. Inflammation: Mechanisms, Costs, and Natural Variation. Annu. Rev. Ecol. Evol. Syst. 43, 385–406. https://doi.org/10.1146/annurev-ecolsys-040212-092530

Aubert, Arnaud, Goodall, G., Dantzer, R., Gheusi, G., 1997. Differential Effects of Lipopolysaccharide on Pup Retrieving and Nest Building in Lactating Mice. Brain. Behav. Immun. 11, 107–118. https://doi.org/10.1006/brbi.1997.0485

Aubert, A., Kelley, K.W., Dantzer, R., 1997. Differential Effect of Lipopolysaccharide on Food Hoarding Behavior and Food Consumption in Rats. Brain. Behav. Immun. 11, 229–238. https://doi.org/10.1006/brbi.1997.0503

Bailly, J., Garnier, S., Khimoun, A., Arnoux, E., Eraud, C., Goret, J.-Y., Luglia, T., Gaucher, P., Faivre, B., 2016. Reduced inflammation in expanding populations of a neotropical bird species. Ecol. Evol. n/a-n/a. https://doi.org/10.1002/ece3.2486

Bellard, C., Cassey, P., Blackburn, T.M., 2016. Alien species as a driver of recent extinctions. Biol. Lett. 12, 20150623. https://doi.org/10.1098/rsbl.2015.0623
Bertolino, S., Montezemolo, N.C. di, Preatoni, D.G., Wauters, L.A., Martinoli, A., 2014. A grey future for Europe: Sciurus carolinensis is replacing native red squirrels in Italy. Biol. Invasions 16, 53–62. https://doi.org/10.1007/s10530-013-0502-3
Blossey, B., Notzold, R., 1995. Evolution of Increased Competitive Ability in Invasive Nonindigenous Plants: A Hypothesis. J. Ecol. 83, 887. https://doi.org/10.2307/2261425
Bonneaud, C., Mazuc, J., Gonzalez, G., Haussy, C., Chastel, O., Faivre, B., Sorci, G., 2003. Assessing the Cost of Mounting an Immune Response. Am. Nat. 161, 367–379. https://doi.org/10.1086/346134
Boughton, R.K., Joop, G., Armitage, S.A.O., 2011. Outdoor immunology: methodological considerations for ecologists. Funct. Ecol. 25, 81–100. https://doi.org/10.1111/j.1365-2435.2010.01817.x
Bradley, J.R., 2008. TNF-mediated inflammatory disease. J. Pathol. 214, 149–160. https://doi.org/10.1002/path.2287
Bronte, V., Pittet, M.J., 2013. The spleen in local and systemic regulation of immunity. Immunity 39, 806–818. https://doi.org/10.1016/j.immuni.2013.10.010
Brown, G.P., Kelehear, C., Shilton, C.M., Phillips, B.L., Shine, R., 2015. Stress and immunity at the invasion front: a comparison across cane toad (Rhinella marina) populations. Biol. J. Linn. Soc. 116, 748–760. https://doi.org/10.1111/bij.12623
Burton, O.J., Phillips, B.L., Travis, J.M.J., 2010. Trade-offs and the evolution of life-histories during range expansion. Ecol. Lett. 13, 1210–1220. https://doi.org/10.1111/j.1461-0248.2010.01505.x
Callaway, R.M., Lucero, J.E., Hierro, J.L., Lortie, C.J., 2022. The EICA is dead? Long live the EICA! Ecol. Lett. 25, 2289–2302. https://doi.org/10.1111/ele.14088
Chaplin, D.D., 2010. Overview of the immune response. J. Allergy Clin. Immunol., 2010 Primer on Allergic and Immunologic Diseases 125, S3–S23. https://doi.org/10.1016/j.jaci.2009.12.980
Close, B., Banister, K., Baumans, V., Bernoth, E.-M., Bromage, N., Bunyan, J., Erhardt, W., Flecknell, P., Gregory, N., Hackbarth, H., Morton, D., Warwick, C., 1997. Recommendations for euthanasia of experimental animals: Part 2. Lab. Anim. 31, 1–32. https://doi.org/10.1258/002367797780600297
Close, B., Banister, K., Baumans, V., Bernoth, E.-M., Bromage, N., Bunyan, J., Erhardt, W., Flecknell, P., Gregory, N., Hackbarth, H., Morton, D., Warwick, C., 1996. Recommendations for euthanasia of experimental animals: Part 1. Lab. Anim. 30, 293–316. https://doi.org/10.1258/002367796780739871
Cornet, S., Brouat, C., Diagne, C., Charbonnel, N., 2016. Eco-immunology and bioinvasion: revisiting the evolution of increased competitive ability hypotheses. Evol. Appl. 9, 952–962. https://doi.org/10.1111/eva.12406
Curone, G., Filipe, J., Cremonesi, P., Trevisi, E., Amadori, M., Pollera, C., Castiglioni, B., Turin, L., Tedde, V., Vigo, D., Moroni, P., Minuti, A., Bronzo, V., Addis, M.F., Riva, F., 2018. What we have lost: Mastitis resistance in Holstein Friesians and in a local cattle breed. Res. Vet. Sci., Periparturient Immune Function and the Acute Phase Response in Dairy Animals 116, 88–98. https://doi.org/10.1016/j.rvsc.2017.11.020
Dantzer, R., O'Connor, J.C., Freund, G.G., Johnson, R.W., Kelley, K.W., 2008. From inflammation to sickness and depression: when the immune system subjugates the brain. Nat. Rev. Neurosci. 9, 46–56. https://doi.org/10.1038/nrn2297
Demas, G.E., Zysling, D.A., Beechler, B.R., Muehlenbein, M.P., French, S.S., 2011. Beyond phytohemagglutinin: assessing vertebrate immune function across ecological contexts. J. Anim. Ecol. 80, 710–730. https://doi.org/10.1111/j.1365-2656.2011.01813.x
Diagne, C., Gilot-Fromont, E., Cornet, S., Husse, L., Doucouré, S., Dalecky, A., Bâ, K., Kane, M., Niang, Y., Diallo, M., Sow, A., Fossati-Gaschignard, O., Piry, S., Artige, E., Sembène, M., Brouat, C., Charbonnel, N., 2017. Contemporary variations of immune responsiveness during range expansion of two invasive rodents in Senegal. Oikos 126, 435–446. https://doi.org/10.1111/oik.03470
Diagne, C., Leroy, B., Vaissière, A.-C., Gozlan, R.E., Roiz, D., Jarić, I., Salles, J.-M., Bradshaw, C.J.A., Courchamp, F., 2021. High and rising economic costs of biological invasions worldwide. Nature 592, 571–576. https://doi.org/10.1038/s41586-021-03405-6

Downs, C.J., Adelman, J.S., Demas, G.E., 2014. Mechanisms and Methods in Ecoimmunology: Integrating Within-Organism and Between-Organism Processes. Integr. Comp. Biol. 54, 340–352. https://doi.org/10.1093/icb/icu082

Dunn, A.M., Perkins, S.E., 2012. Editorial: Invasions and infections. Funct. Ecol. 26, 1234–1237. https://doi.org/10.1111/1365-2435.12022

Dunn, A.M., Torchin, M.E., Hatcher, M.J., Kotanen, P.M., Blumenthal, D.M., Byers, J.E., Coon, C.A.C., Frankel, V.M., Holt, R.D., Hufbauer, R.A., Kanarek, A.R., Schierenbeck, K.A., Wolfe, L.M., Perkins, S.E., 2012. Indirect effects of parasites in invasions. Funct. Ecol. 26, 1262–1274. https://doi.org/10.1111/j.1365-2435.2012.02041.x

Enders, M., Havemann, F., Ruland, F., Bernard-Verdier, M., Catford, J.A., Gómez-Aparicio, L., Haider, S., Heger, T., Kueffer, C., Kühn, I., Meyerson, L.A., Musseau, C., Novoa, A., Ricciardi, A., Sagouis, A., Schittko, C., Strayer, D.L., Vilà, M., Essl, F., Hulme, P.E., van Kleunen, M., Kumschick, S., Lockwood, J.L., Mabey, A.L., McGeoch, M.A., Palma, E., Pyšek, P., Saul, W.-C., Yannelli, F.A., Jeschke, J.M., 2020. A conceptual map of invasion biology: Integrating hypotheses into a consensus network. Glob. Ecol. Biogeogr. 29, 978–991. https://doi.org/10.1111/geb.13082

Falvo, J.V., Tsytsykova, A.V., Goldfeld, A.E., 2010. Transcriptional Control of the TNF Gene. TNF Pathophysiol. 11, 27–60. https://doi.org/10.1159/000289196

Graham, A.L., Allen, J.E., Read, A.F., 2005. Evolutionary Causes and Consequences of Immunopathology. Annu. Rev. Ecol. Evol. Syst. 36, 373–397. https://doi.org/10.1146/annurev.ecolsys.36.102003.152622

Hawley, D.M., Altizer, S.M., 2011. Disease ecology meets ecological immunology: understanding the links between organismal immunity and infection dynamics in natural populations. Funct. Ecol. 25, 48–60. https://doi.org/10.1111/j.1365-2435.2010.01753.x

Hensel, J.A., Khattar, V., Ashton, R., Ponnazhagan, S., 2019. Characterization of immune cell subtypes in three commonly used mouse strains reveals gender and strain-specific variations. Lab. Invest. 99, 93–106. https://doi.org/10.1038/s41374-018-0137-1

Hofmannová, L., Romeo, C., Štohanzlová, L., Jirsová, D., Mazzamuto, M.V., Wauters, L.A., Ferrari, N., Modrý, D., 2016. Diversity and host specificity of coccidia (Apicomplexa: Eimeriidae) in native and introduced squirrel species. Eur. J. Protistol. 56, 1–14. https://doi.org/10.1016/j.ejop.2016.04.008

Holling, T.M., Schooten, E., van Den Elsen, P.J., 2004. Function and regulation of MHC class II molecules in T-lymphocytes: of mice and men. Hum. Immunol. 65, 282–290. https://doi.org/10.1016/j.humimm.2004.01.005

Horrocks, N.P.C., Matson, K.D., Tieleman, B.I., 2011. Pathogen Pressure Puts Immune Defense into Perspective. Integr. Comp. Biol. 51, 563–576. https://doi.org/10.1093/icb/icr011

Jeschke, J.M., Bacher, S., Blackburn, T.M., Dick, J.T.A., Essl, F., Evans, T., Gaertner, M., Hulme, P.E., Kühn, I., Mrugała, A., Pergl, J., Pyšek, P., Rabitsch, W., Ricciardi, A., Richardson, D.M., Sendek, A., Vilà, M., Winter, M., Kumschick, S., 2014. Defining the Impact of Non-Native Species. Conserv. Biol. 28, 1188–1194. https://doi.org/10.1111/cobi.12299

Leary, S., Pharmaceuticals, F., Underwood, W., Anthony, R., Cartner, S., Johnson, C.L., Patterson-Kane, E., 2020. AVMA Guidelines for the Euthanasia of Animals: 2020 Edition 121.

Lee, K.A., 2006. Linking immune defenses and life history at the levels of the individual and the species. Integr. Comp. Biol. 46, 1000–1015. https://doi.org/10.1093/icb/icl049

Lee, K.A., Klasing, K.C., 2004. A role for immunology in invasion biology. Trends Ecol. Evol. 19, 523–529. https://doi.org/10.1016/j.tree.2004.07.012

Lee, K.A., Martin, L.B., Hasselquist, D., Ricklefs, R.E., Wikelski, M., 2006. Contrasting adaptive immune defenses and blood parasite prevalence in closely related Passer sparrows. Oecologia 150, 383–392. https://doi.org/10.1007/s00442-006-0537-6

Lee, K.A., Martin, L.B., Wikelski, M.C., 2005. Responding to inflammatory challenges is less costly for a successful avian invader, the house sparrow (Passer domesticus), than its less-invasive congener. Oecologia 145, 243–250. https://doi.org/10.1007/s00442-005-0113-5

Llewellyn, D., Thompson, M.B., Brown, G.P., Phillips, B.L., Shine, R., 2011. Reduced investment in immune function in invasion-front populations of the cane toad (Rhinella marina) in Australia. Biol. Invasions 14, 999–1008. https://doi.org/10.1007/s10530-011-0135-3

555 Lochmiller, R.L., Deerenberg, C., 2000. Trade-offs in evolutionary immunology: just what is the cost of
556 immunity? Oikos 88, 87–98. https://doi.org/10.1034/j.1600-0706.2000.880110.x
557 Lopes, P.C., Springthorpe, D., Bentley, G.E., 2014. Increased activity correlates with reduced ability to
558 mount immune defenses to endotoxin in zebra finches. J. Exp. Zool. Part Ecol. Genet. Physiol. 321,
559 422–431. https://doi.org/10.1002/jez.1873
560 May, R.M., Anderson, R.M., 1983. Epidemiology and Genetics in the Coevolution of Parasites and Hosts.
561 Proc. R. Soc. Lond. B Biol. Sci. 219, 281–313. https://doi.org/10.1098/rspb.1983.0075
562 Neefjes, J., Jongsma, M.L.M., Paul, P., Bakke, O., 2011. Towards a systems understanding of MHC class I and
563 MHC class II antigen presentation. Nat. Rev. Immunol. 11, 823–836.
564 https://doi.org/10.1038/nri3084
565 Pedersen, A.B., Babayan, S.A., 2011. Wild immunology. Mol. Ecol. 20, 872–880.
566 https://doi.org/10.1111/j.1365-294X.2010.04938.x
567 Phillips, B.L., Brown, G.P., Shine, R., 2010a. Life-history evolution in range-shifting populations. Ecology 91,
568 1617–1627. https://doi.org/10.1890/09-0910.1
569 Phillips, B.L., Kelehear, C., Pizzatto, L., Brown, G.P., Barton, D., Shine, R., 2010b. Parasites and pathogens lag
570 behind their host during periods of host range advance. Ecology 91, 872–881.
571 https://doi.org/10.1890/09-0530.1
572 Phillips, B.L., Perkins, T.A., 2019. Spatial sorting as the spatial analogue of natural selection. Theor. Ecol. 12,
573 155–163. https://doi.org/10.1007/s12080-019-0412-9
574 Prediger, J., Horčičková, M., Hofmannová, L., Sak, B., Ferrari, N., Mazzamuto, M.V., Romeo, C., Wauters,
575 L.A., McEvoy, J., Kváč, M., 2017. Native and introduced squirrels in Italy host different
576 Cryptosporidium spp. Eur. J. Protistol. 61, 64–75. https://doi.org/10.1016/j.ejop.2017.09.007
577 Prendergast, B.J., Freeman, D.A., Zucker, I., Nelson, R.J., 2002. Periodic arousal from hibernation is
578 necessary for initiation of immune responses in ground squirrels. Am. J. Physiol. - Regul. Integr.
579 Comp. Physiol. 282, R1054–R1062. https://doi.org/10.1152/ajpregu.00562.2001
580 Previtali, M.A., Ostfeld, R.S., Keesing, F., Jolles, A.E., Hanselmann, R., Martin, L.B., 2012. Relationship
581 between pace of life and immune responses in wild rodents. Oikos 121, 1483–1492.
582 https://doi.org/10.1111/j.1600-0706.2012.020215.x
583 Prüter, H., Franz, M., Twietmeyer, S., Böhm, N., Middendorff, G., Portas, R., Melzheimer, J., Kolberg, H., von
584 Samson-Himmelstjerna, G., Greenwood, A.D., Lüschow, D., Mühldorfer, K., Czirják, G.Á., 2020.
585 Increased immune marker variance in a population of invasive birds. Sci. Rep. 10, 21764.
586 https://doi.org/10.1038/s41598-020-78427-7
587 Riva, F., Rahman, M.M., Turin, L., Ceciliani, F., Russo, S., Tribbioli, G., Lecchi, C., 2010. TIR8 receptor
588 expression in bovine tissues. Vet. Immunol. Immunopathol. 136, 65–70.
589 https://doi.org/10.1016/j.vetimm.2010.02.009
590 Romeo, C., D'Alessandro, S., Bielli, M., Comazzi, S., Ferrari, N., 2021. Reference intervals for hematological
591 variables in wild Eastern grey squirrels (Sciurus carolinensis). Eur. J. Wildl. Res. 67, 6.
592 https://doi.org/10.1007/s10344-020-01443-5
593 Romeo, C., Ferrari, N., Rossi, C., Everest, D.J., Grierson, S.S., Lanfranchi, P., Martinoli, A., Saino, N., Wauters,
594 L.A., Hauffe, H.C., 2014a. Ljungan Virus and an Adenovirus in Italian Squirrel Populations. J. Wildl.
595 Dis. 50, 409–411. https://doi.org/10.7589/2013-10-260
596 Romeo, C., Lecollinet, S., Caballero, J., Isla, J., Luzzago, C., Ferrari, N., García-Bocanegra, I., 2018. Are tree
597 squirrels involved in the circulation of flaviviruses in Italy? Transbound. Emerg. Dis. 65, 1372–1376.
598 https://doi.org/10.1111/tbed.12874
599 Romeo, C., McInnes, C.J., Dale, T.D., Shuttleworth, C., Bertolino, S., Wauters, L.A., Ferrari, N., 2019. Disease,
600 invasions and conservation: no evidence of squirrelpox virus in grey squirrels introduced to Italy.
601 Anim. Conserv. 22, 14–23. https://doi.org/10.1111/acv.12433
602 Romeo, C., Wauters, L.A., Cauchie, S., Martinoli, A., Matthysen, E., Saino, N., Ferrari, N., 2014b. Faecal egg
603 counts from field experiment reveal density dependence in helminth fecundity: Strongyloides
604 robustus infecting grey squirrels (Sciurus carolinensis). Parasitol. Res. 113, 3403–3408.
605 https://doi.org/10.1007/s00436-014-4005-7
606 Romeo, C., Wauters, L.A., Ferrari, N., Lanfranchi, P., Martinoli, A., Pisanu, B., Preatoni, D.G., Saino, N.,
607 2014c. Macroparasite Fauna of Alien Grey Squirrels (Sciurus carolinensis): Composition, Variability

608 and Implications for Native Species. PLoS ONE 9, e88002.
609 https://doi.org/10.1371/journal.pone.0088002
610 Santicchia, F., Romeo, C., Ferrari, N., Matthysen, E., Vanlauwe, L., Wauters, L.A., Martinoli, A., 2019. The
611 price of being bold? Relationship between personality and endoparasitic infection in a tree squirrel.
612 Mamm. Biol. 97, 1–8. https://doi.org/10.1016/j.mambio.2019.04.007
613 Schilling, A.-K., Avanzi, C., Ulrich, R.G., Busso, P., Pisanu, B., Ferrari, N., Romeo, C., Mazzamuto, M.V.,
614 McLuckie, J., Shuttleworth, C.M., Del-Pozo, J., Lurz, P.W.W., Escalante-Fuentes, W.G., Ocampo-
615 Candiani, J., Vera-Cabrera, L., Stevenson, K., Chapuis, J.-L., Meredith, A.L., Cole, S.T., 2019. British
616 Red Squirrels Remain the Only Known Wild Rodent Host for Leprosy Bacilli. Front. Vet. Sci. 6.
617 https://doi.org/10.3389/fvets.2019.00008
618 Schoenle, L.A., Downs, C.J., Martin, L.B., 2018. An Introduction to Ecoimmunology, in: Cooper, E.L. (Ed.),
619 Advances in Comparative Immunology. Springer International Publishing, Cham, pp. 901–932.
620 https://doi.org/10.1007/978-3-319-76768-0_26
621 Schulze, V., Lurz, P.W.W., Ferrari, N., Romeo, C., Steele, M.A., Marino, S., Mazzamuto, M.V., Calvignac-
622 Spencer, S., Schlottau, K., Beer, M., Ulrich, R.G., Ehlers, B., 2020. Search for polyoma-, herpes-, and
623 bornaviruses in squirrels of the family Sciuridae. Virol. J. 17, 42. https://doi.org/10.1186/s12985-
624 020-01310-4
625 Selechnik, D., West, A.J., Brown, G.P., Fanson, K.V., Addison, B., Rollins, L.A., Shine, R., 2017. Effects of
626 invasion history on physiological responses to immune system activation in invasive Australian cane
627 toads. PeerJ 5, e3856. https://doi.org/10.7717/peerj.3856
628 Simberloff, D., Martin, J.-L., Genovesi, P., Maris, V., Wardle, D.A., Aronson, J., Courchamp, F., Galil, B.,
629 García-Berthou, E., Pascal, M., Pyšek, P., Sousa, R., Tabacchi, E., Vilà, M., 2013. Impacts of biological
630 invasions: what's what and the way forward. Trends Ecol. Evol. 28, 58–66.
631 https://doi.org/10.1016/j.tree.2012.07.013
632 Sonmez, O., Sonmez, M., 2017. Role of platelets in immune system and inflammation. Porto Biomed. J. 2,
633 311–314. https://doi.org/10.1016/j.pbj.2017.05.005
634 Steibel, J.P., Poletto, R., Coussens, P.M., Rosa, G.J.M., 2009. A powerful and flexible linear mixed model
635 framework for the analysis of relative quantification RT-PCR data. Genomics 94, 146–152.
636 https://doi.org/10.1016/j.ygeno.2009.04.008
637 Torchin, M.E., Lafferty, K.D., Dobson, A.P., McKenzie, V.J., Kuris, A.M., 2003. Introduced species and their
638 missing parasites. Nature 421, 628–630. https://doi.org/10.1038/nature01346
639 White, T.A., Perkins, S.E., 2012. The ecoimmunology of invasive species. Funct. Ecol. 26, 1313–1323.
640 https://doi.org/10.1111/1365-2435.12012
641
642