Large wildlife removal drives immune defence increases in rodents

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Summary

1. Anthropogenic disturbances involving land use change, climate disruption, pollution and invasive species have been shown to impact immune function of wild animals. These immune changes have direct impacts on the fitness of impacted animals and, also, potentially indirect effects on other species and on ecological processes, notably involving the spread of infectious disease. Here, we investigate whether the selective loss of large wildlife can also drive changes in immune function of other consumer species.

2. Using a long-standing large-scale exclosure experiment in East Africa, we investigated the effects of selective removal of large wildlife on multiple measures of immune function in the dominant small rodent in the system, the East African pouched mouse, Saccostomus mearnsi.

3. We find support for a general increase in immune function in landscapes where large wildlife has been removed, but with some variation across immune parameters. These changes may be mediated in part by increased pathogen pressure in plots where large wildlife has been removed due to major increases in rodent density in such plots, but other factors such as changes in food resources are also likely involved.

4. Overall, our research reveals that the elimination of large-bodied wildlife – now recognized as another major form of global anthropogenic change – may have cascading effects on immune health, with the potential for these effects to also impact disease dynamics in ecological communities.

Key-words: defaunation, ecoimmunology, Kenya Long-term Exclosure Experiment, rodent, wildlife decline

Introduction

Large wildlife is being selectively lost from ecosystems around the world (Dirzo et al. 2014). This pattern of defaunation has strong consequences for the abundance, composition and behaviour of smaller animal species (Goheen et al. 2010; Kurten 2013; Young et al. 2015a,b). These changes ultimately lead to powerful, often transformative, effects on a wide range of ecosystem functions and services (Estes et al. 2011; Atwood et al. 2013; Dirzo et al. 2014). For example, in African savanna ecosystems, declines in large mammals lead to changes in herbivory, seed predation, plant recruitment, fire intensity and disease dynamics – with most of these responses due to changes in abundance or behaviour of small-bodied mammals and insects (Dirzo et al. 2014; Keesing & Young 2014; Kimuyu et al. 2014; Young et al. 2014). Similarly, in the oceans, loss of great whales is thought to have driven cascading changes in abundance of smaller predators and their prey, triggering profound reductions in carbon sequestration (Springer et al. 2003; Wilmers et al. 2012). Another less explored way in which large animal loss may drive changes in ecosystem functioning is via changes in the physiology of remaining species (Leroux, Hawlena & Schmitz 2012;
Strickland et al. 2013); however, several studies are now beginning to document strong changes in consumer physiology and morphology following removal or addition of other species. For example, variation in the abundance of predators drives changes in tail length in tadpoles (Maher, Werner & Denver 2013), carbon to nitrogen ratios in grasshoppers (Hawlena & Schmitz 2010) and stress hormones across a range of species (Berger et al. 2007; Martin et al. 2010). These physiological changes have in turn been shown to drive changes in a range of ecosystem processes, including decomposition, nutrient cycling and disease control (Martin et al. 2010, Schmitz, Hawlena & Trussell 2010; Hawlena et al. 2012; Strickland et al. 2013).

Thus far, relatively less research has investigated immunological responses to changes in community composition, despite the fact that such changes may have important implications for spread of diseases (including zoonoses). To begin to fill this gap, we examined the effect of large wildlife loss on immune function of wild rodents in a natural setting. Large mammals, both domestic and wild, can greatly impact the density and behaviour of rodents (Heske & Campbell 1991; Keesing and Crawford 2001, Smit et al. 2001; Keesing & Young 2014). These impacts are typically mediated by changes in resource availability or predation risk (Keesing & Young 2014). Additionally, variability in resource availability and host density can both cause changes in the degree or type of investment in immune function (Seiter 2011; Groner et al. 2013; Morosinotto et al. 2013; Zanette, Clinchy & Suraci 2014). Free-ranging animals typically experience much stronger variation in immune function than is observed in laboratory or captive animals, but much remains unknown about both direct and indirect drivers of variation in immune function in the wild (Abolins et al. 2011). Species loss and changes in community structure (e.g. changes in species relative abundances) have the potential to be important drivers of immune variation in the wild. Moreover, the cascading effects of large wildlife loss on immune function of smaller animals could have important direct effects on fitness and even evolutionary trajectories of the smaller species that are left behind (Maizels & Nussey 2013), and indirect effects on many other constituents of the ecosystem, most notably via changes in parasite and pathogen transmission (Hawley & Altizer 2011).

Changes in resource availability may affect immune defence by changing the fitness costs of immune investment. While immune defences are critical to individual survival, they are also energetically and nutritionally costly, leading to measurable effects on fitness, and pressure for individuals to trade off immune defence for growth and reproduction (Lochmiller & Deerenberg 2000; Martin, Weil & Nelson 2008; Graham et al. 2011). Studies in a range of taxa show that investment in immune defence within individuals is highly plastic and that individuals may reduce investment in overall immune defences (Nelson & Demas 1996; Martin, Weil & Nelson 2007b; Martin et al. 2007a) or adjust relative investment in different immune components that have different costs (Ezenwa & Jolles 2011; Gilot-Fromont et al. 2012) when resources are more limited. Effects of change in predation pressure on immune function may be mediated by energetic trade-offs between behavioural defences against predators and immune defences against parasites (Rigby & Jokela 2000; Horak, Tummeleht & Talvik 2006; Raffel, Martin & Rohr 2008; Marino & Werner 2013). Increased predation pressure may also affect immune defence via increased levels of stress hormones in high predator abundance environments, with cascading effects on immune function (Navarro et al. 2004; Thomson et al. 2010; Groner et al. 2013).

Vertebrate immune systems have multiple axes of variation, with an important axis involving innate and acquired components. Innate immune defences provide first-line, relatively non-specific defences against invading pathogens; they also direct subsequent acquired immune responses. Innate immune defences are relatively inexpensive and quick to develop and use, but they may have relatively high immunopathological costs, whereby the response of the immune system itself causes damage to the animal (Klasing 2004; Martin, Weil & Nelson 2008). The innate immune system involves many aspects, including anatomical barriers (mucus, skin), serological components [e.g. natural antibodies (NAbs), lysozymes and complement] and cytological components [e.g. white blood cells (WBC) and natural killer cells; Tizard 2004]. At the other end of the spectrum, acquired immune defences are relatively expensive and generally time-consuming to develop (although in some circumstances may be less expensive to use; Martin, Weil & Nelson 2008). Examples of acquired immune defences are T helper cells, cytotoxic T cells and immunoglobulins produced after antigen exposure (Lee 2006).

In this work, we specifically investigated the impacts of long-term experimental removal of large wildlife on multiple measures of acquired and innate immunity for an ecologically dominant rodent species, *Saccostomus mearnsi* (East African pouched mouse) in Laikipia, Kenya. Many years of study in this African savanna system have shown that the densities of these animals are consistently and significantly elevated (roughly doubled) in sites where large wildlife has been removed (Keesing & Young 2014). The increased density may result from increases in food availability and perhaps quality, leading to elevated reproductive rates (Keesing 2000). In addition, vegetation cover also increases (Young, Palmer & Gadd 2005), providing cover from predators (and, likely, additional food such as seed or palatable foliage). While abundance of at least some small mammal predators does increase in these plots (McCauley et al. 2006), the lack of an overall change in rodent survivorship (Keesing 2000) suggests that the effects of increased cover compensate for increases in predator density. Abundance of small mammal ectoparasites on a per plot (but not per individual) basis has also been shown to increase dramatically in
plots with no large wildlife (MCCAULEY et al. 2008; YOUNG et al. 2014). Similarly, shedding rates of some density-dependent parasites that affect *S. meunasi* (whipworms, hookworms, coccidia) also increase in sites where large wildlife species have been excluded (H. S. Young, unpublished data). Collectively, these findings suggest that pathogen pressure is likely to be much higher in sites with no large wildlife.

Here, we experimentally test the prediction that the combination of increased parasite burdens and increased food availability known to occur in sites with no large wildlife (ARNEBERG et al. 1998; KEESING et al. 2013; YOUNG et al. 2014) would drive overall elevations in immune function in such sites (MARTIN et al. 2010). We argue that such increases in immunity could come as a response to elevated pathogen pressure, similar to a response that has been described as density-dependent prophylaxis (DDP) in insects (COTTER et al. 2004), or by increased resource availability in sites without large wildlife, or the combination thereof.

**Materials and methods**

**STUDY SITE**

This work was conducted in the Kenya Long-term Exlosure Experiment (KLEE; 0°17’N, 36°52’E) in Laikipia County, Kenya. The KLEE experiment, established in 1995, uses electric fences to remove various groups of animals from large (4 ha) plots in an African savanna landscape (YOUNG et al. 1997). KLEE is located in an area with a rich large mammal fauna including elephants (*Loxodonta africana*), giraffes (*Giraffa camelopardalis*), zebras (*Equus quagga* and *Equus grevyi*) and lions (*Panthera leo*), among many other species. The small mammal community in these plots includes at least 12 species, and long-term small-mammal trapping from these sites shows them to be dominated by one species of rodent, *S. meunasi*, which typically accounts for >75% of all captures (YOUNG et al. 2014).

Kenya Long-term Exlosure Experiment includes multiple treatments that simulate various types of wildlife and livestock loss, but here we utilize only two treatments: full exclusion of all treatments that simulate various types of wildlife and livestock species due to ontogeny (PALACIOS et al. 2009), sex (RESTIF & AMOS 2010; PREVITALI et al. 2012), or reproductive condition (i.e. pregnancy or lactation; ARDIA, SCHAT & WINKLER 2003; NORDLING et al. 1998), we used only adult, reproductively mature (testes scrotal) males to minimize this variation. All females and immature animals, and additional animals from species other than *S. meunasi*, were released at point of capture; all adult males were transferred to individual cages and transported to a central holding area for the duration of the study. In total, 128 adult males were utilized in some aspect of the analysis, from a total of 385 individual *S. meunasi* captured in these sampling efforts. Animals were weighed in a bag with a Pesola scale, and body mass was used as an indicator of body condition of these animals. Animals were then allowed to acclimate for 24 h, after which a blood draw was conducted from either the lateral saphenous vein (using Goldner Rod lancet) or caudal vein. Blood was collected in heparinized capillary blood tubes and immediately placed on ice. After blood collection animals received an intraperitoneal injection of 25 μL of a 1% chicken red blood cell (CRBC) solution (solution consisted of red blood cells dissolved in Dulbecco's phosphate-buffered saline) to stimulate antibody production. On day eight, after a second blood draw, animals were permanently marked (to avoid resampling the same animal) and released at point of capture. All animals used in these analyses survived and showed no visible signs of distress prior to their release. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Smithsonian Institution.

**MEASURES OF IMMUNITY**

There is growing consensus that multiple assays are needed to assess varied aspects of immune function and that multiple metrics for a single branch of immune function are preferable (DEMAS et al. 2011; PEDERSEN & BABAYAN 2011; PALACIOS et al. 2012). Based on this, we used four common metrics to assess components of immune function (Table 1), including both descriptive measures (e.g. WBC counts) and more functional measures [e.g. bacteria killing capacity and haemagglutination (HA) assays]. To measure innate cellular

| Innate | 1. Percentage granulocytes | 2. Percentage monocytes |
| Humoral | 3. Bacterial killing capacity | 4. Natural antibodies measured via haemagglutination (HA) score after *ex vivo* challenge with chicken red blood cells (CRBC) |
| Cellular | 5. Percentage lymphocytes | 6. Antibody production in response to CRBC, measured as the difference in HA score before vs. after *in vivo* challenge with CRBCs (anti-CRBC Abs) |


Defaunation drives immune shifts in wild rodents

Innate Capture and Manipulation

The methods for rodent trapping were similar to those detailed in previous studies (YOUNG et al. 2014). In each of the plots, we used Sherman traps baited with peanut butter and oats; traps were opened at dusk and closed at dawn. On the day of capture, animals were marked with individual metal ear tags, weighed and checked for sex and reproductive condition. Animal density at each plot and trapping session was estimated as catch per unit effort, evaluated as the number of unique animals captured per trap night (FUKASAWA et al. 2013).

Because other studies have shown strong variation in immune parameters due to ontogeny (PALACIOS et al. 2009), sex (RESTIF & AMOS 2010; PREVITALI et al. 2012), or reproductive condition (i.e. pregnancy or lactation; ARDIA, SCHAT & WINKLER 2003; NORDLING et al. 1998), we used only adult, reproductively mature (testes scrotal) males to minimize this variation. All females and immature animals, and additional animals from species other than *S. meunasi*, were released at point of capture; all adult males were transferred to individual cages and transported to a central holding area for the duration of the study. In total, 128 adult males were utilized in some aspect of the analysis, from a total of 385 individual *S. meunasi* captured in these sampling efforts. Animals were weighed in a bag with a Pesola scale, and body mass was used as an indicator of body condition of these animals. Animals were then allowed to acclimate for 24 h, after which a blood draw was conducted from either the lateral saphenous vein (using Goldner Rod lancet) or caudal vein. Blood was collected in heparinized capillary blood tubes and immediately placed on ice. After blood collection animals received an intraperitoneal injection of 25 μL of a 1% chicken red blood cell (CRBC) solution (solution consisted of red blood cells dissolved in Dulbecco’s phosphate-buffered saline) to stimulate antibody production. On day eight, after a second blood draw, animals were permanently marked (to avoid resampling the same animal) and released at point of capture. All animals used in these analyses survived and showed no visible signs of distress prior to their release. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Smithsonian Institution.

**Table 1. Immune function parameters measured and the assays used**

| Innate | 1. Percentage granulocytes | 2. Percentage monocytes |
| Humoral | 3. Bacterial killing capacity | 4. Natural antibodies measured via haemagglutination (HA) score after *ex vivo* challenge with chicken red blood cells (CRBC) |
| Cellular | 5. Percentage lymphocytes | 6. Antibody production in response to CRBC, measured as the difference in HA score before vs. after *in vivo* challenge with CRBCs (anti-CRBC Abs) |
immunity, we used proportional granulocyte (neutrophil, eosinophil, basophil) counts and proportional monocyte counts. To measure acquired cellular immunity, we used proportional lymphocyte counts. We also used neutrophil to lymphocyte ratio as an indicator of relative investment in innate vs. acquired cellular defences. To estimate innate humoral immunity, we measured the bacterial killing capacity (BKC) of plasma using a bacterial strain (Escherichia coli ATCC®8739) for which killing is primarily complement mediated (Demas et al. 2011). In addition, we used a HA assay to quantify levels of NABs (Matson, Ricklefs & Klasing 2005). To estimate acquired humoral immunity, we used the same HA assay to measure antibody (Ab) production after an in vivo challenge with CRBCs, by comparing the difference in HA score pre- and post-challenge (anti-CRBC Abs). The three functional metrics (BKC, NABs and anti-CRBC Abs) were only conducted during the final three trapping sessions (June 2013, August 2013 and September 2013) after preliminary data on WBC showed signs of difference among treatments, justifying the more invasive assays. All assays were based on commonly used protocols, and details on each assay are included in Supporting Information.

**Statistical Analyses**

To examine changes in abundance of *S. mearnsi* across treatments, we used repeated-measures ANOVAs with plot as the replicate, with trapping period as a repeated measure and number of unique individuals caught per trap night as response variable. We used linear models to examine effects of treatment (large animals removed vs. large animals present), seasonality (total rainfall 30 days prior to capture), density of rodents (catch per unit effort) and body mass of the captured animal (as an index of body condition) on each of our immune response parameters, treating individual animals as replicates. Body mass was included because other studies have shown correlations between body condition and immune investment (Moller & Petrie 2002; Masello et al. 2011). For proportion of granulocytes, proportion of lymphocytes and BKC, we log-transformed the data prior to analysis, which normalized model residuals. We also log-transformed NABs to meet model assumptions. As some of these predictors might reasonably be expected to be correlated (e.g. body mass, density and seasonality), we first checked for multicollinearity using variance inflation factor analysis and found all values to be <3 and thus much lower than the commonly used cut-off of 10 (Petratis, Dunham & Niewiarowski 1996). All model subsets were compared using Akaike scores. As the best-supported models were frequently within two AICc units from each other, we compared models using a model averaging approach, including only those models with ΔAICc < 2. The relative importance value of each factor was calculated as a sum of Akaike weights over all the models in which the factor occurred, again including only those models with ΔAICc < 2. We report all models that received support at this level, noting the best-fit model, as well as reporting this relative importance metric. All data shown in figures are untransformed.

**Results**

Abundance of *S. mearnsi* (unique individuals caught per trap night) was significantly higher in exclusion (67.6 ± 16.1) as compared to control plots (38.5 ± 11.0; F1,4 = 37.5, P < 0.01). Animals in exclusion plots had higher body mass (95 ± 2 g) than in control plots (88 ± 2 g; d.f. = 1, χ2 = 8.4, P < 0.01). We also found significant variation in body mass by session (d.f. = 4, χ2 = 21.8, P < 0.001).

In total, we gathered immune data for 128 adult male *S. mearnsi*, consisting of 70 from plots with large animals absent (exclusion) and 58 from plots with large animals present (control). Of these 128 animals, 45 were captured in the first two sessions where full sampling was not conducted and thus had only WBC data.

**White Blood Cell Proportions**

For all animals with two slides measured, we found high consistency among values between slides (mean variance = 7%, P < 0.001 for all cell types). Overall, wildlife removal (the ‘treatment’ in our model) was associated with significant reductions in the proportion of lymphocytes among cells counted (d.f. = 1, χ2 = 11.1, P < 0.001; Fig 1); density also had significant negative effects on lymphocyte proportions (d.f. = 1, χ2 = 9.3, P = 0.02; Tables 2 and 3). The best-fit model included treatment and density. This model explained 38% of total variance and received substantially more support than any other model (Table 3).

For granulocytes, we found that the strongest effects were again treatment (d.f. = 1, χ2 = 13.1, P < 0.001;
Relative importance of variables was defined using weighted model average AICc values. Variables that received no support in any of the averaged models have no value shown. Significant relationships are shown in bold.

Table 2. Relative importance of independent variables across models for each measured immune assay (white blood cell proportions, including: % lymphocytes, % granulocytes, % monocytes, and neutrophil to lymphocyte ratio, bacterial killing capacity (BKC), natural antibodies (NABs), and change in Abs following chicken red blood cell (CRBC) antigen exposure anti-CRBC Abs

<table>
<thead>
<tr>
<th>Model</th>
<th>Lymph</th>
<th>Gran</th>
<th>Mon</th>
<th>N : L ratio</th>
<th>BKC</th>
<th>NABs</th>
<th>Anti-CRBC Abs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>1</td>
<td>1</td>
<td>0.24</td>
<td>1</td>
<td>1</td>
<td>0.34</td>
<td>1</td>
</tr>
<tr>
<td>Seasonality</td>
<td>0-28</td>
<td>–</td>
<td>0.13</td>
<td>–</td>
<td>1</td>
<td>0.58</td>
<td>0.2</td>
</tr>
<tr>
<td>Density</td>
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<td>1</td>
<td>0.14</td>
<td>1</td>
<td>0.71</td>
<td>0.63</td>
<td>0.25</td>
</tr>
<tr>
<td>Mass</td>
<td>–</td>
<td>–</td>
<td>0.23</td>
<td>0.29</td>
<td>–</td>
<td>0.13</td>
<td>–</td>
</tr>
</tbody>
</table>

Relative importance of variables was defined using weighted model average AICc values. Variables that received no support in any of the averaged models have no value shown. Significant relationships are shown in bold.

Table 3. Model support for each of the immune parameters measured, ranked (1–8, first column) by the amount of support received. Best fit models are listed in bold

<table>
<thead>
<tr>
<th>Model</th>
<th>LL</th>
<th>ΔAICc</th>
<th>R^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Lymphocyte</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment + density</td>
<td>–147.42</td>
<td>0</td>
<td>0.38</td>
</tr>
<tr>
<td>Treatment + density + season</td>
<td>–145.49</td>
<td>1.93</td>
<td></td>
</tr>
<tr>
<td>% Granulocyte</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment + density</td>
<td>127.29</td>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td>Treatment + density + mass</td>
<td>129.35</td>
<td>2.06</td>
<td></td>
</tr>
<tr>
<td>Treatment + density + season</td>
<td>129.36</td>
<td>2.07</td>
<td></td>
</tr>
<tr>
<td>% Monocyte</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment + density</td>
<td>183.27</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Treatment + season</td>
<td>184.71</td>
<td>1.44</td>
<td></td>
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<tr>
<td>Treatment + density + mass</td>
<td>184.71</td>
<td>1.62</td>
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<tr>
<td>Treatment + density + season</td>
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<td>Bacterial killing capacity</td>
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<tr>
<td>NABs</td>
<td></td>
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<td>Treatment + density</td>
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</tr>
<tr>
<td>Season</td>
<td>80.58</td>
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</tr>
<tr>
<td>Density + season</td>
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<td>1.8</td>
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<tr>
<td>Treatment + season + mass</td>
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<td>1.87</td>
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<tr>
<td>Anti-CRBC Abs</td>
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<tr>
<td>Treatment</td>
<td>738.14</td>
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<td>Treatment + density</td>
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<td></td>
</tr>
<tr>
<td>Treatment + season</td>
<td>740.13</td>
<td>1.99</td>
<td></td>
</tr>
<tr>
<td>N : L ratio</td>
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<td></td>
<td></td>
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<tr>
<td>Treatment + density</td>
<td>252.27</td>
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<td>0.1</td>
</tr>
<tr>
<td>Treatment + density + mass</td>
<td>254.47</td>
<td>2.06</td>
<td></td>
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</table>

Table 3) and density (d.f. = 1, χ^2 = 13.3, P < 0.001). The proportion of granulocytes was higher in exclosure as compared to control treatments and in high-density as compared to low-density plots. The best-fit model included treatment and density and explained 10% of the variation in granulocyte proportions among individuals (Table 3).

For monocytes, we found no support for any of our explanatory variables; the best-supported model was the null model. The next best-supported model included treatment but explained <1% of the overall variation in monocyte proportions among individuals (Table 3).

For neutrophil to lymphocyte (N : L) ratios, we again found significant effects of both treatment (d.f. = 1, χ^2 = 8.4, P < 0.01) and density (d.f. = 4, χ^2 = 6.5, P = 0.01), with N : L levels being higher in exclosure and high-density plots as compared to control plots. The best-fit model included density and treatment (Table 3).

**BACTERIAL KILLING CAPACITY**

After discarding plates with contamination or poor controls, we had 69 animals with BKC data. Data within plates for a single individual showed reasonably high consistency in BKC (R = 0.90, P < 0.0001). Treatment had a highly significant effect (d.f. = 1, χ^2 = 24.1, P < 0.001) in our analyses and appeared in all models with any support, with BKC being significantly elevated in exclosure plots. Higher density also drove significant increases in BKC (d.f. = 1, χ^2 = 7.1, P < 0.01). Higher rainfall tended to cause higher BKC levels, but this effect was not significant (d.f. = 1, χ^2 = 1.5, P = 0.2). The best-fit model included treatment, density and season and explained 18% of variance (Table 3).

**NATURAL ANTIBODY (NAB) AND ANTI-CRBC ANTIBODIES (ABS)**

Haemagglutination assays were highly replicable, with <1% average difference between the two scores calculated for each individual within a sampling period (R^2 = 0.93, P < 0.001). In total, 84 animals were successfully screened for antibodies using HA assays both before and after CRBC exposure. There was a strong positive correlation between antibody responses prior to exposure and after exposure (P < 0.0001, F = 37.49, R = 0.31). However, post-exposure antibody levels (3.68 ± 0.21) were 1.8 times higher than pre-exposure natural antibody levels (2.06 ± 0.17).

We found no significant effect of any of the predictors on NABs (whole model not significant; d.f. = 4, χ^2 = 7.5, P = 0.1). The best-fit model explained only 9% of the variance and included only season (Table 3), but the null model also received substantial support (the only response variable in which this occurred). For the challenge assay,
the best-fit model, which included only treatment, explained just 7% of the variance, but here the whole model was significant. In this case, treatment was significant (d.f. = 1, \( \chi^2 = 11.9, P < 0.001 \)) in explaining anti-CRBC Ab levels with slightly higher levels (i.e. stronger changes in Abs in response to antigen challenge) observed in animals in exclosure plots.

**Discussion**

Consistent with our hypothesis, we found that large wildlife removal was associated with strong and significant changes in immune function in a dominant rodent species. Specifically, we found changes in three of four measures of immune defences in these exclosure sites, as might be anticipated with higher food resources, better body condition and higher parasite risks (due to increased host density). Of the four immune components we quantified, we found that BKC, an innate immune parameter, and anti-CRBC Abs, a measure of the acquired response, both increased in the exclosure plots. We also found that WBC proportions changed drastically. In the exclosure plots, granulocyte proportions increased and lymphocyte proportions decreased, and this was mirrored by an increase in neutrophil to lymphocyte ratios at these sites. Because we do not have absolute values of lymphocytes and granulocytes, we cannot determine whether an overall increase or decrease in the different cell types occurred, or whether the effect reflects a shift in investment from acquired to innate immune effort.

Our manipulations did not allow us to directly identify the causal mechanisms that link wildlife removal to increases in immune investment, which could be due to greater disease exposure or risk, or to better nutrition, or both. However, our data and other studies demonstrate that large animal exclusion is linked to both higher density and higher body mass of rodents (likely due to higher food availability; Keesing 2000; Keesing & Young 2014), suggesting two potential mechanistic pathways driving observed changes in immune profiles. In this study, density repeatedly emerged as a strong predictor of immune function both for individual parameters and for relative investment across branches of immunity. In contrast, body mass was not an important predictor of any immune parameter or in explaining variation across branches of immunity. The strong importance of density is consistent with other studies that have shown DDP, with increased immune function as a response to higher pathogen pressure (Buehler, Piersma & Tieleman 2008; Mugabo et al. 2015). In this system, multiple studies have found sustained differences in plot-level ectoparasite abundance on small mammals in sites with no large wildlife (McCauley et al. 2008; Young et al. 2014) and even stronger effects on macroparasites (H. S. Young, unpublished data), making density dependence one likely mechanism for observed increases in many immune parameters. Clearly, this is an aspect that warrants further research. From our current data, we also cannot tell whether increased pathogen pressure is causing elevation of immunity of individual animals in exclosure plots on one hand, or whether there is some extrinsic cue (e.g. animal density) that is driving elevated responses rather than pathogen pressure itself. Future work in our system will examine in more depth the changes in total parasite levels across seasons and treatments, and the correlation to immune function.

While wildlife treatment and density were the most important variables in most response metrics, for several immune metrics, seasonality was also important, with higher immune levels occurring in wetter seasons. This finding is not surprising as seasonality is known to have strong effects on immune function, and resource stress can cause immune function to be depressed (Martin, Weil & Nelson 2008; Martin 2009). Our study system in particular is highly seasonal, with extended and somewhat irregular dry seasons, punctuated by wet periods of varying intensity. The sites themselves, although in close physical proximity, also vary significantly in amount of total annual rainfall, and in rainfall within a given season. In this system, intra- and interannual variation in rainfall can have dramatic effects on rodent populations, driving strong boom-bust dynamics, ranging from just over zero to >50 mice ha\(^{-1}\) in control plots, with variation equally pronounced in treatment plots (Keesing & Young 2014).

We hypothesize that increased food availability in wet seasons may reduce resource stress and lead to increased levels of immune function. It is also possible that seasonal variation in immunity may be a response to seasonal changes in parasite density (e.g. Young et al. 2015a,b); however, seasonal patterns of parasite density likely vary across parasite species and parasite life cycle stages, and these patterns have not been established for most parasites in this system. Moreover, the one parasite group, fleas, for which seasonal variation in this system has been established, intensity decreases with increasing rainfall (Young et al. 2015a,b). This does not support the idea that changes in parasitism drive increased immune function in the wet season. Yet, given the multiple ways in which seasonality can affect immune function (Martin, Weil & Nelson 2008; Martin 2009), it was somewhat surprising that our seasonality metric was, on average, less important than either treatment or density of animals. It seems likely that the explanation for this is that density may capture seasonal information in a way more relevant to *S. mearnsi*, than does our direct measure of rainfall.

Notably, even after accounting for density, season and body mass, there remain strong effects of treatment on immunity. The differences in immune function among treatments could be due, in part, to some effect of exclosure treatment on male reproductive effort and testosterone. All the animals included in this study were adult males in reproductive condition, based on sexual development. We chose to include only adult males in order to minimize variation due to sex, ontogeny and reproductive status. However, adult males have high levels of testosterone, which is

typically thought to be immunosuppressive (Olsen & Kovacs 1996; Greives et al. 2006; Martin et al. 2006), although more recent evidence suggests that testosterone may drive immune redistribution with high-testosterone males investing more in innate over acquired defences (Ezenwa et al. 2012). It is also possible that food stress in control plots might partially explain the higher levels of immune function we observed for some parameters in enclosure plots. Stress, whether from increased predation risk, elevated food scarcity or other sources, can strongly impact immune responses (Glaser & Kiecolt-Glaser 2005; Martin 2009; Zylberberg et al. 2013). While direction of response can vary based on duration and timing (season, life-history stage), prolonged increases in stress, may drive immune redistribution with high-testosterone males investing more in innate over acquired defences (Ezenwa et al. 2012). We investigated whether selective loss of large wildlife – another major characteristic of the current wave of anthropogenic alteration of ecosystems (Olsen & Kovacs 2007). We considered whether selective loss of large wildlife from other factors, suggesting that the loss of large wildlife from a system may have other effects on immune function beyond those involving host density. Our findings have implications for disease transmission and disease susceptibility in defaunated systems and may inform the ongoing debate about when – and under what conditions – disturbance is likely to cause increases in disease prevalence (Keesing et al. 2013; Young et al. 2013; Wood et al. 2014).

Conclusions and synthesis

Anthropogenic disturbance is known to affect animal immune function through a variety of mechanisms including changes in climatic conditions, pollution levels and invasive species (Martin et al. 2010, Bradley & Altizer 2007). We investigated whether selective loss of large wildlife – another major characteristic of the current wave of anthropogenic alteration of ecosystems – has impacts on animal immune function. We found evidence consistent with this expectation. These changes may in turn affect patterns of disease transmission among these rodents, as well as pathogen spillover to other taxa, including to humans via zoonotic pathogens, an aspect that warrants further investigation.

For the species studied here, the effects of wildlife removal on immune function were characterized by an increase in total immune investment, possibly explained by changes in pathogen pressure. While the effect of treatment was always among the most important factors that drive immune function, rodent density, to a lesser extent seasonality, also covaried with shifts in the immune responses. It is notable, however, that large wildlife removal predicted immune function changes even when accounting for these other factors, suggesting that the loss of large wildlife from a system may have other effects on immune function beyond those involving host density. Our findings have implications for disease transmission and disease susceptibility in defaunated systems and may inform the ongoing debate about when – and under what conditions – disturbance is likely to cause increases in disease prevalence (Keesing et al. 2013; Young et al. 2013; Wood et al. 2014).

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References


Data accessibility

Data deposited in the Dryad Digital Repository http://dx.doi.org/10.5061/dryad.j1t67 (Young et al. 2015a).


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**Supporting Information**
Additional Supporting information may be found in the online version of this article:

**Methods S1.** Detailed methods of immunological assays.