

Supplementary Materials for:

Large wildlife removal drives immune defense increases in rodents

Hillary S. Young, Rodolfo Dirzo, Kristofer M. Helgen, Douglas J. McCauley, Charles L. Nunn, Paul Snyder, Kari E. Veblen, Serena Zhao, Vanessa O. Ezenwa

Supplementary Methods:

White blood cell counts

Two blood smears were taken from all animals 24 hours after recovery from traps. We stained these slides using Wright Giemsa staining and scanned them under 1000x oil immersion using a compound microscope. We counted 200 leukocytes on each slide and differentiated lymphocytes, eosinophils, basophils, neutrophils, and monocytes. The proportion of each type of leukocyte was calculated as the number of each cell type over the total number of leukocytes counted. The average value of both slides was used, except when one slide had a low quality smear or stain (<10% of all plates) in which case only the high quality smear was used. *There was low intra-individual variation for across slides in the proportion of various WBC (7% intra-individual variation).* For analysis we use proportion of lymphocytes and proportion of granulocytes (sum of proportion of

neutrophils, eosinophils, and basophils), and the proportion of neutrophils to lymphocytes as response variables. The same observer counted all slides.

Bacterial killing capacity

Fresh plasma was harvested from whole heparinized blood by centrifugation and immediately used for killing assays. To this end, 7.6 uL of plasma was diluted with a combination of 140ul CO₂ independent media (Gibco and Invitrogen; Life Technologies, Grand Island, NY, USA) and 3ul L-Glutamine, and to each dilution we added 30ul of 3×10^4 CFU/ml suspension of *E. coli*. Lyophilized bacteria pellets (EPower Microorganisms, Microbiologics, St. Cloud, MN, USA, Ref #0483E7, ATCC #8739) were reconstituted in PBS following the manufacturer's specifications to create a stock solution, and the stock solution was diluted prior to each assay run to obtain a 5.6×10^4 CFU working solution used in killing assays. We incubated the plasma-bacteria mixture at 41°C for 30 minutes, and then plated 50 uL of mix onto a tryptic soy agar plate. Each sample was plated in triplicate. Plates were allowed to dry in a sterile hood for two hours, and were then incubated at 37°C for 12 hours. Two positive control plates (PBS in place of plasma in the bacterial solution) and negative control plates (PBS in place of bacterial solution) were run with every set of sample plates. The number of colonies on the plates was counted by hand, and *bacterial killing capacity (BKC)* per sample was calculated as the difference in the average number of colonies between positive control and experimental plates, divided by the average number of colonies on positive control plates. Any plates that showed contamination were not included. Similarly, when the negative controls showed bacterial colonies, all samples from that run were discarded. If there were fewer than two high quality plates per individual those individuals were discarded for analysis.

Natural Antibodies (NABs)

In order to assess levels of natural antibodies, we performed an HA assay following Matson et al. (2005), with the modification that we used chicken red blood cells (CRBCs) in place of rabbit red blood cells as the antigen since laboratory rodents have been shown to mount natural hemagglutinins to CRBCs (Longenecker and Mosmann 1980). We also used sheep plasma as a control on each plate. The CRBCs and sheep plasma used in this study were taken directly from live animals at the study site. A single pooled sample of sheep plasma taken from three individuals at the start of this study was used throughout the study. Plasma was isolated from whole blood by centrifugation at 57g for 7 min. At the time of collection the sheep sample was divided into several aliquots and then frozen until use in the HA assays; a different aliquot was used for each assay to avoid multiple freeze-thaws. For CRBCs, the samples were also a pooled sample of blood from five chickens; however the CRBC samples were collected repeatedly (once at each sampling session) from the same individuals throughout this study. One mL of blood was drawn from each chicken and placed into an equal volume of Alsever's solution. 1.5 mL aliquots of this mixture were then washed four times with PBS (spun at 629g for 5 minutes for each wash). The CRBCs were adjusted to a final cell concentration of 1% in PBS. Suspensions were vortexed immediately prior to use in the assay. At the completion of assays, plates were photographed at high resolution on a white background. From these images we scored agglutination on a scale from 0-11 based on the column number in which agglutination occurred. The column with the lowest concentration of plasma in which agglutination occurred was used. Half scores

were used when it appeared that agglutination likely occurred between two columns. For each animal the agglutination score was determined as the average of two replicates.

Antibody production after in vivo challenge with CRBC

These methods were modified from those described in Palacios et al. (2012). As described in animal handling, after the initial blood draw (taken 24 hours post capture) animals were injected intraperitoneally with a 1% CRBC solution in order to stimulate an immune response. Animals were then held in individual cages for 7 additional days, after which a second blood draw was performed. The HA assay described above was then repeated on these new samples. The difference in average agglutination score between the day 1 and day 8 was used to measure anti-CRBC Abs as an index of acquired immune response.