Relationship between pace of life and immune responses in wild rodents

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Abstract

Life histories of animals tend to vary along a slow to fast continuum. Those with fast life histories have shorter life spans, faster development, and higher reproductive rates relative to animals with slower life histories. These differences in life histories have been linked to differences in investment in immunological defenses. Animals with faster life histories are predicted to invest relatively more in innate immune responses, which include rapidly-deployed, non-specific defenses against a broad spectrum of invaders. On the other hand, animals with slower life histories are predicted to invest relatively more in adaptive immune responses, which are more slowly-deployed and are highly pathogen-specific. These predictions have been confirmed in some taxa, but other studies have not found this association. We tested this prediction by measuring innate and adaptive immunity of white-footed mice (*Peromyscus leucopus*), chipmunks (*Tamias striatus*), and gray squirrels (*Sciurus carolinensis*), three species of rodents that inhabit deciduous forests in the northeastern US. These species exhibit a range of life histories, with mice having a relatively fast life history, squirrels a relatively slow one, and chipmunks an intermediate one. We found mice to have the greatest "bacterial killing capacity", a common measure of innate immunity, and squirrels the lowest, consistent with the pace-of-life immune-defense hypothesis. We also found squirrels to mount the most pronounced antibody response when challenged with lipopolysaccharide (LPS), an immunogenic component of bacteria, while mice had the lowest, again consistent with predictions based on their life histories. These results have implications beyond ecoimmunology because the probability that a host species will transmit an infection – its "reservoir competence" – has been linked to its immune strategy. Understanding the

relationship between immunology and reservoir competence is a critical frontier in the ecology of infectious diseases.

INTRODUCTION

The immune system plays a critical role in prolonging survival by defending against pathogens and cancer cells. However, there is extensive evidence showing that immune defenses are costly, requiring investment of energy, nutrients, and time during the development, maintenance, and use of the immune system (Klasing and Leshchinsky 1999, Lochmiller and Deerenberg 2000). When resources are limited, allocation of energy to immune defenses may be modulated by the need to spend energy on other functions such as growth, reproduction, and maintenance (Nelson and Demas 1996). Stressors other than resource scarcity can also reduce allocation to immune functions (Martin 2009). Reproductive activities are a good example; the combination of breeding stress and energy investment in reproduction is typically associated with a reduction in immune responses (e.g., Nordling et al. 1998, Ardia et al. 2003). Immune function and parasites have also been shown to mediate trade-offs between reproductive effort and survival (Mills et al. 2010).

Further complicating the trade-offs between immunity and other energetically demanding processes are the costs and benefits of the different types of immune responses (Lee 2006). The vertebrate immune system can be divided into two branches: the innate and the adaptive. Under most circumstances, these two branches act in concert to generate a highly integrated defense system. But the benefits provided by these two components of immunity can vary depending on the type of pathogens that the animal is confronting, the need for a rapid response, and the likelihood of future exposures to the same pathogen.

Innate immune responses are relatively rapid, non-specific defenses that act as a first-line of defense against invading pathogens (Sompayrac 2008). Innate immunity includes a constitutive component made of cells and antimicrobial proteins that are present at low levels at all times. The metabolic costs of development, maintenance, and use of innate immune defenses are thought to be relatively low (Lee 2006), but immunopathology costs, due to collateral damage, can be substantial (Råberg et al. 1998).

A second-line of defense is provided by adaptive immune responses, which are typically slower and highly pathogen-specific. B-cells and T-helper cells (Th2) mediate adaptive immune responses involving antibodies, which provide effective defense and immunological memory against pathogens (Sompayrac 2008). Although the energetic cost of operating this type of immunity is thought to be relatively low (Råberg et al. 2002, Lee 2006, Martin et al. 2008a), the developmental costs are believed to be high due to the time and energy invested during lymphocyte proliferation and diversification (Ricklefs 1992). Consequently, a large investment in adaptive immunity is expected only when the fitness consequences of enhanced protection against repeated infections can outweigh those developmental costs. A long-lived animal is likely to face repeated exposures to the same pathogen and to prioritize self-maintenance over short-term reproductive output. Thus, the optimal strategy for these animals would be to allocate more of their limited resources to adaptive immunity.

Considering these differences in costs, we expect an association between the organization of the immune system and certain life-history traits. Life history variation among species can be ordered along a slow to fast continuum (Promislow and Harvey 1990, Ricklefs

and Wikelski 2002, but see Bielby et al. 2007). Under this paradigm, slow-living species with long life spans, high investment per offspring and in future reproduction, should rely more on adaptive immunity, whereas fast-living species with short life spans, fast development, and high reproductive rates should instead favor innate immunity. Although over a dozen years have passed since the idea of a relationship between pace of life and immune strategies was first proposed (Klasing and Leshchinsky 1999, Lochmiller and Deerenberg 2000), few studies have investigated this association. Early studies were mostly conducted in birds (e.g., Martin et al. 2001, Tella et al. 2002, Tieleman et al. 2005, Martin et al. 2006). Then, in 2006, Lee proposed the relationship between pace of life and differential immune investment as a "working framework", and a few additional studies have since adopted this framework. But, while some have found support for some of the predictions of the model (e.g., Martin et al. 2007, 2008b, Sparkman and Palacios 2009), others have found the reverse pattern (e.g., Cutrera et al. 2010). Thus, even though the basic premise is intuitively appealing, the hypothesis that life history shapes the immunological responses of animals remains controversial.

To evaluate the pace of life hypothesis we measured both innate and adaptive immunity in members of a community of rodents inhabiting mixed-hardwood forest of the northeastern United States. We sampled several rodent species that differ in their pace of life, and predicted that slow-living species would rely more on adaptive immunity that will protect them from repeated pathogen exposure and thus should display a strong antibody response when challenged with an antigen. At the same time, we predicted that fast-living species would rely more on innate defenses, such as a high bactericidal activity of plasma proteins and phagocytic cells.

Knowing the immune strategies of the various rodent species examined is of particular interest because they are important hosts for *Borrelia burgdorferi*, the bacterium that causes Lyme disease (Ostfeld 2011), but they differ markedly in their "reservoir competence", the probability that they will transmit the bacterium (LoGiudice et al. 2003). This variation in reservoir competence has been hypothesized to be a consequence of variation in immune strategy (Kurtenbach et al. 2002, 2006, Keesing et al. 2010); however this hypothesis has not previously been tested.

Allocation of resources to different life history components, including immunity, typically differs among male and female mammals due to sex-specific differences in reproductive investment and timing (Bateman's principle; Rolff 2002, Nunn et al. 2009). We therefore noted the sex of each host individual, to be included as a covariate in our analyses. Finally, it has been well established that stress can either enhance or suppress immune function in vertebrates (Martin 2009). Thus, we investigated the potential modulatory effects of stress hormones (glucocorticoids; GCs) on the immune responses of the individuals, and anticipated that GCs relationships with immune measures would vary by species, since they may differ in their sensitivity to stressors.

METHODS

Animal trapping and husbandry

Animals were captured at the Cary institute of Ecosystem Studies, Millbrook, NY, USA (41°50' N, 73°45' W). Trapping took place during five weeks in the fall, from mid September

until the 22nd of October of 2009. White-footed mice (*Peromyscus leucopus*) and eastern chipmunks (*Tamias striatus*) were trapped using Sherman live-traps (23 x 9 x 8 cm; HB Sherman Inc., Tallahassee, FL, USA) baited with oats and covered with a board. Traps were set in the evening and checked and closed in the morning. Captured individuals were marked with a metal ear tag and visually checked for sex and reproductive status of females (pregnant / lactating). Chipmunks, southern flying squirrels (*Glaucomys volans*), red squirrels (*Tamiasciurus hudsonicus*), and eastern gray squirrels (*Sciurus carolinensis*) were captured in small Tomahawk live traps (41 x 13 x 13 cm; Tomahawk Live Trap Company, Tomahawk, WI, USA) set on trees and medium traps (48 x 15 x 15 cm) set on the ground. Each trap was sheltered with a board and outfitted with a nest box built out of milk cartons containing cotton and bait. For bait we used a mixture of molasses, peanut butter, raisins, and rolled oats. Traps were set in the evening and checked in the morning and afternoon. Woodchucks (*Marmota monax*) were trapped using larger Tomahawk live traps (Model 205, 66 x 23 x 23 cm). Traps were also covered with boards and baited with apples, carrots, broccoli, and greens. Woodchuck traps were set in the morning and checked in the afternoon. Animals were used only once in the study and recaptured animals were released at their point of capture. In order to avoid introducing confounders due to age and reproductive condition, and to minimize the impact on pregnancies and nursing young, juveniles and females showing signs of lactation or pregnancy were released without processing. All other captured animals were immediately transported in their trap to the animal rearing facility located on site (within 4 km of any of the trapping locations). Animals were housed in individual cages matched in size to their body sizes. Mice were held in wire mesh cages (20 x 18 x 52 cm) and provided with cotton and cardboard nest

boxes and tubes. Squirrels and chipmunks were housed in wire mesh rack cages (61 x 35.5 x 91 cm) and woodchucks in crates (76 x 48 x 56 cm) containing a wooden nest box. The animals were on a natural light cycle and provided twice a day with *ad libitum* food and water. Diets fed varied by species and were designed to mirror some food items that animals were likely to encounter in their environment. Mice were offered apples, sunflower seeds, peanuts in their shells, rodent lab blocks, and raisins. Chipmunks and gray and red squirrels, we fed apples, sunflower seeds, peanuts in their shells, rodent lab blocks, and walnuts in their shells. Flying squirrels also were offered mushrooms. Woodchucks were fed alfalfa pellets, broccoli, carrots, lettuce, and freshly collected clover, dandelion, and plantain leaves.

Animal manipulation

On day 0, each animal was transferred from its trap to a canvas restraint cone updated from Kowprosky (2002) and anesthetized using Isoflurane (Minrad Inc., Bethlehem, PA, USA) delivered by a 200-4000cc flow meter vaporizer (ACV-1227, Kent Scientific, Torrington, CT, USA). Animals were induced via facemask with 5% Isoflurane (5 l/min 100% O_2). Once fully anesthetized (1-4 min after induction), the percentage of Isoflurane was reduced to 3.5 or lower for the remainder of the anesthetic period. While under anesthesia, animals were individually marked with numbered metal ear tags (1005-1 self-piercing monel tags, National Band and Tag Co., Newport, KY, USA), and were weighed, sexed and inspected for overall health and reproductive condition. If this closer examination revealed signs of lactation or pregnancy, or extremely poor body condition the animal was returned to its trapping location without further processing immediately after recovery from anesthesia.

In anesthetized mice, a blood sample was collected from the retro-orbital sinus using sterile heparinized microcapillary tubes (max. 1 capillary tube ~70 µL). The other species were bled from the lateral or medial saphenous veins via venipuncture using a 25 to 30 gauge needle (approx. volume collected: 200-300uL; Hoff 2000). Blood was collected into heparinized capillary blood collection tubes (Safe-t-fill 076101 and 077221, Ram Scientific Inc., Yonkers, NY, USA) and samples were kept cool on ice until used in the lab.

After blood collection, we administered 0.5 mg/kg lipopolysaccharide (LPS) isolated from the cell wall of *Escherichia coli* (stereotype 026:B6, Sigma-Aldrich Co., St. Louis, MO, USA) dissolved in sterile 0.9% NaCl saline (Abbott Laboratories, North Chicago, IL, USA) via intraperitoneal injection. The animals were monitored carefully during and after their recovery from anesthesia.

On day 4 and 7 we anesthetized, weighed, and drew blood from the animals as described for day 0. We alternated eyes / limbs in successive bleeds to provide time for healing of previously accessed vessels. On day 7, to avoid re-sampling the retro-orbital sinus in animals previously bled in this manner, blood was collected via submandibular puncture in mice. On day 7, animals were returned to their point of capture after fully recovering from the anesthesia. All animal procedures were supervised by a veterinarian, conducted in accordance with the guidelines approved by the American Society of Mammalogists (Sikes et al. 2011), and approved by the Institutional Animal Care and Use Committee at the Cary Institute (Protocol 09-03I).

Measures of response to immunological challenges

As a measure of innate immunity, we quantified bacterial killing capacity (BKC) of whole blood, which represents the integrated bactericidal activity of plasma proteins (including natural antibodies and complement) and phagocytic cells (Matson et al. 2006). As a measure of acquired immunity, we quantified antibody production after challenging the animals with a known antigen, LPS. These two assays are 'challenge techniques' (sensu Norris and Evans 2000) that force individuals to make an investment in an immune response, which some have argued are preferable to monitoring techniques (e.g., leukocyte counts, lymphoid tissue masses), which can vary quantitatively for various reasons and their interpretation as measures of immunocompetence is somewhat problematic (Norris and Evans 2000, Demas et al. 2011).

Bacterial Killing Capacity

A lyophilized pellet of *Escherichia coli* (ATCC# 8739 KWIK-STIK MicroBiologics Inc., Saint Cloud, MN, USA) was reconstituted according to manufacturer's instructions and grown on tryptic-soy agar (TSA; Becton Dickinson, Sparks, MD, USA). Bacterial colonies were kept for up to one week at 4°C on agar plates; we inoculated a new plate the day before the assay was run. The day of the assay, we used the BBL Prompt Inoculation System (Becton-Dickinson) to create a bacterial stock solution containing approximately $1.5x10^8$ colony-forming units (CFU)/ml. From this, a working bacterial solution of roughly $3x10^4$ CFU/ml was generated using sterile Phosphate Buffered Saline (PBS). For this assay we used the blood from day 0, within 6 hours of collection. We diluted 4 µL of whole blood from each animal in 24 µL of sterile PBS, added 2 µL of the bacterial working solution, and then incubated for 30 min at room temperature. We

plated the 30 µL onto a TSA-petri dish. Each blood sample was prepared and plated in triplicate. The number of bacteria in the initial working solution was determined from five control plates, each prepared by mixing 2 µL of the working solution with 28 µL of sterile PBS and plated after the 30 min incubation period. All plates were then incubated inverted at 37 deg. C overnight. The next morning we quantified the total colonies on the plates, averaged across replicates, and calculated the percentage of bacteria killed by dividing the difference in the number of colonies between control plates and experimental plates by the average number of colonies in the control plates.

Antibody production against LPS

Animals were injected with LPS to induce immunoglobulin (IgG) production. Blood samples collected on days 0 and 7 were used in this assay with samples at day 0 collected to ascertain prior exposure to LPS (i.e. baseline IgG levels). Samples were kept on ice until plasma was separated via centrifugation at 6000 rmp for 20 minutes. Plasma was stored at -70°C until we conducted the assay approximately five months later. We used an enzyme-linked immunosorbent assay (ELISA) to detect (IgG) specific to LPS in plasma. Briefly, we coated 96-well plates with LPS, the plate was then blocked with 5% nonfat dry milk in PBS-T (PBS-Tween; Sigma-Aldrich, St. Louis, MO, USA), then aliquots of diluted plasma (1:40 in PBS-T) were plated in duplicate. Positive controls (a mixture of plasma collected on day 7 (126 µL of mice (n=7) + 157 µL of chipmunks (n=5) + 157 µL of squirrels (n=5)) and negative controls (mixture of plasma collected on day 0 (120 µL of mice (n=4) + 161 µL of chipmunks (n=5) + 150 µL of squirrels (n=5)) were added to each plate in triplicate. Plates were incubated at 37°C for 3 hours, washed

232 (PBS-Tween), then secondary antibody (anti-guinea pig IgG alkaline-phosphatase conjugated
233 antibody produced in goat, no. A5062; Sigma-Aldrich, St. Louis, MO, USA) were added to each
234 well (1:750 diluted in PBS-Tween). Plates were incubated at 37ºC for 1 hour, after which they
235 were washed with PBS and treated with p-nitrophenyl phosphate. Exactly 20 min later, optical
236 density (OD) of samples was assessed (405-nm filter, BioRad Benchmark microplate reader,
237 Richmond, CA, USA). We chose a secondary antibody developed for guinea-pigs, *Cavia*
238 *porcellus,* because this rodent is not closely related to any species in the study (Michaux and
239 Catzeflis 2000, Blanga-Kanfi et al. 2009, Churakov et al. 2010), which we assumed would
240 minimize species differences in IgG due to variable affinity of the antibody. We calculated
241 antibody levels as the difference in the mean OD of each sample from the mean OD of the
242 positive controls in each plate. We interpreted antibody levels on day 0 samples as background
243 levels due to prior exposure and those from day 7 samples as a response to current exposure.
244 Intra plate variation was 11.3% and inter plate variation was 20%.

245 Corticosterone levels

246 We measured the levels of corticosterone (the main glucocorticoid of rodents) on a
247 subset of individuals for which we had sufficient plasma samples collected on day 0 and day 4.
248 This subset comprised three females and three males each of mice and squirrels, three male
249 chipmunks, and one female chipmunk.

250 A colorimetric enzyme immunoassay kit (ADI-901-097 Enzo Life Sciences, Inc., Plymouth
251 Meeting, PA, USA) was used to determine levels of corticosterone in plasma in accordance with
252 the manufacturer's protocol (Kuhlman and Martin 2010). Briefly, plasma samples were thawed,

vortexed and diluted 1:100 with Assay Buffer 15. Steroid displacement buffer (SDB) was added and incubated for 5 min to degrade steroid binding proteins that could interfere with corticosterone binding. Due to the limited volume of plasma we obtained from mice we were not able to run optimizations for plasma dilutions and for SDB concentration (see Wada et al. 2007). Samples were aliquoted into separate wells (100 µL per well), run in duplicate, and randomized in the plate. The standard curve was produced using assay standards (32-20 ng) that were run in duplicate on each plate. We then added the conjugate and antibody, and the plate was incubated at room temperature on a plate shaker for 2 hours. The contents of the wells were then rinsed 3 times with wash buffer before an alkaline phosphatase conjugate and a p-nitrophenyl phosphatase substrate were added. The plate was then incubated for 1 hour, at room temperature, in the dark and without shaking. Finally, a stop solution was added and the plate was immediately read using a BioRad Benchmark microplate reader at 405 nm.

Statistical Analysis

Given that the BKC index ranged from 0 to 1 and that the data were highly skewed, we used a beta distribution to model the probability density function of this variable. To explain the variation in BKC we used generalized linear models that included the effect of species, sex, and their interaction. The set of candidate models also included simpler models with only the additive effect of both factors, or with a single factor. A null model with only an intercept was added to assess the explanatory power of the factors in the models. To run these models, we used the Glimmix procedure in SAS statistical software version 9.2 (SAS Institute Inc., Cary, NC, USA). Model comparison was done using the Akaike's Information Criteria corrected for small

sample size (AICc). If the difference in AICc between two models (ΔAICc) is < 2, then both models are equally good at explaining the variation in the response variable; when 4<ΔAICc<7 the model has considerably less support, and models having ΔAICc > 10 have no support from the data (Burnham and Anderson 2002).

To analyze differences among species and sexes in their adaptive immune response to the LPS challenge, we used likelihood models that included the effect of sex and/or species on the levels of antibodies. We ran a set of candidate models to explain the variation in background levels, in the levels observed a week after the challenge, and in the proportional change in the antibody levels between day 0 and 7 [(Antibody levels day 7- Antibody levels day 0)/ Antibody levels day 0]. The flexibility of likelihood models allowed us to explicitly model the variance. As the data appeared heteroscedastic, we tested the possibility that the variance was proportional to the mean as a linear function, whether it was better modeled as a heterogeneous variance being different for every group (for species and/or sex), or whether it was constant (a single variance estimate). In the set of candidate models, we also included a null model that included no group differences. To find the maximum likelihood estimates (MLE) of the parameters of the model, we used a simulated annealing routine (Goffe et al. 1994), implemented in the program R, version 2.10.1 (R Development Core Team 2009) by L. Murphy and C. Canham as part of the Likelihood package. The strength of evidence for the MLEs was expressed by a two-unit likelihood support interval.

To evaluate the relationship between corticosterone levels and immune responses of the individuals, we ran linear regressions using the levels of corticosterone on either day 0 or

day 4, or the percent difference between day 4 and day 0 as the independent variable and the BKC or antibody production as the dependent variable. This was done in R using the data points for all the species combined and in a separate analysis for each of the species.

RESULTS

We captured a total of 59 individuals of 6 different species (Table 1). We were able to obtain a blood sample from all of them on day 0, but complete samples on days 0, 4, and 7 were obtained on only 48 individuals due to early releases, escapes, and deaths. The results that follow are for the three species for which we obtained sufficient sample sizes for all analyses, i.e., white-footed mice, eastern chipmunks, and gray squirrels. Among these three species, pace of life is variable, with mice being fastest, squirrels being slowest and chipmunks being intermediate (Appendix A).

Bacterial killing capacity

We measured BKC for 51 individuals. The modeling approach indicated that species identity was important in explaining the variation in BKC. All the models that contained the effect of species received high support (Table 2). Differences between sexes also received some support; the explanatory power of the interaction term between sex and species was driven by differences between sexes in chipmunks but not in the other two species. However, the simpler model with the effect of species alone was equally good at explaining the variation in BKC (ΔAICc <2 between model 3 and 1; Table 2). The least squares mean estimates predicted by the

314 species only model indicate that mice have a BKC against *E. coli* that is twice as high as that of
315 the two sciurid species (Figure 1).

316 **Antibody production**

317 We were able to collect IgG data for 34 individuals. The results indicate that species but
318 not sexes differed in background levels of LPS-antibodies (Table 3). Background antibody levels
319 were low in mice, intermediate in chipmunks, and high in squirrels (Figure 2A). The variance
320 was best modeled as a linear function of the mean ($s = d \times \mu$; MLE of d (S.I.) = 0.033(0.027-
321 0.044)).

322 Species responded differently to the immunological challenge. The results from
323 modeling the data on antibody levels on day 7 support species but not sex differences (Table 3).
324 Similar to the pattern of the background levels, squirrels were also the ones with the highest
325 antibody levels on day 7 (Figure 2B), and the variance was also best estimated as a linear
326 function of the mean (MLE of d = 0.049 (0.040-0.065)). When modeling the proportional change
327 of antibody levels after the challenge as the response variable the most parsimonious model
328 was again the one with only species differences and a variance proportional to the mean
329 (Model 19, Table 3). Again, squirrels were the species with the strongest adaptive immune
330 response (Figure 2C). The estimated slope of the relationship between the variance and the
331 mean was 0.90 (0.721-1.190).

Corticosterone levels

The levels of corticosterone were an order of magnitude higher in mice than in the other 2 species pre-LPS (Figure 3). Male and female squirrels showed opposite directions in the change of corticosterone levels during the first four days of the study; whereas males decreased their levels, females increased them (Figure 3). The other two species did not show noticeable differences between day 0 and 4 (Figure 3). For mice only, we found that the level of corticosterone in the blood on day 0 had a significant negative relationship with BKC (Linear least squares regression coefficient = -0.001, SE = 0.0005; Figure 4A). The BKC of other two species individually or of all species combined was not affected by corticosterone levels on day 0 ($p > 0.05$ for all linear regressions). On the other hand, the change in the levels of LPS-antibodies from day 0 to day 7 was not affected by corticosterone levels on day 0, day 4 or by the difference between day 0 and day 4 for individual species or all combined ($p > 0.05$ for all linear regressions; Figure 4B,C, and D, respectively).

DISCUSSION

Maximizing adaptive immunity may involve a trade-off in investment in innate immunity (Lee 2006, Martin et al. 2007, Allen et al. 2009, Forsman et al. 2008). The ecoimmunological pace of life hypothesis argues that slow-living species should invest relatively more in the adaptive arm of the immune system, even though it is more developmentally costly, because repeated pathogen exposures are expected over a long life span. On the other hand, fast-living species are expected to rely more strongly on the innate immune system to minimize developmental costs that can be allocated instead to rapid reproduction and to facilitate rapid

353 clearance or resistance of an infection upon first encounter (Martin et al. 2008b). We analyzed
354 immune strategies of wild-caught individuals from rodent species that vary in life history
355 features.

356 Our results support the pace of life hypothesis. We found that adaptive immunity, as
357 measured by the antibody response to the lipopolysaccharide (LPS) challenge, was greatest in
358 squirrels, intermediate in chipmunks, and lowest in mice; whereas bacterial killing capacity, our
359 measure of innate immunity, had the opposite rank order. These results suggest that squirrels
360 can display a strong antibody response that not only may be very effective due to its specificity,
361 but also will confer them protection against future exposures to the same pathogen, which is
362 more likely in this relatively long-lived species. In contrast, perhaps due to immunological trade-
363 offs, squirrels showed the weakest bacterial killing capacity.

364 These findings are consistent with other studies that tested an association between
365 pace of life and immune responses. For instance, Sparkman and Palacios (2009) noted greater
366 constitutive innate response in the fast-living ecotype of garter snakes (*Thamnophis elegans*)
367 than in the slow-living ecotype, in agreement with the idea that fast-living organisms would rely
368 more on inexpensive immune defenses. Supporting evidence for a greater investment in costly
369 immune defenses by slow-living species comes from the study by Tella et al. (2002), which
370 found stronger cell-mediated immunity in bird species with slow-living traits compared to fast-
371 living species. In contrast, Martin et al. (2001) observed an increase in cell-mediated immunity
372 with increasing clutch size (a fast-living trait). Even though cell-mediated immunity is
373 considered a costly response, it is also generally accompanied by the production of
374 inflammatory cytokines which carry a high risk of autoimmune damage; long-lived species may

prevent the accumulation such damage by using antibody-mediated defenses over inflammatory responses (Lee 2006). Martin et al. (2006) found that the fast-living population of house sparrows had stronger cell-mediated immunity and also produced higher antibodies levels (although they showed a slower response to secondary immune challenges) than the slow-living birds. Likewise, in disagreement with the pace of life hypothesis Cutrera et al. (2010), who studied the response to an immunological challenge in Tuco-tucos (*Ctenomys talarum*), noted that the antibody titres produced by this slow-living rodent species were very low in comparison to those reported for fast-living rodents. Additional evidence conflicting with the predictions of the pace-of-life hypothesis comes from Tieleman et al. (2005), who found that tropical bird species with a slower pace-of-life have greater innate immunity than their fast-living counterparts.

Conflicting evidence regarding the pace of life hypothesis may be due to three potential causes: First, the taxonomic breadth of these studies varies from intraspecific, to intrageneric, to intergeneric comparisons, and it is possible that the patterns of immunity along the fast-slow axis may not be consistent across taxonomic levels. Second, the variability in immune strategies is difficult to interpret from single immune measures because trade-offs between different immune components may constrain investment in each defense mechanism (Lee 2006). Martin et al. (2007) observed a trade-off between innate and adaptive immunity among species in the genus *Peromyscus*. They found that species possess either a strong ability to kill bacteria *in vitro* or a strong capacity to produce antibodies when challenged, but not both. Finally, a third potential reason for the mixed support for the pace of life hypothesis is that finding immunological assays that reduce or eliminate taxonomic biases is difficult. For example, we

used an anti-guinea pig IgG antibody because quantitative differences among species in IgG titer could vary due to affinity alone if a murine antibody was selected. Whereas the optimal solution would have been to use antibodies specific to IgG for each host species, such tools are commercially unavailable. Nevertheless, our study provides support for the existence of the relationship between immune strategy and life history based on three rodent species that are not particularly closely related within the Rodentia (families Muridae and Sciuridae), nor are they closely related to guinea pigs (family Caviidae) (Michaux and Catzeflis 2000, Blanga-Kanfi et al. 2009, Churakov et al. 2010), the species for which the secondary antibodies used in the ELISAs were developed. In our study, the differences in immune responses were pronounced: mice were estimated to kill twice as many bacterial colonies as squirrels or chipmunks, and the change in antibody levels was two times greater in squirrels than in the other two species.

It is possible that our results were affected by the influence of stress due to trapping and captivity on immune function. But it is difficult to predict how these stressors would affect the immune responses that we measured. This is because species can differ in their stress responses to being trapped, to the time spent in the trap (Romero et al. 2008, Delehanty and Boonstra 2009), and / or to captivity (Kuhlman et al. 2010, Martin et al. 2011); but they can also differ in how their immune system responds to stress (Martin 2009). In general, mice were in the traps for longer periods of time than chipmunks or squirrels. Our measures of levels of corticosterone on plasma revealed that mice with high concentrations of circulating corticosterone on the first day of the experiment had reduced bacterial killing capacity. If these initial levels of corticosterone are a response to trapping stress, likely perceived as an acute stress, then high corticosterone levels should have had immunoenhancing effects (Sapolsky et

al. 2000, Dhabhar 2002, Martin 2009). Perhaps the corticosterone concentrations that we measured are instead a reflection of different experiences by individuals in the field. On the other hand, of the three studied species we noted that mice had the highest levels of corticosterone in plasma, yet these species differences have to be interpreted cautiously because an optimization of the assay was not possible.

Another factor that could influence the relationship between the pace-of-life and immunity detected in free-ranging animals is the number and types of pathogens encountered at ecological and evolutionary time scales (Lee 2006, Martin et al. 2007). Differences in pathogen exposures are difficult to measure, but we can expect them to be influential if the species differ significantly in their social organization (Altizer et al. 2003, Nunn et al. 2003), feeding habits (Blount et al. 2003), mating system (Nunn 2002, Nunn et al. 2003, Ezenwa et al. 2006), habitat association (Piersma et al. 1997), substrate use (Nunn et al. 2003), abundance (Arneberg et al. 1998, Ezenwa et al. 2006), and / or body size (Poulin 1995, Ezenwa et al. 2006). The three species that we studied are very similar in social organization (mostly solitary with some seasonal sociality), diet (granivorous-omnivorous), mating system (promiscuous/polygynous), habitat association (mixed deciduous forests), and substrate use (terrestrial to semi-arboreal) (Whitaker and Hamilton 1998). They differ in body size and average abundance (Appendix A), but these are strong correlates of the pace of life. Therefore, both pace of life and history of pathogen exposure might be responsible for the different immune strategies we observed.

Prior and current infections are likely important sources of the large individual variation evident in the immune responses that we recorded (Jolles et al. 2008, Pedersen and Fenton

441 2007). This is not surprising given that we studied wild animals instead of laboratory-raised
442 individuals. Our goal was not to try to decouple the immune responses from past and current
443 infections but rather to understand them in the natural context in which they occur. Even with
444 the variation that different exposures may have generated we still detected substantial species
445 differences and they were in agreement with our predictions that immune strategies would be
446 associated with the pace of life.

447 Another important factor that can impact the species immune function is their
448 reproductive status (Lee 2006). Our study was conducted early in the fall, when mice are still
449 showing signs of reproductive activity, but squirrels and chipmunks are no longer breeding.
450 Therefore, it is possible that the species immune responses were affected by the different stage
451 in the breeding season in which the sampling took place. However, we believe that we have
452 minimized this confounding factor by not including reproductive females, for whom the
453 reproductive costs would have been the greatest.

454 Future research should focus on both improving our ability to predict immune strategies
455 from life-history data and understanding the consequences of different immune strategies.
456 There is some evidence that species' reservoir competence is highly influenced by their immune
457 portfolio (e.g., Kurtenbach et al. 2002, 2006). If immune strategy predicts reservoir
458 competence, then we can use information on the life-history traits of the species in a
459 community to hypothesize the role they may play as reservoir for diseases. Measuring roles in
460 disease transmission is difficult and time consuming, whereas data on life-history parameters is
461 sometimes more readily available; thus, a deep understanding of the natural history of the
462 species might be predictive. For instance, by understanding the connections between life

history, immunity, and reservoir competence, we could make predictions on how a disturbance that alters the composition of the host community could influence disease risk and dynamics, depending on whether it favors fast-lived or slower-lived species (Keesing et al. 2010). Understanding patterns of immunity in host species with different paces of life represents a first step in connecting life history, immunity, and reservoir competence. This is crucial, because when faced with accelerating emergence of infectious diseases at a global scale, we need shortcuts to predicting which species are going to be key players in transmitting pathogens in novel ecological contexts. However, pace of life is just one axis in a predictive framework that will clearly need to include multiple host and pathogen characteristics to be of concrete practical use.

Acknowledgements

Julie Ziemba and Sarah Booker assisted in the trapping, manipulation, and husbandry of the animals. Chris Neil also helped with processing the animals. Kathleen LoGiudice, Ray Winchcombe, and Laura Cheney provided valuable advice that facilitated the trapping and care of the animals. We are very grateful to Charlie Canham whose advice improved significantly the data analysis. We thank Andrea Liebl and Courtney Coon for their help in the laboratory at the University of South Florida. Denise Schmidt and Lisa Schwanz provided logistical help at the Cary Institute. Funding for this study came from the National Science Foundation (DEB 0949702 to RSO and C.D. Canham and DEB 0813041 to FK and RSO) and the Centers for Disease Control

and Prevention (CDC Cooperative Agreement 1 U01 CK000107 to M. Gomes-Solecki, D. Brisson, and RSO).

REFERENCES

Allen, L. C., et al. 2009. Roosting ecology and variation in adaptive and innate immune system function in the Brazilian free-tailed bat (*Tadarida brasiliensis*). - J. Comp. Physiol. B-Biochem. Syst. Environ. Physiol. 179: 315-323.

Altizer, S., et al. 2003. Social organization and parasite risk in mammals: Integrating theory and empirical studies. - Annual Review of Ecology Evolution and Systematics 34: 517-547.

Ardia, D. R., et al. 2003. Reproductive effort reduces long-term immune function in breeding tree swallows (*Tachycineta bicolor*). - Proc. R. Soc. Lond. Ser. B-Biol. Sci. 270: 1679-1683.

Arneberg, P., et al. 1998. Host densities as determinants of abundance in parasite communities. - Proc. R. Soc. Lond. Ser. B-Biol. Sci. 265: 1283-1289.

Bielby, J., et al. 2007. The fast-slow continuum in mammalian life history: An empirical reevaluation. - Am. Nat. 169: 748-757.

Blanga-Kanfi, S., et al. 2009. Rodent phylogeny revised: analysis of six nuclear genes from all major rodent clades. - BMC Evol. Biol. 9.

Blount, J. D., et al. 2003. Do individual branches of immune defence correlate? A comparative case study of scavenging and non-scavenging birds. - Oikos 102: 340-350.

Burnham, K. P., and Anderson, D. R. 2002. Model selection and multimodel inference: A practical information-theoretic approach, 2nd ed. New York: Springer-Verlag.

Churakov, G., et al. 2010. Rodent Evolution: Back to the Root. - Mol. Biol. Evol. 27: 1315-1326.

Cutrera, A. P., et al. 2010. Mounting a specific immune response increases energy expenditure of the subterranean rodent *Ctenomys talarum* (tuco-tuco): implications for intraspecific and interspecific variation in immunological traits. - J. Exp. Biol. 213: 715-724.

508 Delehanty, B., and Boonstra, R. 2009. Impact of live trapping on stress profiles of Richardson's
509 ground squirrel (*Spermophilus richardsonii*). - General and Comparative Endocrinology
510 160: 176-182.
511 Demas, G. E., et al. 2011. Beyond phytohaemagglutinin: assessing vertebrate immune function
512 across ecological contexts. - J. Anim. Ecol.: 710–730.
513 Dhabhar, F. S. 2002. A hassle a day may keep the doctor away: Stress and the augmentation of
514 immune function. - Integrative and Comparative Biology 42: 556-564.
515 Ezenwa, V. O., et al. 2006. Host traits and parasite species richness in even and odd-toed
516 hoofed mammals, Artiodactyla and Perissodactyla. - Oikos 115: 526-536.
517 Forsman, A. M., et al. 2008. Immune-challenged house wren broods differ in the relative
518 strengths of their responses among different axes of the immune system. - J. Evol. Biol.
519 21: 873-878.
520 Goffe, W. L., et al. 1994. Global optimization of statistical functions with simulated annealing. -
521 Journal of Econometrics 60: 65-99.
522 Hoff, J. 2000. Methods of blood collection in the mouse. - Lab Animal 29: 47-53.
523 Jolles, A. E., et al. 2008. Interactions between macroparasites and microparasites drive infection
524 patterns in free-ranging African buffalo. - Ecology 89: 2239-2250.
525 Keesing, F., et al. 2010. Impacts of biodiversity on the emergence and transmission of infectious
526 diseases. - Nature 468: 647-652.
527 Klasing, K. C., and Leshchinsky, T. V. 1999. Functions, costs, and benefits of the immune system
528 during development and growth. Pages 2817-2835 in Adams, N. J., Slotow, R. H., eds.
529 Proceedings of the 22nd International Ornithology Congress Durban: BirdLife.

530 Koprowski, J. L. 2002. Handling tree squirrels with a safe and efficient restraint. - Wildl. Soc.
531 Bull. 30: 101-103.
532 Kuhlman, J. R., and Martin, L. B. 2010. Captivity affects immune redistribution to skin in a wild
533 bird. - Funct. Ecol. 24: 830-837.
534 Kurtenbach, K., et al. 2002. Host association of *Borrelia burgdorferi* sensu lato - the key role of
535 host complement. - Trends in Microbiology 10: 74-79.
536 —. 2006. Fundamental processes in the evolutionary ecology of Lyme borreliosis. - Nature
537 Reviews Microbiology 4: 660-669.
538 Lee, K. A. 2006. Linking immune defenses and life history at the levels of the individual and the
539 species. - Integrative and Comparative Biology 46: 1000-1015.
540 Lochmiller, R. L., and Deerenberg, C. 2000. Trade-offs in evolutionary immunology: just what is
541 the cost of immunity? - Oikos 88: 87-98.
542 LoGiudice, K., et al. 2003. The ecology of infectious disease: Effects of host diversity and
543 community composition on Lyme disease risk. - Proc. Natl. Acad. Sci. U. S. A. 100: 567-
544 571.
545 Martin, L. B. 2009. Stress and immunity in wild vertebrates: Timing is everything. - General and
546 Comparative Endocrinology 163: 70-76.
547 Martin, L. B., et al. 2006. Investment in immune defense is linked to pace of life in house
548 sparrows. - Oecologia 147: 565-575.
549 —. 2007. Immune defense and reproductive pace of life in *Peromyscus* mice. - Ecology 88:
550 2516-2528.

551 —. 2008a. Seasonal changes in vertebrate immune activity: mediation by physiological trade-
552 offs. - Philosophical Transactions of the Royal Society B-Biological Sciences 363: 321-339.
553 —. 2008b. Fever and sickness behaviour vary among congeneric rodents. - Funct. Ecol. 22: 68-
554 77.
555 —. 2011. Captivity induces hyperinflammation in the house sparrow. - J. Exp. Biol. 214: 2579-
556 2585.
557 Martin, T. E., et al. 2001. Does clutch size evolve in response to parasites and
558 immunocompetence? - Proc. Natl. Acad. Sci. U. S. A. 98: 2071-2076.
559 Matson, K. D., et al. 2006. Capture stress and the bactericidal competence of blood and plasma
560 in five species of tropical birds. - Physiol. Biochem. Zool. 79: 556-564.
561 Michaux, J., and Catzeflis, F. 2000. The bushlike radiation of muroid rodents is exemplified by
562 the molecular phylogeny of the LCAT nuclear gene. - Mol. Phylogenet. Evol. 17: 280-293.
563 Mills, S.C., et al. 2010. Fitness trade-offs mediated by immunosuppression costs in a small
564 mammal. - Evol. 64: 166-179.
565 Nelson, R. J., and Demas, G. E. 1996. Seasonal changes in immune function. - Quarterly Review
566 of Biology 71: 511-548.
567 Nordling, D., et al. 1998. Reproductive effort reduces specific immune response and parasite
568 resistance. - Proc. R. Soc. Lond. Ser. B-Biol. Sci. 265: 1291-1298.
569 Norris, K., and Evans, M. R. 2000. Ecological immunology: life history trade-offs and immune
570 defense in birds. - Behav. Ecol. 11: 19-26.
571 Nunn, C. L. 2002. A comparative study of leukocyte counts and disease risk in primates. -
572 Evolution 56: 177-190.

573 Nunn, C. L., et al. 2003. A comparative study of white blood cell counts and disease risk in
574 carnivores. - Proc. R. Soc. B-Biol. Sci. 270: 347-356.
575 —. 2009. On sexual dimorphism in immune function. - Philosophical Transactions of the Royal
576 Society B-Biological Sciences 364: 61-69.
577 Ostfeld, R. S. 2011. Lyme Disease: The ecology of a complex system - Oxford University Press,
578 Inc.
579 Pedersen, A. B., and Fenton, A. 2007. Emphasizing the ecology in parasite community ecology. -
580 Trends Ecol. Evol. 22: 133-139.
581 Piersma, T. 1997. Do global patterns of habitat use and migration strategics co-evolve with
582 relative investments in immunocompetence due to spatial variation in parasite
583 pressure? - Oikos 80: 623-631.
584 Poulin, R. 1995. Phylogeny, ecology, and the richness of parasite communities in vertebrates. -
585 Ecol. Monogr. 65: 283-302.
586 Promislow, D. E. L., and Harvey, P. H. 1990. Living fast and dying young - A comparative analysis
587 of life-history variation among mammals. - J. Zool. 220: 417-437.
588 Råberg, L., et al. 1998. On the adaptive significance of stress-induced immunosuppression. -
589 Proc. R. Soc. Lond. Ser. B-Biol. Sci. 265: 1637-1641.
590 —. 2002. Basal metabolic rate and the evolution of the adaptive immune system. - Proc. R. Soc.
591 Lond. Ser. B-Biol. Sci. 269: 817-821.
592 Ricklefs, R. E. 1992. Embryonic -development period and the prevalence of avian blood
593 parasites. - Proc. Natl. Acad. Sci. U. S. A. 89: 4722-4725.

Ricklefs, R. E., and Wikelski, M. 2002. The physiology/life-history nexus. - Trends Ecol. Evol. 17: 462-468.

Rolff, J. 2002. Bateman's principle and immunity. - Proc. R. Soc. Lond. Ser. B-Biol. Sci. 269: 867-872.

Romero, L. M., et al. 2008. Seasonal glucocorticoid responses to capture in wild free-living mammals. - Am. J. Physiol.-Regul. Integr. Comp. Physiol. 294: R614-R622.

Sapolsky, R. M., et al. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. - Endocr. Rev. 21: 55-89.

Sikes, R. S., et al. 2011. Guidelines of the American Society of Mammalogists for the use of wild mammals in research. - J. Mammal. 92: 235-253.

Sompayrac, L. 2008. How the immune system works. - Blackwell Publishing, Inc.

Sparkman, A. M., and Palacios, M. G. 2009. A test of life-history theories of immune defence in two ecotypes of the garter snake, *Thamnophis elegans*. - J. Anim. Ecol. 78: 1242-1248.

Tella, J. L., et al. 2002. Is cell-mediated immunity related to the evolution of life-history strategies in birds? - Proc. R. Soc. Lond. Ser. B-Biol. Sci. 269: 1059-1066.

Tieleman, B. I., et al. 2005. Constitutive innate immunity is a component of the pace-of-life syndrome in tropical birds. - Proc. R. Soc. B-Biol. Sci. 272: 1715-1720.

Wada, H., et al 2007. Development of stress reactivity in white-crowned sparrow nestlings: Total corticosterone response increases with age, while free corticosterone response remains low. – Gen. Comp. Endocrinol. 150: 405-413.

Whitaker, J. O., and Hamilton, W. J. 1998. Mammals of the Eastern United States. - Cornell University Press.

Supplementary material (Appendix o20215 at <www.oikosoffice.lu.se/appendix>).

Appendix A.

Table 1: Number of individuals sampled in each sex and species.

Species \ Sex	white-footed mice (*Peromyscus leucopus*)	southern flying squirrels (*Glaucomys volans*)	eastern chipmunks (*Tamias striatus*)	red squirrels (*Tamiasciurus hudsonicus*)	eastern gray squirrels (*Sciurus carolinensis*)	woodchucks (*Marmota monax*)	Total
Female	8	2	9	1	4	2	26
Male	10	0	15	1	7	0	33
Total	18	2	24	2	11	2	59

Table 2: Generalized linear models for explaining the variation in bacteria killing capacity against *E. coli*. The response variable is the proportion of bacteria colonies killed by the blood of the animals in the study, modeled as a function of species and sex using a beta distribution. AIC_c = Akaike information criteria corrected for small sample size; ΔAIC_c = difference in AICc value between the AICc for the model given in that row and the most parsimonious model (lowest AIC_c, bolded).

Model no.	Model	No. of parameters	AIC_c	ΔAIC_c	Akaike's weight
1	**Species * Sex**	**13**	**-69.14**	**0**	**0.47**
2	Species	5	-68.27	0.87	0.31
3	Species + Sex	7	-67.48	1.66	0.21
4	Sex	4	-60.49	8.65	0.01
5	Intercept	2	-60.63	8.51	0.01

626 **Table 3**: Likelihood models used to investigate the differences among species and sexes on
627 background levels of LPS-specific antibodies, on the levels attained a week after an
628 immunological challenge, and on the change in antibody levels between day 0 and 7. The
629 variance was modeled as proportional to the mean (linear function), or as a heterogeneous
630 variance (different for every group), or as a constant (single estimate). The set of candidate
631 models also includes a null model with no group differences. AIC_c = Akaike information criteria
632 corrected for small sample size; ΔAIC_c = difference in AICc value between the AICc for the model
633 given in that row and the most parsimonious model (lowest AIC_c, bolded).

	Model no.	Model	No. of param.	AIC_c	ΔAIC_c	Akaike's weight
Background antibody levels (day 0)	**1**	**Species proportional variance**	**4**	**92.7**	**0**	**0.86**
	2	Species heterogenous variances	6	97.2	4.5	0.09
	3	Species*Sex proportional variance	7	98.3	5.6	0.05
	4	Species constant variance	4	105.5	12.8	0
	5	Species*Sex constant variance	7	111.7	19	0
	6	Species*Sex heterogenous variances	12	119.3	26.6	0
	7	Null model	2	120.9	28.2	0
Antibody response (day 7)	**8**	**Species proportional variance**	**4**	**182.1**	**0**	**0.94**
	9	Species heterogenous variances	6	187.9	5.8	0.05
	10	Species*Sex proportional variance	7	191.1	9	0.01
	11	Species constant variance	4	199.2	17.1	0
	12	Species*Sex constant variance	7	206.3	24.3	0
	13	Species*Sex heterogenous variances	12	212.3	30.2	0
	14	Null model	2	216.6	34.5	0
Change in antibody levels (day 7-day 0) /day 7	**15**	**Species proportional variance**	**4**	**136.7**	**0**	**0.65**
	16	Species*Sex proportional variance	7	138.5	1.9	0.26
	17	Species heterogenous variances	6	140.7	4	0.09
	18	Species constant variance	4	148	11.3	0
	19	Null model	2	149.7	13.1	0
	20	Species*Sex heterogenous variances	12	149.9	13.2	0
	21	Species*Sex constant variance	7	153.8	17.2	0

634

635 Figure legends

636 **Figure 1.** Least square means estimates (and 95% CI) of the species bacteria killing capacity
637 against *E. coli* predicted by the generalized linear model that included only species differences.

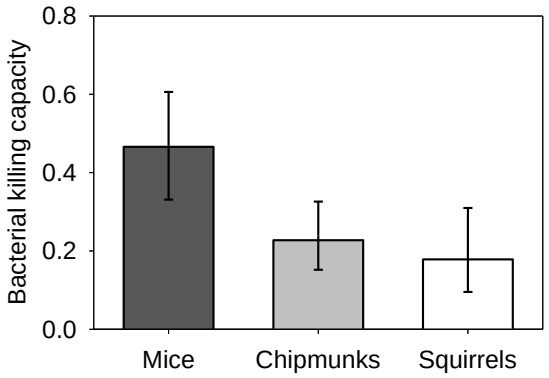
638 **Figure 2.** Maximum likelihood parameter estimates predicted by the most parsimonious models
639 for LPS-specific antibody levels detected on samples collected on (A) day 0 (background levels,
640 in absorbance units (AU)) or on (B) day 7 (response to LPS challenge, in AU), and (C) their
641 proportional change (day 7 – day 0) / day 0. Error bars are lower and upper likelihood support
642 intervals.

643 **Figure 3.** Observed changes in corticosterone levels in blood (mean ± 1 S.E.) collected on day 0
644 and on day 4 for females (circles) and males (triangles) of (A) mice, (B) Chipmunks, and (C)
645 Squirrels.

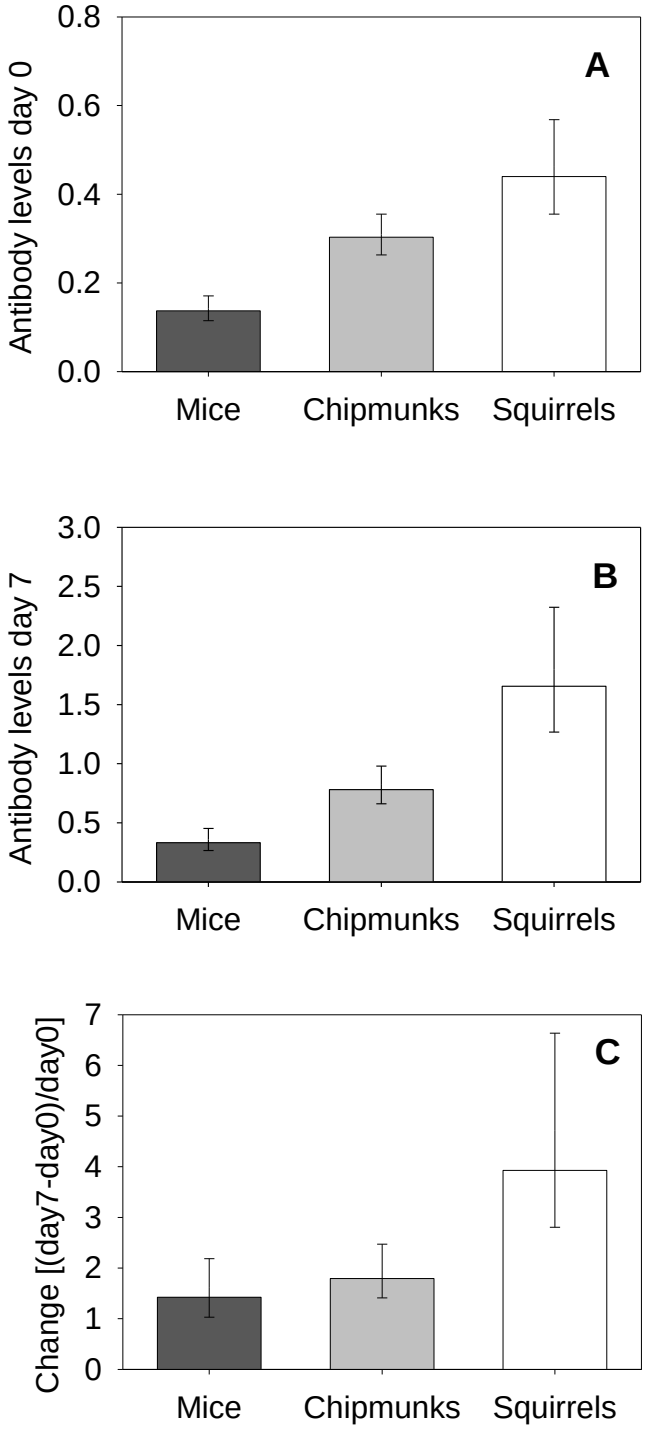
646 **Figure 4.** Relationship between corticosterone levels in blood and immunological responses: (A)
647 Bacterial killing capacity, (B-D) change in LPS-antibody levels. Showing individual samples
648 corresponding to females (circles) and males (triangles) of mice (dark gray), chipmunks (gray),
649 and squirrels (white).

650

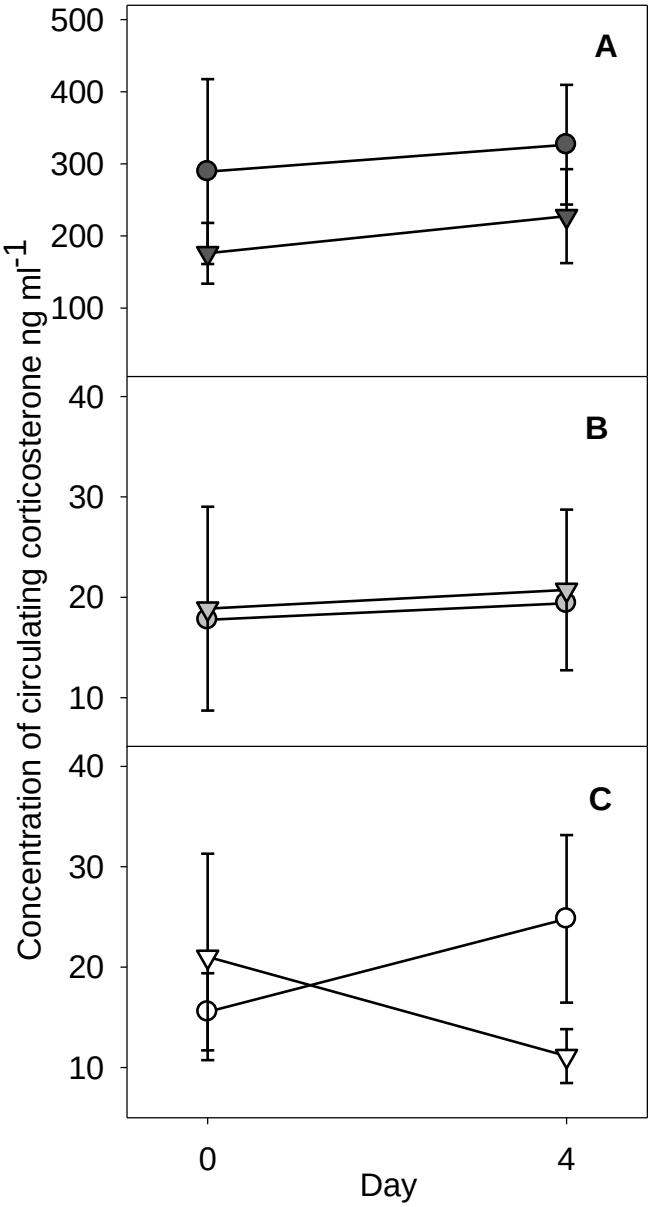
651 **Figure 1**


Bacterial killing capacity
0.8
0.6
0.4
0.2
0.0
Mice
Chipmunks
Squirrels

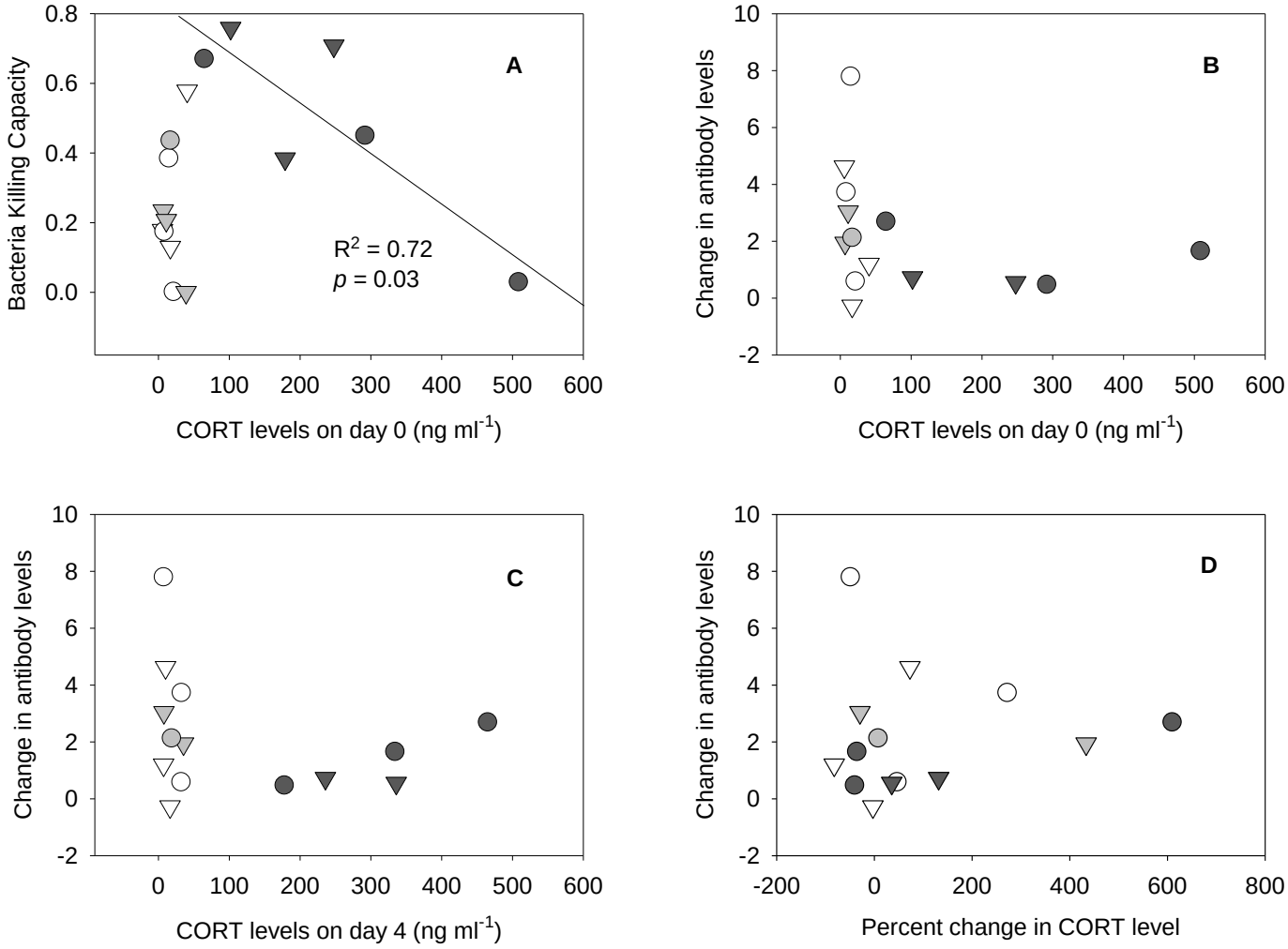
652 **Figure 2**


A
Antibody levels day 0
0.0
0.2
0.4
0.6
0.8
Mice
Chipmunks
Squirrels
B
Antibody levels day 7
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Mice
Chipmunks
Squirrels
C
Change [(day7-day0)/day0]
0
1
2
3
4
5
6
7
Mice
Chipmunks
Squirrels

653 **Figure 3**


A
B
C
500
400
300
200
100
40
30
20
10
40
30
20
10
Concentration of circulating corticosterone ng ml-1
0
4
Day

654 **Figure 4**


A
Bacteria Killing Capacity
0.8
0.6
0.4
0.2
0.0
R2 = 0.72
p = 0.03
0
100
200
300
400
500
600
CORT levels on day 0 (ng ml-1)
B
Change in antibody levels
10
8
6
4
2
0
-2
CORT levels on day 0 (ng ml-1)
C
CORT levels on day 4 (ng ml-1)
D
-200
800
Percent change in CORT level