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The effect of environmental differences on the immune response of rodents

Inaugural-Dissertation
to obtain an academic degree of
Doctor of Philosophy (PhD) in Biomedical Sciences
under the Dahlem Research School (DRS) Program
of Freie Universität Berlin

submitted by
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Berlin 2015
Journal-Nr.: 3821

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The work described in this dissertation was performed in the Leibniz Institute for Zoo and Wildlife Research in Berlin from September 02, 2011 to July 23, 2015 under the supervision of Prof. Dr. Alex D. Greenwood and Dr. Gábor Á. Czirják, and has been submitted to the Department of Veterinary Medicine of Freie Universität Berlin.

Front cover image: A young brown rat (*Rattus norvegicus*) photographed at Wangwu County, Jiyuan, Henan, China (Credit: Jundong Tian, August 13, 2014).
Back cover image: Altocumulus clouds (Credit: Jundong Tian, September 16, 2014).

Aus der Abteilung für Wildtierkrankheiten
im Leibniz-Institut für Zoo- und Wildtierforschung
im Forschungsverbund Berlin e.V.

eingereicht beim Fachbereich Veterinärmedizin
der Freien Universität Berlin

The effect of environmental differences on the immune response of rodents

Gedruckt mit Genehmigung des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

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4.19 The Master said, When one's parents are alive, make no distant journeys; when you travel, have a set destination.

15.12 The Master said, A man who does not think far ahead will have troubles near at hand.

In memory of my grandfather

In memory of my grandmother

Dedicated to

> *my mother and father*
>
> *my brother and sister*

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This thesis is based on the following manuscripts:

1. **Jundong Tian**, Alexandre Courtiol, Karin Schneeberger, Alex D. Greenwood, Gábor Á. Czirják. 2015. Circulating white blood cell counts in captive and wild rodent are influenced by body mass rather than testes mass, a correlate of mating promiscuity. Functional Ecology, 29:823–829. doi:10.1111/1365-2435.12394.

2. **Jundong Tian**, Heribert Hofer, Alex D. Greenwood, Gábor Á. Czirják. Colonization history shaped the immunity of the western house mouse. Oecologia, 179:679–686. doi:10.1007/s00442-015-3397-0.

3. **Jundong Tian**, Alex D. Greenwood, Yifeng Zhang, Anca Dorhoi, Heribert Hofer, Gábor Á. Czirják, Jiqi Lu. Urbanization modulates the condition and health of wild brown rats. In preparation.

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List of Abbreviations

Abbreviation	Full term
ABM	Adult body mass averaged across sexes
CD4/ 8	cluster of differentiation 4/ 8 cells
CI	Confidence interval
CNKI	China national knowledge infrastructure
CO_2	Carbon dioxide
df	Degree of freedom
F	Female
IgA/ IgD/ IgE/ IgG/ IgM	Immunoglobulin A/ D/ E/ G/ M
ISIS	International species information system
KLH	Keyhole limpet hemocyanin
ln	Natural logarithmic transformation
LR	Likelihood ratio
M	Male
MBM	Mean adult body mass of males
MHC	Major histocompatibility complex
NDV	Newcastle disease virus
PGLS	Phylogenetic generalized least-squares
SE	Standard error of estimate
se	Standard error of mean
SRBC	Sheep red blood cells
STDs	Sexually transmitted diseases
TLR	Toll-like receptor
TM	Mean testes mass
WBC	White blood cell

Chapter 1 General introduction

Macro- and micro-parasites are ubiquitous and present a constant challenge to their hosts. However, host animals actively resist microbes with the immune system representing one of the most important defenses. While previous medical immunological studies have provided substantial information on how hosts control pathogens and parasites, why certain species or individuals are more or less susceptible to a given pathogen remains unclear (Sheldon & Verhulst, 1996). With the emergence of eco-immunology as a field, it has become clear that the immune defense is context dependent and is a result of host's interaction with a given biotic (e.g., host-parasite interaction) and abiotic (e.g., temperature, humidity) factors (Schulenburg et al., 2009; Martin et al., 2011; Malagoli & Ottaviani, 2014).

Although the immune system is very complex, its components can be clustered into two categories, innate and adaptive, both branches having cellular and humoral effectors (Fig.1.1) (Murphy, 2012). The innate immune system has low specificity and reacts quickly to foreign antigens, representing the host's first line of defense. In contrast, adaptive immunity requires an initial contact with an antigen, and it is slow but very antigen specific. The two branches of the immune system are connected via cytokines, a biologically active protein group which act as intercellular messengers (Murphy, 2012).

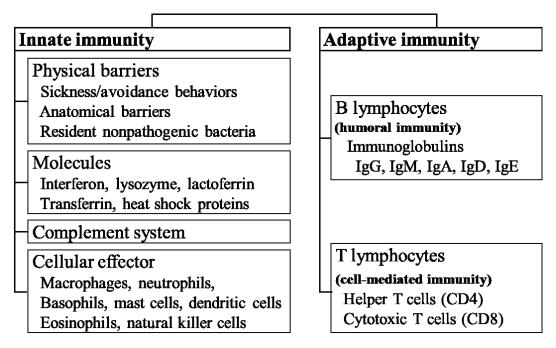| Innate immunity	Adaptive immunity
Physical barriers Sickness/avoidance behaviors Anatomical barriers Resident nonpathogenic bacteria	
Molecules Interferon, lysozyme, lactoferrin Transferrin, heat shock proteins	**B lymphocytes** **(humoral immunity)** Immunoglobulins IgG, IgM, IgA, IgD, IgE
Complement system	
Cellular effector Macrophages, neutrophils, Basophils, mast cells, dendritic cells Eosinophils, natural killer cells	**T lymphocytes** **(cell-mediated immunity)** Helper T cells (CD4) Cytotoxic T cells (CD8)

Fig.1.1 The major immune mechanisms in vertebrates (modified from Muehlenbein, 2010)

1.1 Introduction to ecological immunology

Most of the immunological literature describes studies on animals with limited genetic diversity and living under highly regulated laboratory conditions where variation in biotic (e.g., exposure to pathogens and parasites) and abiotic (e.g., food and water resources, temperature) factors is minimized (Lazzaro & Little, 2009; Pedersen & Babayan, 2011; Babayan et al., 2011; Maizels & Nussey, 2013). However, most organisms, including humans, live in variable natural environments which will influence the immune phenotype at the level of individual and species (Sheldon & Verhulst, 1996; Schulenburg et al., 2009; Brock et al., 2014). A new field has emerged called ecological immunology or eco-immunology, which attempts to address variation in immunological function taking into account the variability of natural environments (Sheldon & Verhulst, 1996; Demas & Nelson, 2012; Malagoli & Ottaviani, 2014).

Eco-immunology derives primarily from life-history theory, stating that the immune response is energetically costly and thus will be traded-off with other life-history traits such as reproduction (Sheldon & Velhust, 1996). Several costs have been described for the different immune effectors such as developmental, use and maintenance costs (Schmid-Hempel & Ebert, 2003; Armitage et al., 2003; Lazzaro & Little, 2009). Accordingly the immune phenotype of an individual will be influenced by its physiological status, environment and specific pathogen pressure. Moreover, immune up- or down-regulation will be affected by the costs and benefits of developing a specific immune phenotype (Schmid-Hempel & Ebert, 2003; Gause et al., 2003). It is also postulated that the immune system is under sexual selection. Accordingly, the immuno-competence handicap hypothesis suggests that only males in good health can invest in both immunity and secondary sexual traits, which is mediated mainly by testosterone (Roberts et al., 2004). Therefore not only natural selection in general but specifically sexual selection shapes the evolution of the immune system (McKean & Nenney, 2005). The effects of sex, age or seasonality on the within and among species variation of immune responses constitute the main research topics in eco-immunology (Hasselquist, 2007; Demas & Nelson, 2012; Malagoli & Ottaviani, 2014). Understanding applied aspects of immunological variation, incorporating immunological questions in invasion biology, understanding the association between immuno- and reservoir-competence, or how we can use immunological knowledge in conservation biology are current areas of intensive study (Lazzaro & Little, 2009; Pedersen & Babayan, 2011; Babayan et al., 2011).

Eco-immunology is a relatively new field and therefore many recent studies focus on methodological aspects, not only developing or validating new methods or assays originating from medical immunology but also reducing sample volumes needed, describing new sample storage techniques or the use of non-invasive samples (e.g. faeces, saliva) (Schulenburg et al., 2009; Boughton et al., 2011; Demas & Nelson, 2012; Malagoli & Ottaviani, 2014). These methodological aspects are important in remote sites or in small or endangered species. The assessment of protective immune

phenotype is feasible in commonly studied species, such as humans, domestic species and model laboratory animals. For these species a myriad of measurements are available, both commercial and/or laboratory-developed tools. However, this is not the case for free-living wildlife (Lotze & Thomson, 2005; Viney & Riley, 2014). Moreover, in many cases researchers have to rely on a single or low volume samples. Despite these obstacles, several assays are available for assessing both the structure and the function of the immune system. Some of these parameters are influenced by several factors (e.g., circadian rhythm, stress) and change quickly which make interpretations difficult (Boughton et al., 2011; Pedersen & Babayan, 2011). Some measurements are suitable for characterizing within species immunological variation, while others are more suitable for between species comparisons (Matson et al., 2006). Initial studies focused on measuring one or limited number of immune parameters and extrapolating the obtain results to the immuno-competence of the individual or species (Sheldon & Verhulst, 1996). Recent studies on the other hand have been able to include more variables which is important since the variation in immune effectors depends on the context and trade-off that exist also among different parts of the immune response (for example T helper cell 1 *versus* T helper cell 2 responses) (Boughton et al., 2011; Pedersen & Babayan, 2011). The commonly measured immune parameters for wild species include the size of lymphoid organs (e.g., spleen, thymus, and bursa of Fabricuis for birds), total and differential leukocyte counts (e.g., lymphocyte, neutrophil, monocyte, eosinophil, and basophil), total immunoglobulin concentrations (e.g., IgA, IgD, IgE, IgG, and IgM), and complement levels. Functional tests, such as bacterial killing assays, lymphocyte proliferation assays or challenges with different non-pathogenic antigens (e.g., KLH, SRBC), with generic stimulants (e.g., phytohaemagglutinin) or vaccines (e.g., NDV, diphtheria-tetanus) are also frequently applied (Sheldon & Verhulst, 1996; Norris & Evans, 2000; Boughton et al., 2011; Demas et al., 2011; Viney & Riley, 2014). In addition to these organismal approaches, several studies apply genetics, mainly major histocompatibility complex (MHC) variation, as a potential reflection of immuno-competence. This has recently been complemented with Toll like receptor (TLR) diversity analysis (Acevedo-Whitehouse & Cunningham, 2006; Takeda et al., 2003; Tschirren et al., 2013). The application of proteomics and transcriptomics in eco-immunology is also a recent promising development (Calis & Rosenberg, 2014). For instance, the transcriptome of the wild white-footed mouse (*Peromyscus leucopus*) revealed that there significantly differentially expressed genes involved in immune responses between urban and rural populations (Harris et al., 2015).

Avian species have been the main eco-immunological models since the publication of key articles in the field (Sheldon & Verhulst, 1996; Salvante, 2006). Recently, however, the number and diversity of taxa has been extended to include molluscs (Chen & Yang, 2007), insects (Tripet et al., 2008), amphibians (Brown et al., 2015), reptiles (French et al., 2007), and mammals (Demas & Nelson, 2012; Malagoli & Ottaviani, 2014). These studies integrate a diverse array of measurements (e.g., life history traits, parasite infections, environmental variation, and immune phenotypes)

from both observational and, in some cases, experimental approaches in order to understand what factors drive immune phenotypes and parasite burdens in natural populations (Babayan et al., 2011; Boughton et al., 2011).

Many ecosystems experience significant human-driven alterations (e.g., pollution, habitat changes, urbanization) especially since the beginning of the last century, which has already demonstrated significant consequences (e.g., climate change, emergence of infectious diseases) (Jones et al., 2008; Martin et al., 2010; Gortazar et al., 2014; Becker et al., 2015). However, limited knowledge exists about the effects of these anthropogenic changes on the immune responses of wildlife, especially in mammals, on how the individuals, populations, or species respond to these new environments; how it affects their health status, and ultimately what the consequences are for example on their reservoir competence or their conservation status (Martin et al., 2010; Gottdenker et al., 2014; Jolles et al., 2015).

1.2 The effect of captivity on immunity

Life history theory suggests that trade-offs exist between energetically costly traits, such as immune response and reproduction, which is dependent on the abundance and availability of resources (Lochmiller & Deerenberg, 2000; Schmid-Hempel & Ebert, 2003). Therefore, identifying relationships between immune function and other traits could provide and/or extend the knowledge of how the evolutionary process likely has shaped the evolution of immunity (Sheldon & Verhulst, 1996). Comparative immunological studies have suggested that basal immune investment significantly increases with levels of mating promiscuity in primates and carnivores (Nunn, 2002; Nunn et al., 2003). This is referred to as the 'Sexually transmitted diseases (STDs)' hypothesis (Nunn, 2002; Nunn et al., 2003). No such association has been detected between immunity and other investigated social (e.g., group size and population density), ecological (e.g., risk of soil-borne pathogens, diet) and life-history factors (e.g., pace of life, longevity) (Nunn, 2002; Nunn et al., 2003; Cooper et al., 2012). However, all of these studies were performed on captive animal populations.

Whether these conclusions derived from captive animals can be generalized to free-ranging populations is unclear. Differences between captive and wild populations in terms of behavior and physiology have been described (Calisi & Bentley, 2009; Mason, 2010; Maizels & Nussey, 2013), which occurs mainly due to the differences in biotic (e.g. host-parasite interaction) and abiotic (e.g., resources, hygiene) factors. In accordance, differences in immune functions (e.g., levels of IgE, IgG, and activation of splenic leukocytes) have been observed between wild and captive house mouse and brown rat populations (Devalapalli et al., 2006; Abolins et al., 2011).

Comparative studies of immune function in wild animals have been mainly conducted in avian species. In birds, comparative analyses have revealed that immune defense correlates positively with sociality (Møller et al., 2001), specialization by

avian fleas (Møller et al., 2005) and developmental period (Pap et al., 2015), but negatively with eggshell bacterial load (Soler et al., 2011) and basal metabolic rate (Pap et al., 2015) for example. The only comparative eco-immunological study of wild mammals was conducted on bats, showing that ecological factors such as diet, roosting habits indeed can relate to the species specific immune responses (Schneeberger et al., 2013). However, there is lack of such comparative study on other wild mammals, especially the most diverse group, the rodents.

Obtaining immunological data from several free-living species in a standardized manner is time and resource consuming. However, previous published data can be used for such purposes. In eco-immunology, most of the methods (e.g., immunoglobulin levels, bacterial killing assay) are validated to the species-specific requirements of the assay (Boughton et al., 2011, Demas et al., 2011) or to the volume of the collected materials. One immunological variable which was systematically collected and reported is the total and differential WBC counts, which is used for both research and veterinary purposes (Weiss & Wardrop, 2010). These parameters can be measured under standard conditions and thus are ideal for comparative eco-immunological studies.

Another methodological issue is controlling for the non-independence of traits resulting from shared ancestry among species using phylogenetic comparative methods (Felsenstein, 1985; Grafen, 1989; Pagel, 1999; Freckleton et al., 2002). The most common phylogenetic comparative methods to investigate the correlations among various traits include phylogenetically independent contrasts, phylogenetic generalized least squares (PGLS), and phylogenetic eigenvector regression (Paradis, 2012; Swenson, 2014). Among these methods, the PGLS method considers the variation within a data set by weighing the measurement error (e.g., standard error of the mean) which makes the statistics more robust (Paradis, 2012; Swenson, 2014). The uncertainty of within-species and/or within-population data sets may derive from experimental procedures and/or the nature of the characteristics of the species/population and therefore often needs to be corrected for e.g. PGLS (Ives et al., 2007; Silvestro et al., 2015).

1.3 Colonization history and immunity

Since the late 15th century, biological invasions have become a pervasive component of global change due to the accelerated globalization of trade and travel which has resulted in a drastic breaching of biogeographic barriers that previously had isolated continental and islandic biotas for millions of years (Mooney & Cleland, 2001; Arim et al., 2006). Numerous studies have investigated the effects of biological invasions from various perspectives (e.g., economical, ecological, and public health), and demonstrate negative consequences such as decrease in biodiversity, negative changes of ecosystem functions and services, economic damage, and impacts on wildlife and public health (Nentwig, 2008; Keller et al., 2011; Morand et al., 2015). In

order to develop proper mitigation strategies for the effects of invasive species and predict the novel invasion patterns, there is a need to understand which traits are crucial to becoming a successful invader (Arim et al., 2006; Nentwig, 2008; Keller et al., 2011).

During invasions, species are exposed to different environmental and biological challenges compared to their native habitats, thus facing novel selection pressures from both biotic (e.g., lack of predator and native parasites, encounter novel parasites and pathogens) and abiotic (e.g., temperature, humidity, food resources) sources (Phillips et al., 2010; White & Perkins, 2012). The process of becoming a successful invader is a result of strong selection, specific 'founder events' from various source populations and/or invasion-enhancing phenotypes (e.g., specific morphological, physiological, behavioral traits) (Arim et al., 2006; Travis & Dytham 2002; Shine et al., 2011). Therefore, studies of invasive species not only have importance for conservation biology, but can contribute to understanding rapid evolutionary change in invading species (Carroll, 2008; Brown et al., 2015).

In invasion biology, several hypotheses have been formulated to characterize the success and failure of some species, many of them being associated with parasites (e.g., enemy release, immuno-competence advantage or novel weapons hypothesis) (Prenter et al., 2004; Jeschke et al., 2012a; Prins & Gordon, 2014; Morand et al., 2015). The enemy release hypothesis suggests that the abundance or impact of some alien species is related to the scarcity of natural enemies in the introduced range compared with their native range (Colautti et al., 2004; Liu & Stiling, 2006; Jeschke et al., 2012a; Heger & Jeschke, 2014). The novel weapons hypothesis on the other hand assumes that invasive species carry pathogens which are harmless for them but are highly pathogenic for the native species (Jeschke et al., 2012a; Callaway & Aschehoug, 2000). Several of these hypotheses have been tested in plants (Jeschke et al., 2012a), avian (Lee et al., 2005) and amphibian species (Llewellyn et al., 2012); however, there is lack of studies on mammalian species, despite mammals playing a crucial role in the biological invasions, for instance the globally spread house mice (*Mus musculus*) or *Rattus* species (Berry, 1970; Jeschke et al., 2012b; Gray et al., 2014; Morand et al., 2015).

Due to the primary role of the immune system in defending the host against parasites and other pathogens, immunity is likely to play a key role in determining the success of an invading species (Lee & Klasing, 2004; White & Perkins, 2012). Life-history theory suggests that trade-offs shall favor invader's trait(s) with low energy costs but providing resistance, because the abundance and availability of resources for survival and reproduction are not infinite in a natural environment (Lochmiller & Deerenberg, 2000). Accordingly, successful invaders would rely on energy cheap immune effectors, such as antibody mediated or constitutive innate immunity (Lee & Klasing, 2004; White & Perkins, 2012). In term of developmental and use costs, systemic inflammation and T-cell mediated immunity are the most costly (Lee, 2006). Therefore, one would expect them to play a lesser role in invasive individuals/species compared to their conspecifics from their native habitats or closely

related species from the novel environment. In line with these predictions, by comparing different sparrow (*Passer* sp.) species, it has been shown that dampened inflammatory response have been involved in the range expansion of the house sparrow *P. domesticus* (Lee et al., 2005). Similar decreases in inflammatory response were described in invading cane toads (*Rhinella marina*) from Australia (Llewellyn et al., 2012), with invaders relying mainly on constitutive innate, immune effectors (e.g., neutrophils, phagocytic cells) that are not energetically costly (Brown et al., 2015). However, information about the shift towards antibody-mediated immunity, especially in mammals, is still lacking.

1.4 Urbanization and immunity

Human activities such as agricultural development, deforestation, and urbanization are associated with landscape changes with consequences for biodiversity, ecosystem functions and services and both animal and public health. Some of these effects are local or regional, but over the last century have become global due to rapid technical developments and globalization. Among human activities, urbanization is the major change affecting and transforming native environments, urban areas becoming the dominant environment in both developed and developing countries (McKinney, 2002; Elmqvist et al., 2013; Seto et al., 2013). Several effects, both positive and negative, of urbanization have been described (e.g., pollution, local climate change, increased food resources, decreased predation), and urbanization has been recognized as a major cause of regional biodiversity loss (Grimm et al., 2008; Elmqvist et al., 2013; Seto et al., 2013; Forman, 2014). While many of wildlife species are negatively impacted by the direct and indirect effects of urbanization, the so-called urban exploiters such as some rat (*Rattus* sp.) and mouse (*Mus* sp.) species or red fox (*Vulpes vulpes*) can thrive in these new, human-dominated ecosystems (McKinney, 2002; Kark et al., 2007; Becker et al., 2015).

Urban wildlife can serve as reservoirs/vectors for numerous pathogens (Himsworth et al., 2013; Reyes et al., 2013; Hegglin et al., 2015). High seroprevalence (over 50%, in total 6059 birds) of avian influenza virus in urban birds suggest that they play an important role in disease transmission at the human-wildlife interface (Verhagen et al., 2012). The prevalence of *Bartonella* sp. in urban black rats *Rattus rattus* (60%, 19/32) is more than four times higher compared to the rural individuals (13%, 2/16) (Halliday et al., 2015). Differences in terms of the distribution and intensity of anthropogenic disturbance, urban–rural environmental gradient could modify host–parasite/pathogen interactions and thus lead to emergence of infectious diseases threatening both animal and public health in the context of 'One Health', concept which recognizes the link between environmental, animal and human health (Shochat et al., 2006; Weaver, 2013; Reyes et al., 2013; Gottdenker et al., 2014; Mackenzie et al., 2014). Despite an observed increased incidence of pathogens and

parasites in urban wildlife populations, there is virtually no information on which mechanisms lead to the increases. Though not thoroughly investigated, chronic stress and modified immunity have been suggested to play an important role. The effect of urbanization on animal physiology has been studied mainly in reptiles and birds (Forman, 2014). Tree lizards (*Urosaurus ornatus*) showed that urban individuals may suppress their overall corticosterone concentrations but in parallel increase their immuno-competence compared with their rural counterparts, which might be the outcome of repeated immune challenges, such as wounding or the benefits of increased food resources in cities (French et al., 2008). Comparative study of passerine birds showed urban exploiters generally exhibit less blood parasite infection than rural individuals and this correlates with changes in leukocyte numbers (Fokidis et al., 2008). However, conflicting data has also been obtained for house sparrows (*Passer domesticus*) (Bókony et al., 2012). Another proposed scenario is that the increased level of some chemical pollutants or physical stressors (e.g., light or noise) could suppress the immunity of urban wildlife, which ultimately will shed more parasites and pathogens and will thus suffer higher mortality rates (Bradley & Altizer, 2007). However, apart from limited studies in humans (Amoah et al., 2014; Mbow et al., 2015), similar investigations in urban mammalian species have not yet been conducted.

Resulting shifts in wildlife ecology due to anthropogenic disturbance (e.g., urbanization) can alter the dynamics of infectious disease, and potentially enhance the risk of cross-species transmissions (Martin et al., 2010; Becker et al., 2015). This may be due to biodiversity loss and dilution effects, increasing the contact rates or via pollutants and stress (Bradley & Altizer, 2007; Civitello et al., 2015). Each of these factors is associated with the immune system. Urbanization both benefits (e.g., increased food resources) and hinders (e.g., increased exposure to physical and chemical stressors) animal condition in urban/suburban areas compared with rural/natural environments (McKinney, 2002). Investigating the immunity, parasite load, and allostatic load of urban and rural rodents could address whether urbanization influences their epidemiological role, or alternatively decreases body condition and depresses the immune responses of urban mammals.

1.5 Study aims

The aims of this study were to describe how wildlife adapts immunologically to human-dominated environments, which has relevance not only for evolutionary ecology but both for conservation biology and public health. I used rodents as a model, as they constitute the most diverse group of mammals, accounting for over 40% of all the extant mammalian species (Wolff & Sherman, 2007). They are one of the most important reservoir groups for zoonotic emerging pathogens due to their life-history traits (e.g., short lifespan with multiple litters) and close proximity to human populations (Avenant, 2011; Honeycutt et al., 2007). The vast majority of

immunological knowledge has been generated from studies of laboratory rodent models, mainly house mouse *Mus musculus* and rats *Rattus rattus* and *R. norvegicus*. In general, for these studies, animals have been selectively bred (e.g., genetically homogeneous) and maintained under specific and controlled environments (e.g., common hygiene, specific-pathogen free condition). However, most organisms, including humans, experience more dynamic, uncontrolled conditions in nature. In the first part of my dissertation, I examined whether the 'Sexually-transmitted diseases' hypothesis (Nunn et al., 2000; Nunn et al., 2003) can be generalized to the most diverse mammalian group, the rodents. The hypothesis suggests that differential spread of sexually transmitted diseases will be associated with variation in mating behavior and will drive the baseline structure of the mammalian immune system. Since support for this hypothesis was obtained from captive animals, I examined whether environment (captive *versus* free-living) influences the results. To do so, I performed a phylogenetic comparative analysis on the relationships between immunological data of both captive and free-living rodents and their relative testes mass—a proxy for mating promiscuity (Chapter 2).

Since invading species have to adapt to the newly colonized habitat, in the following chapter (Chapter 3) I investigated the effect of colonization history on the immunity of the western house mouse (*M. m. domesticus*) using wild-derived outbred laboratory individuals from their original range (Iran) and historically recently colonized European populations (from France and Germany). By comparing the immune phenotype of the source and invader populations, I investigated whether immunological differences occur among populations and what mechanism can explain the differences with respect to colonization success.

As urbanization is a both locally and globally influential human-driven process and develops rapidly in developing areas (Yang, 2013), in Chapter 4 I describe how this process impacts the condition and health of urban wildlife using brown rats (*R. norvegicus*) as a model species in Henan Province, China.

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Chapter 2 Circulating white blood cell counts in captive and wild rodent are influenced by body mass rather than testes mass, a correlate of mating promiscuity

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Chapter 3 Colonization history shaped the immunity of the western house mouse

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Chapter 4 Urbanization modulates the condition and health of wild brown rats

Publication 3: Tian JD, Greenwood AD, Zhang YF, Dorhoi A, Hofer H, Czirják GÁ, Lu JQ. Urbanization modulates the condition and health of wild brown rats. In preparation.

Full title: Urbanization Modulates the Condition and Health of Wild Brown Rats

Short title: Urbanization and Health of Urban Rodents

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Abstract

Urbanization has considerable side effects such as overpopulation and environmental pollution, favoring the emergence and re-emergence of various infectious agents, including those originating from wildlife species. Commensal brown rats (*Rattus norvegicus*) represent a reservoir for numerous pathogens of major public health concern. It is currently unclear whether urbanization amplifies their epidemiological role, or alternatively decreases body condition and depresses their immune status. We addressed these questions by measuring morphological, physiological and pathophysiological traits (morphometric, allostatic load, immunity, parasite load) in free-living brown rats sampled from forest-rural, agro-rural and urban areas along a gradient of increasing urbanization and human population density in Henan Province, China. Urban rats (1) were bigger in body length, shorter in tail length, heavier in body mass and had a higher body mass index than rural rodents; (2) had larger spleens and higher leukocyte counts than rural counterparts; (3) tended to have a higher allostatic load as they showed enlarged adrenal glands and a higher neutrophil/lymphocyte ratio; and (4) had a relatively higher parasite prevalence. We conclude that urban brown rats are larger and enjoy a superior nutritional status but

also face more challenges (allostatic load, stress) and a higher parasite load than rural counterparts.

Keywords urbanization; immunity; white blood cell; stress; parasite; brown rat

Introduction

Over the last one hundred years, urbanization has dramatically altered natural landscapes [1]. Numerous studies have focused on wildlife populations and their community ecology in order to understand how urbanization influences biodiversity and ecosystem functioning and services [2–4]. Such investigations underline the massive negative consequences of ecological changes, including the emergence and re-emergence of infectious diseases originating from wildlife [5–6]. Currently it is largely accepted that anthropogenically altered land changes increase species to species transmission of pathogens [7]. Yet, there is scant knowledge regarding the underlying physiological condition in reservoir species. Whether and how urbanization affects host–pathogen interactions and the immune competence in free-living species inhabiting altered environments [5,8] remains to be elucidated.

Recent investigations attempted to establish the impact of environmental differences (e.g., rural *versus* urban) on the health of wildlife species which serve as pathogen reservoirs [9–10]. Studies in free-living tree lizards (*Urosaurus ornatus*) and passerine species (*Pipilio aberti*, *P. fuscus*, *Mimus polyglottos*, *Toxostoma curvirostre*, and *Passer domesticus*) revealed differences in morphometric, immunological and parameters indicative of differences in challenges and allostatic load [11–12] along with divergent blood parasite abundance between rural and urban populations [9,13–14]. Apart from studies in humans [15–16], to the author's knowledge, similar investigations in mammalian species have not yet been conducted so far.

Rodents represent the most diverse mammalian group and account for over 40% of extant mammalian species. They are distributed across a wide variety of habitats, from tropical to sub-arctic zones, and populate both urban and rural areas [17]. As rodents (especially rats, genus *Rattus*) harbor numerous zoonotic agents and have a close commensal relationship with humans [18–22], within the context of the 'One Health' approach understanding the factors that influence their health status and immune response is essential.

In this study, we examined how the rural–urban gradient in terms of urbanization and increasing human population density changes the condition and health in wild brown rats (*R. norvegicus*), which have been described as urban 'exploiter' [2]. Morphometric, hematological, immunological parameters and variables relevant to the allostatic load of individuals [11–12] were monitored. Animals inhabiting forest landscape (forest-rural), agricultural landscape (agro-rural), and urban areas were included in the analysis. We predicted that the condition and health of brown rats should improve with increasing levels of urbanization because they would benefit from the additional food and water resources and the decrease in predator presence

associated with urban environments [2]. Here we extend these investigations to a widely distributed mammal, the brown rats.

Materials and methods
Ethics statement

Brown rats, a major pest, can be caught without a specific license in China. Trapping was performed with informed consent of settlement owners from the rural areas (Jiyuan and Luoyang) and staff of the zoological garden in Zhengzhou Zoo, Zhengzhou, China. This study was approved by The School of Life Sciences of Zhengzhou University's Institutional Animal Care and Use Committee. Additionally, all the experimental procedures were approved by the Animal Welfare and Ethic Committee of the Leibniz Institute for Zoo and Wildlife Research (Permit Number: 2013-05-02). All the procedures were carried out in a tranquil environment, and animals were euthanized individually using diethyl-ether anesthesia and exsanguination with all efforts to minimize discomfort.

Sampling sites and rat trapping

During August and September 2013 and 2014 we captured brown rats with steel-wire live traps (30 cm × 13 cm × 12 cm) baited with peanuts and apple pieces in three sampling sites in Henan Province (E 110°21′ – 116°39′, N 31°23′ – 36°22′), China: in Jiyuan, a forest rural landscape (hereafter forest-rural), in Luoyang, an agricultural rural landscape (hereafter agro-rural) and in Zhengzhou, a urban area (hereafter urban). We selected the sampling sites according to their developing process of urbanization, especially the resident human population density: Jiyuan (E 112°16'40.95", N 35°08'10.31") 370 people/km^2, Luoyang (E 112°24'55.72", N 34°31'52.82") 614 people/km^2 and Zhengzhou city center (E 113°40'56.00", N 34°47'21.61") 13,347 people/km^2. The density of the cantonal population of Zhengzhou was collated from the Henan Statistical Yearbook 2014 [23], and the resident human population densities of Jiyuan and Luoyang were calculated on the basis of data of population and area compiled from Henan Statistical Yearbook 2014 [23] and the website of the Ministry of Civil Affairs of the People's Republic of China (http://qhs.mca.gov.cn).

Traps were set before sundown and checked at sunrise the following morning. Live-trapped brown rats were individually housed with standard plastic cages (37 cm × 26 cm × 17 cm) and carefully transported to the animal house of the Institute of Biodiversity and Ecology, Zhengzhou University. The cages were systematically coded in the animal house, and rats were allowed to get accustomed to the captive environment before the start of experiments. They were kept under conditions of ambient temperature and natural light, and given *ad libitum* access to water and standard laboratory rat diet (Henan Laboratory Animal Center, Zhengzhou, China). We excluded several individuals from the study as they either gave birth or turned out to be juveniles, and finally obtained 10 (2013: 3 F + 3 M; 2014: 3 F + 1 M), 12 (2013: 2 F + 4 M; 2014: 2 F + 4 M) and 24 (2013: 1 F + 6 M; 2014: 8 F + 9 M) adult rats for

Jiyuan, Luoyang, and Zhengzhou, respectively.

Sample collection and analytical procedures

Rats were euthanized individually using diethyl-ether anesthesia and exsanguination. Blood samples were collected from the heart with a 21G vacutainer blood collection set (Becton Dickinson Medical Devices Co., Ltd., Shanghai, China) and pumped to a 2-mL EDTA-K$_2$ vacuum blood collection tube (Shandong Aosaite Medical Devices Co., Ltd., Heze, China). The collected blood (approximate 2 ml blood per individual) was stored on ice until transported to the Drug Safety Evaluation and Research Center of Zhengzhou University, where hematological parameters such as total and differential white blood cell (WBC) counts were measured using an ABX Pentra 80 Hematology Analyser (HORIBA ABX, Ltd., Montpellier, France). After euthanasia, standard biometrical parameters were measured including body mass, body length and tail length using an SD-168 electronic balance (0.1 g accuracy), ruler (0.1 cm accuracy) and micrometer (0.02 mm accuracy), respectively. Body mass index was calculated by dividing body mass (grams) by body length (centimeters) squared, which was adopted to assess body fat mass [24].

Following blood collection and morphological measurements, the rats were dissected, the spleen and the right adrenal gland of each rat were individually removed and weighed with a milligram balance (0.001 g accuracy) (Sartorius AG, Göttingen, Germany). The presence of the larva stage of the cestode *Taenia taeniaeformis* (Cestoda: Taeniidae) was identified as pea-sized whitish cysts (i.e. breaking the cyst and exposing the larva) on the surface of the liver.

Statistical analyses

Non-parametric analyses were employed because of the relatively small sample size for each group (S1 Table). Specifically, the permutation one-way analysis of variance test with general scores (permutation test) [25] was applied to compare differences between the three groups. The number of Monte Carlo simulations to establish the P value was set to 10^4. If the difference was significant, then the permutation test with general scores, known as Pitman's test, was used as a post hoc test for multiple comparisons [25] for which we obtained exact P values. To control the type I error originating from post hoc multiple testing, we adjusted the significance threshold α by controlling the false discovery rate as suggested by Benjamini and Hochberg [26]. All these analyses were performed with StatXact 10 (Cytel, Cambridge, MA, USA), with the tests conducted as two-sided tests and a significance level of α at 0.05. Figures were plotted with R version 3.1.2 for Windows [27]. Given the potential effect of allometry, we checked for possible effects of body mass and populations on rat immunological variables and found no obvious interaction of any immunological parameters with body mass (S1 Fig).

Results
Morphometric measurements

Significant differences in body length, tail length, body mass and body mass index were observed among the three sampling sites (permutation test: body length, statistic = 17.20, df = 2, P < 0.0001; tail length, statistic = 10.54, df = 2, P = 0.0024; body mass, statistic = 14.96, df = 2, P = 0.0001; body mass index, statistic = 8.83, df = 2, P = 0.01; Fig 1).
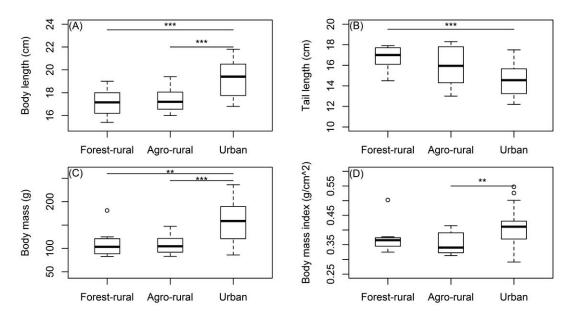
Fig 1. Morphological variables of brown rats sampled from forest-rural, agro-rural and urban areas.
(A) Body length (cm). (B) Tail length (cm). (C) Body mass (g). (D) Body mass index (g/cm^2). Measurements are shown as boxplots with median (bold solid lines), first and third quartiles (top and bottom of the box), ± 1.5 × interquartile range between the upper and lower quartiles (upper and lower whiskers), and values of more than 1.5 × upper quartile (unfilled cycles). *, 0.01 < P ≤ 0.05; **, 0.001 < P ≤ 0.01; ***, P ≤ 0.001.

Urban rats were bigger in body length than forest-rural (Pitman's test: statistic = 171.2, P < 0.0001, adjusted α = 0.0167) and agro-rural individuals (Pitman's test: statistic = 464.7, P = 0.00052, adjusted α = 0.033), but no significant difference between forest-rural and agro-rural individuals was detected (Pitman's test: statistic = 171.3, P = 0.60, adjusted α = 0.05) (Fig 1A). The urban rats had a shorter tail than the forest-rural individuals (Pitman's test: statistic = 167.4, P = 0.00069, adjusted α = 0.0167). Tail length was similar between urban and agro-rural individuals (Pitman's test: statistic = 352.4, P = 0.067, adjusted α = 0.033), and between forest-rural and agro-rural groups (Pitman's test: statistic = 167.4, P = 0.19, adjusted α = 0.05) (Fig 1B). The urban rats had a heavier body mass than the forest-rural counterparts (Pitman's test: statistic = 1024, P = 0.0033, adjusted α = 0.033) and agro-rural individuals (Pitman's test: statistic = 3.782, P = 0.00072, adjusted α = 0.0167). There

were no differences between forest-rural and agro-rural rats (Pitman's test: statistic = 1105, P = 0.85, adjusted α = 0.05) (Fig 1C). The urban rats (mean = 0.41 g/cm^2; 95% CI [0.395, 0.429]) showed a higher body mass index than the agro-rural individuals (mean = 0.36 g/cm^2; 95% CI [0.331, 0.381]) (Pitman's test: statistic = 9.886, P = 0.0052, adjusted α = 0.0167). No distinct differences between urban and forest-rural rats (mean = 0.37 g/cm^2; 95% CI [0.343, 0.399]) (Pitman's test: statistic = 3.71, P = 0.063, adjusted α = 0.033), or between forest-rural and agro-rural individuals were revealed (Pitman's test: statistic = 3.71, P = 0.46, adjusted α = 0.05) (Fig 1D).

Immunological measurements

Absolute and relative spleen mass are commonly used to assess the immunity of animals (e.g., avian [28]; mammals [29]) as they can reflect their immune-competence and/or acute pathogen and parasite exposure and current infections [30]. Significant differences in spleen mass, both absolute (permutation test: statistic = 16.45, df = 2, P = 0.0002) and relative (permutation test: statistic = 10.49, df = 2, P = 0.0028) from the three sites were detected (Fig 2). The urban rats displayed a heavier spleen than the forest-rural (Pitman's test: spleen mass, statistic = 2.043, P = 0.0011, adjusted α = 0.033; relative spleen mass, statistic = 1.773, P = 0.0055, adjusted α = 0.0167) and agro-rural rats (Pitman's test: spleen mass, statistic = 14.65, P = 0.00093, adjusted α = 0.0167; relative spleen mass, statistic = 2.659, P = 0.017, adjusted α = 0.033). No significant differences between forest-rural and agro-rural rats were observed (Pitman's test: spleen mass, statistic = 2.043, P = 0.65, adjusted α = 0.05; relative spleen mass, statistic = 2.659, P = 0.54, adjusted α = 0.05) (Fig 2A, 2B).
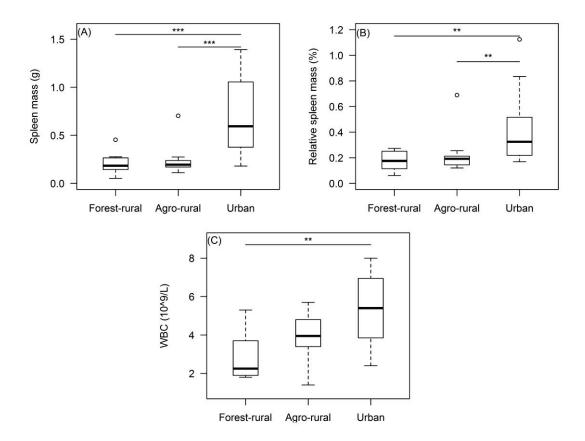
Fig 2. Immunological variables of brown rats sampled from forest-rural, agro-rural, and urban areas.
(A) Absolute spleen mass (g). (B) Relative spleen mass (%). (C) Total white blood cell (WBC) counts ($\times 10^9$ cells/L). Measurements are shown as boxplots with median (bold solid lines), first and third quartiles (top and bottom of the box), $\pm$ 1.5 $\times$ interquartile range between the upper and lower quartiles (upper and lower whiskers), and values of more than 1.5 $\times$ upper quartile (unfilled cycles). *, $0.01 < P \leq 0.05$; **, $0.001 < P \leq 0.01$; ***, $P \leq 0.001$.

Total white blood cell counts (WBC) are indicative of pathogen exposure levels with different classes of WBC diagnostic for exposure to specific pathogen classes [31]. Brown rats from all three sites displayed significant differences in total WBC counts (permutation test: statistic = 11.14, df = 2, P = 0.0012), neutrophils (permutation test: statistic = 7.687, df = 2, P = 0.015), monocytes (permutation test: statistic = 7.784, df = 2, P = 0.0121), and eosinophils (permutation test: statistic = 8.198, df = 2, P = 0.009), but not lymphocytes (permutation test: statistic = 4.84, df = 2, P = 0.0785) or basophils (permutation test: statistic = 3.902, df = 2, P = 0.11) (Fig 2C, Table 1). The urban rats presented significantly higher numbers of circulating WBC counts than the rats inhabiting forest-rural (Pitman's test: statistic = 27.5, P = 0.0013, adjusted α = 0.0167) and agro-rural (Pitman's test: statistic = 114.4, P = 0.039, adjusted α = 0.033) areas. In contrast, no significant difference in total WBC between forest-rural and agro-rural individuals (Pitman's test: statistic = 27.5, P = 0.085, adjusted α = 0.05) was observed (Fig 2C). The differential WBC counts displayed

complex patterns of differences or the lack of them between the three groups (Table 1).

Table 1. Numbers of immune cells (× 10^9 cells/L) of brown rats sampled from forest-rural, agro-rural and urban areas.

	Forest-rural (n=10)	Agro-rural (n=10)	Urban (n=20)
Total WBC	2.750±0.373^a	3.840±0.459^{a,b}	5.720±0.586^b
Neutrophil	0.380±0.099^a	0.936±0.181^b	1.107±0.175 (18)^b
Lymphocyte	2.015±0.315^a	2.186±0.303^a	2.979±0.318^a
Monocyte	0.326±0.088^a	0.645±0.188^{a,b}	1.376±0.287 (19)^b
Eosinophil	0.008±0.003^a	0.029±0.009^{a,b}	0.051±0.010 (19)^b
Basophil	0.034±0.007 ^{a}	0.036±0.008 ^{a}	0.084±0.023^a

Number in brackets indicates the sample size. WBC, white blood cells. Values in cells are presented as mean ± standard error. Superscripts (a, b) indicate the statistical analyses (see main text for statistical analyses for details): same letter means no significant difference, otherwise significant difference occurs between groups.

Allostatic load and *Taenia* sp. larvae prevalence

The size of right adrenal gland was used to assess the level of allostatic load, possibly caused by 'social stress' generated by potentially increasing population density and sociality with increasing urbanization [32–33]. The neutrophil/lymphocyte ratio in rats, which could reflect the impact of aggressive or agonistic interactions [34], was adopted as an indicator to estimate chronic stress. Absolute adrenal gland mass differed among groups (permutation test: statistic = 10.68, df = 2, P = 0.0028) whereas relative adrenal gland mass (permutation test: statistic = 0.007, df = 2, P = 1.0) and neutrophil/lymphocyte ratio (permutation test: statistic = 3.475, df = 2, P = 0.17) did not differ between the three groups (Fig 3).
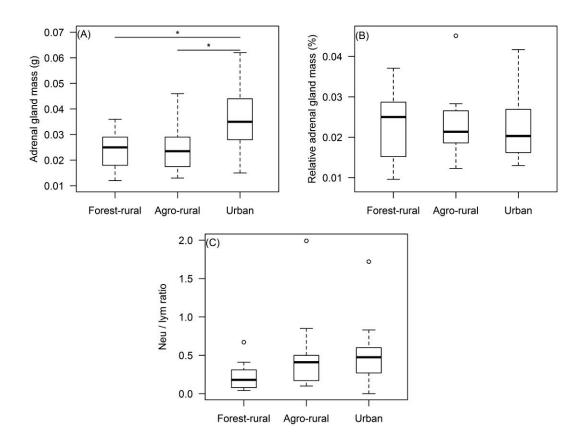
Fig 3. Variables of allostatic loads of brown rats sampled from forest-rural, agro-rural, and urban areas.
(A) Absolute adrenal gland mass (g). (B) Relative adrenal gland mass (%). (C) Neutrophil/lymphocyte ratio. Measurements are shown as boxplots with median (bold solid lines), first and third quartiles (top and bottom of the box), $\pm 1.5 \times$ interquartile range between the upper and lower quartiles (upper and lower whiskers), and values of more than $1.5 \times$ upper quartile (unfilled cycles). *, $0.01 < P \le 0.05$.

The absolute adrenal gland mass of urban rats was higher than those of the forest-rural (Pitman's test: statistic = 0.243, P = 0.0097, adjusted α = 0.033) and agro-rural rats (Pitman's test: statistic = 0.759, P = 0.0067, adjusted α = 0.0167). This parameter did not differ between forest-rural and agro-rural rats (Pitman's test: statistic = 0.243, P = 1.0, adjusted α = 0.05) (Fig 3A).

Rats are common intermediate host for the cestode *Taenia taeniaeformis* of whom the larval stage is described as *Cysticercus fasciolaris* [35–36]. The prevalence of *Taenia* sp. larvae in urban rats (21.7 %, 5/23) was twice the value recorded for rural rats (forest-rural 10.0 %, 1/10; agro-rural 8.3 %, 1/12), although this was statistically not significant (Chi-Square test, Pearson χ^2 = 1.38, df = 2, P = 0.50).

Discussion

Studies of free-ranging urban wildlife suggest that increased food availability and lower predator pressure contribute to better body condition (e.g., bigger size and higher weight) than in rural wildlife [37–39]. Brown rats living in urban areas were

bigger in body size, heavier in body mass and had a higher body mass index than forest-rural and agro-rural rats. This is consistent with previous work demonstrating that abundant food resources in urban environments [40] results in increased body mass and positively correlates with fat tissue storages in rats [10].

The urban rats also displayed elevated values of several immune parameters such as total WBC counts, neutrophil, monocyte and eosinophil numbers and spleen size (Fig 2, Table 1). There are several explanations for these findings that are not mutually exclusive. Because of the energetic costs of immune responses, individuals in better conditions or inhabiting environments with abundant resources can invest more in immunity [41]. Thus, urban rats can allocate more resources to immune activity than the rural individuals. This is partially consistent with our prediction. Enhanced immunity has been also observed in an avian urban 'exploiter', the house sparrow *Passer domesticus* [9]. A second explanation is that elevated immune parameters in urban rats reflect higher pathogen exposure than in their rural counterparts. Although the observed trends did not reach statistical significance, the prevalence of larvae of *Taenia* sp. in urban rats was twice as high as in forest-rural and agro-rural individuals. This observation is consistent with studies demonstrating that prevalence of cestodes in urban rats exceeded that detected in rodents inhabiting rural areas [42–43]. Cysticerci of *T. taeniarformis* initiate an immune response [44], and more frequent exposure would be reflected in a higher prevalence. The larger spleens detected in urban rats could therefore reflect prior parasite exposure or ongoing infections rather than a heightened immuno-competence [45–46]. This is consistent with results from studies examining the prevalence and consequences of exposure to nematodes. Investigations conducted in the same province concluded that the prevalence of the nematode *Capillaria hepatica* in rats collected from urban settlements (20%, 142/707) was six times higher than in rural environments (3.36%, 15/446) [47]. Experimental infections demonstrated that the spleen plays a major role in the defense against nematodes in rats [48]. Our results on increased spleen size therefore suggest a direct influence of parasitic burden in urban brown rats. In line with these findings, increased number of eosinophils also reinforces the higher exposure of urban rats to parasites compared to their rural counterparts. In comparative immunological studies, total and differential white blood cell counts are used as proxies for species-specific immune investment [49], however these parameters are used both in human and in veterinary medicine to diagnose current, ongoing infections [31,50]. While high total WBC counts (leukocytosis) suggest a pathologic process, and increases in certain lymphocyte types indicate exposure to specific pathogen group or antigen class. For example, neutrophilia (high neutrophil numbers) could indicate bacterial infection, lymphocytosis (increase in lymphocyte counts) an acute viral infection, eosniphilia (increase in eosinophils) parasitic infestations; monocytosis (high monocyte numbers) chronic inflammation and basophilia (increase in basophils) is common during allergic reactions [50]. In addition to the higher observed parasite exposure, our results on differential WBC counts indicate a higher prevalence of bacterial infections and chronic inflammatory

processes. Although pathogen surveillance was not undertaken, future microbiological studies associated with histopathology may represent a future line of research to clarify the basis of the observed higher monocyte and neutrophil numbers in urban rats.

Although no statistically significant differences in relative adrenal gland sizes were found, absolute adrenal gland size in urban rats was greater than in rural rats (Fig 3). Enlarged adrenal glands suggest an increase in long-term allostatic load (e.g., 'stress' from crowded social living conditions) independent of weight [32–33,51]. No direct analysis of aggressive behaviors in rural and urban rats was performed. However, tail length, an indicator of aggressive behavior [52], of urban rats was significantly shorter than in forest-rural rats, suggesting that urban rats experienced more frequent aggressive interactions. This could be indicative of density-dependent effects such as an increase in competition because of the possibly higher rat densities in urban environment [53]. The trend towards a higher neutrophil/lymphocyte ratio, a reliable indicator of chronic stress [34], in urban rats than in rural individuals would be consistent with such an idea. Common voles (*Microtus arvalis*) inhabiting areas with heavy industrial emissions exhibited elevated total WBC counts and displayed larger spleens than animals sampled from non-industrial areas [54]. Urban pollution might therefore also contribute to the allostatic load of urban rats.

The results of the current study suggest the increased access to food, the higher parasite burden and the increased allostatic load associated with urbanization all modulate the condition and health of brown rat populations. As the emerging picture is complex, since urbanization is not only associated with improvements to body condition but also increases in allostatic and parasite load, it will be important to monitor the intensification of urbanization process on wildlife and, particularly, on known reservoirs of zoonotic pathogens such as rodents.

Supporting Information
S1 Fig. Graphical assessment of interactive effects.
    (DOCX)
S1 Table. Data table used for the analysis.
    (PDF)

Author Contributions

Conceived and designed the experiments: JT ADG AD GÁC. Performed the experiments: JT YZ JL. Analyzed the data: JT HH. Contributed reagents/materials/analysis tools: GÁC JL. All authors contributed to the writing of the manuscript.

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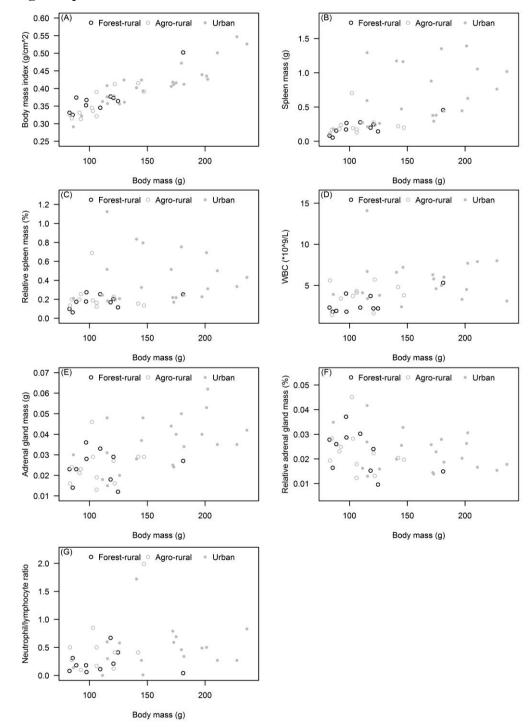15   Amoah AS, Obeng BB, May L, Kruize YC, Larbi IA, Kabesch M, et al. Urban–

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Supporting Information
S1 Fig. Graphical assessment of interactive effects.

S1 Fig. The possible influence of body mass across three sampling sites, representing a gradient of increasing urbanization and human population density (from forest-rural via agro-rural to urban), on the immunological variables measured. No obvious interactions of body mass of brown rats was observed with body mass index (A), absolute (B) and relative (C) spleen mass, total white blood cell (WBC) counts (D), absolute (E) and relative (F) adrenal gland mass, and neutrophil/lymphocyte ratio (G).

S1 Table. Data table used for the analysis.

Year	Location	Sex	BL	TL	BM	BMI	SP	RSP	WBC	Neu	Lym	Mon	Eos	Bas	AG	RAG	Neu/Lym	Tapeworm
2013	Agro-rural	F	16.4	14.1	84.7	0.315	0.168	0.198	1.4	0.29	1.02	0.10	0.01	0.01	0.024	0.0283	0.28	No
2013	Agro-rural	F	16.6	15.7	91.3	0.331	0.180	0.197	NA	NA	NA	NA	NA	NA	0.021	0.0230	NA	No
2013	Agro-rural	M	17.2	15.0	121.8	0.412	0.241	0.198	5.7	1.31	3.18	1.10	0.03	0.07	0.016	0.0131	0.41	Yes
2013	Agro-rural	M	19.4	16.4	147.1	0.391	0.198	0.135	3.8	1.91	0.96	0.87	0.02	0.02	0.029	0.0197	1.99	No
2013	Agro-rural	M	17.2	17.8	92.5	0.313	0.236	0.255	3.4	0.29	2.80	0.23	0.00	0.03	0.023	0.0249	0.10	No
2013	Agro-rural	M	17.9	18.3	120.9	0.377	0.275	0.227	1.6	0.15	1.24	0.15	0.00	0.03	0.027	0.0223	0.12	No
2013	Forest-rural	F	16.6	17.6	97.0	0.352	0.171	0.176	4.0	0.56	3.15	0.23	0.03	0.03	0.036	0.0371	0.18	No
2013	Forest-rural	F	16.3	17.9	97.4	0.367	0.266	0.273	1.8	0.10	1.63	0.04	0.00	0.01	0.028	0.0287	0.06	No
2013	Forest-rural	F	15.4	17.7	88.6	0.374	0.154	0.174	1.9	0.27	1.53	0.12	0.01	0.02	0.023	0.0260	0.18	No
2013	Forest-rural	M	19.0	17.5	181.1	0.502	0.454	0.251	5.3	0.19	4.36	0.74	0.00	0.03	0.027	0.0149	0.04	Yes
2013	Forest-rural	M	16.2	16.1	85.4	0.325	0.053	0.062	1.8	0.38	1.23	0.19	0.00	0.02	0.014	0.0164	0.31	No
2013	Forest-rural	M	17.7	15.6	118.2	0.377	0.198	0.168	3.7	1.17	1.75	0.74	0.01	0.03	0.018	0.0152	0.67	No
2013	Urban	F	17.2	15.4	86.0	0.291	0.181	0.210	3.9	0.47	3.34	0.07	0.00	0.04	0.030	0.0349	0.14	No
2013	Urban	M	17.5	14.4	111.3	0.363	0.273	0.245	4.1	0.00	3.76	0.33	0.00	0.03	0.018	0.0162	0.00	No
2013	Urban	M	21.2	16.2	236.2	0.526	1.018	0.431	3.1	1.18	1.42	0.48	0.03	0.03	0.042	0.0178	0.83	No
2013	Urban	M	19.3	13.7	146.3	0.393	1.163	0.795	7.2	0.07	6.14	0.95	0.00	0.04	0.048	0.0328	0.01	Yes
2013	Urban	M	18.7	14.6	140.7	0.402	1.173	0.834	6.6	3.09	1.80	1.67	0.05	0.03	0.028	0.0199	1.72	Yes
2013	Urban	M	17.0	12.8	93.2	0.322	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	No
2013	Urban	M	17.5	17.0	129.9	0.424	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	No
2014	Urban	M	20.4	17.5	227.6	0.547	0.762	0.335	8.0	1.26	4.72	1.94	0.09	0.04	0.035	0.0154	0.27	No
2014	Urban	M	20.3	15.8	172.1	0.418	0.377	0.219	6.3	2.26	2.85	1.10	0.06	0.08	0.025	0.0145	0.79	No
2014	Urban	M	16.8	12.8	115.1	0.408	0.594	0.516	14.1	NA	6.50	4.79	NA	0.21	0.031	0.0269	NA	No
2014	Urban	M	19.0	15.5	130.2	0.361	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
2014	Urban	M	21.0	14.5	181.7	0.412	0.441	0.243	6.0	0.87	2.53	2.09	0.01	0.47	0.034	0.0187	0.34	Yes
2014	Urban	M	20.5	12.8	172.9	0.411	0.293	0.169	5.8	1.40	2.37	1.87	0.06	0.05	0.024	0.0139	0.59	No
2014	Urban	M	18.0	13.8	115.6	0.357	0.212	0.183	3.4	0.63	2.07	0.62	0.01	0.05	0.015	0.0130	0.30	No
2014	Urban	M	20.5	12.2	210.7	0.501	1.056	0.501	7.9	0.87	3.23	3.68	0.05	0.07	0.035	0.0166	0.27	No
2014	Urban	M	18.8	12.7	126.0	0.356	0.262	0.208	3.8	0.97	1.68	0.97	0.11	0.08	0.020	0.0159	0.58	No
2014	Urban	F	17.5	14.5	115.2	0.376	1.295	1.124	6.7	1.36	2.28	2.73	0.17	0.17	0.048	0.0417	0.60	No
2014	Urban	F	18.5	12.8	145.0	0.424	0.471	0.325	2.4	0.42	1.54	0.45	0.01	0.04	0.037	0.0255	0.27	No
2014	Urban	F	20.5	14.8	170.7	0.406	0.879	0.515	NA	NA	NA	NA	NA	NA	0.044	0.0258	NA	Yes
2014	Urban	F	19.5	14.5	179.5	0.472	1.352	0.753	5.0	1.19	2.61	1.04	0.04	0.10	0.050	0.0279	0.46	No
2014	Urban	F	21.5	15.5	201.3	0.435	1.392	0.692	4.5	1.32	2.65	0.49	0.05	0.04	0.053	0.0263	0.50	No
2014	Urban	F	21.8	15.8	202.3	0.426	0.627	0.310	7.7	NA	3.86	NA	0.07	0.05	0.062	0.0306	NA	Yes
2014	Urban	F	20.5	15.5	174.9	0.416	0.382	0.218	4.6	1.64	2.36	0.43	0.11	0.02	0.040	0.0229	0.69	No
2014	Urban	F	21.2	17.3	197.4	0.439	0.446	0.226	3.3	0.92	1.87	0.44	0.04	0.03	0.040	0.0203	0.49	No
2014	Agro-rural	F	17.2	13.0	102.1	0.345	0.703	0.689	NA	NA	NA	NA	NA	NA	0.046	0.0451	NA	No
2014	Agro-rural	F	17.5	14.5	103.0	0.336	0.193	0.187	3.7	1.17	1.37	1.03	0.06	0.07	0.029	0.0282	0.85	No
2014	Agro-rural	M	18.5	17.8	142.1	0.415	0.219	0.154	4.8	1.24	3.01	0.41	0.07	0.02	0.029	0.0204	0.41	No
2014	Agro-rural	M	18.2	17.8	106.2	0.321	0.128	0.121	4.1	1.22	2.45	0.36	0.02	0.01	0.019	0.0179	0.50	No
2014	Agro-rural	M	16.5	16.2	106.1	0.390	0.173	0.163	4.3	0.60	3.48	0.23	0.01	0.03	0.013	0.0123	0.17	No
2014	Agro-rural	M	16.0	13.0	83.1	0.325	0.112	0.135	5.6	1.18	2.35	1.97	0.07	0.07	0.016	0.0193	0.50	No
2014	Forest-rural	M	18.5	17.8	124.7	0.364	0.144	0.115	2.2	0.44	1.08	0.68	0.01	0.04	0.012	0.0096	0.41	No
2014	Forest-rural	F	17.8	16.5	109.3	0.345	0.277	0.253	2.3	0.19	1.77	0.20	0.01	0.09	0.033	0.0302	0.11	No
2014	Forest-rural	F	18.0	16.2	120.7	0.373	0.245	0.203	2.2	0.35	1.66	0.18	0.00	0.02	0.029	0.0240	0.21	No
2014	Forest-rural	F	15.8	14.5	82.7	0.331	0.081	0.098	2.3	0.15	1.99	0.14	0.01	0.05	0.023	0.0278	0.08	No

Note: BL: body length (cm); TL: tail length (cm); BM: body mass (g); BMI: body mass index (g/cm^2); SP: spleen (g); RSP: relative spleen mass (%); WBC: total WBC (10^9/L); Neu: neutrophil (10^9/L); Lym: lymphocyte (10^9/L); Mon: monocyte (10^9/L); Eos: eosinophil (10^9/L); Bas: basophil (10^9/L); AG: adrenal gland mass (g), right side; RAG: relative adrenal gland mass(%); Neu/Lym: neutrophil/lymphocyte ratio; Tapeworm: present or not of tapeworm (*Taenia* sp.).

Chapter 5 Concluding remarks

In comparison to the mechanistic view of the immune system in the defense of the host, ecological immunology puts the organism in a broader context, where the defense is only one trait with costs and benefits relative to other life-history traits due to limited resources (Schulenburg et al., 2009; Demas & Nelson, 2012; Viney & Riley, 2014). Despite the great progress in the last decades, methodological issues have hindered progress (Martin et al., 2011). Limited sample volumes, difficult and variable field conditions or extrapolations from simple assays despite the complexity of the immune system all hinder progress. Nevertheless, eco-immunology as a field has diversified extensively both in terms of new methods developed and applied (Demas et al., 2011), new non-model species and research questions introduced (Schulenburg et al., 2009). While early years were dominated by avian eco-immunological studies, which dealt with general questions such as effect of sex, age, season or latitude/environment on immunity (Hasselquist, 2007), recent mammalian studies have described both generalizable, and in some cases, contradictory processes.

During my PhD study, I was primarily interested in how environmental differences in human-dominated habitats influence the immunity of rodents. In the first part of this dissertation, I tested whether the previously described 'Sexually-transmitted diseases (STDs)' hypothesis, which states a positive correlation between species-specific basal immunity (e.g., leucocyte numbers) and the level of mating promiscuity (Nunn et al., 2000), holds true in rodents. Moreover, since the previous evidence for this hypothesis was derived from studies of captive primates and carnivores, I also compared free-living and captive rodents (Chapter 2). The results suggested that the hypothesis is not generalizable to either captive or free-living rodents, because the total and differential WBC counts did not co-vary with the residual testes mass – a correlate of mating promiscuity. Moreover, species with greater adult body mass averaged across sexes had elevated total WBC and differential WBC (neutrophils and lymphocytes) counts, and captive animals presented higher lymphocyte cell counts than free-ranging ones, indicating a clear captivity effect though this did not affect the conclusion about the STDs hypothesis. Based on these findings, further comparative studies are needed on free-living mammalian species.

Since invasive species are among the most important factors contributing to biodiversity loss and several studies target on understanding which traits define a successful invader. Understanding these questions has implication for both conservation biology and evolutionary ecology. In Chapter 3, I studied how the colonization history shaped the immunity of western house mouse (*Mus musculus domesticus*) using laboratory outbred but wild-derived individuals. Using this common garden approach, the impacts of current environments (e.g., temperature,

exposure to pathogens) were minimized but the effects of genetic variation were highlighted. This study provided the first empirical evidence that successful invasive rodent species down regulate their costly cellular immune mechanisms in new habitats, which is compensated by developing high antibody-mediated immunity. The mechanism underlying the observed patterns could be a 'founder effect' in western house mouse and/or selection pressure exerted by the composition of local parasitic helminth communities. My findings were in accordance with previous avian and amphibian studies, showing a shift from the costly immune parameters and thus maximizing their competitiveness or reproduction (Lee & Klasing, 2004; Lee et al., 2005; Llewellyn et al., 2012).

Finally, I was interested in the condition and health of wild brown rats sampled from forest-rural, agro-rural and urban areas along a gradient of increasing urbanization and human population density in Henan Province, China (Chapter 4). The findings showed that urban rats (1) were bigger in body length, shorter in tail length, heavier in body mass and had a higher body mass index than rural rodents; (2) had larger spleens and higher leukocyte counts than rural counterparts; (3) tended to have a higher allostatic load as they showed enlarged adrenal glands and a higher neutrophil/lymphocyte ratio; and (4) had a relatively higher parasite prevalence. These findings suggest that urban brown rats are larger and enjoy a superior nutritional status but also face more challenges (allostatic load, 'stress') and a higher parasite load than rural counterparts. Although the results of this pilot study are very promising and provide a background for further studies on the effect of urbanization on wildlife health, the findings should be interpreted cautiously. First, the small sample sizes of the two rural groups limited the use of multivariate parametric analysis (e.g., general linear models), which allows for parallel testing of the effect of both sampling site and gender on the measured parameters. Since the human population density between the two rural sites (370 people/km^2 and 614 people/km^2) is of the same magnitude compared to the urban area (13,347 people/km^2), merging the rural groups could be a solution for this issue. Moreover, only adult rats were sampled during this experiment, however, the exact age was not determined (e.g., weight of eye lens, growth of molar teeth; Sengupta, 2013). Integrating information of precise age estimation could strengthen the conclusion regarding the rat morphological analysis, because it has been shown that morphometric traits (e.g., body mass; Hughes & Tanner, 1970; Hofstetter et al., 2006) but not the hematological parameters (e.g., leukocyte counts; Matsuda et al., 2000) are associated with the age of laboratory rats. On the other hand, the differences in morphometry among the sampling sites could be a result of non-random distribution of age groups among the rat locations tested. These factors should be considered in future studies of the relationship between health status and environmental differences in wild rats. As the physiological mechanisms associated with urbanization seems to be very complex, further studies involving comprehensive pathogen screen (using both molecular and serological methods) associated with histopathology, more elaborated immune tests (e.g., flow cytometry, functional immune assays) and newer OMICS (e.g., proteomics,

transcriptomics) approaches will allow for a better understanding of the effect of urban environment on wildlife physiology. Moreover, similar to my study on the effects of invasion, a common garden approach would be required to determine how plastic these traits are.

In conclusion, when investigating the effect of anthropogenic environments (e.g., free-living *versus* captive, native *versus* invaded, rural *versus* urban) on immunity of rodents, I found that they all demonstrated significant impacts. When considering the influence of human presence or activity on the immunity of rodents, based on the experiments of my thesis, it could be generally concluded that human dominated habitats (e.g., urban and captive environment) augment the immunity of rodents, while introduction to novel areas causes a shift between cellular and humoral immunity rather than a decrease or increase in their overall immunity. Since immune function is an energetically costly trait, most of these findings could be explained by increased access to food, whereas negative effects could be explained by relatively higher pathogen exposure in human dominated areas. Wild rodents are very important reservoirs for zoonotic pathogens, and their immunity is associated with reservoir competence. Therefore, it is necessary to understand the effect of various scales of human-associated (e.g., captivity, invasion, urbanization) and natural (e.g., latitudinal, altitudinal) environmental differences on the immunity of wild rodents in the context of "One Health". Moreover, as most of these species are also models for medical immunology, the existing tools developed make them an ideal model for answering evolutionary immunological questions. The comparison of the immune- and reservoir-competence in rodent species is needed, and the effect of environment on the epidemiological role of rodents at population and species level needs to be performed. My dissertation will serve as a basis for this future work.

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Summary

In comparison with medical immunology, which focuses on mechanistic descriptions of defense mechanisms against pathogens and parasites, ecological immunology hypothesizes that variation in immune defense, both within and among species, is a result of biotic and abiotic factors of an animal's environment.

Macro-environment can greatly influence immunity in terms of both resources and pathogen pressure. In this thesis, I attempted to compare the immunity of animals living in different human-influenced environmental gradients. By using different study designs (comparative, experimental and correlative approaches), I examined whether captivity, colonization history and urbanization have an effect on the immunity of rodents. Rodents constitute the most diverse group of mammals, accounting for over 40% of all the extant mammalian species. They are one of the most important reservoirs for zoonotic emerging pathogens due in part to their life-history traits (e.g., short lifespan with multiple litters) and close proximity to human populations. Moreover, the vast majority of immunological knowledge has been generated by studies on laboratory model rodents (e.g., house mouse *Mus musculus*, lab rats *Rattus rattus* and *R. norvegicus*), and the developed immunological techniques and tools can be generally applied to free-living conspecifics.

Previous comparative eco-immunological studies have suggested that mating promiscuity is one of the main factors explaining the variation in basal immune investment (total and differential white blood cell (WBC) counts) in captive primates and carnivores. In Chapter 2, I examined whether this conclusion is generalizable to other mammalian groups, how the observed patterns are related to the living environment and if there are differences between free-living and captive animals. Using phylogenetic generalized least-squares statistical models considering non-independence resulting from shared ancestry, I confirmed that species with greater adult body mass averaged across sexes had elevated total and differential (neutrophils and lymphocytes) WBC counts, and found that captive animals presented higher lymphocyte numbers than free-ranging ones. However, I found that the total and differential WBC counts did not co-vary with the residual testes mass, a correlate of mating promiscuity, suggesting that previous conclusion generated from studies of captive primates and carnivores is not generalizable to all mammals. These results also emphasize the need for further comparative studies on free-ranging animals.

Parasites play an important role in the invasion processes and success of their hosts through multiple biological mechanisms such as 'parasite release', 'immuno-competence advantage' or 'novel weapon', among others. In Chapter 3, I examined whether colonization history had an effect on the immunity of western house mouse (*M. m. domesticus*). Using a common garden experimental approach of wild-derived outbred laboratory populations, I compared the cellular and humoral immunity of European and Iranian mice, representing the newly colonized and source

populations respectively. I found that European mice had lower total WBC counts but higher immunoglobulin E concentrations than their Iranian counterparts, providing the first empirical evidence that successful invasive species downregulate their costly cellular immune mechanisms in new habitats, which is compensated by developing high antibody-mediated immunity. Invasive species capable of such immunological shift can be successful in novel habitats by increasing fitness both in term of survival (e.g., efficient immunity, low costs) and reproduction (e.g., investing more resources).

Since the beginning of last century, urbanization has had considerable global effects such as overpopulation and environmental pollution, favoring the emergence and re-emergence of various infectious agents, including those originating from wildlife. It is unclear, however, which physiological mechanisms are behind these emergence events. I attempted to address these questions in the Chapter 4 by measuring the morphological, physiological and pathophysiological traits (morphometry, allostatic load, immunity, parasite load) of free-living brown rats (*R. norvegicus*) sampled from forest-rural, agro-rural and urban areas along a gradient of increasing urbanization and human population density in Henan Province, China. Urban rats had increased body condition, but also relatively higher allostatic (stress) and parasite load compared to rural individuals, indicating the complex effect of urbanization on the physiology of wildlife.

The results of this dissertation demonstrate that anthropogenic activities (captive maintenance, colonization history, and urbanization) have a clear effect on the immuno- and reservoir-competence of rodents, information which may be crucial in the context of the 'One Health', concept which recognizes human, animal and environmental health as connected and interlinked.

Zusammenfassung

Titel: Der Einfluss von umweltbedingten Unterschieden auf die Immunantwort von Nagetieren.

Im Vergleich mit der medizinischen Immunologie, die sich auf die mechanistische Beschreibung der Abwehrmechanismen gegen Krankheitserreger konzentriert, geht die ökologische Immunologie davon aus, dass die Variation der Immunabwehr, sowohl innerhalb einer Tierart als auch zwischen den Tierarten, von biotischen und abiotischen Umweltfaktoren beeinflusst wird.

Das Makroumfeld hat dabei generell einen Einfluss auf die Immunität in Hinblick auf die Reserven und den pathogenen Druck. In der vorliegenden Arbeit wird die Immunität von Tieren aus verschieden stark durch den Menschen beeinflussten Umgebungen miteinander verglichen. Durch den Gebrauch unterschiedlicher Studienansätze (komparative, experimentelle und korrelative) untersuchte ich, ob Gefangenschaft, Kolonisationsgeschichte sowie Urbanisierung einen Effekt auf die Immunität von Nagetieren hat. Innerhalb der Säugetiere sind Nagetiere die Gruppe mit der höchsten Diversität, welche mehr als 40% der gesamten Säugetierarten stellt. Infolge ihrer lebensgeschichtlichen Merkmale (z.B. kurze Lebensspanne mit vielen Nachkommen) und ihrer engen Nähe zur menschlichen Bevölkerung stellen Nagetiere eines der wichtigsten Reservoire von neu aufkommenden Zoonose-Erregern dar. Ein großer Teil des heutigen Wissens über die Immunologie wurde an Modell-Labornagetieren wie z.B. der, Hausmaus (*Mus musculus*) und Laborratten (*Rattus rattus* und *R. norvegicus*) gewonnen. Die hierbei entwickelten immunologischen Techniken und Methoden sind leicht auf wild lebende Artgenossen anwendbar.

Frühere vergleichende Arbeiten der Öko-Immunologie deuteten darauf hin, dass Promiskuität ein Hauptfaktor zur Erklärung der Varianz unterschiedlicher relativer Investitionen in die grundlegende Immunantwort (Gesamtzahl weißer Blutkörperchen und Differentialblutbild) bei in Gefangenschaft gehaltenen Primaten und Karnivoren darstellt. Im zweiten Kapitel wird geprüft, ob diese Schlussfolgerung auf andere Säugetierarten generalisierbar bzw. übertragbar ist und wie das beobachtete Muster in Verbindung zu Umweltbedingungen gebracht werden kann. Auch wird untersucht ob und in wie fern Unterschiede zwischen wild lebenden und in Gefangenschaft gehaltenen Tieren bestehen. Durch Einsatz der phylogenetisch generalisierten statistischen Methode der kleinsten Fehlerquadrate, welche unabhängige Resultate gleicher Herkunft berücksichtigt, konnte ich bestätigen, dass Tiere mit größerer adulter Körpermasse (bei gleichem Anteil beider Geschlechter) eine höhere absolute Anzahl weißer Blutkörperchen und im Differentialblutbild einen hören Anteil neutrophiler Granulozyten und Lymphozyten aufwiesen. Tiere in Gefangenschaft wiesen im Vergleich zu wild lebenden eine höhere Anzahl an Lymphozyten auf. Es fand sich jedoch keine Korrelation mit der Hodenmasse als Maß für promiskuitive

Paarungssysteme. Dies legt nahe, dass sich die Schlussfolgerungen von Studien an in Gefangenschaft lebenden Primaten und Karnivoren nicht generell auf alle Säugetiere übertragen lassen. Die Ergebnisse der vorliegenden Studie unterstreichen den Bedarf für weitere vergleichende Studien an wild lebenden Tieren.

Parasiten spielen eine wichtige Rolle beim Invasionsprozess und dem Wirtserfolg durch multiple biologische Mechanismen wie unter anderem dem der Parasitenfreisetzung, des Immunkompetenzgewinns oder ihrer neuartigen Waffen. In Kapitel 3 untersuchte ich, ob die Kolonisierungsgeschichte einen Effekt auf die Immunität der westlichen Hausmaus (*M. m. domesticus*) hat. Mittels aus der Wildnis stammender ausgezüchteter Labormauspopulationen verglich ich die zelluläre und humorale Immunität der Europäischen und der Iranischen Maus, welche die neu kolonialisierte, respektive die Ausgangspopulation repräsentieren. Die vorliegenden Studien zeigten, dass Europäische Mäuse im Vergleich zu ihren iranischen Verwandten eine niedrigere Anzahl weißer Blutkörperchen aufwiesen aber zugleich eine höhere Konzentration an Immunglobulin E. Dies stellt den ersten empirischen Beweis für eine erfolgreiche invasive Art dar, ihre kostenintensiven zellulären Immunmechanismen in einem neuen Habitat herabzuregulieren und zum Ausgleich eine höhere antikörpervermittelte Immunität zu entwickeln. Invasive Arten, die die Fähigkeit einer derartigen Verlagerung der Immunabwehr besitzen, verfügen in einem neuen Habitat über einen Vorteil sowohl im Überleben (z.B. effizienteres Immunsystem, geringere Kosten), als auch bei der Reproduktion (z.B. mehr freie Ressourcen investierbar).

Die seit Beginn des letzten Jahrhunderts durch zunehmende Urbanisierung entstandenen massiven globalen Effekte, insbesondere der Überbevölkerung und Umweltverschmutzung, begünstigen das Auftauchen und Wiederaufkeimen verschiedener infektiöser Erreger inklusive solcher, welche ihren Ursprung in Wildtieren nehmen. Bis dato ist unklar, welche physiologischen Mechanismen dieser Entwicklung zugrunde liegen. Diesen Fragen versuchte ich in Kapitel 4 nachzugehen, indem ich die morphologischen, physiologischen und pathophysiologischen Merkmale (Morphometrie, allostatische Last, Immunität, parasitäre Belastung) der wild lebenden braunen Ratte (*R. norvegicus*) in abgelegenen Wald-, Agrar-, sowie urbanisierten Regionen entlang eines Urbanisierungsgradienten und in Abhängigkeit der der Bevölkerungsdichte der chinesischen Provinz Henan untersuchte. Urbane Ratten wiesen dabei eine höhere Körpermasse, aber auch eine höhere allostatische- und Parasitenblast im Vergleich zu Ratten aus abgelegeneren Regionen auf. Dies weist auf einen komplexen Effekt von Urbanisierung auf die Physiologie der Wildtiere hin.

Die in dieser Dissertation vorliegenden Ergebnisse zeigen, dass anthropogene Aktivitäten (Gefangenschaftshaltung, Kolonisierungsgeschichte und Urbanisierung) einen deutlichen Effekt auf die Immunkompetenz und den Gehalt an Pathogenen (Anzahl und Arten) von Nagetieren ausüben. Diese Informationen sind essentiell im Hinblick auf das „One Health" Konzept, welches der engen Verknüpfung zwischen der Gesundheit von Menschen und Tieren und der Umwelt Rechnung trägt.

List of publications

1. **Tian JD**, Hofer H, Greenwood AD, Czirják GÁ. Colonization history shaped the immunity of the western house mouse. Oecologia, 179:679–686. (doi:10.1007/s00442-015-3397-0).

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3. Wang BS, Wang ZL, **Tian JD**, Cui ZW, Lu JQ. 2015. Establishment of a microsatellite set for noninvasive paternity testing in free-ranging *Macaca mulatta tcheliensis* in Mount Taihangshan area, Jiyuan, China. Zoological Studies, 54:8. (doi:10.1186/s40555-014-0100-9).

4. **Tian JD***, Wang BS*, Wang ZL, Liu JD, Lu JQ. 2015. Effects of social and climatic factors on birth sex ratio in *Macaca mulatta* in Mt. Taihangshan area. Integrative Zoology, 10:199–206. (doi:10.1111/1749-4877.12118). (* These two authors contributed equally to this study).

5. **Tian JD**, Wang ZL, Lu JQ, Wang BS, Chen JR. 2013. Reproductive parameters of female *Macaca mulatta tcheliensis* in the temperate forest of Mount Taihangshan, Jiyuan, China. American Journal of Primatology, 75:605–612. (doi:10.1002/ajp.22147).

Acknowledgements

I would like to start by expressing my sincere gratitude to my supervisors Prof. Dr. Alex D. Greenwood and Dr. Gábor Á. Czirják from the Leibniz Institute for Zoo and Wildlife Research, Berlin (IZW) who offered me the opportunity to conduct my PhD study in their labs. I thank for their supervision and the discussions we had; they always found time for me or my work, to revise and improve the manuscripts and my dissertation. I am also greatly indebted to my supervisory committee members, Prof. Dr. Heribert Hofer (IZW) for his great statistical support and guidance, and Dr. Anca Dorhoi (Max-Planck Institute for Infection Biology) for her support and guidance in the perspective of immunology.

I am thankful to Prof. Dr. Diethard Tautz (Max-Planck Institute for Evolutionary Biology) for providing access to his house mouse collection. Thanks to Dr. Natascha Hasenkamp, Heike Harre, Christine Pfeifle, Heinke Buhtz, Cornelia Burghardt and the mouse housing team from Max Planck Institute for Evolutionary Biology for their technical and logistic assistance.

I am grateful to my Chinese supervisor Prof. Dr. Jiqi Lu (Institute for Biodiversity and Ecology and School of Life Sciences, Zhengzhou University) for his endless support in both my scientific research and studying abroad. I also would like to thank Prof. Dr. Lu and Prof. Dr. Zhenlong Wang for offering me the opportunity to carry out experiments at Institute for Biodiversity and Ecology and School of Life Sciences, Zhengzhou University. I am greatly indebted to Ms. Shujie Zhang (School of Life Sciences, Zhengzhou University) for her kindness and supporting me in studying abroad. Thanks to Dr. Yifeng Zhang (Institute for Biodiversity and Ecology & School of Life Sciences, Zhengzhou University), Shuliao Tian (Zhengzhou Zoo), Jiafu Hou (Jiyuan State-owned Yugong Forest Farm) for helping during trapping sessions. I am also thankful to Shizheng Wang (School of Life Sciences, Zhengzhou University), Prof. Chunyao Wang and Dr. Ning Wang (Henan Laboratory Animal Center, Zhengzhou University), and Linping Zhao (Zhengzhou Zhongdao Biotechnology Co., Ltd.) for permission to use their laboratories.

I wish to express my gratitude to Katja Pohle (IZW) for her support in the laboratory as well as in logistic questions, and to Dr. Claudia Szentiks, Dr. Mirjam Grobbel, Dr. Gudrun Wibbelt, Dr. Oliver Krone, and Dr. Susanne Holtze for their patience and kindness.

I heartily thank Dr. Alexandre Courtiol (IZW) for his patience and statistical supports. Thank Dr. Karin Schneeberger for statistical help.

Thanks to Dr. Kristin Mühldorfer, Dr. Zaida Melina Rentería-Solís, Paula Ortega Pérez, and Sanatana-Eirini Soilemetzidou for the good time we had while sharing the office at the IZW and for their friendship.

Ms. Angela Daberkow (secretary of Dahlem Reseach School Biomedical Sciences) helped me with administrative questions and her guidance through the

Dahlem Research School, Freie Universität Berlin during my doctoral study was of great help; I also appreciate the time and help from Ms. Christine Gaede (secretary of Department of Veterinary Medicine), Ms. Stefanie Böhler (International Office), Ms. Aada Šilde (Welcome Center of Dahlem Research School), and other administrative staff of Dahlem Research School and Department of Veterinary Medicine of Freie Universität Berlin.

The administrative staff of the IZW was always very supportive and helped me in logistic questions and always had friendly words: Ms. Sike Ehle, Ms. Dagmar Boras, Ms. Gabriele Liebich, Ms. Katrin Hohenstädter, Ms. Stephanie Vollberg, and Ms. Stefanie Lenz; thank you all for everything. Many thanks to the librarians from the IZW, Ms. Beate Peters-Mergner and Ms. Cornelia Greulich who provided me with literature search supports; to Mr. Wolfgang Tauche and Mr. Paul Sobtzick for their great technical support and help in all questions concerning computers; to Mr. Krebs for the always friendly atmosphere.

I would like to thank Hui Zhang, Yunhua Lou, and Lianping Huang (Department of Education of the Embassy of the People's Republic of China in the Federal Republic of Germany) for their support in the management of regular report, stipend release, and also daily life.

I would like to express my special thanks to all PhD students from the IZW for the fun we had at the IZW. Special thanks go to Dr. Kyriakos Tsangaras, Pin Cui, Alexander Markus Hecht, Niccoló Alfano, Luis Eduardo Flores Landaverde, Marina Escalera Zamudio, Olga Amelkina, Dr. Sebastian Menke, Ines Lesniak, Azza Abdelgawad, Sonja Heinrich, and Dr. David Alquezar for their overall support, the wonderful time and friendship we have.

All my friends: Ning Liu and Ningning Li, Dr. Zhenya Wang, Dr. Shuwen Yu, Dr. Lu Meng, Ruifang Jiang, Wei Ding, Dr. Chao Yu, Jin Zhang, Thierry Kombou, Dr. Tao Liu, Dr. Ping Liu, Dr. Mingyang Wang, Dr. Xiaoying Hu, Yue Zheng, Zhigao He, Dr. Huaming Wu, Shujuan Li, Xianwen Chen, Dr. Weidong Chai, Chengpeng Cui, Jieqi Chen, Yun Lu, Taiyun Li, Chao Gong, Dr. Yide He, Bin Yue, Ping Lu, Teng Qiu, Kuangxin Zhou, and others supported me during my stay in Germany, thank you for your amity.

I want to thank and dedicate this thesis to the most important people in my life: my father and mother, and other members of my whole family for their patience, their support, their endless love, and believing in me; Hong Li for her love and support. They helped me during both good and bad times and supported me incredibly with their love and encouragement.

I am grateful to the China Scholarship Council (CSC) who funded my stay in Germany.

I wish to reserve a last word for all the rodents that were involved in my study as without them none of my experiments would have been possible.

Selbständigkeitserklärung

Ich erkläre, dass ich die vorliegende Dissertation selbständig, ohne unzulässige fremde Hilfe und nur unter Verwendung der angegebenen Literatur und Hilfsmittel angefertigt habe.

Jundong Tian
Berlin, July 23, 2015