Development and application

of novel immunological approaches

to chiropteran immunology

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2. **Hecht-Höger, A. M.**, Braun, B. C., Krause, E., Meschede, A., Krahe, R., Voigt, C. C., Zychlinsky, A., Greenwood, A. D., & Czirják, G. Á. **Plasma proteomic profiles differ between European and North American Myotid bats with White-Nose Syndrome.** *In preparation*.

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Content

CHAPTER 1

General Introduction

CHAPTER 1 – General Introduction

All organisms live in an environment where they are continuously exposed to invading microbes and parasites. In consequence different forms of host protection have evolved with the immune system as the most important defense mechanism in metazoa. Even though the immune system represents a complex combination of different physiological processes, it has been grouped in two main types, innate and adaptive immunity, both with cellular and humoral effectors (Murphy 2016). The innate immune system is the first line of defense, which provides quick, unspecified protection against all invading pathogens. The adaptive immunity is characterized to be a more specific response, though it is requiring a first exposure based on which a memory is developed for later encounters.
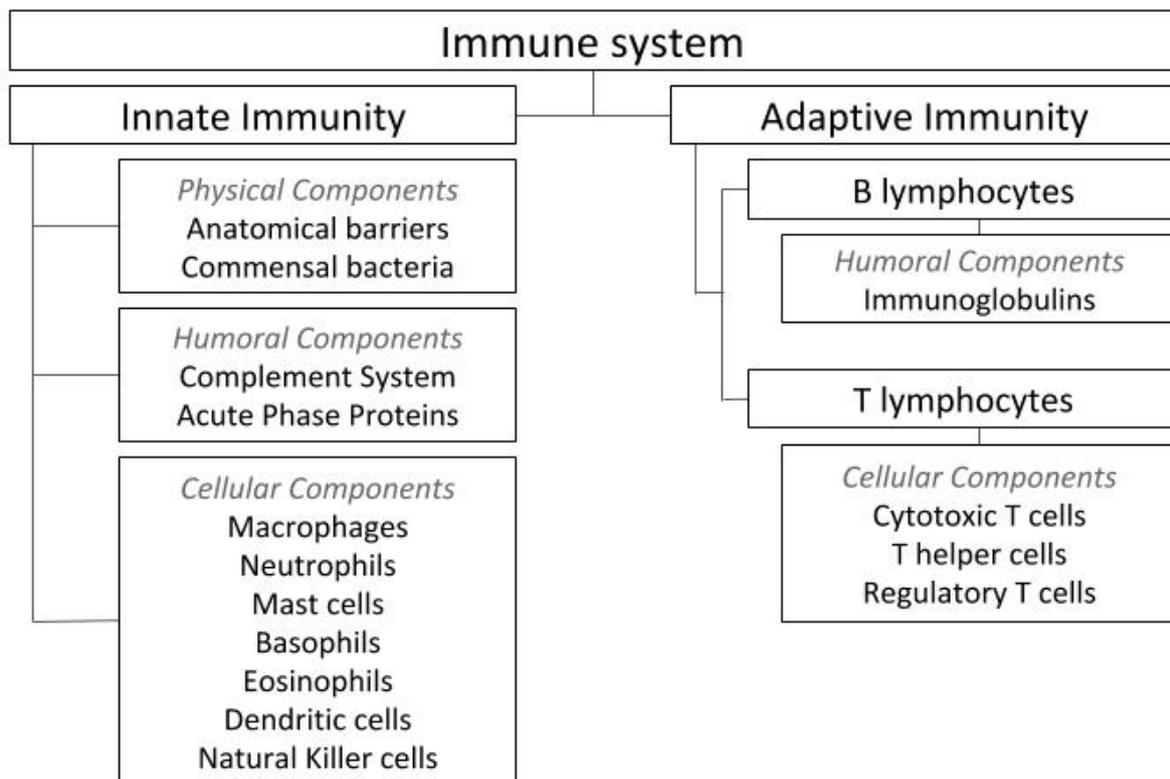
Figure 1. Major components of the mammalian immune system (modified from Muehlenbein, 2010)

Our substantial knowledge about the complexity of the immune system, its regulation and variable responses to infection by parasites or pathogens was mostly revealed by immunological research in controllable laboratory environments. Experimental designs with a high reproducibility and a narrow genetic diversity allowed the detailed investigation of

immune mechanisms with a simplified influence of environmental conditions, where variation between groups and individuals were not desired (Lazzaro & Little 2009; Maizels & Nussey 2013; Pederson & Babayan 2011). In contrast most organisms live in highly diverse natural environments, with variable resources and different eco-physiological context. Influenced by ecological and evolutionary factors, this leads to differences in pathogen susceptibility and immunity in individuals, populations and species (Brock et al. 2014; Martin et al. 2011). Focusing on immune processes in the context of natural populations and how immunity can be driven by abiotic and biotic factors the research field of ecological or wildlife immunology has emerged over the last decades (Schulenburg et al. 2009; Sheldon & Verhulst 1996). Knowledge achieved from the immunological research on free-ranging species will contribute to the integrative understanding of emerging diseases and their possible threats on both animals and humans.

1.1. Background of eco-immunology

Eco-immunology as a discipline investigates variations in the immune system of living organisms and how they have been shaped by physiological and ecological mechanisms in an evolutionary framework (Martin et al. 2011; Schulenburg et al. 2009; Demas & Nelson 2012). Focusing on the divergence of immunity among and within species, eco-immunological studies allow insights on interactions between physiological mechanisms (Downs et al. 2014) and under evolutionary perspectives such as life history (Lee 2006; Lochmiller & Deerenberg 2000) and disease ecology (Alizon & Baalen 2008; Boots et al. 2008).

The life history theory states that divergent strategies evolved in different species in order to maintain reproduction and survival which are correlated with trade-offs among life history traits (Ricklefs and Wikelski 2002; Stearns 1992). The immune system has evolved to facilitate survival and fitness in a pathogenic environment, however developing and maintaining an adequate immunity is an energetically costly process (Lochmiller & Deerenberg 2000; Viney & Riley 2014). Moreover, excessive and unregulated immune reactions can have negative effects on the host fitness known as immunopathology (Graham et al. 2005). Thus other life-history traits are likely to be traded off with immune responses including sexually selected traits, reproduction and growth (Lee 2006; Nunn et al. 2008; Schmid-Hempel & Ebert 2003; van der Most et al. 2011; Zuk & Stoehr 2002). In addition the occurrence of trade-offs between immunity and other physiological systems influences the evolution of host-pathogen interactions (Best et al. 2009).

The immune system is an important physiological mechanism therefore the influence of proximate physiological systems on immune variation has been focused. An early approach was the immunocompetence handicap hypothesis suggesting an immunosuppressive capability of the endocrine system in favor of sexual traits (Folstad & Karter 1992). Rather than a direct influence a more complex interaction of both systems was revealed by subsequent studies (Ashley & Wingfield 2012; Demas et al. 2011; Roberts et al. 2004). In addition physiological factors associated with the neuroendocrine system have been described to induce variation of immunity, e. g. stress responses (Martin 2009), reproductive physiology (McKean & Nunney 2007) and lactation (East et al. 2015). The temporary or long-term availability of energetic resources affects the interaction among physiological systems as not all processes can be maximized at the same time (Stearns 1992). This is leading to subsequent trade-offs between these systems under the principle of energy allocation (Ardia et al. 2011; Lochmiller & Deerenberg 2000; Norris & Evans 2000). Accordingly variations of the individual immune phenotype can be influenced by the physiological status and the energy availability (Viney & Riley 2014). Independent from resource availability physiological and genetic control mechanisms allow a more permanent perspective on the expression of different immune phenotypes in terms of natural selection (Ardia et al. 2011; Downs et al. 2014; Swallow et al. 2009). Systems of signaling molecules such as cytokines are building the base of regulatory networks which can interfere with physiological processes and will be influenced by environmental conditions (Cohen et al. 2012; Martin et al. 2011b).

Besides the physiologic and environmental influence, the exposure and interaction of the immune system to a wide variety of organisms including parasites, fungi, bacteria and viruses is another important factor that drives variation. Considering that not all of these organisms are a potential thread to the host, distinguishable mechanisms of antigenic recognition and immunity have evolved in animals (Schulenburg et al. 2009; Viney & Riley 2014). Moreover co-evolution between the immune system and microorganisms contributes not only towards effective defense mechanisms against pathogens but also to possible beneficial mutualism with commensal gut or skin microbiota (Macpherson & Harris 2004), which can support the immune system. In natural habitats animals are facing a consistent exposure to pathogens with patterns of simultaneous and sequential infections (Pedersen & Babayan 2011) thus resulting in a high and diverse antigenic load (Viney & Riley 2017). As different pathogens are able to induce different immune responses understanding host-pathogen interactions under different environmental circumstances is another important part of eco-immunological research (Brock et al. 2014).

1.2. Methods used in eco-immunology

Investigations of immunity in the context of natural conditions and populations need to consider the possible variations caused by a general immune-heterogeneity, a wider physiological state and pathogen pressure. In theory a wide variety of immunological methods and tools is available. However the majority of these methods were developed for the usage in humans, laboratory model-species and domestic species which impedes a simple adaptation to free-ranging wildlife species (Pedersen & Babayan 2011; Viney & Riley 2014). Especially due to the lack of species-specific antibodies and annotated genomes eco-immunological studies are often depending on classic methods that only allow a measurement of few immune parameters (Boughton et al. 2011; Demas et al. 2011b). Moreover, field conditions, lack of laboratory setup in remote places and the missing storage conditions hamper the proper characterization of the immunity in wild animals and lead to a focus on the humoral immune effectors. Commonly used methods are descriptive and quantitative, which has clear limitations. These methods include size measurements of lymphoid organs (e.g. spleen and thymus), counts of innate and adaptive immune cells by hematology, determination of complement proteins, antibacterial enzymes (lysozyme, transferins), acute phase proteins (haptoglobin or serum amyloid A) and total immunoglobulins (Ig, mainly IgG and IgM). A more desired and informative approach is the use of functional tests such as bacterial killing assay for the constitutive innate immunity, the lymphocyte proliferation assay for the adaptive immunity, or immune challenges by injection of different non-infectious antigens (e.g. phytohaemagglutinin, sheep red blood cells, key-hole limpet haemocyanin), pyrogenic molecules (e.g. lipopolysacharids) or vaccines (e.g. diphtheria-tetanus vaccine) (Boughton et al. 2011; Demas et al. 2011b; Norris & Evans 2000; Sheldon & Verhulst 1996; Viney & Riley 2014). The limitation of these measurements is that they only allow momentary observations which can be influenced by several factors such as stress or circadian rhythm (Boughton et al. 2011; Zylberberg 2015). Nevertheless a combination of these methods makes a characterization of immune differences in-between or among wildlife species possible (Matson et al. 2006). The investigation of immune gene differences helps to understand variation of immune responses. Genetic analysis of innate and adaptive immune components e.g. the major histocompatibility complex (Acevedo-Whitehouse & Cunningham 2006) and Toll like receptors (Piertney & Oliver 2006) revealed insights on how host-pathogenic coevolution may contribute to differences in immune-competence and pathogen susceptibility (Tschirren et al. 2013; Wilfert & Schmid-Hempel 2008).

The recent development of high-throughput analysis in the –omics field enabled more detailed research on systemic interactions between innate and adaptive immunity (Calis & Rosenberg 2014). Genomic, transcriptomic and proteomic approaches impacted a more detailed understanding of how different expression of immune parameters is influenced by physiological and environmental factors (Babayan et al. 2018; Harris et al. 2015; Field et al. 2015, Flies & Wood 2019). Although the range of methodological approaches for eco-immunology increased the central challenge of how to best measure immune response is still existent (Siva-Jothy 1995). Single measurements will not demonstrate the whole capability and complexity of the host's immune system (Adamo 2004; Demas et al. 2011b) therefore a combination of different techniques assessing both innate and adaptive immunity is essential (Boughton et al. 2011; Downs & Stewart 2014b; Norris & Evans 2000). As studies on free-ranging species are aggravated by the availability and quality of samples the advancement of methods on reduction and storage of samples, or the use of non-invasive samples is important (Boughton et al. 2011; Demas & Nelson 2012; Schulenburg et al., 2009).

1.3. Bats as a model system in eco-immunology

Inside the mammalian class bats are building the second largest order with more than 1300 species in 21 families (Simmons 2005). The traditional taxonomical classification system divides bats in two suborders, Megachiroptera and Microchiroptera, based on their morphology and unique characteristics (e.g. vision, laryngeal echolocation). However recent molecular phylogenetic data reclassified bats into the suborders Yinpterochiroptera and Yangochiroptera which remains controversial (Teeling et al. 2000, 2005, 2016). Bat species are ecological highly diverse, abundant and widely distributed on all continents except Antarctica. They are the only mammals that evolved active flight and occupy diverse ecological niches (Teeling et al. 2005) providing important ecosystem services e.g. insect consumption and pollination (Kunz et al. 2011). The behavior of flight is energetically costly and bats exhibit high oxygen consumption and metabolic rates (Speakman et al. 2013). Genes involved in energy metabolism are positive selected in bats allowing a physiological modification to higher energetic demands during flight (Shen et al. 2010). Identification of positive selected genes in DNA damage control overlapping with innate immunity, further indicates that evolutionary adaptions to flight also influenced bat immunity (Zhang et al. 2013). Despite their relatively small body size bats have a long lifespan which exceeds those

of similar sized terrestrial mammals (Wilkinson & South 2002). The absence of strong senescence until high ages (Fleischer et al. 2017) suggests the evolution of protective molecular mechanism against age-related diseases and cancer (Wang et al. 2011). Peculiarities in expression of growth hormones (Seim et al. 2013) and mechanisms preventing oxidative stress and cellular damage (Huang et al. 2016) might contribute to an extraordinary longevity.

The most crucial characteristic of bats is their role as natural reservoir for a wide variety of emerging viruses (Chalisher et al. 2006, Wang 2009). About 200 different viruses have been described in different bat species including high pathogenic types for humans and livestock such as coronavirus (Wang et al. 2006), filoviruses (Towner et al. 2007) and paramyxoviruses (Drexler et al. 2012). A clinical manifestation of virus infection in bats is seemingly absent (Moratelli & Calisher 2015; Wynne & Wang 2013). Most likely due to general high levels of interferones, a group of chemokines secreted by tissue and immune cells in order to control viral infection and activating host immune response (Subudhi et al. 2019). However, in a few cases bats showed susceptibility against rabies (Almeida et al.2005), Australian bat lyssa virus (McColl et al. 2002) and Tacaribe virus (Cogswell-Hawkinson et al. 2012) after experimental infection. Nevertheless a long co-evolutionary history in favor for peculiar control mechanism of viral replication is evident (Baker et al. 2013, Brook & Dobson 2015).

In contrast bats may be vulnerable to other pathogens such as bacteria and fungi (Brook & Dobson 2015; Mühldorfer 2013). The most famous example is the white-nose syndrome caused by psychrophilic fungi *Pseudogymnascus destructans* (*Pd*) resulting in mass mortalities and population declines in North American bat species (Blehert et al. 2009). *Pd* invades exposed skin regions of hibernating bats causing alterations in the hibernating behavior leading to more frequent arousals and resulting in early depletion of energy reserves (Lorch et al. 2011). The infection is associated with severe inflammatory responses (Field et al. 2015) but cellular infiltration of innate immune cells is missing (Meteyer et al. 2009). During hibernation the immune system of bats is suppressed (Moore et al. 2011) similar to other hibernating mammals (Bouma et al. 2010). However, immune responses can occur during arousal periods and may elicit an exaggerated inflammatory response in WNS affected bats (Meteyer et al. 2012). The fungus was introduced from Europe (Warnecke et al. 2012) where it is widely distributed and was also found recently in Asia (Hoyt et al. 2016). In contrast to North American bat species naturally infected bats in Europe and Asia show only mild clinical signs and no associated mortality suggesting a tolerance towards *Pd* (Zukal et al.

2016). Inflammatory responses (Davy et al. 2017) and antibody production (Johnson et al. 2015) against *Pd* seem absent in European bat species. However, higher numbers of eosinophil immune cells in *Pd* infected European individuals indicate a basic immune defense towards the fungus (Banduchova et al. 2018).

Considering the general species diversity in terms of life-history traits (e. g. flight, echolocation, hibernation) and pathogenic load of different viruses bats are important models for comparative and within-species eco-immunological studies (Becker et al. 2019; Ruoss et al. 2019; Schneeberger et al. 2013, 2014). Although knowledge about general bat immunity is slowly increasing (Baker & Schountz 2018) immune response may differ due to their great diversity. Within species, immune responses of bats can vary between colonies and types of roosts (Allen et al. 2009) and it has been shown that the immune cell composition is different among species (Schinnerl et al. 2011). Current knowledge remains restricted to innate immunity and only partially to adaptive responses, therefore an overall understanding of systemic adaptive immune reactions and regulative immune mechanisms in bats is needed.

1.4. Thesis Objectives

The challenge in eco-immunology is to characterize a possible impact of ecological factors on the immune response in a context of potentially extensive genetic and environmental diversity. Such investigations are limited by the lack of reagents and annotated genomes. Research in this field is mostly done on the innate immune system based on functional assays and simple experimental approaches to test the total immune response of an animal (Demas *et al.* 2011, Graham *et al.* 2011). There is little known about development and processes in adaptive immune response and the link between innate and adaptive immunity. The main reason for this is lack of experimental procedures which can be used easily in the field and the difficulty of sampling wildlife (recapture of individuals, number and quality of samples). With my thesis I addressed these particular issues and focused on the development and application of novel immunological approaches for investigating bat immunology.

My findings are presented in three manuscripts, comprised in the following chapters:

CHAPTER 2

Plasma proteomic analysis of active and torpid greater mouse-eared bats (*Myotis myotis*)

In human medicine proteomic studies of blood plasma allow efficient insights in general immune processes during disease with a relative small amount of sample (Geyer et al. 2017). Therefore I used for the first time blood plasma proteomics in the European bat species *Myotis myotis* in order to gain knowledge about general regulative immune processes during hibernation. The energy conserving behavior of hibernation is associated with physiological changes and immune suppression in mammals (Bouma et al. 2010). I hypothesized that immunological and physiological changes will manifest in the blood plasma of hibernating bats and proteomic profiles will differ from the active state.

CHAPTER 3

Plasma proteomic profiles differ between European and North American Myotid bats with White Nose Syndrome

Based on the establishment of the plasma proteomic analysis in hibernating bats I used the technique for investigating white-nose syndrome affected and healthy individuals in Europe (*Myotis myotis*) and North America (*Myotis lucifugus*). Whereas North American bat species show a high susceptibility including high mortality rates towards the disease European bat species are mildly affected with the absent of mortality (Wibbelt 2018). Here I hypothesized that the plasma proteomic profile would differ between the diseased and healthy state in both species and that differences among the species would be detected.

CHAPTER 4

Cross-reactivity of human and murine antibodies against major lymphocyte markers in three chiropteran species

In order to advance understanding and description of bat adaptive immunity an essential requirement is the availability of bat specific antibodies. Novel techniques allow the generation of antibodies based on single B lymphocytes (Tiller et al. 2008) but single cell sorting with cell specific antibodies is required. As available antibodies against different bat species are rare (Periasamy et al. 2019) I performed a cross-reaction study with 19 commonly used human and murine antibodies with isolated blood cells from three bat species (*Myotis myotis, Pyllostomus discolor, Rousettus aegyptiacus*) representing the distinct bat families Vespertilionidae, Phyllostomidae and Pteropodidae.

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CHAPTER 2

Plasma proteomic analysis
of active and torpid
greater mouse-eared bats
(*Myotis myotis*)

SCIENTIFIC REP♦RTS

Plasma proteomic analysis of active and torpid greater mouse-eared bats (*Myotis myotis*)

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Hibernation is a physiological adaptation to overcome extreme environmental conditions. It is characterized by prolonged periods of torpor interrupted by temporary arousals during winter. During torpor, body functions are suppressed and restored rapidly to almost pre-hibernation levels during arousal. Although molecular studies have been performed on hibernating rodents and bears, it is unclear how generalizable the results are among hibernating species with different physiology such as bats. As targeted blood proteomic analysis are lacking in small hibernators, we investigated the general plasma proteomic profile of European *Myotis myotis* and hibernation associated changes between torpid and active individuals by two-dimensional gel electrophoresis. Results revealed an alternation of proteins involved in transport, fuel switching, innate immunity and blood coagulation between the two physiological states. The results suggest that metabolic changes during hibernation are associated with plasma proteomic changes. Further characterization of the proteomic plasma profile identified transport proteins, coagulation proteins and complement factors and detected a high abundance of alpha-fetoprotein. We were able to establish for the first time a basic myotid bat plasma proteomic profile and further demonstrated a modulated protein expression during torpor in *Myotis myotis*, indicating both novel physiological pathways in bats in general, and during hibernation in particular.

In order to overcome food limitation and high metabolic energy demands during winter periods, animals in temperate climate zones have evolved strategies such as diet switching, annual migration, winter resting or hibernation. Hibernating species undergo a circannual rhythm between homeothermy (activity) and heterothermy (hibernation), in which the heterothermic hibernation cycle alters between extended phases of deep torpor interspersed by short rewarming phases called arousals[1]. During the torpid phase, which lasts between 6 and 40 days, the metabolic rate drops to 2% of normal coinciding with lowered body temperatures between 10 °C and −2 °C, decreased heart rate and longer breath intervals[1–3]. During arousal, torpor-associated physiological changes are restored to euthermic values for 10–15 hours[1]. As a consequence of metabolic suppression, the immune system is also functionally suppressed during torpor and restored during arousals in order to clear infections[4].

To describe the mechanism underlying the circannual rhythm of hibernation a two-transition model has been proposed, which designates a transition from homeothermy to heterothermy and another cyclic transition within the heterothermic state between torpor and arousal[5,6]. Although the precise genetic and molecular regulation underlying this mechanism remains unclear, it was shown that the switch from homeothermy to heterothermy is associated with a broad differential expression of existing genes rather than with the evolution and expression of hibernation-specific genes[7–9]. These expression

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differences occur at the protein level in heart[10], intestine[11], liver[12] and skeletal muscles[13] of ground squirrels (*Ictidomys tridecemlineatus*). In these tissues, increased expression levels of proteins involved in gly- colysis, glycogenesis and amino acid catabolism were observed during the active state, while hibernation was characterized by increased expression of proteins involved in fatty acid catabolism. These findings were consistent with the shift from carbohydrates to lipid oxidation during hibernation[14]. Another study investigating the kidney proteome in the same species found evidence for a turnover of plasma proteins alpha-2-macroglobulin, albumin and apolipoprotein during torpor-arousal cycles[15].

Plasma proteins and their composition in the blood are known to be an important indica- tor of physiological changes including disease recognition or pathogen infection in humans[16]. Thus hibernation-associated changes in blood plasma protein composition may also be detectable. In Asian chipmunks (*Tamias sibiricus asiaticus*), for example, specific proteins associated with hibernation have been identified in plasma[17,18], while in ground squirrels (*Ictidomys tridecemlineatus*), altered plasma metabolites (e.g. amino acids, regulatory lipids) during the heterothermic hibernation cycle were characterized using non-targeted metabolomic approaches[19,20]. A targeted proteomic analysis of the blood serum in hibernating American black bears (*Ursus americanus*) revealed differential expression of proteins involved in immunity, coagulation and bone metabolism[21]. These results provide molecular support for the peculiarities of ursid hibernation, including wound healing and active immune function during hibernation[22]. In contrast to bears, rodent hibernation is associated with immunosuppression[4], highlighting the importance of research on blood proteomics in small mammalian hibernators given the lack of a universal mammalian hibernation proteomic profile.

Research on mammalian hibernation has focused on rodents. Although there is information available on general regulatory processes from tissue proteomic studies in rodents, targeted analysis of plasma and serum proteome profiles is lacking. Even if the hibernating phenotype between species is similar across taxa, differences in the mechanisms leading to hibernation associated changes are unclear, especially if physiological adaptations during homeothermy are considered. Accordingly, similarities in physiological aspects of hibernation may be expected for rodents and bats, e.g. fuel change from carbohydrate to lipid metabolism, reduction in protein synthesis, among others. However other processes may be unique due to basic differences in their physiology. Bats are able to enter a heterothermic state throughout the year by entering bouts of prolonged torpor also including the summer period[23] while most hibernating rodents exhibit heterothermy only during the winter season. Bats are also the only mammalian group capable of powered flight, which has selected for increased metabolic capacity and elevated antioxidant levels[24]. Moreover, they are unusually long-lived species[25], traits which are different from the hibernating rodents studied to date. Furthermore, the reproduction patterns of hibernating bats[26,27] or the role of wing membrane in maintaining the water balance[28], makes chiropteran hibernation related physiology unique. It has been suggested that some bat species are not immunosuppressed during hibernation as observed in rodents and that bats maintain specific defenses against psychrophilic pathogens such as *Pseudogymnoascus destructans*[29]. Therefore, we hypothesized that some of the regulatory mechanisms of hibernating bats should be distinct from those of rodents.

In order to improve our knowledge on chiropteran hibernation and small mammalian hibernation in general, we compared the blood plasma proteomic profile of a hibernating European bat species, the greater mouse-eared bat (*Myotis myotis*) by using a two-dimensional gel electrophoresis approach to characterize differential expressed proteins between homeothermic and heterothermic, torpid individuals. In comparison to rodent hibernation we predict for bats similar pattern of protein expression in general regulatory mechanisms but differences in the regulation of proteins involved in specific physiological processes such as immune function or reproduction.

Materials and Methods

Ethics statement. All experimental procedures described in the materials and methods section were approved by the animal welfare and ethics committee of the Leibniz Institute for Zoo and Wildlife Research (permit #2010-05-01). All experiments were carried out in accordance with the approved guidelines of the Leibniz Institute for Zoo and Wildlife Research.

Sample collection. Greater mouse-eared bats (*M. myotis*) were captured in Northern Bavaria (Germany), under the license of regional governments (permits 54-2532.2-9/10, 55.1-8642G062/10 and 55.1-8642-01-17/10). Blood samples from 14 male individuals in the homeothermic active state (n = 7) were captured in September 2010 and torpid individuals (n = 7) during the hibernation period in March 2011. Active individuals were captured using mistnets (Ecotone, Poland), while torpid individuals were picked by hand from the walls of the hibernacula. Blood samples were collected in active bats from the uropatagial vein using sterile needles and by transferring blood droplets into heparinized microcapillary tubes and in torpid bats from the jugular vein using a sterile heparinized needle and syringe. All bats were released at the site of capture after bleeding was completed. Structural and functional immunological measurements were performed on fresh blood while in case of surplus blood the plasma was separated by centrifugation and stored at $-80\,°C$ until further analysis.

2-Dimensional Fluorescence Difference Gel Electrophoresis (2-D DIGE). Proteomic profiles were determined from blood plasma samples of 14 *M. myotis* male individuals (7 homeothermic, active individuals and 7 heterothermic, torpid individuals) using 2-D DIGE.

Serum albumin is the most abundant plasma protein in humans[16] and can impede the detection and quantification of low abundance plasma proteins[30]. Therefore plasma investigation studies often deplete albumin prior to analysis. However the depletion of albumin also can remove untargeted proteins[31]. Albumin depletion was not performed in this study but serum albumin was excluded during mass spectrometry identification.

Total plasma protein concentration was determined using a NanoDrop and diluted to the required concentration of 0.55 µg protein/µL in labeling buffer [50 mM tris, 5 mM EDTA, 5% v/v glycerol, pH 7.2; final volume = 9 µL] for fluorescent protein labeling using S-Dye300 of the Saturn-2D™ labeling kit (NH DyeAGNOSTICS GmbH, Germany) according to manufacturer´s protocol. An internal standard (IS) consisting of all samples used in each experimental procedure was diluted to the required concentration of 0.55 µg protein/µL in labeling buffer (final volume = 9 µL) and fluorescent labeled using S-Dye200 of the Saturn-2D™ labeling kit.

Labeled samples (per gel: 9µL of an individual S-Dye300-labeled sample + 9µL of S-Dye200-labeled IS) were diluted in 432 µL rehydration buffer [8 M Urea, 1% w/v 3-[(3-Cholamidopropyl)dimethyl- ammonio]-1-propanesulfonate hydrate (CHAPS), 13 mM Dithiothreitol (DTT), 0.5% v/v Servalyt (SERVA Electrophoresis GmbH, Germany)] and loaded on IPG *Blue*Strips pH 3–10 NL/24 cm (SERVA Electrophoresis GmbH, Germany) for active (50 V, 15 h) sample-in-gel rehydration using PROTEAN IEF Cell tray (Bio-Rad, USA). Isoelectric focusing was performed under the following conditions: step 1, 300 V, 150 V/h rapid; step 2, 600 V, 300 V/h rapid; step 3, 1500 V, 750 V/h rapid; step 4, 3000 V, 48000 V/h

rapid; step 5, 6000 V, 10000 V/h rapid; step 6, 300 V, 5 h; total 60700 V/h.

Prior to second dimension separation, IPG stripes were equilibrated in equilibration buffer [EB: 6 M Urea, 2% SDS, 0.375 M Tris, 20% v/v glycerol] with first 20 mg/mL DTT for 15 min, followed by EB with 25 mg/mL iodoacetamide (IAA) for 15 min. After equilibration, stripes were placed on 15% SDS gels in 27.5 × 22 cm low fluorescence glass cassettes (NH DyeAGNOSTICS GmbH, Germany) and overlaid with 1% agarose including bromphenol blue. Gel electrophoresis was performed in a SE900 electro- phoresis unit (Hoefer Inc., USA) for a minimum of 1900 V/hours and a maximum of 2200 V/hours at 80 mA/ gel, 100 W and 100 V. Imaging of the gels was performed by fluorescence scanning on a Typhoon 9400 Imager (GE Healthcare, USA) at excitation/emission wavelengths of 532/576 nm (S-Dye200) and 633/664 nm (S-Dye300).

To evaluate the expression pattern of protein spots separated by 2-D DIGE, all sample gels were analyzed using the Delta2D software (DECODON, Germany). An IS S-Dye200 image was designated as the master gel based on the largest number of detectable spots, and then connected to all images by a "sample in gel" warping strategy in the Delta2D software. Warping of gels was done by defining matched vectors between distinct protein spots chosen automatically and manually. For expression analysis of protein spots, a fused image of all sample images (S-Dye300; gel images of each sample are shown in sup- plementary Figure S1) was generated and a consensus spot pattern for normalization against IS images was applied. Matched protein spots present in all sample images and with a minimum of 1.5 fold change between active and torpid state were statistically analysed using a non-parametric Wilcoxon Rank Sum test (alpha: $p < 0.05$) with the Delta2D statistic software TMeV (Decodon).

Preparative 2-D gel for protein identification. Preparative gel separation was employed using pooled samples of all 14 individuals. For the first dimension, unlabeled pooled plasma (total protein concentration = 240 µg) was loaded onto an IPG stripe and separated according to isoelectric points as described above. Separation according to molecular weight in the second dimension was also performed as described above with the exception that a 28 × 21 cm hinged glass cassette (Hoefer Inc., USA) was used instead of low fluorescence glass cassettes. After 2-D gel electrophoresis the gel was Coomassie blue dye stained [0.02% Coomassie blue G-250; 5% w/v aluminum sulfate; 10% v/v ethanol; 2% v/v ortho-phosphoric acid in dH₂O] for 4 h and then destained [10% ethanol; 2% v/v ortho-phosphoric acid in dH₂O] to remove unbound Coomassie blue[32]. Spots with a fold change difference of ≥1.5 and a statistical significance of $p < 0.05$ between the active and torpid state were picked for protein identification. It was not possible to pick all differentially expressed spots as not all spots were distinguishable on the Coomassie blue stained gel. Additional spots for a basic survey of the bat plasma proteome were chosen based on a distinct appearance in the preparative gel and co-localization with protein spots that were differentially expressed. Protein spots of interest were picked manually and stored in Eppendorf tubes containing 5% v/v acetic acid (in dH₂O) at 4 °C until analysed by mass spectrometry.

Protein identification by mass spectrometry (MS). Excised gel spots were washed with water, 25 mM ammonium bicarbonate in acetonitrile/water (1:1) and 50 mM ammonium bicarbonate, shrunk by dehydration in acetonitrile and dried in a speed-vacuum centrifuge. The dry gel pieces were re-hydrated in 20 µL of 50 mM ammonium bicarbonate containing 50 ng trypsin (sequencing grade modified, Promega). After incubation at 37 °C overnight, the enzymatic reaction was terminated by addition of 20 µL of 0.5% (v/v) trifluoroacetic acid in acetonitrile, the liquid was separated, evaporated to dryness

under vacuum, and the tryptic peptides were re-dissolved in 6 μL 0.1% (v/v) trifluoroacetic acid , 5% (v/v) acetonitrile in water.

MALDI mass spectrometry was performed as previously described[33]. In brief, the peptides were puri-fied on a C18 RP minicolumn (ZipTip C18, Millipore, Bedford, MA) and eluted directly onto the MALDI target plate using alpha-cyano-hydroxycinnamic acid matrix solution. MS and MS/MS measurements were performed using a MALDI-TOF-TOF instrument (AB SCIEX TOF/TOF 5800; Applied Biosystems, Framingham, MA, USA) equipped with a Neodymium-doped yttrium lithium fluoride laser (Nd:YLF, 349 nm). MS spectra were acquired in positive ion reflector mode by accumulating 5000 consecutive laser shots. For MS/MS, a maximum of 20 precursor ions were selected automatically. GPS Explorer (version 3.6, Applied Biosystems) was used to process the spectra.

LC-MS/MS analyses were performed on an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher) equipped with an Ultimate 3000 nanoLC system (Thermo Scientific). For separation of tryptic peptides, a capillary column (PepMap100, C18, 3 μm, 100 Å, 250 mm × 75 μm i.d., Thermo Scientific) was used. Elution was performed at a flow rate of 300 nL/min using a gradient of 3–50% B in 30 min. Mobile phase A contained 0.1% formic acid in water, and mobile phase B contained 0.1% formic acid in acetonitrile. Mass spectra were acquired in a data-dependent mode with one MS survey scan (with a resolution of 60,000) in the Orbitrap and MS/MS scans of the five most intense precursor ions in the linear trap quadrupole. The dynamic exclusion time for precursor ions was set to 90 s and automatic gain control was set to 1×10^6 for Orbitrap-MS and 10,000 for LTQ-MS/MS scans. The Mascot Distiller Quantitation Toolbox (Matrix Science) was used to generate peak lists.

The processed MS data were analysed on a MASCOT (mass spectral search algorithm) server (ver-sion 2.2.2, Matrix Science Ltd, London) and searched in-house against the mammalian subset of the NCBI database (version 221013; 33,055,681 sequences). For MALDI-MS, the mass tolerance of precursor and sequence ions was set to 100 ppm and 0.35 Da, respectively. For LC-MS/MS, the mass tolerance of precursor and sequence ions was set to 10 ppm and 0.35 Da, respectively. A maximum of two missed cleavages was allowed. Methionine oxidation and the acrylamide modification of cysteine were used as variable modifications. A protein was accepted as identified if the total MASCOT score was greater than the significance threshold and at least two peptides appeared the first time in the report and were the top ranking peptides (peptide matches of all proteins identified are listed in supplementary Table S2). For MALDI data, the protein score was $-10*\log(p)$, where p is the probability that the observed match is a random event, e.g. protein scores greater than 75 are significant($p < 0.05$). For LC-MS/MS data, the ions score was $-10*\log(p)$, where p is the probability that the observed match is a random event, e.g. individual ions scores >41 indicate identity or extensive homology ($p < 0.05$).

Results

Differential protein expression of plasma proteins in *M. myotis*. When considering all gel images, we detected a total of 204 matched protein spots present in all individual samples by using a consensus spot pattern. Of 204 protein spots, 13 protein spots (6.4%) showed a significant ($p < 0.05$) differential expression with a minimum of a 1.5 fold difference between the two physiological states, nine protein spots being down regulated and four being up regulated in torpor compared to the active state (Fig. 1 and Table 1).

We were not able to determine and pick all differential expressed protein spots on the Coomassie stained gel. Protein identification (IDs) were successful for 5 out of 13 differential expressed protein spots (S1/C7, S4/C15, S5/C16, S6/C17 down regulated; S2/C3 up regulated), in which MS data obtained mul- tiple protein IDs for 3 protein spots (S1/C7, S5/C16, S6/C17) and single protein IDs for 2 protein spots (S2/C3, S4/C15). For multiple protein IDs, the top 3 ranked MS peptide matches are shown in Table 1, excluding matches to serum albumin.

Protein matches to those identified in *Myotis davidii* and *Myotis brandtii* based on a MASCOT search of the NCBI database. The significantly down regulated proteins during the torpid state were identified as vitamin D-binding protein, vascular non-inflammatory molecule 3, anti-thrombin III (multiple pro-tein IDs of spot S1/C7), serotransferrin (S4/C15; S5/C16; S6/C17), ß-chain of fibrinogen (S6/C17), and alpha-fetoprotein (S5/C16). The significantly up regulated protein in torpor (S2/C3) was identified as Kininogen-1 (Table 1).

Survey of expressed plasma proteins in *M. myotis*. To describe a general proteomic profile of bat plasma, 27 protein spots co-localizing with differentially expressed protein spots were picked for analysis (Fig. 2). For 18 out of 27 picked protein spots, MS data yielded multiple protein IDs. The top 3 ranks of MS peptide match results are shown in Table 2 excluding matches to serum albumin. Identified proteins matched those identified in *Myotis davidii* and *Myotis brandtii* based on MASCOT searches of the NCBI protein database. In total, MS yielded 20 different protein IDs of which 12 protein IDs appeared in more than one protein spot. Protein IDs appearing in numerous protein spots included alpha-fetoprotein (20 protein spots (PS)), serotransferrin (7 PS), anti-thrombin III (6 PS), vitamin D-binding protein (6 PS), hemoglobin subunit beta (5 PS), complement C4A (3 PS), fibrinogen beta chain (3 PS), vascu- lar non-inflammatory molecule 3 (3 PS), alpha-1-antitrypsin (2 PS), hemoglobin subunit alpha (2 PS), hemoglobin alpha chain (2 PS) and kininogen-1 (2 PS). Protein IDs found in single protein spots were kininogen-2 (C1), complement C3 (C13), NSFL1 cofactor p47 (C18), apolipoprotein A-V (C19), chain
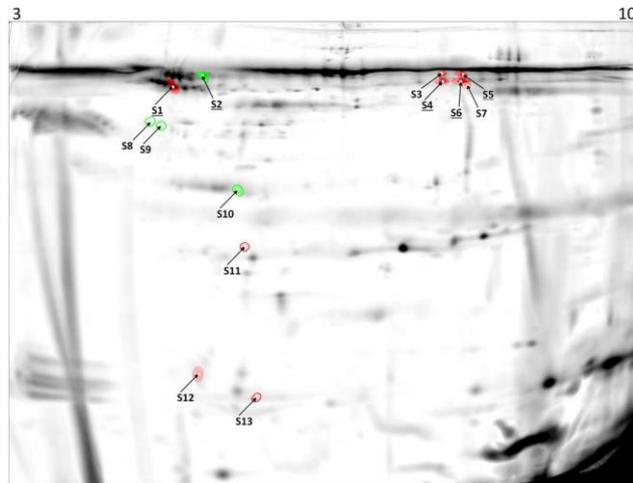
Figure 1. *Myotis myotis* plasma proteome. Fused image of representative images of S-dye labeled *M. myotis* active and torpid plasma samples separated by 2-DIGE. Differential expressed protein spots (p < 0.05; minimum fold-change 1.5) are displayed in either green for up regulated or red for down regulated proteins during torpor and indicated by numbers corresponding to Table 1. Protein spots which were identified via mass spectrometry are underlined. The IPG stripe pH range is indicated on top of the image.

Spot number	Fold Change	*p* value	Protein ID	MS protein score	MS method	Accession number‡
S1 [C7]	−1.7	0.012	Vitamin D-binding protein	615	2	gi432093474
			Vascular non inflammatory molecule	566		gi521021447
			Anti-thrombin III	564		gi432097679
S2 [C3]	+2.4	0.001	Kininogen-1	103	1	gi521019686
S3*	−2.2	0.001	*no protein picking possible*	/		/
S4 [C15]	−2.9	0.001	Serotransferrin	490	1	gi432108417
S5 [C16]	−2.4	0.002	Serotransferrin	457	1	gi432108417
			Alpha-fetoprotein	242		gi521031276
S6 [C17]	−3.3	0.001	Serotransferrin	215	1	gi432108417
			Fibrinogen beta chain	84		gi521022331
S7*	−1.6	0.044	*no protein picking possible*	/		/
S8*	+5.9	0.001	*no protein picking possible*	/		/
S9*	+3.1	0.005	*no protein picking possible*	/		/
S10*	+2.6	0.045	*no protein picking possible*	/		/
S11*	−8.0	0.033	*no protein picking possible*	/		/
S12*	−1.5	0.024	*no protein picking possible*	/		/
S13*	−6.6	0.015	*no protein picking possible*	/		/

Table 1. Differential protein expression in the *Myotis myotis* plasma proteome. Protein spots exhibiting differential expression (p < 0.05; minimum fold-change 1.5) using Delta2D software are shown. Fold change reflects differences in protein spot volume comparing active to torpid samples. Identified proteins via MS techniques MALDI-TOF/TOF (1) and LC-MS/MS (2) are displayed with the total MS protein score based on MASCOT searches on NCBI database and affiliated accession numbers of *Myotis davidii* and *Myotis brandtii* (‡). Protein IDs listed are the top 3 ranked protein matches based on the MASCOT score excluding protein matches of serum albumin. Spots S3, S7, S8, S9, S10, S11, S12 and S13 were not possible to relate to a protein spot on preparative gels and thus could not be picked and identified (*).

A profilin-Beta-Actin (C20), carbonic anhydrase 2 (C24), adenine phosphoribosyltransferase (C26), apolipoprotein M (C29) and dihydroorotate dehydrogenase (C30) (Table 2). Protein IDs appearing in more than one protein spot were located in similar gel regions (C2, C4, C6, C8 and C9; C11 and C12)
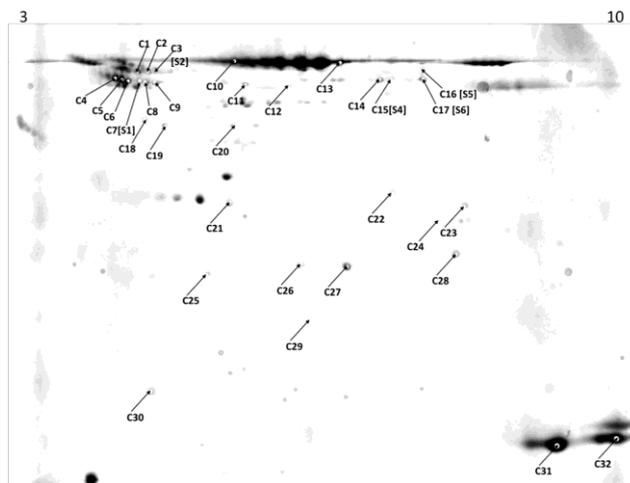
Figure 2. Coomassie Gel of *Myotis myotis* plasma proteome. Preparative Coomassie gel of all 14 plasma samples (pooled) separated by two-dimensional gel electrophoresis. The IPG stripe pH range is indicated on top of the image. Protein numbers display spots that were picked based on co-localization with hibernation specific differentially expressed protein spots distinct appearance in the preparative gel.

expect protein IDs alpha-fetoprotein, fibrinogen, serotransferrin and hemoglobin subunit beta which were located in protein spots distributed over all gel regions.

Discussion

We observed differential expression for 13 of 204 detectable spots between the active and torpid state representing 6.4% of the total detected protein spots, a similar proportion observed by targeted serum proteomic analysis in American black bears[21]. The protein gel spot pattern exhibited electrophoretic accumulation of protein spots, suggesting an occurrence of multiple protein isoforms. The plasma protein signature clearly differentiated the torpid state from the active homeothermy which is consistent with observed variation in tissues of other hibernating mammalian species[10–14]. Mass spectrometric analysis identified seven differentially regulated proteins. Most of them were down regulated (Vitamin D-binding protein (DBP), Serotransferrin (TF), Vascular non inflammatory molecule 3 (VANIN-3), Alpha-fetoprotein (AFP), Fibrinogen beta chain (FIG-β) and Anti-thrombin III (AT)), whereas Kininogen-1 (KNG1) was up regulated.

Down regulated proteins TF, AFP and DBP are classified as transport proteins based on their primary function of binding essential body metabolites, vitamins or metal ions. The iron binding glycoprotein TF plays an important role in cellular and systemic iron homeostasis particularly during long fasting periods[34]. DBP and AFP belong to the albumin gene family in humans and have multifunctional roles in plasma. DBP is a major transporter of Vitamin D3 and its metabolites, and is found as a free plasma protein and also on the surface of many cell types including blood cells[35,36]. In contrast, AFP occurs primarily as a free plasma protein in numerous polymeric forms[37]. AFP is involved in the binding and transport of several metabolites including fatty acids, bilirubin and estrogens or metal ions in human fetuses and other mammals[37–39]. The down regulation of these transport proteins might reflect the decreased metabolic rate of hibernating species[3]. Consistent with this hypothesis, studies on diet-restricted rats demonstrated lower mRNA levels of DBP and a decrease of expressed DBP in the liver during fasting[40,41]. Furthermore TF and DBP are involved in the modulation of innate immunity[42–44] and DBP has a particu- larly important role in the activation of macrophages[45]. Therefore, down regulation suggests a reduction of innate immunity during hibernation[4].

VANIN-3 is an amidohydrolase involved in the catabolism of CoenzymeA (CoA) from pantoth- enic acid (Vitamin B5). CoA, and its thioester form acetyl-CoA, are essential cofactors in maintaining fatty acid balance[46]. During hibernation, a switch from glycolysis to the oxidation of triacylglycerols is observed[14] making fatty acids of adipose tissue the primary energy source. However, the function of VANIN-3 in CoA catabolism is poorly understood. Down regulation during hibernation might suggest an alternate catabolism of CoA reflecting the switch from glucose to triacylglycerols as an energy source. Differentially expressed proteins FIG-β, AT and KNG1 are part of the blood coagulation system.

FIG-β together with other protein domains forms the soluble glycoprotein fibrinogen which is converted by thrombin into insoluble fibrin during formation of blood clots[47]. The serine protease inhibitor AT degrades proteases of the coagulation cascade in order to regulate coagulation and to prevent throm- bosis[48]. KNG1 in contrast is part of the kallikrein-kinin system and is known to be essential in many pathways including thrombosis, vascular permeability, and inflammation[49]. During hibernation, plate- let aggregation is reduced in brown bears (*Ursus arctos*)[50]. Also an elevation of the protease inhibitor

Spot number	Protein ID	MS protein score	MS method	Accession number‡
C1	Kininogen-1	84	1	gi432105324
	Kininogen-2	60		gi521032202
C2	Vitamin D-binding protein	690	2	gi432093474
	Antithrombin-III	594		gi432097679
	Alpha-fetoprotein	550		gi521031276
C3 [S2]	Kininogen-1	103	1	gi432105324
C4	Alpha-1-antitrypsin	633	2	gi432096197
	Antithrombin-III	461		gi432097679
	Vitamin D-binding protein	434		gi432093474
C5	Alpha-1-antitrypsin	374	1	gi432096197
C6	Vitamin D-binding protein	658	2	gi432093474
	Antithrombin-III	607		gi432097679
	Alpha-fetoprotein	545		gi521031276
C7 [S1]	Vitamin D-binding protein	615	2	gi432093474
	Vascular non-inflammatory molecule 3	566		gi521021447
	Antithrombin-III	564		gi432097679
C8	Vitamin D-binding protein	494	1	gi432093474
	Vascular non-inflammatory molecule 3	55		gi521021447
C9	Vitamin D-binding protein	425	1	gi432093474
	Vascular non-inflammatory molecule 3	76		gi521021447
C10	Alpha-fetoprotein	299	1	gi521031276
C11	Alpha-fetoprotein	724	2	gi521031276
	Anti-thrombin III	576		gi432097679
	Fibrinogen beta chain	566		gi521022331
C12	Alpha-fetoprotein	587	2	gi521031276
	Anti-thrombin III	506		gi432097679
	Serotransferrin	394		gi432108417
C13	Alpha-fetoprotein	1111	2	gi521031276
	Serotransferrin	819		gi432108417
	Complement C3	535		gi521031112
C14	Fibrinogen beta chain	677	1	gi521022331
C15 [S4]	Serotransferrin	490	1	gi432108417
C16 [S5]	Serotransferrin	457	1	gi432108417
	Alpha-fetoprotein	242		gi521031276
C17 [S6]	Serotransferrin	215	1	gi432108417
	Fibrinogen beta chain	84		gi521022331
C18	Alpha-fetoprotein	596	2	gi521031276
	Complement C4-A	462		gi432089459
	NSFL1 cofactor p47	339		gi431894242
C19	Alpha-fetoprotein	614	2	gi521031276
	Complement C4-A	356		gi432089459
	Apolipoprotein A-V	329		gi432105735
C20	Alpha-fetoprotein	969	2	gi521031276
	Complement C4-A	534		gi432089459
	Chain A, Profilin-Bet-Actin	487		gi313507212
C21	Alpha-fetoprotein	175	1	gi521031276

Continued

Spot number	Protein ID	MS protein score	MS method	Accession number‡
C22	Alpha-fetoprotein	987	2	gi521031276
	Serotransferrin	786		gi432108417
	Hemoglobin, subunit beta	296		gi432107589
C23	Alpha-fetoprotein	166	1	gi521031276
C24	Carbonic anhydrase 2	88	1	gi432088987
	Alpha-fetoprotein	87		gi521031276
C25	Alpha-fetoprotein	303	1	gi521031276
C26	Alpha-fetoprotein	720	2	gi521031276
	Hemoglobin, subunit beta	356		gi432107589
	Adenine phosphoribosyltransferase	314		gi432104870
C27	Alpha-fetoprotein	739	2	gi521031276
	Hemoglobin, subunit beta	308		gi432107589
	Serotransferrin	268		gi432108417
C28	Alpha-fetoprotein	109	1	gi521031276
C29	Alpha-fetoprotein	297	2	gi521031276
	Hemoglobin, subunit beta	277		gi432107589
	Apolipoprotein M	217		gi432089435
C30	Hemoglobin, subunit beta	355	2	gi432107589
	Alpha-fetoprotein	317		gi521031276
	Dihydroorotate dehydrogenase	257		gi521035253
C31	Hemoglobin subunit alpha	347	1	gi110831911
	Hemoglobin alpha chain	274		gi189909345
C32	Hemoglobin subunit alpha	356	1	gi122428
	Hemoglobin alpha chain	335		gi189909345

Table 2. Identified proteins in *Myotis myotis* plasma proteome. Protein IDs obtained using MS techniques MALDI-TOF/TOF (1) and LC-MS/MS (2) are displayed with the total MS protein score based on MASCOT searches on the NCBI database and affiliated accession numbers of *Myotis davidii* and *Myotis brandtii* (‡). Protein IDs listed are the top 3 ranked protein matches based on the MASCOT score excluding protein matches to serum albumin.

alpha-2-macroglobulin in serum of hibernating ground squirrels and black bears was observed, consistent with reduced coagulation[21,51]. In addition, KNG1 was found to be down regulated in hibernating black bears[21]. A decrease of coagulation activity during hibernation could protect the individual from blood clotting during periods of low heart rate and reduced blood flow[1,52]. However, the expression pattern of down regulated AT and up regulated KNG1 in *M. myotis* could suggest an elevated coagulation capacity. Similarly, up regulation of coagulation associated genes have been described in hibernating *Myotis brandtii* at the transcription level[25]. In contrast a down regulation of FIG-β during torpor in *M. myotis* may result in a reduction of coagulation capacity[53]. Based on the contradictory findings of proteins involved in coagulation in *M. myotis* we conclude that the detected coagulation associated proteins might be involved in other physiological processes relevant to hibernation. This may imply that bats have a different coagulation cascade compared to other mammals as it is unlikely that increased coagulation during hibernation would be advantageous.

In order to establish a basic plasma proteomic profile in bats, twenty-seven clearly definable protein spots co-localizing with hibernation specific differentially expressed protein spots were identified. MS protein identification yielded multiple IDs per protein spot (Table 2) and IDs found in more than one protein spot including differential expressed protein spots suggesting the co-occurrence of different isoforms, which can be regulated differentially[54]. Most proteins identified are known to be the most abundant proteins in human plasma[16] including TF, FIG-β, alpha-1-antitrypsin and complement C3. Based on their primary functions, further identified proteins could be categorized as transport proteins (e.g. hemoglobin, apolipoprotein A-V and M), coagulation proteins (e.g. AT, kininogen I and II), proteins of the complement system (e.g. complement factor C4A and C3) and proteins involved in a variety of regulative processes (e.g. carbonic anhydrase). All identified proteins in *M. myotis* can be found in a general plasma profile of humans[16] indicating similarities in the plasma protein composition across mammalian taxa. However, AFP exhibited an unusual profile by identification in 21 of 34 picked protein

spots suggesting a high abundance in plasma of adult *M. myotis*. In humans, AFP is the most abundant protein during fetal development and has a similar function to serum albumin in adults including the binding of hydrophobic ligands such as fatty acids, metabolites and as well metal ions[35–37]. The abundance of AFP in the adult bat plasma suggests that this protein may play an important functional role in different mechanisms in *M. myotis*, maybe in bats in general, thus deserving further scientific attention.

Protein identification was hindered by the lack of protein databases for non-model, wildlife species. Homologous proteins of other species do not always share 100% sequence similarity with unknown *M. myotis* proteins, reducing the amount of potential analysable tryptic peptides per protein depending on the level of similarity which influences the peptide match ranking. This lack of knowledge will certainly change as proteomic research on non-model species develops further[55]. Nonetheless, we were able to establish a basic plasma proteome profile for *M. myotis*. Moreover, we showed differential expression of plasma proteins in hibernating bats compared to active bats demonstrating a modulation of proteins involved in transport, fuel switching from carbohydrate to triacylglycerol oxidation, innate immunity and blood coagulation cascade. In addition protein identification of further protein spots demonstrated evidence for an alternate composition of high abundant plasma proteins in *M. myotis* with AFP as a possible prominent protein in adult bat plasma. Chiropteran hibernation proteome profile was generally consistent with other hibernating species at the pathway level as hypothesized except for coagulation which appears to be myotid bat specific. Further comparisons with rodents and bears will clarify the general similarities and differences among hibernating species proteomic profiles particularly as protein databases improve and identification becomes facilitated.

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Author Contributions

G.Á.C. conceived the study; A.M.H. performed all laboratory experiments expect MS under the supervision of B.C.B. and E.K. performed the MS experiments; A.M.H., E.K. and B.C.B analysed the data; C.C.V. worked on the permissions and collected together with G.Á.C the samples; A.M.H., B.C.B., E.K., A.D.G. and G.Á.C. discussed the results and wrote the manuscript. All authors commented, read and approved the final manuscript.

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CHAPTER 3

Plasma proteomic profiles

differ between

European and North American Myotid bats

with White-Nose Syndrome

(*in preparation*)

Plasma proteomic profiles differ between European and North American Myotid bats with White-Nose Syndrome

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Abstract

Emerging infectious diseases induced by fungi have become a growing threat for wildlife health. In North American cave-hibernating bat species the psychrophilic fungus *Pseudogymnascus destructans* (*Pd*) is associated with the disease called White-nose syndrome (WNS) causing significant population decline by high mortality rates. In contrast European bat species are only mildly affected with the absence of mortality. Differences in epidemiology and pathology indicate an evolution of tolerance mechanisms towards *Pd* in European bats. We compared for the first time the proteomic profile of blood plasma of healthy and *Pd* colonized European *Myotis myotis* and North American *Myotis lucifugus* in order to identify patho-physiological changes during WNS. Expression analyses of plasma proteins revealed no significant differences between healthy and *Pd* colonized *Myotis myotis* whereas in *Myotis lucifugus* a differential expression was observed. We could identify differential expressed proteins for acute phase response, constitutive and general immunity, oxidative stress control, metabolism, exosomes and desmosomes suggesting a systemic response against *Pd* infection. Differences in the plasma proteomic profile between European and North American bat species during WNS are supporting the hypothesis that European bats have evolved tolerance mechanisms towards infection with *Pd*.

Keywords

Introduction

Emerging infectious diseases (EIDs) are most often associated with viruses and bacteria (Daszak et al. 2000). However, fungal pathogens are known to be major threats to plants (Anderson et al. 2004). Although fungi are generally thought to be less of an EID concern for vertebrates, there are increasing wildlife examples of fungal EIDs (Scheele et al. 2019; Fisher et al. 2012). Pathogenic fungi have led to massive declines in snakes (*Ophidiomyces ophiodiicola*) (Lorch et al. 2016) and amphibians (*Batrachochytrium dendrobatidis* and *B. salamandrivorans*) (Berger et al. 2016). White-nose syndrome (WNS), caused by the psychrophilic fungus *Pseudogymnascus destructans* (*Pd*), is an emerging fungus of cave-hibernating bats. Since its initial outbreak in 2006, the fungus has killed millions of North American bats, fatalities reaching up to 90-99% per hibernacula (Langwig et al. 2015). The associated mortality has resulted in local extinction of several previously common myotid bat species (Blehert et al. 2009; Coleman & Reichard 2014). Seven bat species have been diagnosed with WNS in North America and five more species exhibit a fungal growth without disease confirmation (Wibbelt 2018). Susceptibility varies among bat species, with the little brown bat (*Myotis lucifugus*) exhibiting mortality rates as high as 91% (Frick et al. 2015; Turner et al. 2011). In contrast the big brown bat (*Eptesicus fuscus*) appears to be resistant to *Pd* (Frank et al. 2014; Moore et al. 2018).

WNS is characterized by growth of *Pd* on exposed skin regions of hibernating bats (Lorch et al. 2011; Warnecke et al. 2012). The fungus invades the epidermis and dermis which leads to distinctive cupping erosions (Cryan et al. 2010; Meteyer et al. 2009). Clinical pathology of *Pd* infection in North American species is associated with abnormal behaviour, a disturbed natural hibernation-cycle characterized by increased arousal frequency resulting in early fat store depletion during hibernation (Reeder et al. 2012). Diseased animals show disturbed electrolyte and hydration balance (Cryan et al. 2013; Willis et al. 2011), oxidative stress (Moore et al. 2013), chronic respiratory acidosis (Verant et al. 2014), altered complement protein activity (Moore et al. 2011) and fever response (Mayberry et al. 2018). *Pd* infection does not produce primary inflammatory cellular infiltration into infected tissues (Meteyer et al. 2009), which be associated with leukopenia (e.g. low white blood cell counts) during mammalian hibernation (Bouma et al. 2010). However, the expression of inflammatory, wound healing and metabolic genes increase without the recruitment of neutrophils and T cells to the site of invasion (Field et al. 2015). WNS may also elicit an exaggerated inflammatory response during arousal (Meteyer et al. 2012). How WNS causes

death in bats is not clear (Wibbelt 2018) but is likely a combination of physiological disturbances provoked by *Pd* resulting in a multi-stage progression of the disease (Verant et al. 2014).

In contrast to North America, *Pd* is widely distributed among hibernating bat species in Europe (Puechmaille et al. 2011; Zukal et al. 2014) but without associated clinical signs or mortality (Wibbelt et al. 2013). Seventeen bat species are colonized by the fungus in Europe, with the greater mouse-eared bat (*Myotis myotis*) being most often infected (Wibbelt 2018). Recently four additional species tested positive for *Pd* in North-eastern China extending the host and the geographic range of the pathogen (Hoyt et al. 2016). Lesions associated with *Pd* infection in European bats are less pronounced than in North America (Wibbelt et al. 2013). However, deep cutaneous invasion associated with neutrophil infiltration has been observed in some species (Bandouchova et al. 2015; Wibbelt et al. 2013). The differences in epidemiology and pathology of *Pd* infection in European and North American bats suggest that European bats have co-evolved with the fungus. Considering the possible origin of *Pd* in Europe (Leopardi et al. 2015), European bats may be immunologically or behaviorally resistant (Puechmaille et al. 2011; Wibbelt et al. 2010).

Natural and experimental infection studies of the physio- and immuno-pathological aspects of WNS have been performed on susceptible and resistant North American bat species. However, similar studies are rare for European species. The different approaches and experimental setups used among studies complicates data interpretation and among study comparison (Johnson et al. 2015, Moore et al. 2018, Davy et al. 2017). To address the paucity of European and North American comparative *Pd* infection studies, we compared the plasma proteomic profile of hibernating North American *Myotis lucifugus* and European *Myotis myotis*. Blood plasma composition is an important indicator of physiological changes during infection in human (Anderson et al. 2004b) and veterinary medicine (Bilić et al. 2018; Ghodasara et al. 2017). Proteomic approaches have been used to understand the pathways associated with hibernation in several model and non-model organisms (Grabek et al. 2015), including bats (Hecht et al. 2015). We hypothesized that healthy and *Pd* colonized individual plasma proteomic profiles will vary among species and reflect patho-phyisological changes.

Materials and Methods

Ethics statement

Capture, handling and sample collection protocols for this study were reviewed and approved by the animal welfare and ethics committee of the Leibniz Institute for Zoo and Wildlife Research (permit #2011-12-01) and the Macdonald Campus Facility Animals Care Committee, McGill University. All protocols complied with existing guidelines from Germany and Canada.

Sample collection

During March-April of 2012, greater mouse-eared (*M. myotis*) and little brown bats (*M. lucifugus*) were hand collected from hibernacula in Germany and Canada, respectively. In Germany, bats were collected from two mines and two cellars in Northern Bavaria (n = 12, 6 female and 6 male). In Canada, individuals (n = 12, 6 female and 6 male) were collected from the Trou de la Fée caverne and Laflèche caves. Equal numbers of bats with and without clinical signs of *Pd* colonization were collected for both species. To avoid cross-contamination gloves were exchanged between the processes of each animal and all clothes, shoes and gear were disinfected before moving to the next hibernacula following accepted decontamination protocols (www.whitenosesyndrome.org).

Immediately after removing the bats from the hibernacula, adhesive tape was used to collect samples for mycological analysis (Wibbelt et al. 2010, Wibbelt 2018). The localization of fungal colonization was registered and the bats were euthanized using isoflurane overdose followed by exsanguination. Necropsy was performed locally and blood and tissues taken. Blood plasma was separated by centrifugation and all samples stored in liquid nitrogen. Samples were transported to the Leibniz Institute of Zoo and Wildlife Research Berlin, Germany (IZW), where they were stored at -80°C until further analysis.

Although histopathological lesions characteristic of WNS have been described in European species, European bats cannot be considered diseased (Wibbelt 2018). We assigned collected bats from Europe and North America to healthy and *Pd* colonized groups according to their *Pd* colonization status. Colonization status was defined by analyzing the samples collected with adhesive tapes. After collection, the tape samples were transferred to glass slides which were examined under light microscope for characteristic *Pd*'s conidia. If observed, isolation and mycological confirmation of the fungus was subsequently performed

(Wibbelt et al. 2010). In the case of *M. lucifugus*, *Pd* infection was also diagnosed histologically (Meteyer et al. 2009).

2-D Fluorescence Difference Gel Electrophoresis (2-D DIGE)

Plasma proteomic profiles of 12 *M. myotis* individuals (6 healthy and 6 *Pd* colonized) and 12 *M. lucifugus* individuals (6 healthy and 6 diseased; *Pd* colonized and WNS positive) were determined. Albumin depletion was not performed but albumin was excluded during mass spectrometry identification process, as described in a prior study (Hecht et al. 2015).

2-D DIGE analyses was performed across 12 SDS gels for each species with fluorescent protein labeling of the individual samples using the G-Dye Refraction-2D^{TM} labeling kit (NH DyeAGNOSTICS GmbH, Germany) according to the manufacturer´s protocol. Briefly, total plasma protein concentration was determined using a NanoDrop® and diluted to the required concentration of 5 µg protein/µL in labeling buffer [30 mM Tris, 7 M urea, 2 M thiourea, 4% w/v 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS) pH 8.5; final volume = 10 µL] for G-Dye labeling. Individual samples were labeled with G-Dye200 or G-Dye300 using dye swaps between the samples. An internal standard consisting of a pool of all samples was always labeled with G-Dye100. Each gel contained 2 individual samples (one stained with G-Dye200 and one stained with G-Dye300) and the internal standard. The stained samples plus the internal standard were diluted in a total volume of 450 µL rehydration buffer [8 M Urea, 1% w/v 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS), 13 mM Dithiothreitol (DTT), 0.5% v/v Servalyt (SERVA Electrophoresis GmbH, Germany)] and loaded on IPG BlueStrips pH 3-10 NL/24 cm (SERVA Electrophoresis GmbH, Germany) for active (50 V, 15 h) sample-in-gel rehydration using PROTEAN® IEF Cell tray (Bio-Rad, USA). Isoelectric focusing was performed using the following conditions: step 1, 300 V, 150 V/h rapid; step 2, 600 V, 300 V/h rapid; step 3, 1500 V, 750 V/h rapid; step 4, 3000 V, 48000 V/h rapid; step 5, 6000V, 10000 V/h rapid; step 6, 300 V, 5 h; total 60700 V/h.

Prior to second dimension separation, IPG stripes were equilibrated in equilibration buffer [EB: 6 M Urea, 2% SDS, 0.375 M Tris, 20% v/v glycerol] with first 20 mg/mL DTT for 15 min, followed by EB with 25 mg/mL iodoacetamide (IAA) for 15 min. After equilibration, stripes were placed on 12,5 % SDS gels in 27.5 x 22 cm low fluorescence glass cassettes (NH DyeAGNOSTICS GmbH, Germany) and overlaid with 1 % agarose including bromophenol blue. Gel electrophoresis was performed in a SE900 electrophoresis unit (Hoefer Inc., USA) for a minimum of 2400 V/hours and a maximum of 2550 V/hours at 80

mA/ gel, 100 W and 100 V. Imaging of the gels was performed by fluorescence scanning on a Typhoon 9400 Imager (GE Healthcare, USA) at excitation/emission wavelengths of 498/524 nm (G-Dye100), 554/575 nm (G-Dye200) and 648/663 nm (G-Dye300).

To evaluate the expression pattern of protein spots separated by 2-D DIGE for healthy and *Pd* colonized individuals, sample gels were analyzed using the Delta2D software (DECODON, Germany). Briefly, an IS G-Dye100 image was designated as the master gel based on the largest number of detectable spots, and then connected to all images by a "sample in gel" warping strategy in the Delta2D software. Warping of gels was done by defining matched vectors between distinct protein spots chosen automatically and manually. For expression analysis of protein spots, a fused image of all sample images (G-Dye200 and G-Dye300) was generated and a consensus spot pattern for normalization against the internal standard images was applied. Matched protein spots present in all sample images with a minimum of 1.5 fold change between the healthy and Pd colonized state were statistically analyzed using a non-parametric Wilcoxon Rank Sum test (alpha: $p < 0.05$) with the Delta2D statistic software TMeV (Decodon). Protein spots on the edges and in the upper region of the gels where no adequate protein separation was achieved were excluded from spot normalization and analysis in the Delta2D software.

Preparative 2-D gel for protein identification

Spot picking on preparative gel was performed by NH DyeAgnostics Services (NH DyeAGNOSTICS GmbH, Germany). Preparative gel separation was performed with pooled samples of all *M. lucifugus* or *M. myotis* individuals. A standardized company protocol was used. Pooled plasma was labeled with G-Dye300 and mixed with unlabeled sample. A total protein concentration of 900 µg was applied per IPG stripe (pI 3 – 10NL) and passively rehydrated. Isoelectric focusing was performed on an IEF 100 Focusing Unit (Hoefer Inc, USA) and second dimension separation was performed on an SE900 electrophoresis unit (Hoefer Inc., USA) with a 12,5 % SDS gel. After 2-D electrophoresis the gel was stained with Coomassie® Brilliant Blue and fixed (40% ethanol, 10% acetic acid). Preparative gel images were matched with sample images in the Delta2D software. According to analyzed expression profiles proteins spots with a fold change difference of ≥1.5 and a statistical significance of $p < 0.05$ between healthy and *Pd* colonized individuals were picked for protein identification. It was not possible to pick all differentially expressed spots as not all spots were distinguishable on the Coomassie blue stained gel.

Protein identification by liquid chromatography-mass spectrometry (LC-MS).

Excised gel spots were washed with water, 25 mM ammonium bicarbonate in acetonitrile/water (1:1) and 50 mM ammonium bicarbonate, shrunk by dehydration in acetonitrile and dried in a speed-vacuum centrifuge. The dry gel pieces were re-hydrated in 20 µL of 50 mM ammonium bicarbonate containing 50 ng trypsin (sequencing grade modified, Promega). After incubation at 37 °C overnight, the enzymatic reaction was terminated by addition of 20 µL of 0.5% (v/v) trifluoroacetic acid in acetonitrile, the liquid was separated, evaporated to dryness under vacuum, and the tryptic peptides were re-dissolved in 6 µL 0.1% (v/v) trifluoroacetic acid , 5% (v/v) acetonitrile in water.

LC-MS/MS analyses were performed on an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher) equipped with an Ultimate 3000 nanoLC system (Thermo Scientific). For separation of tryptic peptides, a capillary column (PepMap100, C18, 3 µm, 100 Å, 250 mm × 75 µm i.d., Thermo Scientific) was used. Elution was performed at a flow rate of 300 nL/min using a gradient of 3-50 % B in 30 min. Mobile phase A contained 0.1 % formic acid in water, and mobile phase B contained 0.1 % formic acid in acetonitrile. Mass spectra were acquired in a data-dependent mode with one MS survey scan (with a resolution of 60,000) in the Orbitrap and MS/MS scans of the five most intense precursor ions in the linear trap quadrupole. The dynamic exclusion time for precursor ions was set to 90 s and automatic gain control was set to 1×10^6 for Orbitrap-MS and 10,000 for LTQ-MS/MS scans. The Mascot Distiller Quantitation Toolbox (Matrix Science) was used to generate peak lists.

The processed MS data were analysed on a MASCOT (mass spectral search algorithm) server (version 2.2.2, Matrix Science Ltd, London) and searched in-house against a combination of NCBI database of 3 Myotis species (*M. brandtii, M. davidii, M.lucifugus*) (FASTA files; 87021 entries). For LC-MS/MS, the mass tolerance of precursor and sequence ions was set to 10 ppm and 0.35 Da, respectively. A maximum of two missed cleavages was allowed. Methionine oxidation and the acrylamide modification of cysteine were used as variable modifications. A protein was accepted as identified if the total MASCOT score was greater than the significance threshold and at least two peptides appeared the first time in the report and were the top ranking peptides. For LC-MS/MS data, the ions score was -10*log(p), where p is the probability that the observed match is a random event, e.g. individual ions scores >41 indicate identity or extensive homology ($p < 0.05$).

Results

Analysis of protein expression profiles revealed a total of 157 matched protein spots, present in all samples of *M. myotis* by using a consensus spot pattern. No significant differential protein expression was observed between healthy and *Pd* infected bats thus protein identification was not performed.

Of 157 protein spots identified in M. lucifugus, 11 protein spots (7,01%) showed a significant (p<0.05) differential expression with a minimum of a 1.5 fold difference between the healthy and diseased bats. Compared to the healthy state a significant up regulation of eight protein spots and a significant down regulation of three protein spots could be detected in WNS positive *M. lucifugus* (Figure 1 and Table 1).

Protein identification (IDs) was successful for 8 of 11 differential expressed protein spots (Ml1, Ml2, Ml3, Ml5, Ml8, Ml9 up regulated; Ml4, Ml10 down regulated) (Table 1). For all protein spots MS data obtained multiple protein IDs. Excluding serum albumin matches, the top 5 ranked protein IDs with recurrent protein IDs under the top ranks based on MS peptide scores are shown in Table 1.

Protein IDs for up regulated protein spots include proteins involved in acute phase response (Alpha-2-macroglobulin-like, Serotransferrin and Fibrinogen chains alpha, beta, beta isoform X1, gamma isoform X2), complement system (Complement C3-like), immunity (Immunoglobulin lambda-like polypeptide 5-like), lipoproteins (Apolipoprotein A-I, Serum paraoxonase/arylesterase 1 isoform X1), metabolism (Alpha-2,8-sialyltransferase 8F, S-adenosylmethionine synthetase isoformtype-2), oxygen transport (Hemoglobin subunit beta-like) and reactive oxygen species (6-phosphogluconate dehydrogenase, Protein DJ-1 isoform X1). For down regulated protein spots protein IDs involved in acute phase response (Alpha-2-macroglobulin-like, Fibrinogen alpha chain, Serotransferrin), desmosomes (Desmoplakin, Junction plakoglobin Isoform X1), exosomes (Actin, cytoplasmic 2, Tubulin beta-2B chain-like isoform X1, 14-3-3 protein sigma), immunity (Putative V-set and immunoglobulin domain-containing-like protein IGHV4OR15-8-like) and reactive oxygen species (α 1-microglobulin/bikunin precursor (AMBP) isoform X1), were obtained. Hereby proteins Alpha-2-macroglobulin-like, Serotransferrin, Fibrinogen, Apolipoprotein A-I, Hemoglobin subunit beta-lik and Immunoglobulin lambda-like polypeptide 5-like appear in more than one protein spot under the top 5 ranked protein IDs.

Discussion

The comparative expression analysis of plasma proteins between healthy and *Pd* colonized European *M. myotis* did not reveal significant differential protein expression. This is in agreement with the lack of pathological changes associated with infection and the lack of WNS associated mortality in *M. myotis* (Davy et al 2017; Johnson et al. 2015; Wibbelt et al. 2010). However, North American *M. lucifugus* showed significant differences in expression indicating a very different response to WNS infection than observed in *M. myotis*.

Of the 11 differentially expressed protein spots identified in North American *M. lucifugus*, MS analysis yielded multiple protein IDs from 8 spots which are linked to physiological mechanisms including acute phase response (A2M-like, TF, PF chains α, β, γ), constitutive innate immunity (C3-like), immune gene products (IGLL5, IGHV4OR15-8), oxidative stress control (6PGD, DJ-1-X1, AMBP-X1), metabolism (SIAT8-F, MAT2) and further proteins of desmosomes (DP, JPG-X1) and exosomes (ACT2, TB-β-2B-like-X1, 14-3-3 protein). These findings support an active host response against *Pd* on a systemic level and concur with transcriptomic findings at the local site of infection of WNS-affected *M. lucifugus* (Field et al. 2015) and functional studies (Moore et al. 2011, Moore et al. 2013).

By activation of pattern recognition receptors *Pd* infection in *M. lucifugus* shows similarities to other fungal skin infections in euthermic animals (Field et al. 2015). Host response against fungi is initiated by the recognition of fungal components by epithelian and innate immune cells resulting in the activation of the immune system through the production of inflammatory cytokines (Romani 2011). In euthermic animals, the production of pro-inflammatory cytokines activates the acute phase response (APR), a systemic immune reaction characterized by the production of acute phase proteins (APPs), fever, leucocytosis (e.g. increase in white blood cell counts) and sickness behaviour (anorexia, lethargy) (Cray et al. 2009). Several aspects of the acute phase response in hibernating mammals have been shown to be constrained by the immunosuppression during the torpid state, which includes lack of fever (Prendergast et al. 2002) and the lack of leucocytosis (Bouma et al. 2010). Accordingly, it was described that naturally infected WNS positive *M. lucifugus* lack the recruitment of innate immune cells and T cells to the site of *Pd* infection despite the apparent expression of inflammatory cytokines and chemokines (Field et al. 2015). We identified several APPs, like A2M-like, TF and protein chains of PF (α, β, γ), in the majority of differential regulated protein spots in *M. lucifugus* plasma. The physiological function of APPs is to reestablish homeostasis, promote healing, kill different pathogens and mitigate

reactive oxygen species. They also have important roles in the promotion of the host's immune response (Cray et al. 2009). Iron-binding glycoprotein TF is part of the systemic iron homeostasis which is alternated during infections with effects on the modulation of innate immune defenses and prevention of pathogen survival (Ganz & Nemeth 2015). It has a similar activity to haptoglobin, another important APP in bats (Costantini et al. 2019) which has been identified in the wing tissue transcriptome of WNS positive *M. lucifugus* (Field et al. 2015). As major protease inhibitor, A2M is involved in regulation of inflammatory processes by scavenging defensins and inhibiting proteases of host and non-host origin which protects inflamed tissues against excessive damage (Rehmann et al. 2013). Also glycoprotein PF is described with regulatory properties during inflammation in different tissues (Davalos & Akassoglou 2012).

Up regulated C3 complement component, together with other complement factors, is part of the constitutive innate immune system, which is considered the first line of defense against pathogens (Becker et al. 2019). C3 has a central role in the complement system, as it is involved in phagocytosis, inflammation and cell lysis and is required in both classical and alternative complement activation pathways (Sarma & Ward 2011). Complement associated defense is one of the few parts of the immune system which is not affected by hibernation (Maniero 2002). Increased C3 production may represent an attempt to control *Pd* infection using one of the only available active immune responses during hibernation.

The observed differential regulation of innate immune proteins (A2M, C3, PF and TF) in the plasma of WNS-affected *M. lucifugus* samples suggest an activated systemic innate immunity triggered by *Pd*. These results support previous transcriptomic and functional immunological studies, where *Pd*-colonized *M. lucifugus* showed an up regulation of gene expression of PF chains, C3 and other innate immune genes in the affect tissues (Field et al. 2015), and showed altered complement activity (Moore et al. 2011). An activated systemic immune response may also explain the detection of up regulated gene product IGLL5 associated with different immune functions in cancer (Ascierto et al. 2013; White et al. 2018) and down regulated IGHV4OR15-8-like, a gene product of the immunoglobulin heavy chain variable region associated with immune response (Dammalli et al. 2017; Matsuda et al. 1998).

Activation of the immune system by fungal and bacterial pathogens initiates phagocytosis associated with the production of reactive oxygen species (ROS) (Brown 2011). Increasing level of ROS during host defense leads to oxidative imbalances in affected tissues and thus an up regulation of oxidative stress markers (Schneeberger et al. 2013). During *Pd* infection an altered expression of oxidative stress related genes was detected (Field et al.

2015) and the total antioxidant defense is compromised (Moore et al. 2013). Accordingly we found up regulated plasma proteins DJ-1-X1, 6PGD and down regulated AMBP-X1 which are associated with host protection during oxidative stress. LPS-induced inflammatory conditions in mice showed an up regulation of protein DJ-1 in response to ROS (Mitsumoto & Nakagawa 2001). The pentose phosphate pathway with 6PGD as essential factor is known to have protective properties against oxidative damage during infections (Riganti et al. 2012) as well as alpha-1-microglobulin the derivative of protein AMBP (Olsson et al. 2008). Interestingly alpha-1-microglobulin also has protective effects against increased levels of extracellular hemoglobin associated with different diseases (Rother et al. 2005; Olsson et al. 2012) and elevated in our samples of WNS-affected *M. lucifugus*.

One of the energetic costs of the APR during infection is the changed lipid metabolism (Khovidhunkit et al. 2004). The identified up regulated plasma proteins APO-AI and PON-X1 are associated with the high-density lipoprotein complex which is essential for cholesterol trafficking between peripheral cells and the liver (Tall 1990). An increased level of lipoproteins in WNS-affected *M. lucifugus* may suggest a changed triglyceride metabolism through the fungal infection as also gene expression of apolipoproteins were increased at affected wing membrane (Field et al. 2015).

The down regulation of exosomal proteins (ACT2, TB-β-2B-like-X1 and 14-3-3) may represent the disruption of *Pd* affected tissues (Cryan et al. 2010; Meteyer et al. 2009) and associated wound healing (Field et al. 2015). Exosomes function as mediator for intercellular communication (Théry et al. 2009) and it has been shown that exosome release can be supressed during wound healing (Zhou et al. 2017).
Other differentially expressed proteins identified in *Pd* infected *M. lucifugus* plasma were up regulated SIAT8-F and MAT2 which are associated with metabolic cycles of oligosaccharides (Wang et al. 2016) and amino acid methionine (Finkelstein & Martin 2000). Further we identified down regulated desmosome proteins DP and JPG-X1 involved in cell signaling (Johnson et al. 2014). Alternated genes involved in different metabolic pathways and cell signaling were also found on the transcriptomic level displaying a changed metabolism most likely associated with disturbance of host homeostasis due to WNS (Field et al 2015; Verant et al. 2014).

In contrast to North American *M. lucifugus*, European *M. myotis* did not exhibit any changes in proteomic profile, suggesting they tolerate *Pd*. Tolerance in a host-pathogen system is context dependent and influenced by both host and pathogen factors (King & Li 2018, Mandl et al. 2018). *Pd* in Europe and North America shows very little divergence and it

has been demonstrated that it was recently introduced to North America, likely by European cave divers (Leopardi et al. 2015). Therefore, pathogen factors likely play little role in the differences observed between North American and European bats. Our results suggest that the lack of response of *M. myotis* to *Pd* reflects a long term co-existence of pathogen and host which has resulted in an equilibrium state that does not result in high host mortality rates. The change in proteomic profile of North American *M. lucifugus* and extremely high mortality rates suggests this equilibrium state has not been reached in North America.

Acknowledgments

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Figure 1. *Myotis lucifugus* **plasma proteome of healthy and diseased individuals.** Fused image of representative images of G-dye labeled *M. lucifugus* plasma samples separated by 2-DIGE. Differential expressed protein spots ($p < 0.05$; minimum fold-change 1.5) are displayed in either green for up regulated or red for down regulated proteins in *Pd* infected individuals. Spot numbers correspond to Table 1.
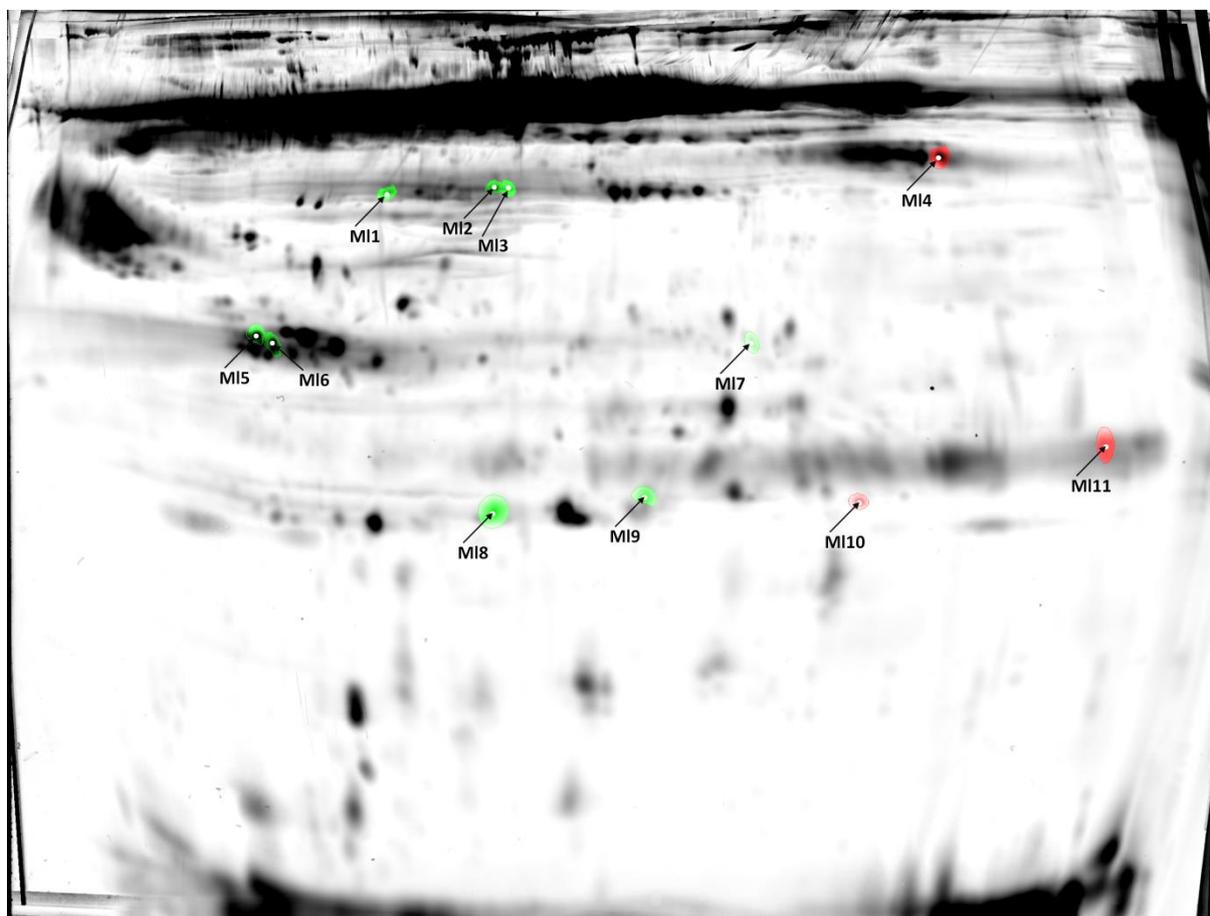
Table 1. Differential protein expression in the *Myotis lucifugus* plasma proteome Protein spots exhibiting differential expression (p < 0.05; minimum fold-change 1.5) using Delta2D software are shown. Fold change reflects differences in protein spot volume comparing healthy to diseased individuals. Identified proteins via LC-MS/MS are displayed with the total MS protein score based on MASCOT searches on NCBI database. Protein IDs listed are the top 5 ranked protein matches based on the MASCOT score excluding protein matches of serum albumin.

Spot number	Fold change	Protein ID	MS protein score	Accession number
Ml1	+ 1.57	A2M-like	1277	XP_006084289.1
		TF	849	XP_006083771.1
		PFα	443	XP_006081874.1
		PON1-X1	369	XP_006085046.1
		6PGD	304	XP_006104980.1
Ml2	+2.03	A2M-like	1415	XP_006084289.1
		PFγ-X2	810	XP_006081876.1
		TF	652	XP_006083771.1
		PFα	434	XP_006081874.1
		PFβ	293	XP_006081872.1
Ml3	+2.74	PFγ-X2	568	XP_006081876.1
		A2M-like	483	XP_006084289.1
		PFβ-X1	238	XP_006081872.1
		PFα	212	XP_006081874.1
		TF	184	XP_006083771.1
Ml4	-3.15	PFα	2121	XP_006081874.1
		TF	1138	XP_006083771.1
		A2M-like	172	XP_006084289.1
		IGHV4OR15-8-like	113	XP_006776697.1
		AMBP-X1	76	XP_005880980.1
Ml5	+4.39	Apo-AI	2160	XP_006103027.1
		C3-like	945	XP_006107818.1
		A2M-like	928	XP_006084289.1
		Hb-β-like	742	XP_005880462.1
		IGLL5	652	XP_005871762.1

Ml6	+3.37	*no protein identification possible*	/	/
Ml7	+5.17	*no protein identification possible*	/	/
Ml8	+1.8	Apo-AI	241	XP_006103027.1
		TF	184	XP_006083771.1
		A2M-like	184	XP_006084289.1
		DJ-1-X1	151	XP_006102968.1
		Hb-β-like	90	XP_005880461.1
Ml9	+1.57	IGLL5	76	XP_005871755.1
		PFα	67	XP_005860555.1
		A2M-like	63	XP_006084289.1
		SIAT8-F	36	XP_005856790.1
		MAT2	33	XP_005882476.1
Ml10	-37.04	DP	978	XP_006105268.1
		JPG-X1	569	XP_005875826.1
		ACT2	508	XP_006753072.1
		TB-β-2B-like-X1	221	XP_005869692.1
		14-3-3 protein sigma	197	XP_005875205.1
Ml11	-1.94	*no protein identification possible*	/	/

Abbreviation protein IDs

6PGD = 6-phosphogluconate dehydrogenase; A2M-like = Alpha-2-macroglobulin-like; ACT2 = Actin, cytoplasmic 2; AMBP-X1 = α 1-microglobulin/bikunin precursor (AMBP) isoform X1; ApoAI = Apolipoprotein A-I; C3-like = Complement C3-like; DJ-1-X1 = Protein DJ-1 isoform X1; DP = Desmoplakin; Hbβ-like = Hemoglobin subunit beta-like; IGHV4OR15-8-like = Putative V-set and immunoglobulin domain-containing-like protein IGHV4OR15-8-like; IGLL5 = Immunoglobulin lambda-like polypeptide 5-like; JPG-X1 = Junction plakoglobin Isoform X1; MAT2 = S-adenosylmethionine synthetase isoformtype-2; PFα = Fibrinogen alpha chain; PFβ = Fibrinogen beta chain; PFγ-X2 = Fibrinogen gamma chain isoform X2; PON1-X1 = Serum paraoxonase/arylesterase 1 isoform X1; SIAT8-F = Alpha-2,8-sialyltransferase 8F; TB-β-2B-like-X1 = Tubulin beta-2B chain-like isoform X1; TF = Serotransferrin

CHAPTER 4

Cross-reactivity of human and murine antibodies against major lymphocyte markers in three chiropteran species

(*in preparation*)

Cross-reactivity of human and murine antibodies against major lymphocyte markers in three chiropteran species

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Abstract

Chiropteran species are a known important reservoir for different emerging viruses, and act as natural hosts for zoonotic diseases. Despite their epidemiologic role, little is known about immunity in bats and how their immune system control viral infections. Hindering investigation of bat immunity is the lack of bat specific molecular tools. Such shortcomings prompted us to test the cross-reactivity of 19 anti-murine and anti-human antibodies against different lymphocyte surface-markers (CD3, CD4, CD5, CD8, CD19, CD45 and CD79) with lymphocytes isolated from three different bat species (*Myotis myotis; Phyllostomus discolor; Rousettus aegyptiacus*). We were able to identify cross-reaction for 5 antibodies in *M. myotis*, 1 antibody in *P. discolor* and 1 antibody in *R. aegyptiacus*. Our findings provide further tools for the characterization of adaptive immunity in these bat species.

Keywords

Myotis myotis, Phyllostomus discolor, Rousettus aegyptiacus, Flow Cytometry, cross-reaction, non-model species

Introduction

The order Chiroptera (bats) is a highly diverse, abundant and widely distributed mammalian group with over 1300 species across 21 families (Simmons 2015). Based on size and unique characteristics (e.g. vision, echolocation), bats are taxonomically classified in the two suborders, Megachiroptera and Microchiroptera. They are the only mammals capable of flight and provide important ecosystem services (e.g. insect consumption, seed-dispersion and pollination) for silviculture and agriculture (Kunz et al. 2011; Ghanem & Voigt 2012). Bats are also important reservoirs for emerging viruses (Calisher et al. 2006), including coronaviruses (Wang et al. 2006), filoviruses (Olival & Hayman 2014) and paramyxoviruses (Drexler et al. 2012). Although bats can host different pathogenic viruses clinical manifestation is rare (Baker et al. 2013). While they play subclinical epidemiological roles for viruses and other intracellular pathogens (e.g *Bartonella*) (Brook & Dobson 2015; Kosoy et al. 2010), bats may be vulnerable to other pathogens, such as extracellular bacteria and fungi (Mühldorfer 2013). For example, white-nose syndrome caused by psychrophilic fungi *Pseudogymnascus destructans* causes unprecedented mass mortalities and population declines in hibernating North American bats (Blehert et al. 2009). Bats may have a peculiar immune system which is able to control and contain intracellular, but not extracellular pathogens (Brook & Dobson 2015). The mechanisms involved are unclear. In addition to their epidemiological role, bats are a used as important model for comparative eco-immunological studies because of their general diversity (Schneeberger et al. 2013; Becker et al. 2019; Rouss et al. 2019). However, knowledge on the bat immune system is limited and relates mostly to innate immunity (Baker et al. 2018). The majority of immunological studies in bats focused on humoral and cellular immune responses using functional and hematological methods, in part due to the limitations of working under field conditions (Rouss et al. 2019). Recent genomic and transcriptomic studies revealed a more systemic understanding on immunity in bats (Field et. al 2015; Pavlovich et al. 2018). However, there is a general paucity of information on the bat adaptive immune response due to the lack of bat specific antibodies and poor cross-reaction of bat immune cells with antibodies developed for other species (Gómez et al. 2016, Periasamy et al. 2019). This results in a strong bias towards general understanding of innate immunity and a general lack of information on how bat adaptive immune responses develop.

We tested a set of established human and mouse specific antibodies against different immune cell markers in order to identify possible cross-reactivity with lymphocytes isolated from

three different chiropteran species, the greater mouse-eared bat (*Myotis myotis,* Vespertilionidae), the pale spear-nosed bat (*Phyllostomus discolor,* Phyllostomidae) and the Egyptian fruit bat (*Rousettus aegyptiacus,* Pteropodidae). *Myotis myotis* is one of the most common bat species in the Palearctic and a reservoir for several viruses (Drexler et al. 2011). It is the species most frequently colonized by the fungi *Pseudogymnascus destructans*, the causal agent of white-nose syndrome (Wibbelt 2015) and an important model for eco-physiological study (Hecht et al. 2015). The pale spear-nosed bat is associated with several pathogens e.g. *Bartonella* (Stuckey et al. 2017), or coronaviruses (Corman et al. 2013) in South America and a common model for neuroscientific and eco-physiology studies. *Rousettus aegyptiacus* is one of the best studied bat species in terms of immunology, mainly due to its epidemiological role for the spread of pathogenic viruses such as Ebola or Marburg virus (Mandl et al. 2018; Pourrut et al. 2009).

The three species are not closely related and distinguishable in their diet and behavior, therefore span a broad taxonomic spectrum of the potential immune response diversity of bats despite the limited number of species tested. The results are discussed in the context of developing research on adaptive immunological processes in both healthy and diseased bats.

Materials and Methods

Sample collection

We collected blood samples from bat species *Myotis myotis* (*M. myotis*), *Phyllostomus discolor* (*P. discolor*) and *Rousettus aegyptiacus* (*R. aegyptiacus*). All samples were collected during population management actions (see below). Since immediate analysis of fresh blood was logistically impossible, the collected blood was pooled and conserved with FACS Lysing Solution (BD BioSciences, San Jose, USA) in order to fix peripheral leukocytes and lyse red blood cells. This method has been used with success for marsupial mammals (Lau et al. 2012). Pooled blood was mixed with FACS Lysing Solution at a ratio of 1:10 and incubated for 15 min at room temperature. Afterwards sample aliquots were frozen with liquid nitrogen and stored at -80°C until further analysis.

Free-living juvenile, male *M. myotis* (n=39) were captured in Northern Bavaria and Thuringia, Germany (Davy et al. 2017). Blood was collected via paracentesis of the uropatagial vein using capillaries with heparin. *P. discolor* (n=15) and the *R. aegypticus* (n=20) were from captive colonies. *P. discolor* belonged to an experimental colony held at the

Ludwig-Maximilian-Universität (LMU) Munich, Germany and *R. aegypticus* to a colony in the Copenhagen Zoo, Denmark. All animals were euthanasized for population management purposes by the responsible veterinarians of the institutions, following the animal welfare legislation of the respective countries. For both species, blood samples were collected via cardiocentesis with heparin washed syringes after euthanasia.

Sample preparation

Frozen aliquots were thawed in a 37°C warm water bath and centrifuged at 188 x G. The supernatant was removed and the cell pellet was resuspended in phosphate-buffered saline (PBS) containing 5% fetal bovine serum (FBS; Sigma Aldrich, St. Louis, USA) followed by two washing steps with 5% FBS/PBS and a centrifugation speed of 188 x G. Cells were counted using a Neubauer Counting Chamber and adjusted to a final cell concentration of 5×10^5 cells/mL for fluorescence staining.

Antibodies and staining

A total of 19 monoclonal antibodies (mAbs) (Table 1) against different human and murine lymphocyte CD-markers were donated for the study by AbD Serotec (Raleigh, USA), Miltenyi Biotec (Bergisch Gladbach, Germany) or were used in different experiment of the Deutsches Rheuma-Forschungszentrum Berlin (DRFZ). Based on the type of mAbs a direct or indirect staining procedure was performed with the recommended concentrations. Directly labeled mAbs were incubated with the cell suspension for 45 min at 4°C and washed twice with 5% FBS/PBS. Unconjugated mAbs (Isotypes: mouse IgG1, mouse IgG3, rat IgG1) were incubated with the cell suspension accordingly, washed twice and then incubated with FITC conjugated secondary goat IgG (Thermo Fisher, Waltham, USA) for 45 min at 4°C and washed twice with 5% FBS/PBS. Afterwards all samples were resuspended in 1% FBS/PBS and flow cytometry analysis was performed. A control staining was performed with the anti-human antibodies using the same protocol. Human blood was sampled from the vein using heparin washed tubes, fixated with FACS Lysing Solution, frozen in liquid nitrogen and stored at -80°C until analysis. No control staining was performed with anti-murine antibodies.

Flow Cytometry

Data acquisition of each sample was performed on a FACScalibur (BD BioSciences, San Jose, USA) and analyzed using BD CellQuest™ Pro (BD BioSciences, San Jose, USA). Based on their characteristic scatter profile of size (forward scatter, FSC) and cellular complexity (side scatter, SSC) isolated cells were gated for lymphocytic cell populations and

a minimum of 10,000 events in the chosen gate were counted. Gated cell events were then analyzed for cross reaction by fluorescence signals in histogram plots.

Results and Discussion

Although several bat species are commonly kept under laboratory conditions and in zoological collections, for most field studies it is logistically not possible to perform cell sorting and staining on fresh blood bat samples. We used a protocol for fixation of blood samples in wild animal species (Lau et al. 2012) and were able to isolate the peripheral lymphocyte blood cell population from three chiropteran species. Determination and gating of the lymphocyte population was possible by a characteristic flow cytometric scatter profile in the region FSC-Height 150-500 and SSC-Height 20-200 in all three bat species (Figure 1). Lymphocytes were clearly detected in samples from all three bat species. Monocyte and granulocyte populations were visible in *P. discolor* isolates. However all samples had large amount of cell debris presumably caused by the sample fixation, indicating a possible limitation for detection of other immune cell types. Further storage method development is clearly needed for field based studies of wildlife.

We tested 19 monoclonal antibodies for possible cross-reaction with different bat lymphocytes (Table 1). We defined a 5% threshold in the gated lymphocytic cell population as a positive cross-reaction. Thirteen of 19 tested antibodies failed to stain bat cells specifically. Six antibodies specifically stained cells from at least one of the three bat species. Five of the tested antibodies reacted with cells from *M. myotis* whereas *P.discolor* and *R. aegyptiacus* reacted with one antibody each (Table 2). The low cross-reaction success rate is consistent with observations from other wildlife species (Heinrich et al. 2015; Periasamy et al. 2019). Despite the low cross reactivity success rates, even with few cross-reacting antibodies functional and phenotypical characterization of major T cell subsets, B and NK cells in fruit bats can be performed (Gómez et al. 2016, Periasamy et al. 2019). It can therefore be worthwhile to identify the few cross reacting antibodies in wildlife.

For *M. myotis* positive cross-reactivity in the gated lymphocyte population could be observed with T cell markers anti-human CD3/clone UCHT1 (43.29 %) and anti-human CD4/clone TT1 (7.39%). B cell marker anti-murine CD19/clone 1D3 (35.57%), anti-murine CD19/clone 6D5 (6.48%) and anti-human CD79a/clone HM57 (5.52%) cross reacted in the same species. In *P. discolor* anti-human CD4/clone TT1 (8.14%) and in *R. aegyptiacus* anti-

murine marker CD79b/clone AT107-2 (10.66%) showed a positive reaction in the gated lymphocytes.

The used antibodies showing a positive cross-reaction with bat lymphocytes are commonly used in immunological research in model species and specific against important immune cell markers. The multi-polypeptide membrane receptor CD3-T cell complex (TCR) is located on the surface of all T lymphocytes and essential for T cell activation in the adaptive immune response (Kalergis et al. 2001). As a co-receptor of the TCR, CD4 is found mainly on T-helper cells which are important for the regulation of adaptive immunity (Zhu & Paul 2008). The transmembrane protein CD19 present on all B lymphocytes plays a major role in the signaling pathways of B cells during B cell development (Fujimoto et al. 1999)**.** CD79 is a transmembrane protein found on the B cell surface and involved in the cellular signal transduction during antigen recognition (Chu & Arber 2001). Therefore these cross-reacting antibodies are potential for characterization of B- and T-cells in bats. Taking into account the taxonomic depth of our study and the importance of these species in eco-evolutionary and medical studies, our results provide further tools for studying the adaptive immunity of these different bat species.

Understanding of bat adaptive immunity in terms of activation and composition is hindered by the lack of specific antibodies. Although generating specific antibodies is the best option, it is time and resource demanding (Periasamy et al. 2019) and hampered by lack of species specific reagents. For example, species specific antibodies can be generated using single B cell antibody technology which is based on cloning and expression of Ig genes from single B cells sorted by flow cytometry (Tiller *et al.* 2008, 2009; Wardemann *et al.* 2003). Since this method uses single B cells as a basis for antibody generation, it also allows the characterization of the whole antibody repertoire of an individual. In the future this method could be applied to bats which could further define the development of acquired immunity in bats. Additionally, identifying cross-reactive antibodies in combination with computational design strategies improves the affinity of antibodies (Farady et al. 2009) and could be a future perspective approach in wildlife immunology. Until simple methods for the generation of species specific antibodies are established, research on bat adaptive immunity will be limited to the utilization of classic hematological and immunological methods using the limited number of cross-reacting antibodies available. Given their medical and ecological importance, identifying further cross reacting antibodies is warranted.

Acknowledgments

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Figure 1. Scatter profile of size (SSC) and cellular complexity (FSC) of isolated PBMCs from fixed blood in three different bat species. Representative images display the gated lymphocyte population (FSC 150-500, SSC 20-200) in the overall profile of isolated cells from *Myotis myotis* (A), *Phyllostomus discolor* (B), and *Rousettus aegyptiacus* (C).

A   B   C
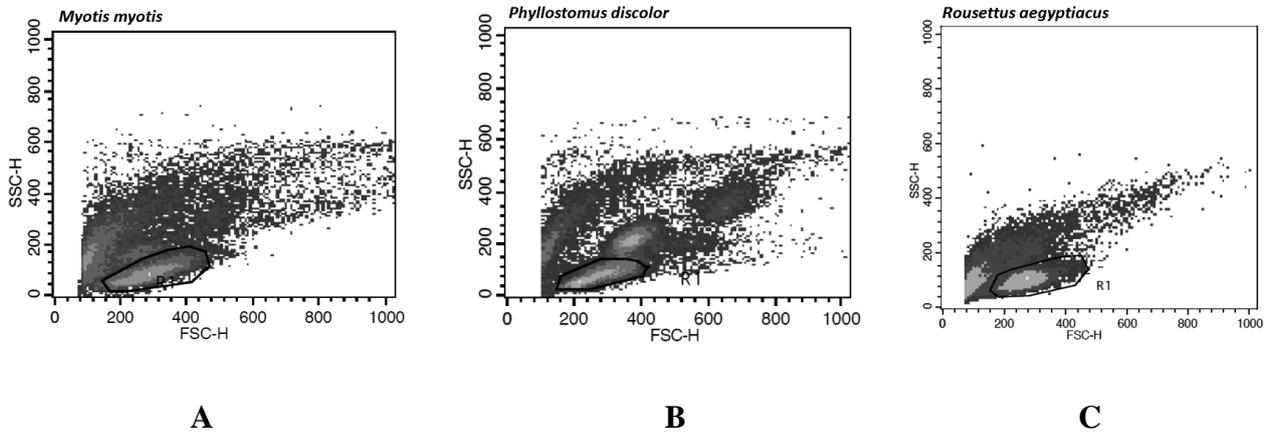
Table 1. Overview of the tested antibodies used in flow-cytometric analysis in bat blood-derived lymphocytes.

Target cell	Antigen	Clone	Isotype	Specifity	Flourochrome	Source
	CD3	17A2	rat IgG2b	mouse	FITC	Miltenyi
T cells	CD3	OKT3	mouse IgG2a	human	FITC	DRFZ
	CD3	UCHT-1	mouse IgG1	human	FITC	DRFZ
	CD3	145-2C11	hamster IgG1	mouse	FITC	DRFZ
T helper cells	CD4	GK1.5	rat IgG2b	mouse	FITC	DRFZ
	CD4	TT1	mouse IgG1	human	unlabelled	DRFZ
T cells	CD5	19_3	-	mouse	Cy5	DRFZ
	CD5	53-7.3	rat IgG2a	mouse	FITC	Miltenyi
Cytotoxic T cells	CD8	53-6.72	rat Ig G2a	mouse	Cy5	DRFZ
	CD8	GN11/134D7	mouse IgG1	human	Cy5	DRFZ
	CD19	1D3	rat IgG2a	mouse	A488	DRFZ
	CD19	6D5	rat IgG2a	mouse	FITC	Miltenyi
B cells	CD19	BU12	mouse IgG1	human	unlabelled	DRFZ
	CD19	LT19	mouse IgG1	human	FITC	Miltenyi
	CD19	N6B1	mouse IgG3	human	unlabelled	DRFZ
Leukocytes	CD45	30F11.1	rat IgG2b	mouse	FITC	Miltenyi
	CD45	5B1	mouse IgG2a	human	FITC	Miltenyi
B cells	CD79a	HM57	mouse IgG1	mouse	unlabelled	AbDSerotec
	CD79b	AT107-2	rat IgG1	mouse	unlabelled	AbDSerotec

Table 2. Cross-reactivity results with lymphocytes subsets of bat species
***M. myotis*, *P. discolor*, and *R. aegyptiacus*.** A minimum of 10.000 events were counted in the gated lymphocyte region of each species and a 5% positive threshold of labeling was defined. Results below the threshold are displayed as "-". Values above the threshold are displayed as a percentage. Control staining values of anti-human antibodies with human blood are shown. Percentage of human lymphocyte staining lays in the expected values for each anti-human marker. A murine staining control was not performed.

Antigen	Clone	*M. myotis*	*P. discolor*	*R.aegyptiacus*	Human CTR
CD3	17A2	-	-	-	/
CD3	OKT3	-	-	-	73.18%
CD3	UCHT-1	43.29%	-	-	76.27%
CD3	145-2C11	-	-	-	/
CD4	GK1.5	-	-	-	/
CD4	TT1	7.39%	8.14%	-	36.16%
CD5	19_3	-	-	-	/
CD5	53-7.3	-	-	-	/
CD8	53-6.72	-	-	-	/
CD8	GN11/134	-	-	-	39.55%
CD19	1D3	35.57%	-	-	/
CD19	6D5	6.48%	-	-	/
CD19	BU12	-	-	-	10.88%
CD19	LT19	-	-	-	8.63%
CD19	N6B1	-	-	-	32.27%
CD45	30F11.1	-	-	-	/
CD45	5B1	-	-	-	95.95%
CD79a	HM57	5.52%	-	-	10.88%
CD79b	AT107-2	-	-	10.66%	/

CHAPTER 5

General Discussion

CHAPTER 5 – General Discussion

Immunological research under classical medical aspects focuses on understanding the mechanisms of the immune system, the host's responses and interaction with pathogens during infection. Eco-immunology expands this focus on understanding the intra- and interspecific variation of immune defenses under the influence of evolutionary and ecological factors, focusing mainly on free-living, non-model organisms (Martin et al. 2011; Schulenburg et al. 2009). Methods commonly used in this field are functional assays and simple experimental approaches allowing only singular immune measurements (Demas et al. 2011; Graham et al. 2011). Although recent development of high-throughput techniques advanced research on systemic interactions within the immune system and with other physiological systems (Calis & Rosenberg 2014; Flies & Wood 2019) knowledge about development and processes of adaptive immunity is limited in wild animals. Therefore the lack of suitable techniques and specific tools for non-model organisms remains a sustaining challenge in the field. Moreover, many of the assays used focus on specific molecules (e.g. total antibody levels), while overall molecular mechanisms or smaller molecules remain understudied.

The main objective of this thesis was the advancement of immunological research in wildlife populations focusing on the methodological transfer and establishment of common used laboratory techniques in bats. Due to their high species diversity and the eco-physiological characteristics of bats, this group has a great potential for eco-immunological research. Bats is an intensively studied group in ecophysiology, including hibernation phenology, energetics, movement and vocalization, however recently they become an important target for medical research, especially discovering novel viruses with zoonotic potential. Bats are known to harbor several virulent viruses without any clinical symptoms (Baker et al. 2013). Accordingly, in the last decades several groups have focused on the chiropteran immunity, to better understand how these species tolerate and resist these pathogens. In parallel, bats have been screened for several non-viral pathogens and it is apparent that they harbor several other parasite groups (e.g. Bacteria, Protozoa, Fungi) (Brook & Dobsen 2015). One of these pathogens is *Pseudogymnoascus destructans*, a fungus which causes unprecedented mortality and population declines in North American cave-hibernating bat species. Species-differences in pathogen susceptibility and the factors affecting the variance of immune response is a field of interest for both animal and public health and conservation biology. Most of the current knowledge on bat immunity focus on innate antiviral defenses which show peculiarities in the production of interferons (Zhou et al. 2011)

and suppression on inflammatory molecules (Banerjee et al. 2017). However, the in depth understanding of bat immunity is still in its infancy (Baker and Schountz 2018) and is exemplary for the limitations of wildlife immunology through lack of tools and specific reagents.

In **Chapter 2** I was able to demonstrate that a common used method in disease diagnostics of humans (e.g. cancer, cardiovascular disorders) the blood plasma proteomic analyses (Geyer et al. 2017) can be applied to healthy bats. A main advantage of this technique is that even small volumes of plasma are sufficient for precise examinations (Issaq et al. 2007). Besides the lack of tools, immunological studies under field conditions are also limited by the quantity and quality of samples collected, the storage conditions, and thus the focus of many studies are determined by all these aspects (Boughton et al. 2011; Demas & Nelson 2012). Accordingly, most of the eco-immunological studies focus on humoral immune aspects, with plasma and serum being the most common used sample types (Moore et al. 2011; Schneeberger et al. 2013, 2013b). Before understanding the hots' immune response to certain pathogens, description of the immunity of healthy individuals and its variation under various physiological stages (e.g. reproduction, migration, hibernation) is essential. The analysis of the blood plasma proteome was performed in the European hibernating bat species *M. myotis* comparing the active and torpid state of hibernation. The energy-conserving behavior of hibernation is associated with modulated metabolic pathways and immunosuppression in mammalian species (Bouma et al. 2010). For the first time in bats I was able to show that a modulated physiology during hibernation will manifest in the plasma proteomic profile. I found an altered expression of proteins involved in innate immunity, blood coagulation and metabolic pathways between the active and torpid state. My findings on the differential expression of proteins involved in immunity and metabolism suggested a suppression of these pathways as known from other mammalian hibernators (Bouma et al. 2010). However I found unexpected expression differences for proteins involved in blood coagulation contradictory to other mammals (Srere et al. 1995) but were supported by transcriptomic findings in a different bat species (Seim et al. 2013) indicating possible differences in the coagulation cascade of bats. An additional basic characterization of the proteomic plasma profile identified proteins involved in the complement system, coagulation and transport of different molecules.

Proteomic investigations have the capability to identify and specify single components from a complex composition of proteins from different biological sources. Blood plasma in particular is rich in various proteins and used increasingly in veterinary studies of livestock

(Almeida et al. 2015) and recently in few wildlife species (Bertile et al. 2016; Chow et al. 2013). The reflection of physiological and pathological conditions in differential protein expression allows the identification of biomarkers for animal disease pathogenesis and diagnostics (Ceciliani et al. 2014). Thus the establishment of plasma proteome analyses in a bat species gives the potential to characterize proteins and peptides associated with the presence of a disease or infection.

On this basis, in **Chapter 3** I used plasma proteomics for investigating immunological and pathological differences between susceptible and resistant Myotis species to *Pd* colonization. *Pd* is a fungi species responsible to white-nose syndrome, a disease specific to hibernating bats (Blehert et al. 2009). It caused mass mortalities and population declines in North America whereas European populations seem to be unaffected (Lorch et al. 2009; Wibbelt et al. 2010). In this chapter, I compared healthy and diseased individuals in species from both continents. For the North American species, I studied *M. lucifugus*, which is the most affected species and my comparison revealed a differential expression of plasma proteins associated with acute phase response, constitutive innate immunity, immune gene products, oxidative stress control, metabolism, exosomes and desmosomes in diseased individuals compared to the healthy ones. Similar processes were described on the transcriptome levels in the wing membrane (Frick et al. 2015). My findings suggest that a systemic immune response towards *Pd* is also reflected in the plasma proteome. As identified complement and acute phase proteins are recognized as biomarkers of pathological changes in veterinary medicine (Eckersal & Bell 2010; Kuleš et al. 2014) a future perspective in research on WNS towards the understanding of exaggerated inflammatory responses (Meteyer et al. 2012) and physiological disturbances (Verant et al. 2014) in response to *Pd* can be emphasized. In contrast, the European species *M. myotis* showed no differential plasma protein regulation among the two states. My novel findings on the plasma level are supportive to other comparative findings on the transcriptome level of immune genes in *M. myotis* (Davy et al. 2017). Due to missing mass mortalities and a general mild clinical outcome in WNS affected European bats (Wibbelt et al. 2010) an evolved tolerance against *Pd is* suggested (Zukal et al. 2016).

Using a gel-based 2D-DIGE proteomic technique I was able to identify differential protein expression in response to a physiological state and disease for the first time in bats. Gel-based proteomics are an economical approach for the separation and detection of expression changes in complex protein samples (Van den Bergh & Arckens 2004). Further the observation of post-translational modifications also in response to disease (Heegaard et al.

2013) is an important advantage of this technique. However, several limitations have to be considered such as under-representation of different protein classes (e.g. hydrophobic proteins) and low abundant proteins (Garbis et al. 2005). A particular challenge is the presence of high abundant proteins, which complicate the identification of other relevant proteins in smaller concentrations. Even protein identification via mass spectromic analyses is possible the observation of differential regulation might be influenced (Garbis et al. 2005). Addressing this issue the depletion of different protein fractions e.g. albumin is possible but also has own limitations as not only targeted proteins will be removed (Chromy et al. 2004). Independent from these challenges gel-free proteomic shotgun approaches were developed recently (Zhang et al. 2007). These will allow simultaneous broad discovery of proteins by mass-spectromic screening in a biological sample. Though rely on complete protein databases to avoid false identifications hindering an extensive use in animal science (Ghodasara et al. 2017). The increasing effort on the identification of genomes and proteomes especially in non-model species (e.g. Comparative Mammalian Proteome Aggregator Resource – CoMPARe- Program) will give future perspectives for the use of gel-free approaches in eco-immunological studies.

The two previous chapters primarily focused on a methodology that would allow systematic investigations of immune responses in bats. In order to gain further knowledge about the general composition of the bat immune system and how innate and adaptive immunity interact other methodological approaches are required. Most of the immune studies on bats investigate mainly the functional and structural aspects of innate and only partially of the adaptive immunity (Baker et al. 2013, 2018). Moreover, due to field conditions, these studies focus on humoral effectors, while cellular immunity is quantified using classic hematological methods. A more detailed research on the functional outcome of adaptive immunity and its activation is limited by available tools, especially antibodies. As a first step to resolve this issue I performed in **Chapter 4** a flow cytometric cross-reactivity study with human and murine monoclonal antibodies in blood lymphocyte populations of three different bat species, *M. myotis*, *P. discolor* and *R. aegyptiacus*. Of 19 tested antibodies I only observed a positive cross-reaction for 5 antibodies in *M. myotis* and only for 1 antibody in *P. discolor* and *R. aegyptiacus*. Cross-reaction was shown for general B and T-cell marker. Although with a small number of known cross-reactive antibodies for bat species a characterization of adaptive immune cell populations is possible (Periasamy et al. 2019). Thus the use of the identified cross-reactive antibodies might have a potential in the further characterization of adaptive immunity in bats.

A future perspective on improving the availability of specific agents is the generation of monoclonal antibodies against different bat species. Besides the classical technique for generation of antibodies through hybridomas (Shirahata et al. 1998) also alternative methodological approaches are available. For example the Single B cell antibody technology which is using cloning and expression of Ig genes from single B cells (Tiller et al. 2008, 2009; Wardemann et al. 2003). Hereby the basic requirement is the availability of single B cells which can be achieved by flow cytometric cell sorting. The identification of cross-reactive B cell antibodies can be used as a foundation for subsequent steps. In contrast the phage display antibody generation (Hudson & Souriau, 2003) is based on the expression of cell specific cDNA in phages and an affinity based purification of binding antigens or antibodies. This method would allow the generation on non-characterized proteins based on cDNA sequences. Amplification of cDNA for genes of interests will be facilitated by the further identification and annotation of different bat genomes (Teeling et al. 2018).

Since its emergence in the mid 90's, the field of eco-immunology experienced a steep diversification in taxonomic depth, factors influencing the immune variance and available methodologies. While the first studies predominantly used one measurement to characterize the "immunocompetence" of an individual or species, recently the complexity of the immune system and within interactions is also considered. Moreover, it is recognized, that in order to fully understand the immune system, information on all organizational levels, from molecules to cells, from individuals to species is essential (Downs et al. 2014). Despite eco-immunology is not a novel field of biology anymore, due to the above mentioned complexities the development of novel methods is still a major topic. Dealing with such complexity, clearly also comes with limitations, however, as showed by the different approaches in my thesis, application of state-of-art and modern methodologies developed for model organisms can provide valuable insights in wildlife species. The use of these methods, not only opens several new, interesting interdisciplinary avenues, but has clear benefits for animal health and conservation biology.

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Summary

Classical immunological research investigates immune defense mechanisms in a controlled environment with a narrow genetic diversity and high reproducibility. Aiming the understanding of host immune variations and disease susceptibility in the broad framework of evolution, ecology and life-history, the field of eco-immunology is expanding the focus under the influence of high genetic diversity and abiotic and biotic factors in wild animals. Moreover, immunological research in non-model species is facing methodological challenges by lack of reagents and annotated genomes. Limited by experimental approaches that can easily be used under field conditions eco-immunological research focus mainly on innate and only partially on adaptive immunity. Despite the recent availability of high-throughput technologies, little is known about the development and processes of acquired immunity and how interactions with innate immune cells affect systemic immune responses. In my thesis I focused these issues using chiropteran species which represent an ideal model for eco-immunology due to their high diversity, physiological peculiarities and their prominent role as natural reservoir for pathological viruses.

In Chapter 2 I used gel based plasma proteomics, a common methodology of human medicine for the investigation of physiological and immunological changes in healthy bats. I compared the protein expression of active and hibernating *Myotis myotis (M. myotis)* hypothesizing that modulation of physiological pathways and suppression of immunological functions during hibernation will manifest in the plasma. For the first time in bats I could show that proteins involved in immunity, coagulation and metabolism are differently regulated during hibernation.

The establishment of plasma proteomics in bats enabled in Chapter 3 the investigation of susceptible and resistant Myotid species during white-nose syndrome (WNS). The disease associated with the fungus *Pseudogymnascus destructans* (*Pd*) is responsible for population declines and mass mortalities in North American bat species whereas European species are only mildly affected. I assumed that physiological and pathological conditions during infection with *Pd* will reflect in the composition of blood plasma proteins and compared the expression profile of healthy and diseased animals in North American *Myotis lucifugus* and European *M. myotis*. No significant differences were found in *M. myotis* in agreement with the hypothesis that European bat species have evolved tolerance mechanisms against the fungus as its origin lays in Europe. In contrast *Myotis lucifugus* exhibit expression differences of proteins involved in acute phase response, immunity, oxidative stress, metabolism,

exosomes and desmosomes suggesting a systemic response towards *Pd*. My novel findings on the protein level are consistent with studies on the transcriptomic level that emphasize a recent introduction of the fungus to North America where bats presumably couldn´t develop a specific immune response.

With my findings from Chapter 2 and 3 I could show that gel-based proteomics are applicable for the investigation of physiological states and diseases in bats. This technique allows a systemic investigation of immunity by using small sample volumes. In order to investigate adaptive immune responses antibodies against immune cells are crucial. As for bat species only a handful of antibodies are available I performed in Chapter 4 a flow cytometric cross-reaction study with human and murine antibodies in three different bat species. I isolated the blood lymphocyte population from the distinct species *M. myotis*, *Phyllostomus discolor* and *Rousettus aegyptiacus* and tested antibodies specific for major B- and T-lymphocyte marker. Of 19 tested antibodies I could only observe cross-reaction for a total of 6 antibodies, most of the reacting with *M. myotis* lymphocytes. Although small numbers of available antibodies would allow a characterization of adaptive immune cells there is an urgent need for the generation of specific antibodies for bat species.

In conclusion this thesis could show that the used approaches could overcome methodological limitations in bat immunology and eco-immunology in general. The application of state-of-the-art techniques originally developed for model organisms may in the future enable more detailed integrative studies on immune variation through the use in wildlife species.

Zusammenfassung

Die klassische immunologische Forschung untersucht die Abwehrmechanismen des Immunsystems in einer kontrollierten Umgebung mit enger genetischer Vielfalt bei hoher Reproduzierbarkeit. Mit dem Ziel Variationen des Immunsystems und Krankheitsanfälligkeit im weiten Rahmen der Evolution, Ökologie und Lebensgeschichte zu verstehen, erweitert der Bereich der Öko-Immunologie den Fokus unter dem Einfluss hoher genetischer Vielfalt und abiotischen und biotischen Faktoren bei Wildtieren. Darüber hinaus steht die immunologische Forschung an Nicht-Modellarten vor methodischen Herausforderungen durch den Mangel an molekularen Werkzeugen und bekannten Genomen. Begrenzt durch experimentelle Ansätze, die unter Feldbedingungen leicht anwendbar sind, konzentriert sich die öko-immunologische Forschung hauptsächlich auf die angeborene und nur teilweise auf die adaptive Immunantwort. Trotz der jüngsten Verfügbarkeit von Hochdurchsatztechnologien ist nur wenig über die Entwicklung und Prozesse der erworbenen Immunität bekannt und wie Wechselwirkungen mit angeborenen Immunzellen systemischen Immunantworten beeinflussen. In meiner Dissertation beschäftigte ich mich mit diesen Herausforderungen unter der Verwendung von Fledermaus-Arten, die aufgrund ihrer hohen Vielfalt, ihren physiologischen Besonderheiten und ihrer herausragenden Rolle als natürliches Reservoir für pathologische Viren ein ideales Modell für die Öko-Immunologie darstellen.

In Kapitel 2 benutzte ich die gel-basierte Proteomik von Plasma, eine verbreitete Methodik der Humanmedizin, zur Untersuchung physiologischer und immunologischer Veränderungen bei gesunden Fledermäusen. Ich verglich die Expression von Plasma Proteinen bei aktiven und im Winterschlaf befindlichen großen Mausohren, *Myotis myotis* (*M. myotis*). Dabei ging ich davon aus, dass sich die Veränderung physiologischer Prozesse und die Unterdrückung immunologischer Funktionen während des Winterschlafs im Plasma manifestieren. Zum ersten Mal konnte ich bei Fledermäusen zeigen, dass Proteine, die an Immunität, Koagulation und Stoffwechsel beteiligt sind, im Winterschlaf unterschiedlich reguliert werden.

Die Etablierung der Plasma-Proteomik bei Fledermäusen ermöglichte es mir in Kapitel 3 empfindliche und resistente Myotid-Arten während des Weißnasen-Syndroms (WNS) zu untersuchen. Die mit dem Pilz *Pseudogymnascus destructans* (*Pd*) verbundene Krankheit ist für den Bevölkerungsrückgang und die Massensterblichkeit bei nordamerikanischen Fledermausarten verantwortlich, während europäische Arten nur geringfügig betroffen sind. Ich nahm an, dass sich physiologische und pathologische Besonderheiten während der

Infektion mit *Pd* in der Zusammensetzung der Blutplasmaproteine widerspiegeln und verglich dazu das Expressionsprofil von gesunden und kranken Tieren bei der nordamerikanischen Art *Myotis lucifugus* und der europäischen Art *M. myotis*. In Übereinstimmung mit der Hypothese, dass die europäischen Fledermausarten Toleranzmechanismus gegenüber dem Pilz entwickelt haben, da er seinen Ursprung in Europa hat, wurden keine signifikanten Unterschiede bei *M. myotis* festgestellt. Im Gegensatz dazu wurden in *Myotis lucifugus* Expressionsunterschiede von Proteinen beobachtet, welchen an Immunmechanismen, oxidativem Stress, Stoffwechsel beteiligt sind. Desweitern wurden Proteine von Desmosomen und Exosomen identifiziert. Dies lässt auf eine systemische Reaktion gegenüber *Pd* schließen. Diese neuen Ergebnisse auf Proteinebene stehen im Einklang mit Studien auf transkriptomischer Ebene, die eine kürzlich erfolgte Einführung des Pilzes in Nordamerika hervorheben, wo Fledermäuse höchstwahrscheinlich keine spezifische Immunantwort entwickeln konnten.

Mit meinen Erkenntnissen aus den Kapiteln 2 und 3 konnte ich zeigen, dass gelbasierte Proteomik für die Untersuchung von physiologischen Zuständen und Krankheiten bei Fledermäusen geeignet ist. Diese Technik ermöglicht eine systemische Untersuchung der Immunität mit kleinen Probenvolumina. Um adaptive Immunantworten zu untersuchen, sind Antikörper gegen Immunzellen unerlässlich. Da für Fledermausarten nur eine Handvoll Antikörper verfügbar sind, habe ich in Kapitel 4 eine durchflusszytometrische Kreuzreaktionsstudie mit menschlichen und murinen Antikörpern in drei verschiedenen Fledermausarten durchgeführt. Ich isolierte die Blutlymphozytenpopulation von den verschiedenen Spezies *M. myotis*, *Phyllostomus discolor* und *Rousettus aegyptiacus* und testete Antikörper, die spezifisch für die wichtigsten B- und T-Lymphozytenmarker sind. Von 19 getesteten Antikörpern konnte ich nur bei insgesamt 6 Antikörpern Kreuzreaktionen beobachten, wobei die meisten mit Lymphozyten von *M. myotis* reagierten. Obwohl eine geringe Anzahl verfügbarer Antikörper eine Charakterisierung adaptiver Immunzellen ermöglichen würde, ist es dringend notwendig, spezifische Antikörper für Fledermausarten zu entwickeln.

Abschließend konnte diese Arbeit zeigen, dass die verwendeten Ansätze die methodischen Einschränkungen in der Fledermausimmunologie und der Ökoimmunologie im Allgemeinen überwinden konnten. Die Anwendung modernster Techniken, die ursprünglich für Modellorganismen entwickelt wurden, können in der Zukunft durch den Einsatz bei Wildtieren detailliertere integrative Studien in Bezug auf Immunvariationen ermöglichen.

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Selbständigkeitserklärung

Hiermit versichere ich, dass ich die vorliegende Doktorarbeit eigenständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Berlin, 29.04.2019

Alexander Hecht-Höger