Immunological and physiological responses of hibernating bats to fungal infections

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Summary

White-nose syndrome, or white-nose disease, is a fungal disease that is fatal to North American bats. The pathogen, a psychrophilic fungus called *Pseudogymnoascus destructans*, infects bats during hibernation. It grows on the animals while they are in torpor - a state in which all physiological functions including the immune system are downregulated. The fungus infests all hairless body parts and invades deep into the skin layers where it causes great damage. It has been observed that infected bats arouse more often from torpor than normal in order to clean off the fungus and elicit an immune response. Due to the increased energy consumption resulting from the multiple arousals and the attempts to mount an immune response, the animals prematurely deplete their fat reserves, which are essential for survival during hibernation, ultimately leading to death. Since the 2006 outbreak near New York, the fungus has spread across half of the North American continent and several million bats have died.

The pathogen fungus *P. destructans* was also discovered on bats in Europe and Asia after the disease became known in North America. However, no increased mortality in relation to the fungus has been observed in Europe so far, and ring recaptures proved that individuals survive the fungal infections even over several winter periods. Molecular genetic analyses have shown that the *P. destructans* is native to Europe and has been carried to North America, under unknown circumstances. Historical data sources have also shown that the fungus has been present on bats in Europe for at least 100 years. Therefore, it is reasonable to assume that European bats have already evolved defences to the fungal disease, while American bats still lack this adaptation.

To shed light on the question what mechanisms enable European bats to survive *P. destructans* infections, the present work investigated physiological and immunological responses occurring in fungus-infected European hibernating bats. For this purpose, I studied greater mouse-eared bats (*Myotis myotis*) in laboratory and field experiments, as this species is most frequently infected with *P. destructans* in Europe.

Since previous attempts failed to effectively infect greater mouse-eared bats with *P. destructans* in the laboratory, I mimicked a fungal infection under laboratory conditions in a

first experiment (Chapter 1) by subcutaneously inoculating zymosan into the bats. This substance is often used as a fungal antigen in laboratory experiments to elicit antifungal immune responses. Skin temperature recordings from data loggers attached to the bats revealed that the animals did not respond to zymosan with fever or with more frequent arousals, and blood levels did not show excessive immune responses - contrasting to American bats which are infected with *P. destructans*. However, I found that although the animals treated with zymosan did not react with an adequate immune response as would be expected outside of the hibernation period (e.g., change in white blood cell concentration and composition), they did only respond with an increase in haptoglobin, an acute-phase protein that is secreted during inflammatory responses and is effective against microbial pathogens. From the results, I concluded that there seems to be a hibernation-adapted immune response in great mouse-eared bats that allows them to limit the negative effects of infection with pathogens such as *P. destructans* without completely eliminating the pathogen to conserve energy (tolerance response). This moderate immune response apparently occurs during regular arousals without change in their duration or frequency. This reaction could be a general adaptation for the hibernation period, whereas a full immune response and recovery does not occur until the following spring, when sufficient food or energy resources are available.

To perform immunological studies on bats which are actually infected with *P. destructans*, I conducted a field experiment (Chapter 2). Thus, I first placed temperature loggers on wild hibernating greater mouse-eared bats before any visible fungal infections could be observed. After approximately one month, I revisited the hibernacula to determine disease symptoms of the bats, retrieved the temperature loggers, and collected blood samples. Based on the cutaneous temperature recordings and the documented disease symptoms, I was able to show that the greater mouse-eared bats continued their hibernation despite fungal infections and also did not show fever symptoms, as is the case with North American bats. In addition, I found that body weight, an indicator of stored energy in the form of fat, has a decisive influence on how often the animals can arouse from torpor during hibernation period. The heavier the animals, the more frequently they can afford the energy-consuming arousals during which they also reduce the fungus. Interestingly, we found signs of an inflammatory response in the blood of torpid severely infected animals. In doing so, they use baseline concentrations of some immune parameters present in the blood (including haptoglobin)

without synthesizing new immunological substances. I assume that the use of baseline concentrations in response to the infection is a strategy to avoid additional arousals which are presumably necessary for protein synthesis since most immune functions are downregulated during torpor. In conclusion, these results also suggest immunological tolerance of European bats to the pathogen *P. destructans* during hibernation. Additionally, I found tendencies of increased oxidative stress in animals that exhibited severe symptoms of the fungal infection. This result indicates that there is still a need for research on the ultimate cost of white-nose disease in European bats.

As a helpful tool for further white-nose disease studies, I developed a non-invasive method (Chapter 3) to assess white-nose disease symptoms on hibernating bats without the need to disturb the animals, as it was the case for previous standard methods. This assessment system, which uses visual classification of the fungal infestation to determine the severity of the disease, was compared and validated against the other invasive standard methods used to date. With the help of this method it is henceforth possible to determine white-nose disease including its severity in torpid animals and due to its non-invasiveness it is even possible to do this with the help of many other bat researchers and conservationists on a large scale and in a standardized way during routine winter censuses, as it is requested on international level.

Zusammenfassung

Das Weißnasen-Syndrom oder auch Weißnasenkrankheit genannt, ist eine für nordamerikanische Fledermäuse tödliche Pilzkrankheit. Der Erreger, ein psychrophiler Pilz namens *Pseudogymnoascus destructans*, befällt Fledermäuse während der Überwinterung. Er wächst auf den Tieren solange sie im Torpor sind - ein Zustand, in dem alle physiologische Funktionen inklusive des Immunsystems weitgehend heruntergefahren sind. Er befällt alle unbehaarten Körperteile und dringt tief in die Hautschichten ein, wo er große Schäden verursacht. Es wurde beobachtet, dass befallene Fledermäuse öfter als normal aus dem Winterschlaf erwachen, um während dieser Wachphasen sich den Pilz von der Haut zu putzen und eine Immunreaktion hervorzurufen. Durch den erhöhten Energieverbrauch infolge der häufigen Aufwachphasen und das hochfahren des Immunsystems verbrennen die Tiere vorzeitig ihre Fettreserven, die für das Überleben während des Winterschlafes essentiell sind,

was letztendlich zum Tode führt. Seit dem Ausbruch der Krankheit im Jahr 2006 in der Nähe von New York hat sich der Pilz über den halben nordamerikanischen Kontinent ausgebreitet und es sind mehrere Millionen Fledermäuse gestorben.

Der pathogene Pilz *P. destructans* wurde nach dem Bekanntwerden der Krankheit in Nordamerika auch auf Fledermäusen in Europa und Asien entdeckt. Allerdings wurde hier bislang keine erhöhte Mortalität durch den Pilz verursacht und Ring-Wiederfunde belegten, dass die Individuen die Pilz-Infektionen selbst über mehrere Winterperioden überleben. Molekular-genetische Analysen haben gezeigt, dass der Pilz in Europa heimisch ist und auf ungeklärtem Wege nach Nordamerika verschleppt worden ist. Auch historische Datenquellen haben gezeigt, dass der Pilz schon seit mindestens 100 Jahren auf Fledermäusen in Europa vorkommt. Deshalb liegt die Annahme nahe, dass Europäische Fledermäuse bereits an die Pilzkrankheit angepasst sind, während den amerikanischen Fledermäusen diese Anpassung noch fehlt.

Um zu untersuchen, welche Anpassungen es europäischen Fledermäusen ermöglicht, die Infektionen mit *P. destructans* zu überleben, wurde in der vorliegenden Arbeit untersucht, welche physiologischen und immunologischen Reaktionen bei pilzbefallenen winterschlafenden europäischen Fledermäusen auftreten. Hierfür habe ich Große Mausohren (*Myotis myotis*) in Labor- und Freilandexperimenten untersucht, da diese Art in Europa am Häufigsten mit *P. destructans* befallen wird.

Da es bislang nicht gelungen ist, Große Mausohren im Labor effektiv mit *P. destructans* zu infizieren, habe ich in einem ersten Experiment unter Laborbedingungen eine Pilzinfektion simuliert (Kapitel 1), in dem ich den Großen Mausohren subkutan Zymosan injiziert habe. Diese Substanz ist ein pilzliches Antigen, welche in Laborversuchen häufig als genutzt wird um antifungale Immunreaktionen auszulösen. Temperaturaufzeichnungen von Datenloggern, die auf den Tieren angebracht waren, haben ergeben, dass die Tiere weder mit Fieber noch mit häufigeren Aufwachphasen auf Zymosan reagierten und auch die Blutwerte zeigten keine übermäßigen Immunreaktionen - anders als bei den amerikanischen Verwandten, die mit *P. destructans* infiziert wurden. Ich habe festgestellt, dass die mit Zymosan behandelten Tiere zwar nicht mit einer adäquaten Immunreaktion reagierten wie sie außerhalb der Winterschlafphase zu erwarten wäre (z.B. Veränderung der Konzentration und der Zusammensetzung der weißen Blutzellen), jedoch mit einer Erhöhung von Haptoglobin, ein

Akute-Phase-Protein, das bei Entzündungsreaktionen ausgeschüttet wird und effektiv gegen mikrobielle Erreger wirkt. Aus den Resultaten schlussfolgerte ich, dass es bei Großen Mausohren eine winterschlaf-angepasste Immunreaktion gibt, die es ihnen erlaubt, die negativen Auswirkungen einer Infektion mit Pathogenen wie *P. destructans* zu begrenzen, ohne den Erreger komplett zu beseitigen, um Energie zu sparen (Toleranzreaktion). Diese moderate Immunreaktion findet offenbar während regulärer Aufwachphasen statt, ohne dass diese sich hinsichtlich ihrer Dauer oder Häufigkeit verändern. Dies könnte eine generelle Anpassung für die Winterschlafphase sein, während eine vollständige Immunreaktion und eine Heilung erst im darauffolgenden Frühling stattfindet, wenn wieder ausreichend Nahrung bzw. Energieressourcen zur Verfügung stehen.

Um immunologische Untersuchungen an Fledermäusen durchzuführen, die tatsächlich mit *P. destructans* infiziert sind, habe ich ein Freilandexperiment durchgeführt (Kapitel 2). Dafür habe ich zunächst Temperaturlogger auf wildlebenden winterschlafenden Großen Mausohren angebracht - zu einem Zeitpunkt, an dem noch keine Pilzinfektionen sichtbar waren. Nach zirka einem Monat habe ich erneut die Winterquartiere aufgesucht, um Krankheitssymptome der Fledermäuse zu bestimmen, die Temperaturlogger zu entfernten und Blutproben zu entnehmen. Anhand der kutanen Temperaturaufzeichnungen und dem erfassten Krankheitsbild konnte ich zeigen, dass die Mausohren trotz Pilzinfektionen ihren Winterschlaf fortsetzten und auch keinerlei Fiebersymptome zeigten, wie es bei amerikanischen Fledermäusen der Fall ist. Darüber hinaus ließ sich feststellen, dass das Körpergewicht, ein Indikator für die gespeicherte Energie in Form von Fett, einen entscheidenden Einfluss darauf hat, wie oft die Tiere während der Winterschlafperiode aus dem Torpor erwachen können. Je schwerer die Tiere sind, desto häufiger können sie sich die energieaufwändigen Aufwachphasen leisten, in denen sie auch den Pilzbefall reduzieren. Interessanter Weise haben wir in dem Blut der torpiden Tiere festgestellt, dass schwer infizierte Tiere Anzeichen für eine Entzündungsreaktion zeigen. Dabei scheinen Sie Basiskonzentrationen einiger im Blut vorhandenen Immunparameter zu nutzen (u.a. Haptoglobin), ohne neue immunologische Substanzen zu synthetisieren. Ich gehe davon aus, dass die Nutzung dieser Basiskonzentrationen eine Strategie ist, um zusätzliche Aufwachphasen zu vermeiden, die für die Proteinsynthese vermutlich notwendig sind, da die meisten Immunfunktionen während des Torpors herunterreguliert sind. Auch diese Ergebnisse suggerieren eine immunologische Toleranz der europäischen Fledermäuse gegenüber dem Erreger *P. destructans* während des

Winterschlafes. Darüber hinaus habe ich bei den Tieren mit schweren Infektionen Anzeichen für erhöhten oxidativen Stress gefunden. Dies zeigt, dass es noch Forschungsbedarf bei den tatsächlichen Kosten der Weißnasenkrankheit bei Europäischen Fledermäusen gibt.

Als hilfreiches Instrument für weitere Untersuchungen der Weißnasenkrankheit habe ich eine nicht-invasive Methode entwickelt (Kapitel 3), mit der man Symptome der Weißnasenkrankheit auf den winterschlafenden Fledermäusen bewerten kann, ohne dass man die Tiere hierfür stören muss - wie es bei bisherigen Standardmethoden der Fall war. Dieses Bewertungssystem, welches mittels visueller Klassifizierung des Pilzbefalls den Schweregrad der Erkrankung ermittelt, wurde mit den anderen bislang verwendeten invasiven Standardmethoden verglichen und validiert. Mit Hilfe der neuen Methode ist es fortan möglich, die Weißnasenkrankheit inklusive ihrer Schweregrade an torpiden Tieren zu bestimmen und aufgrund ihrer Nichtinvasivität ist es sogar möglich, dies mit Hilfe vieler andere Fledermausforscher und -schützer großflächig und standardisiert während routine-mäßiger Winterzählungen zu tun, wie es bereits international gefordert wird.

1 General introduction

1.1 White-nose disease - a serious threat to hibernating bats

The immune system of animals and especially of bats has recently received broad attention in science due to zoonotic diseases and especially to the recent Covid-19 crisis. Indeed, several mammal species including bats have been suggested to be the source of SARS-Cov-2, the viral agent of Covid-19 (Andersen et al., 2020; Boni et al., 2020; Damas et al., 2020). Besides viruses, bacteria and fungi represent potential pathogens which may cause zoonotic diseases (Bueno-Marí et al., 2015; Rahman et al., 2020). Those pathogens can become major threats to animals and their populations, especially in combination with or due to anthropogenic environmental changes (Daszak, 2000; Fisher et al., 2012; Pedersen et al., 2007; Seyedmousavi et al., 2018).

Mechanisms explaining how animal hosts cope with diseases are relevant for species conservation as well as for human medical research since biodiversity including pathogens, ecosystems, and humans are linked (Cunningham et al., 2017; Daszak et al., 2001; Sandifer et al., 2015). Animal species evolved different physiological features which lead to different adaptations characterizing their immune systems. For example, hibernation is a physiological state in which mammal species downregulate physiological functions in order to save energy during adverse winter periods (Carey et al., 2003; Geiser, 2013; Lyman and Chatfield, 1955). Among them, immunological functions are also downregulated (Bouma et al., 2010a) which raises the question, how hibernators cope with pathogens during winter.

A prominent example and contribution to the scientific knowledge about immune responses of hibernators is the current research on White-nose syndrome in bats. The outbreak of the dramatic pandemic was in 2006 when hundreds of bats were found dead on the ground in Howes Cave near New York (Blehert et al., 2009; Veilleux, 2008). After this event, more fatalities were found in several other caves around the first outbreak and researches noticed specific symptoms on the bats - a mouldy fungus around the noses ('white nose', see figure 1.1) of the bats as well as on the wing membranes

Figure 1.1: Little brown bat with white-nose syndrome (photo: Marvin Moriarty/USFWS)

(Veilleux, 2008). It became evident that the fungus which was originally named *Geomyces destructans* (Blehert et al., 2009; Gargas et al., 2009) and later renamed to *Pseudogymnoascus destructans* (Minnis and Lindner, 2013) was the causative agent of the mysterious disease which was called henceforth White-nose syndrome (Blehert et al., 2009; Veilleux, 2008). The ongoing spread of the disease reached rapidly across the North American continent (figure 1.2) and millions of bats died (Frick et al., 2015, 2010; Froschauer and Coleman, 2012).

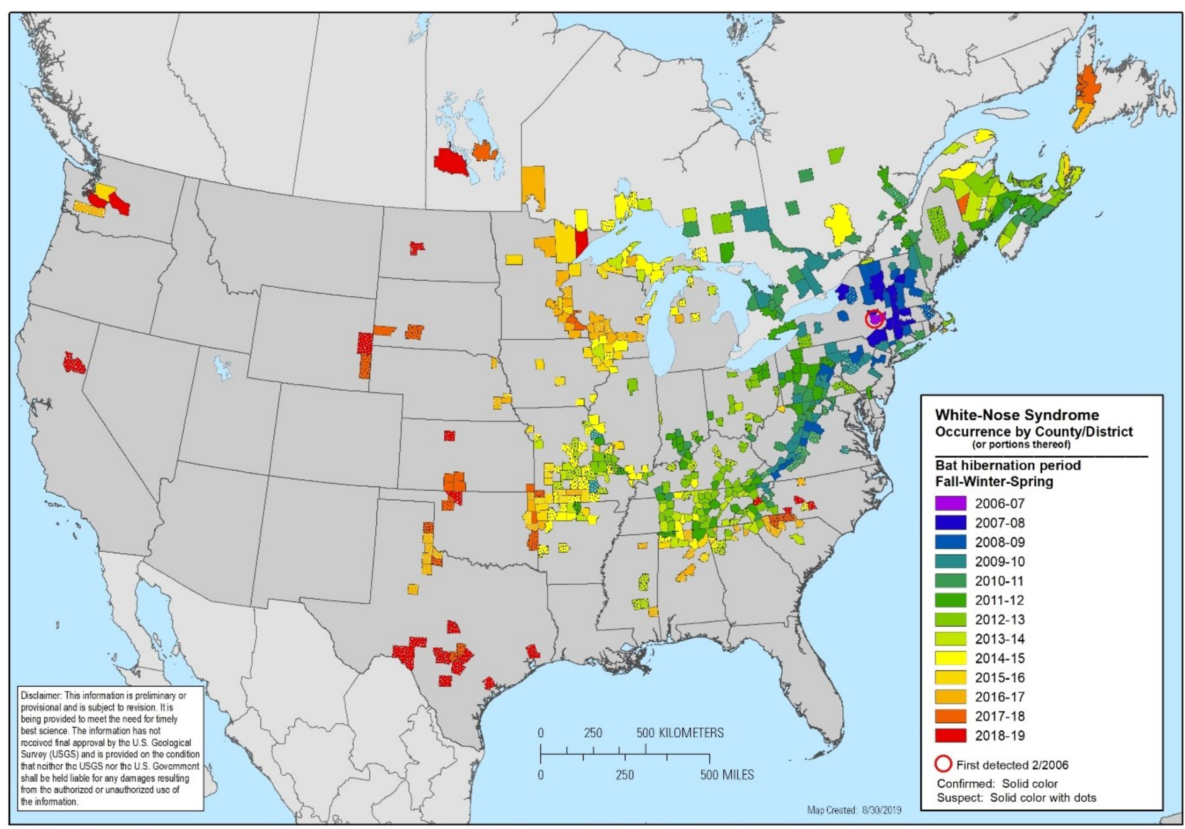

Figure 1.2: White-nose syndrome occurrence map - by year (2019). Data last updated: 8/30/2019, accessed January 2021. Available at: https://www.whitenosesyndrome.org/static-spread-map/august-30-2019

The reasons why bats die due to *P. destructans* infections are manifold. Investigations on infected bats have shown the fungus invades hair follicles, sebaceous glands, and apocrine glands, destroys connective tissues, causing widespread structural damage (lesions) on all furless areas of the bats (Meteyer et al., 2009; Pikula et al., 2017) and severe physiological disruptions due to the damage of the skin (Cryan et al., 2010; Verant et al., 2014). The sick bats show unusual behaviour like changed torpor patterns and even show unusual day flights which leads to depletion of fat reserves before the winter ends (Brownlee-Bouboulis and Reeder, 2013; Field et al., 2015; Moore et al., 2013; Reeder et al., 2012; Warnecke et al., 2012).

After the outbreak of white-nose syndrome in North America, *P. destructans* has also been identified on bats in Europe (Martinkova et al., 2010; Puechmaille, 2010; Puechmaille et al., 2011; Wibbelt et al., 2010). However, no mortality associated to the fungus has been found so far (Fritze and Puechmaille, 2018; Puechmaille et al., 2011b; Wibbelt et al., 2013) and ring recaptures confirmed infected but surviving bats over several winters (Fritze et al., 2012a; Ohlendorf et al., 2011; Wibbelt et al., 2013).

Although *P. destructans* also causes lesions in European bats (Pikula et al., 2012; Pikula et al., 2017; Wibbelt et al., 2013), there are differences in the pathological characteristics between the North American and European continent, e.g. European bats are not dying due to the infection and show less severe damages in the skin membranes compared to North American bats (Pikula et al., 2017; Wibbelt et al., 2013). Therefore, researches started to use the term 'white-nose disease' in Europe instead of 'white-nose syndrome', because the white-nose syndrome was originally termed in association with the distinct symptoms including mortality described for bats in North America (Breitenbach et al., 2011; Chaturvedi and Chaturvedi, 2011; Frick et al., 2016; Paiva-Cardoso et al., 2014; Wibbelt, 2018). The reason for the pathological differences in the infections between the continents is probably due to the native origin of *P. destructans* in Europe, and its most likely accidental transport to North America where it established as a new pathogen (Drees et al., 2017; Leopardi et al., 2015; Zukal et al., 2016a). Therefore, it is suggested that European bats co-evolved with the fungus and developed adaptations to cope with the pathogen which bats in North America are still missing (Harazim et al., 2018, 2021; Puechmaille et al., 2011b).

To shed light on the question which mechanisms enable European bats to cope with the pathogen while North American bats do not, I investigated in physiological and immunological responses to fungal infections in hibernating European bats.

1.2 Reduced physiological and immunological host responsiveness during hibernation - A gateway for the psychrophilic pathogen *Pseudogymnoascus destructans*

Mammalian hibernation is characterized by extended bouts of torpor that allow mammals to survive adverse conditions during winter. Torpor is a controlled reduction in body temperature, energy expenditure, water loss, and other physiological functions and is the most effective mean for energy conservation in endotherms (Geiser, 2013; Lyman and Chatfield, 1955). Hibernators use multiday torpor bouts throughout the winter which substantially increases winter survival (Geiser, 2013; Geiser and Turbill, 2009; Lyman and Chatfield, 1955). Despite the low energy requirement during torpor, hibernators still need to build a body fat store which is generated by the oxidation of fatty acids in adipose tissue (Boyer and Barnes, 1999; Carey et al., 2003; Florant, 1998; Speakman and Rowland, 1999). In small hibernating mammals like ground squirrels or bats, body temperature drops down to levels slightly above ambient temperatures and the resting metabolic rate is reduced to 2–4% of normal rates for periods of 4 to 30 days (Bouma et al., 2010a, 2010b; Carey et al., 2003). Consequently, the immune system of hibernating mammals is also down regulated to a large extent (Bouma et al., 2010a). For example, it has been shown in European ground squirrels (*Spermophilus citellus*) that white blood cell concentrations decreased by 90% within 24h torpor (Bouma et al., 2010b).

However, torpor bouts are periodically interrupted by short arousal events during which animals increase their body temperature (Hut et al., 2002). It is suggested that during arousals most of the physiological functions are restored, and that arousals are necessary for initiation of immune responses (Carey et al., 2003; Hut et al., 2002; Prendergast et al., 2002). Therefore, it is suggested that one main function of arousals is to clear accumulated pathogens (Prendergast et al., 2002). On the other hand, interbout arousals are energetically costly (Humphries et al., 2003; Speakman et al., 1991; Thomas et al., 1990) as well as immune responses are costly in general (Svensson et al., 1998). Therefore, arousals during which hibernators additionally mount immune responses are particularly detrimental during hibernation because fat deposits are excessively consumed (Canale and Henry, 2011; Demas et al., 1997; Martin et al., 2003). Thus, hibernators should be thrifty with the frequency of

arousals as well as with immune responses (Bouma et al., 2010a; Boyles et al., 2020; Humphries et al., 2003).

However, besides the general energy saving strategy associated with impaired immunity in hibernators, activities of most pathogens are also decreased at low temperatures, except psychrophilic pathogens such as some bacterial and fungal species (Bižanov and Dobrokhotova, 2007; Brunet et al., 2018; Dempster et al., 1966). A prominent example is the fungus *Pseudogymnoascus destructans* which has its optimal growth rate at temperatures approximately between 8-15°C and relative air humidity above 80% (Fritze et al., 2012a; Gargas et al., 2009; Marroquin et al., 2017; Verant et al., 2012). Since this temperature and humidity range corresponds to the optimum hibernation conditions of many cave-dwelling bat species (Dietz and Kiefer, 2014; Kulzer, 2008; Webb et al., 1996), *P. destructans* is able to infest hibernating bats across the temperate zone in Nearctic and Palearctic (Hoyt, 2021; Hoyt et al., 2016; Kovacova et al., 2018; Martínková et al., 2018; Pikula et al., 2017; Puechmaille et al., 2011; Zukal et al., 2016a). However, genetic studies and historical data revealed that *P. destructans* is native to Europe for long time; at least more than 100 years (Campana et al., 2017; Feldmann, 1984; Martinkova et al., 2010; Zahradníková et al., 2018), and has been introduced to North America after the last millennium (Drees et al., 2017; Leopardi et al., 2015; Puechmaille et al., 2011). Thus, *P. destructans* is a novel pathogen on the North American continent and bat species living there are highly susceptible because they likely lack physiological and immunological adaptations for which European bats were probably selected.

North American bats infected with *P. destructans* show increased arousal frequencies and show abnormal behavior (Bohn et al., 2016; Brownlee-Bouboulis and Reeder, 2013; Reeder et al., 2012; Warnecke et al., 2012; Wilcox et al., 2014). Additionally, these bats mount various immune responses and fever during these arousals (Field et al., 2018, 2015; Hecht-Höger et al., 2020; Lilley et al., 2017; Mayberry et al., 2018; Moore et al., 2013; Rapin et al., 2014; Reeder et al., 2017). These attempts of the host to resist against the pathogen leads to premature depletion of fat reserves and are thus maladaptive (Blehert et al., 2009; Courtin et al., 2010; Field et al., 2015; Meteyer et al., 2009; Storm and Boyles, 2011; Warnecke et al., 2012).

In Europe, neither abnormal winter activity, nor body mass changes, nor mortalities have been detected in bats infected with *P. destructans* so far (Bandouchova et al., 2018; Fritze and Puechmaille, 2018; Puechmaille et al., 2011; Zukal et al., 2016b). However, studies on arousal patterns of European bats similar to studies on North American bats infected with *P. destructans* have never been conducted so far. Concerning immunity, recent studies have shown different transcriptomic immune responses during arousals between bats infected with *P. destructans* in North America and in Europe (Lilley et al., 2019). In contrast to highly susceptible North American little brown bats (*Myotis lucifugus*) showing expressions of inflammatory, immune cell activation and migration, wound healing, and metabolic genes (Field et al., 2015; Lilley et al., 2019, 2017), European *Myotis myotis* did not activate a transcriptional response (Lilley et al., 2019). Moreover, European bats do neither show a cellular immune response (Bandouchova et al., 2018), nor an antibody-mediated adaptive immune response to the infection contrasting to various responses including cellular responses in North American bats (Johnson et al., 2015; Moore et al., 2013). It is suggested that in contrast to unsuccessful resistance response in North American bats, European bats co-evolved with the fungus and developed a tolerance strategy (Harazim et al., 2018; Hecht-Höger et al., 2020; Lilley et al., 2019; Zukal et al., 2016a). However, the mechanisms behind this suggested tolerance are still not understood.

Previous immunological studies on European bats have not particularly focused on the humoral aspect of the induced and constitutive innate immunity. Such studies are crucial because innate immunity represents the first line of defense against pathogen invasions, especially against bacteria and fungi (Tizard, 2008). Fungal pathogens are recognized by immune cells through toll-like receptor 2 (TLR2), toll-like receptor 4 (TLR4), dectin-1, C-type lectins, and NLRP3 inflammasome (Brown, 2006; Calich et al., 2008; Durai et al., 2013; Salazar and Brown, 2018). After recognition, immune cells such as monocytes, macrophages, fibroblasts, endothelium, platelets, keratinocytes, and T cells release cytokines and chemokines (Cray et al., 2009; Tizard, 2008). These pro-inflammatory signals, including different interleukins and TNF, have numerous effects throughout the body including inducing an acute-phase response which is a core part of the innate immune response (Calich et al., 2008; Cray et al., 2009; Young et al., 2001). Moreover, acute phase responses can be regarded as systemic emergency responses which come into play when the adaptive immune system of the host failed to cope with an infection (Berczi and Szentivanyi, 2003; Tizard, 2008). In locally

infected tissue of aroused bats infected with *P. destructans*, transcriptomic investigations identified activated immune genes constituting to an inflammation including those responsible for activating acute phase responses (Field et al., 2018; Lilley et al., 2019). Additionally, bats in North America have shown limited gene expressions associated with inflammatory response even during torpor, nevertheless, this indication for a weak immune response during torpor was related and followed by gene expressions showing a robust immune response during arousal (Field et al., 2018). Since transcriptomic approaches on European bats only used locally infected wing tissues of aroused bats so far, it is still unclear whether European bats can mount systemic immune responses as suggested by Hecht-Höger et al. (2020) and if such responses occur during arousals or even during torpor (Lilley et al., 2019). Additionally, links between immune gene expressions and actual changes in concentrations of immunological effective substances are still understudied (Hecht-Höger et al., 2020). For example, haptoglobin has been suggested to play an important role in hibernation immunology (Chow et al., 2013; Mominoki et al., 2005) and can be used as an indicator for an acute phase response (Godson et al., 1996; Gruys et al., 2005; Matson et al., 2012).

At the same time, acute phase responses lead to noxious effects that are harmful to the host, e.g. immune cells release cytotoxic chemicals with pro-oxidant activity, which damage pathogens but also cause cell damage to the host (Costantini, 2014; Schneeberger et al., 2013; Sorci and Faivre, 2009). Therefore, excess pro-oxidants need to be balanced by anti-oxidants (Costantini and Møller, 2009; Schneeberger et al., 2013). The imbalance between pro- and antioxidants, called oxidative stress, has been found to contribute to the mortality related to white-nose syndrome (Moore et al., 2013). However, it is unclear whether oxidative stress occurs in European bats infected with *P. destructans* or if pro- and antioxidants are involved in the immune defence which may be mounted during arousals and/or during torpor.

To sum up, in the present work, I was interested in closing some general knowledge gaps concerning immune responses and resulting consequences of hibernating European bats which get regularly infected with the fungal pathogen *P. destructans*. In detail, I have focussed on systemic innate immune responses towards fungal infections which may occur both during arousals and torpor, and how these possible responses relate to torpor patterns. These investigations may help to answer the question whether European bats use tolerance or

resistance responses to cope with *P. destructans* and possibly other pathogens bats are exposed to during hibernation.

By definition, tolerance is the ability to limit the negative impact of an infection on the hosts fitness without directly affecting the pathogen burden (Medzhitov et al., 2012; Schneider and Ayres, 2008). If European bats show tolerance towards fungal pathogens such as *P. destructans* during hibernation, immune responses should be characterized by mechanisms that limit the damage caused by the infections but without showing energetically costly responses such as increased arousal frequencies or full immune responses. Instead, bats may show hibernation-adjusted, limited immune responses that reduce damages caused by the fungal pathogens until they can be fully cleared after the hibernation period. Alternatively, resistance is the ability to reduce the pathogen burden (Medzhitov et al., 2012; Schneider and Ayres, 2008). Hence, bats that are resistant towards pathogens such as *P. destructans* should build responses that are characterized by reactions that directly affect the pathogen load. In this case, infected individuals should increase arousal frequencies, during which they reduce the fungal infection by grooming and by mounting a full immune response to completely clear the pathogen. Thus, such response would be energetically costly and ultimately would reduce the fat stores and thus the weight of hibernating bats, as observed in North American bats (Warnecke et al., 2012).

1.3 Methodological approaches to investigate physiological and immunological responses of hibernating bats to infections with *Pseudogymnoascus destructans*

Previous studies on European bats exposed to *P. destructans* compared infected with (obviously) non-infected bats without taking into the consideration the disease progression, pathogen load or disease severity (Bandouchova et al., 2018; Hecht-Höger et al., 2020; but see Harazim et al., 2018). In a comparative laboratory study, individuals of *Myotis lucifugus* and *Myotis myotis* were exposed to *P. destructans* and immune gene expressions were analysed and compared irrespective of the actual pathogen load (Davy et al., 2017), in which the authors did not detect an immune response of *M. myotis* in contrast to *M. lucifugus*. In fact, in this study, *M. myotis* was exposed to P. destructans but did not show typical disease

symptoms. The work was therefore criticized because the title indicates different responses of the two host species infected with *P. destructans* but since the typical symptoms of one host species have not developed, immune response are hardly comparable (Field, 2018). Since several previous attempts failed to infect *M. myotis* with *P. destructans* under laboratory conditions (Davy et al., 2020, 2017; Field, 2018; Wibbelt, unpublished), I used zymosan in my laboratory approach. Zymosan is an antigen widely used in laboratory research to stimulate antifungal immune responses (Bastos-Pereira et al., 2018; Payá et al., 1996; Underhill, 2003; Volman et al., 2005). Zymosan is derived from a cell wall material of *Saccharomyces cerevisiae*, a single-cell fungus, and is typically used as a model microbial particle in studies on the innate immune system including the stimulation of inflammatory responses (Underhill, 2003; Volman et al., 2005). Zymosan it is recognized by typical fungal receptors (e.g. TLR2) and activates pathways which induce an innate immune response (Dillon, 2006; Field et al., 2015; Underhill, 2003; Volman et al., 2005). I inoculated zymosan subcutaneously in *M. myotis* to induce a systemic antifungal response. Thus, I measured physiological and immunological responses in a comparative approach by using an experimental and a control group as well as repeated sampling before and after inoculation.

Under field conditions, it is not possible to sample bats several times because once you disturb a bat infected with *P. destructans* for sampling, it will arouse and clean off the fungus (Bohn et al., 2016; Brownlee-Bouboulis and Reeder, 2013; Fritze et al., 2012a; Wilcox et al., 2014). Therefore, to conduct a comparative experiment, I had to develop an experimental design with only one sampling event per bat, including bats at different disease stages. Since we knew that in middle and western Europe most infections with *P. destructans* occur in late winter season, with visible growth starting approximately in the beginning of February and a growth peak in March (Fritze et al., 2012a; Puechmaille et al., 2011b), I attached temperature data loggers to the wild-living bats before appearance of disease symptoms, in January. Then, I waited until mid of March to start to retrieve the data loggers and to select bats to which I collected blood. To choose bats with different disease stages, I used a visual estimation to categorise levels of the *P. destructans* colonization before we selected bats for blood sampling. Additionally, I used a combination of visually detecting the fungal growth and quantified the actual damage by lesions counts since different physiological disruptions and damage levels have been described for different stages of disease progression (Cryan et al., 2010; Pikula et al., 2017; Wibbelt et al., 2013). I used this information to group the

experimental bats into three categories: asymptomatic bats (no symptoms), mildly infected bats (fungal growth but no lesions) and severely infected bats (fungal growth and lesions).

Additionally, I took swabs from the bats to estimate the pathogen load by quantifying the DNA of *P. destructans* by qPCR (Langwig et al., 2015; Muller et al., 2013) in order to validate the visual assessments. Moreover, as a separate aspect, I compared the data between the three methods: visual quantification based on classifications (Fritze et al., 2012a; Horáček et al., 2014), fungal load using qPCR technique and lesion counts using UV light transillumination (Turner et al., 2014). I used this data to check whether the visual classification system (namely 'Visual Pd-score') can replace the other two methods because in contrast to the Visual Pd-score, bat swabbing or lesion counts require to disturb the bats during hibernation. The two latter methods are invasive and thus not recommendable for large-scale applications, e.g. in disease surveillance programs which are crucial to control emerging infectious diseases (Daszak, 2000; Fritze and Puechmaille, 2018; Leendertz et al., 2006). Such methods have to be carefully evaluated concerning level of invasiveness, animal welfare, conservation criteria and the specific value of the obtained data, especially when dealing with endangered species heavily impacted by a disease. Therefore, I compare and discuss advantages and disadvantages of the aforementioned methods concerning their usability during routine hibernacula counts and their data quality in context to further research questions.

1.4 Thesis outline

In chapter one, I investigated in the immune response of hibernating greater mouse-eared bats (*Myotis myotis*) to a fungal challenge by using the fungal antigen zymosan in a laboratory-based experiment. In this chapter I particularly focus on body temperature patterns and the immune response during arousals from torpor.

In chapter two, I conducted a field-based experiment in which I sampled wild-living hibernating greater mouse-eared bats naturally infected with *Pseudogymnoascus destructans*. Here, I tracked body temperatures of some individuals to investigate in torpor patterns in relation to their health status and investigated in the immune responses during torpor.

In chapter three, I developed a field-based, non-invasive method to assess the health status of bats exposed to *P. destructans*. I validated this approach by comparing with gold-standard methods of white-nose research and evaluated pros and cons of these clinical assessment instruments.

2 Chapter One - Immune response of hibernating European bats towards fungal pathogens during arousals from torpor

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As

Immune response of hibernating European bats to a fungal challenge

by

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2.1 Abstract

Immunological responses of hibernating mammals are suppressed at low body temperatures, a possible explanation for the devastating effect of the white-nose syndrome on hibernating North American bats. However, European bats seem to cope well with the fungal causative agent of the disease. To better understand the immune response of hibernating bats, especially against fungal pathogens, we challenged European greater mouse-eared bats (*Myotis myotis*) by inoculating the fungal antigen zymosan. We monitored torpor patterns, immune gene expressions, different aspects of the acute phase response, and plasma oxidative status markers and compared them with sham-injected control animals at 30 min, 48 h and 96 h after inoculation. Torpor patterns, body temperatures, body masses, white blood cell counts, expression of immune genes, reactive oxygen metabolites, and non-enzymatic antioxidant capacity did not differ between groups during the experiment. However, zymosan injected bats had significantly higher levels of haptoglobin than the control

animals. Our results indicate that hibernating greater mouse-eared bats mount an inflammatory response to a fungal challenge, with only mild to negligible consequences for the energy budget of hibernation. Our study gives a first hint that hibernating European bats may have evolved a hibernation-adjusted immune response in order to balance the trade-off between competent pathogen elimination and a prudent energy-saving regime.

2.2 Introduction

Hibernation is a prolonged state of reduced metabolic activity and lowered body temperature in mammals and has evolved as a strategy to overcome winter periods with adverse weather conditions and low resource availability (Boyer and Barnes, 1999). During hibernation, all major physiological functions are reduced to save energy, which is generated by the oxidation of fatty acids from adipocytes (Florant, 1998). In small hibernating mammals, body temperature drops down to levels slightly above ambient temperatures for periods of 4 to 30 days, a stage called torpor (Bouma et al., 2010a). These torpor bouts are periodically interrupted by short arousal events during which animals increase their core body temperature and during which most of the physiological functions are restored (Hut et al., 2002).

In general, immune responses are energetically costly as they involve significant increases in the metabolic rate, which may ultimately deplete fat deposits during hibernation (Canale and Henry, 2011; Demas et al., 1997; Martin et al., 2003). Consequently, immune functions were found to be down regulated in hibernating mammals (Bouma et al., 2010a). Besides the general energy saving strategy associated with impaired immunity in hibernators, there is no need for an active immune system during torpor bouts because activities of most pathogens are also decreased at low temperatures. An exception are psychrophilic pathogens, especially some bacteria and fungi, that grow at low temperatures (Bižanov and Dobrokhotova, 2007; Brunet et al., 2018; Dempster et al., 1966). How hibernators cope with these potential pathogens when physiological functions are constrained is not yet fully understood. This question is even more important since the emergence of white-nose syndrome, a disease affecting hibernating North American bats after having contracted the psychrophilic fungus *Pseudogymnoascus destructans* (Frick et al., 2016; Wibbelt, 2018).

Pathogens which enter organisms are first recognized by the innate immune receptors, of which Toll-like receptors (TLRs) are highly relevant (Doan et al., 2013). Fungi are typically recognized by TLR2 and TLR4 on macrophages, as well as dectin-1, C-type lectins, and NLRP3 inflammasome (Calich et al., 2008; Salazar and Brown, 2018). Positive signals from these receptors expressed on the surface of sentinel immune cells such as macrophages, stimulate cells to secrete pro-inflammatory cytokines (e.g. interleukin 1β (IL-1β), interferon gamma (IFN-γ) and tumor necrosis factor α (TNF-α)), and anti-inflammatory (e.g. interleukin 10 (IL-10)) as well as inflammatory mediators like nitric oxide (NO) (Calich et al., 2008; Young et al., 2001). Under the influence of cytokines, the brain responds via modified behaviors (e.g. sickness behavior including anorexia, lethargy and sleepiness) and development of fever. During this acute phase, the bone marrow will increase white blood cell production and the liver will initiate the synthesis and secretion of defense proteins; e.g. acute phase proteins such as haptoglobin, serum amyloid A or C-reactive protein (Tizard, 2008). All these modifications constitute the acute phase response, which is part of the innate immune defense known to be energetically costly (Lee, 2006). As a consequence of the acute phase response, immune cells release cytotoxic chemicals with pro-oxidant activity, which damage pathogens (Costantini, 2014; Schneeberger et al., 2013a; Sorci and Faivre, 2009). To avoid cell damage of the host, excess pro-oxidants need to be balanced by anti-oxidants. An imbalance between pro-oxidants and anti-oxidants leading to increased oxidative damage may occur as a consequence of an immune response (Costantini and Moller, 2009; Schneeberger et al., 2013).

Compared with adaptive immunity, relatively little is known about innate immunity in hibernating mammals, specifically when challenged by an antigen or an infectious agent. In vitro studies on hibernating rodents report a decrease in pro-inflammatory cytokine production and phagocytosis in innate immune cells (Kandefer-Szerszen, 1988; Novoselova et al., 2000). Moreover, golden-mantled ground squirrels (*Spermophilus lateralis*) challenged with bacterial lipopolysaccharide during a torpid phase developed fever only during the subsequent arousals, indicating that arousal events evolved, at least partly, in order to clear the pathogens accumulated during hibernation (Prendergast et al., 2002).

To better understand, how hibernating European bats generally cope with fungal challenges, we followed aspects of the acute phase response over a 4-day period after challenging greater mouse-eared bats (*Myotis myotis*) with Zymosan, a homoglucan of n glucose molecules in ß-

1,3-glycosidic linkage. Zymosan is a fungal antigen that activates macrophages via TLR2 and thus is regularly used in experimental studies to induce immune responses (Bastos-Pereira et al., 2018; Underhill, 2003; Volman et al., 2005). Besides the general interest to better characterize the acute phase response of hibernators against fungal pathogens, studies on European hibernating bats are important because of their apparent resistance against the white nose-syndrome causing agent *P. destructans* (Puechmaille et al., 2011; Wibbelt et al., 2013). Considering the energetic aspects of hibernation and the acute phase response and also the apparent resistance of this bat species against local fungal infections, we hypothesized that zymosan-challenged hibernating *M. myotis* will show an immunological response but with hibernation-related adjustments in the composition of the response in order to avoid activating processes that are energetically costly. Therefore, we predicted that zymosan-treated bats will not increase arousal frequencies and durations but will show a response dominated by acute-phase proteins. However, experimental bats may develop a fever response during arousals, and immune gene expressions in response to the zymosan challenge (Young et al., 2001). We expected the acute phase response in zymosan-treated bats to be characterized by an increase in haptoglobin and oxidative damage or antioxidant levels, but not in white blood cells (WBCs) in order to save energy. If zymosan-treated bats do not bear higher energetic costs than their untreated conspecifics, then their loss in body mass during hibernation will not be higher than in bats of the control group.

2.3 Material and methods

Bat capture and housing

We captured sub-adult yearlings (14 males, 2 females) of greater mouse-eared bats (*Myotis myotis*) during autumn at a swarming site in Northern Bavaria (Germany) using two 10m long mist nets (Solida, Steinbach). Bats were transported to the field station of the Leibniz Institute for Zoo and Wildlife Research Berlin, where all individuals hibernated in individual boxes in a climate chamber, mimicking conditions in natural hibernacula (temperature 7-10 °C and relative humidity 90-100 % (Kulzer, 2008)). Animal experiments were approved of by the Animal Experiments Committee of Brandenburg (permit no. 2347-43-2015) and the authority for conservation of protected species of Franconia, Germany (permit no. 55.1-8642.01-11/15).

Immunological challenge

After five months of hibernation, bats were randomly assigned to either the zymosan treatment group (N=7 males, 1 female) or to the control group (N=7 males, 1 female). Bats of the zymosan treatment group received a subcutaneous injection of 0.7 mg/kg zymosan (Merck KGaA, former Sigma Aldrich, Darmstadt) dissolved in 100 µl sterile, isotonic phosphate buffered saline solution (PBS). The dose was derived from previous studies conducted on house sparrows (*Passer domesticus*) (Coon et al., 2011) and active *M. myotis* (Seltmann et al. unpublished results). The bats of the control group received 100 µl of PBS. The injections were conducted immediately after individuals had been taken out of hibernation chambers to ensure that body temperature was low in order to challenge bats in the state of hibernation before they aroused.

Body temperature monitoring

To monitor body temperature, torpor and arousal patterns of bats, we used iButton® temperature loggers (Thermochron iButton®, Maxim, San Jose, USA). Immediately after injection, the loggers (±0.5 °C accuracy, factory programmed and calibrated) were attached to the dorsal skin of the interscapular region of the bats, right above to the injection area, by using waterproof medical sticking plaster (WUNDmed, Abenberg). We shaved the fur from this area in order to record the superficial skin temperature (T_{sk}) as a proxy for the core body temperature (Currie et al., 2015; Warnecke et al., 2012). The metal surface of the iButtons had direct contact to the bat skin, without glue in between to avoid isolation effects and variations in contact with the body surface. Loggers were programmed before attachment (one measurement per 5 min interval) and the data was extracted after the experiment by using the 1-Wire® system (Dallas Semiconductor, Dallas, USA).

Blood sampling

We collected blood samples 30 min after the initial injection, placed all bats back into hibernation conditions and collected additional blood samples after 48 h and 96 h (Fig. 2.2). For the sampling procedure, we removed bats from the climate chamber and allowed the animals to arouse (30 min). We collected about 50 µl blood from the uropatagial vein of aroused bats using a sterile needle (20GX1", WDT, Garbsen) and heparinized micro haematocrit tubes (BRAND, Wertheim). We used 2.5 µl of each sample to prepare a blood smear and dissolved additional 5 µl in 100 µl Tuerk's solution (Merck, Darmstadt, Germany)

for counting leukocytes. The remaining blood was centrifuged to separate plasma and blood pellet (buffy coat and erythrocytes) and the samples were stored for approximately one year at -80°C until further analysis.

Expression of immune genes

RNA was extracted from blood pellets following the manual of the NucleoSpin® RNA Blood extraction kit (Macherey & Nagel GmbH & Co. KG, Düren) with slight modification due to different type of sample used: We added 150 µl H2O to the blood pellet during the lysis step and added a second elution step to increase the RNA yields. The cDNA was synthesized using the 'RevertAid H Minus First Strand cDNA Synthesis Kit' (ThermoFisher Scientific, Waltham, USA) according to the manufacturer's instructions. For the qPCR we used bat specific primers (Kacprzyk et al., 2017) for actin Beta (ACTB, housekeeping gene), IL-1β, TNFα, IL-10 and iNOS2 (table 1). The qPCR was carried out on a CFX96 cycler (Bio-Rad, Munich, Germany) applying the 'Real Time PCR iQ SYBR Green Supermix' kit (Bio Rad, Hercules, USA) according to manufacturer's protocol. Prior to the analysis, we optimized the primer-specific annealing temperature (T_a, table 2.1). Reaction conditions were 3 min at 95 °C; 40 cycles: 20 s at 95 °C, 20 s at T_a, 30 s at 72 °C; melting curve analysis: 10 s at 95 °C, 5 s at 60 °C to 95 °C in increments of 0.5 °C. Quantitation cycle values (Cq) determined during the quantitative real time PCR were used to express the levels of the immune genes relative to that of ACTB present as the relative expression ratio (rER): immune gene/ACTB. To assess RNA integrity, RIN values of the extracted total RNAs were determined on a 2200 TapeStation (Agilent) according to the manufacturer's instructions.

Table 2.1: Primer sequences (Kacprzyk et al., 2017) and optimized annealing temperatures (Ta) for selected immune genes of *M. myotis*

Primer	Sequence (5' → 3')	T_a
ACTB	F: AAATGCTTCTAGGCGGACTG	63 °C
	R: AGCCATGCCAATCTCATCTC	
IL1b	F: GAATCCATCGACTGCATGTG	63 °C
	R: GACAGCACCAGGGATTTTTG	
IL10	F: TTCAAGGGTTACCTGGGTTG	*Not amplified*

	R: TCAGCGTCTTCAGCTTCTCC	
TNFα	F: ATCAACCTCCTCTCTGCCATC	61 °C
	R: ACTGAGCCGATCACCCTTC	
iNOS2	F: CTGTGAGGCGTTCGATGTC	56 °C
	R: GTCCCAGGTCACATTGGTG	

Haptoglobin

Haptoglobin was measured following the standard procedure of the commercial "PHASE"™ Haptoglobin Assay (Tridelta, Maynooth, Ireland) which was previously used in other bat species (Costantini et al. 2019). As an acute phase protein, haptoglobin reduces oxidative damage by binding hemoglobin released during hemolysis, has immune-modulatory effects and inhibits bacterial growth. Haptoglobin binds to haemoglobin and maintains its peroxidase activity at a low pH. The measured peroxidase activity of haemoglobin is directly proportional to the amount of haptoglobin in the sample. After we diluted the plasma samples (1:4) with PBS, we added haemoglobin. Haptoglobin concentrations (mg/ml) were calculated according to the standard curve on each plate.

Oxidative status

We measured reactive oxygen metabolites (ROMs) and non-enzymatic antioxidant capacity (OXY), two markers of oxidative status known to be associated with immune responses (e.g., Costantini and Møller 2009; Schneeberger et al. 2013). We determined the ROMs using the d-ROMs test (Diacron, Grosseto, Italy) - a test that quantifies the number of damaged molecules generated early in the oxidative cascade (e.g., organic hydroperoxides, endoperoxides). We diluted 4 µl of plasma in 200 µl of a solution containing 0.01 M acetic acid/sodium acetate buffer (pH 4.8) and N,N-diethyl-p-phenylenediamine as chromogen. This mixture was incubated for 75 minutes at 37°C. Analyses were performed in duplicates on the same plate (CV = 4.8%). After incubation, absorbance was measured at 505nm with a spectrophotometer (µQuant Microplate Spectrophotometer, Biotek). ROM concentrations were calculated by plotting values onto a calibration curve obtained by measuring the absorbance of a standard solution. Values were expressed in mM of H2O2 equivalents. The OXY-adsorbent test (Diacron, Grosseto, Italy) was used to quantify the non-enzymatic antioxidant capacity of

plasma. This test is based on an in vitro reaction of non-enzymatic antioxidants (e.g., both lipophilic and hydrophilic antioxidants, such as vitamins, protein thiols, etc.) that occur in a biological matrix (e.g., plasma, haemolysate, tissue homogenate) with hypochloric acid (HOCl). We carried out our analyses using 200µl of diluted plasma (2 µl of plasma 1:100 with distilled water). To the diluted plasma we added 200 µL of HOCl followed by incubation at 37°C for 10 minutes. Analyses were carried out in duplicates on the same plate (CV = 4.4%). After incubation, absorbance was also measured at 505 nm. We calculated the antioxidant capacity using a reference standard. Values were expressed as mM of HOCl neutralized.

White blood cell counts

Total numbers of white blood cells (total WBCs) were calculated by using the cell counts obtained from diluted blood on a Neubauer chamber using the formula: number of cells in the fields × 10,000 / number of fields × dilution = WBCs/ ml (Bastidas, 2014; Weise et al., 2017). The number of WBCs per different cell type was determined by counting the different WBC types among 100 immune cells on a May-Grünwald-Giemsa stained blood smear under a microscope at 1000× magnification (Blumenreich, 1990; Schneeberger et al., 2013b; Seltmann et al., 2017). The concentrations of the different white blood cell types (lymphocytes, neutrophils, eosinophils, monocytes, basophils) were then determined by calculating the proportion of each WBC type in the total WBC concentration. All WBC counts are reported in number of cells per 1µl blood.

Calculations and statistical analysis

Torpor and arousal events were recognized by changes of skin temperature (T_{sk}) recorded at 5min intervals. Arousals were determined by a T_{sk} increase of at least 10 °C and lasting at least 20 min (Lilley et al. 2016). Torpor was determined by T_{sk} falling below 11 °C for at least 2 hours. Artificial arousals due to bat handling (inoculation, blood sampling) and natural arousals were analyzed separately.

All statistics were done in R (version 3.6.0). Data from all response variables was tested for normal distribution (Shapiro-Wilk test and Lillie test). Non-normal distributed data was transformed per square root (sqrt), natural logarithm (log_e) and 10 (log_{10}) into normal distributions (for details see 'Results'). The number of arousals could not be transformed into normal distribution and was analyzed by Kruskal-Wallis rank sum test. Mean body temperature during torpor, mean body temperature during arousal and maximum

temperatures during arousal were obtained from individual Tsk records. Differences between zymosan-treated and control bats were calculated by ANOVA. Body mass data, WBC counts, concentrations of haptoglobin, ROMs, OXY, and rER were analyzed by linear mixed-effects models (lmer) fitted by the Restricted Maximum Likelihood (REML) method from the R package ‘lmerTest’ (Kuznetsova et al., 2017). As factors we here included treatment groups, sampling days, interaction between treatment group and sampling days, and individual body mass and forearm length. The individual was included as random factor. For the body mass lmer-analysis we set treatment group, sampling days, and interaction between them as factors, forearm length as covariate and individual as random effect. All fitted models were analyzed by ANOVA and stochastic dominances among factors were calculated by t-tests using Satterthwaite's method (Kuznetsova et al., 2017). P values were adjusted for multiple comparisons by using Tukey’s method in the ‘emmeans’ package (Lenth et al., 2019). Because haptoglobin may correlate with ROM (Corsetti et al., 2018; Sauerwein et al., 2005; van de Crommenacker et al., 2010), the relationship was calculated by using the Pearson's rank correlation for each treatment group.

2.4 Results

Body condition

We observed changes in body mass (loge transformed) in individuals across sampling days ($SS=0.007$, $F=8.33$, $p=0.001$) (fig. 2.1). However, we neither detected significant effects in body mass changes between treatment group ($SS<0.001$, $F=0.76$, $p=0.399$) nor in the interaction between sampling day and treatment group ($SS<0.001$, $F=0.34$, $p=0.713$). Forearm length was also not a significant covariate ($SS<0.001$, $F=0.41$, $p=0.536$).

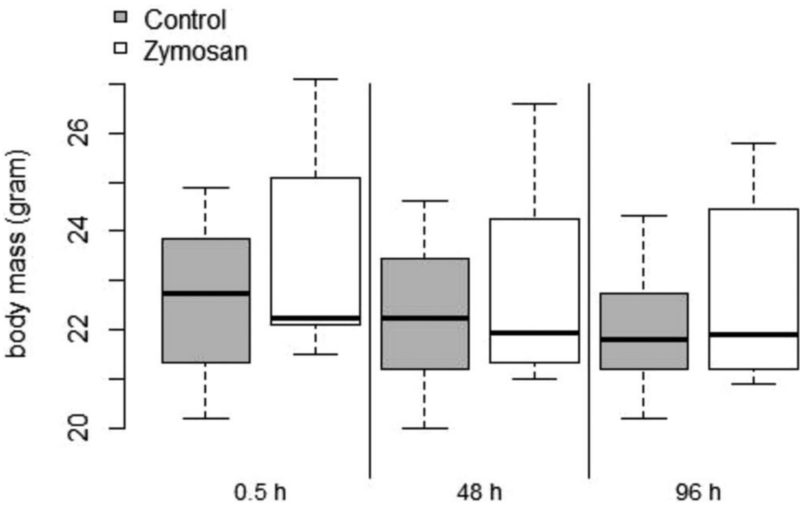


Figure 2.1: Boxplot displaying the body masses of the zymosan-treated bats (white) and the bats of the control group (grey) during 5 experimental days. Upper and lower borders of boxes indicate first and third quantiles, respectively, whiskers represent maximum and minimum, the median is shown as a solid horizontal line within boxes.

Body temperature monitoring

Following the treatment-specific injections, all bats entered torpor, resulting in an average torpid skin temperature (T_{sk}) of 7.6 ± 0.5°C. There was no difference between treatment groups in the time required for aroused bats to decrease T_{sk} from euthermic state (Tsk~36.0°C) to the torpor state (T_{sk}<10.5°C) (Ø 598 min, SS=1506, F=0.12, p=0.738, ANOVA). We then excluded all T_{sk} data from artificial arousal events from further analysis, i.e. we considered only T_{sk} data from the intervals between 1st and 2nd blood sampling and between 2nd and 3rd blood sampling (fig. 2.2) for further evaluating natural arousals. Data collected by temperature loggers amounted to about 1,000 T_{sk} measurements per individual for analysis. The frequency of natural arousals did not vary between treatment groups ($\chi 2$=1.65, Df=3, p=0.649), i.e. in total, we observed five arousals in bats of the zymosan-treated group and four in the control group. The maximal T_{sk} during arousal periods did not differ between treatment groups (SS=1.190, F=0.07, p=0.806) suggesting no fever response after zymosan inoculation. We also did not find differences in the mean T_{sk} during arousal (SS= 0.009, F= 0.002, p= 0.965) and mean T_{sk} during torpor (SS=0.005 F=0.11, p=0.748, fig. 2.3) between treatment groups. The durations of arousals neither differed between treatment groups (SS=1650, F=0.15, p=0.712) nor between artificially induced and natural arousals (SS=0.072, F=0.01, p=0.928). On average,

arousal events lasted for 190 min (table 2.2) Also, the time required to increase body temperature by +10°C during rewarming was not different between treatment groups (SS=1.540, F=0.181, p=0.674). On average, bats required about 21 min to return to the euthermic state (see appendix 7.1.1).

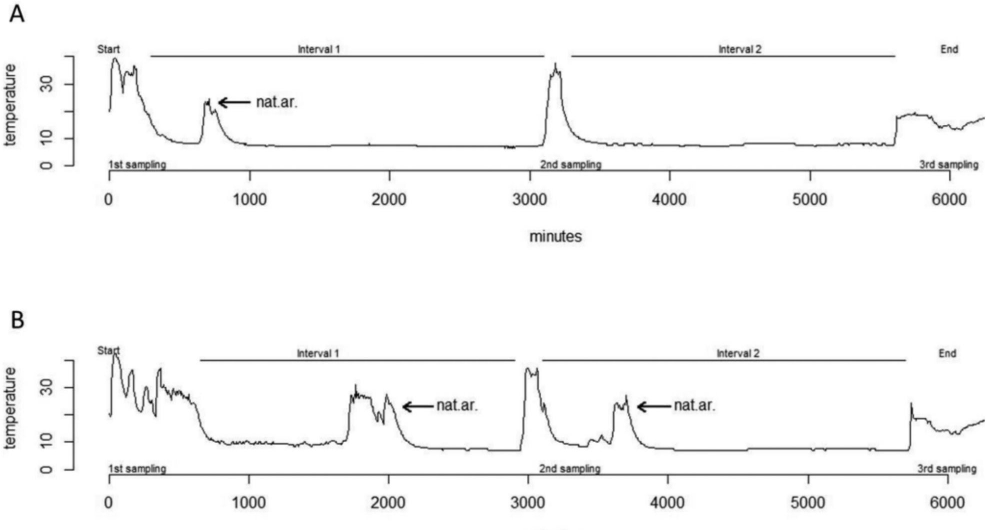


Figure 2.2: Representative examples of a Tsk profile (°C) of a zymosan-treated bat (A) and a control bat (B). The starting point (at 0 min) represents the arousal during the first injection and the first blood sampling on day 1; the second sampling took place 48 h (2880 min) after inoculation and the third sampling 96 h after the injection (5760 min). In the example shown here, the zymosan-treated bat (A) had one natural arousal (nat. ar.) in interval 1 (between the start of the experiment and the second sampling), but remained torpid in interval 2 (between the second and third sampling). The control bat (B) had two natural arousals, one in the first interval and one in the second.

Table 2.2: Comparison of the number and duration of natural arousal events, and of maximum and mean skin temperature (max Tsk, mean Tsk) during arousal between animals of the control (n=8) and the zymosan-treated group (n=8).

Individual	**Group**	**number of arousals (n)**	**arousal duration interval 1 (min)**	**arousal duration interval 2 (min)**	**max T_{sk} during arousal (°C)**	**mean T_{sk} during arousal (°C)**
Mmyo_3	CONTROL	0	-	-	-	-
Mmyo_4	CONTROL	0	-	-	-	-
Mmyo_11	CONTROL	1	95	-	19	14.9

Mmyo_12	CONTROL	0	-	-	-	-
Mmyo_13	CONTROL	0	-	-	-	-
Mmyo_14	CONTROL	1	105	-	24	19.3
Mmyo_15	CONTROL	0	-	-	-	-
Mmyo_16	CONTROL	2	430	195	31	20.9
	Mean	**0.5**	**210**	**195**	**24.7**	**18.4**
Mmyo_1	ZYMOSAN	0	-	-	-	-
Mmyo_2	ZYMOSAN	1	-	205	24.5	18.3
Mmyo_5	ZYMOSAN	2	135	145	29	18
Mmyo_6	ZYMOSAN	1	210	-	24	19.3
Mmyo_7	ZYMOSAN	0	-	-	-	
Mmyo_8	ZYMOSAN	1	200	-	24.5	18.1
Mmyo_9	ZYMOSAN	0	-	-	-	-
Mmyo_10	ZYMOSAN	0	-	-	-	-
	Mean	**0.625**	**181.67**	**175**	**25.5**	**18.4**

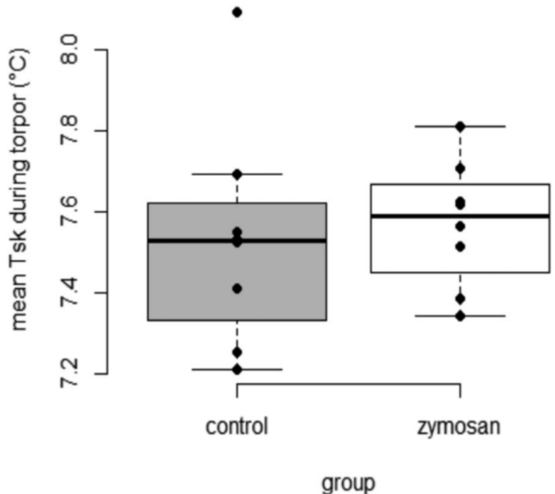


Figure 2.3: Boxplot of the mean skin temperatures (mean Tsk) during torpor (°C) of bats of the control (grey) and of the zymosan-treated group (white). Upper and lower borders of boxes indicate first and third quantiles,

respectively, whiskers represent maximum and minimum, solid horizontal line within boxes shows the median. Raw data are shown as solid circles.

Inflammatory gene expressions

We found significant increases in the gene expression levels of IL-1β across the sampling days in both treatment groups (SS=0.052, F=5.70, p=0.009). However, neither the treatment (SS<0.001, F=0.19, p=0.666) nor the interaction between treatment and sampling day (SS=0.004, F=0.43, p=0.655), nor the interaction between body mass and forearm lengths (SS=0.002, F=0.34, p=0.564) differed between treatment groups (fig. 2.4). For iNOS2 expression, we also found significant increases across the sampling days in both groups (SS=0.11, F=3.87, p=0.031). Further, we detected a significant influence of the body condition on iNOS2 expression: mass (SS=0.067, F=4.91, p=0.034), forearm length (SS=0.066, F=4.85, p=0.034) and the interaction between body mass and forearm length (SS=0.064, F=4.70, p=0.037). However, there was no significant difference in iNOS2 expression between treatment groups (SS<0.001, F=0.037, p=0.848). TNF expression significantly increased across the sampling days (SS=0.059, F=5.016, p=0.012). However, neither treatment group affiliation (SS=0.006, F=0.967, p=0.332), nor the interaction between treatment and day (SS=0.012, F=1.043, p=0.362) nor the body condition of animals (interaction mass and forearm: SS=0.011, F=1.915, p=0.175) had a significant effect on TNF expression. IL-10 primers failed to amplify any fragment in a PCR and thus its expression could not be assessed in a qPCR. The mean of the RIN values of the RNA samples was 3.9 (SD=0.89). A full table with RIN values of all measured gene expressions is available in appendix 7.1.2.

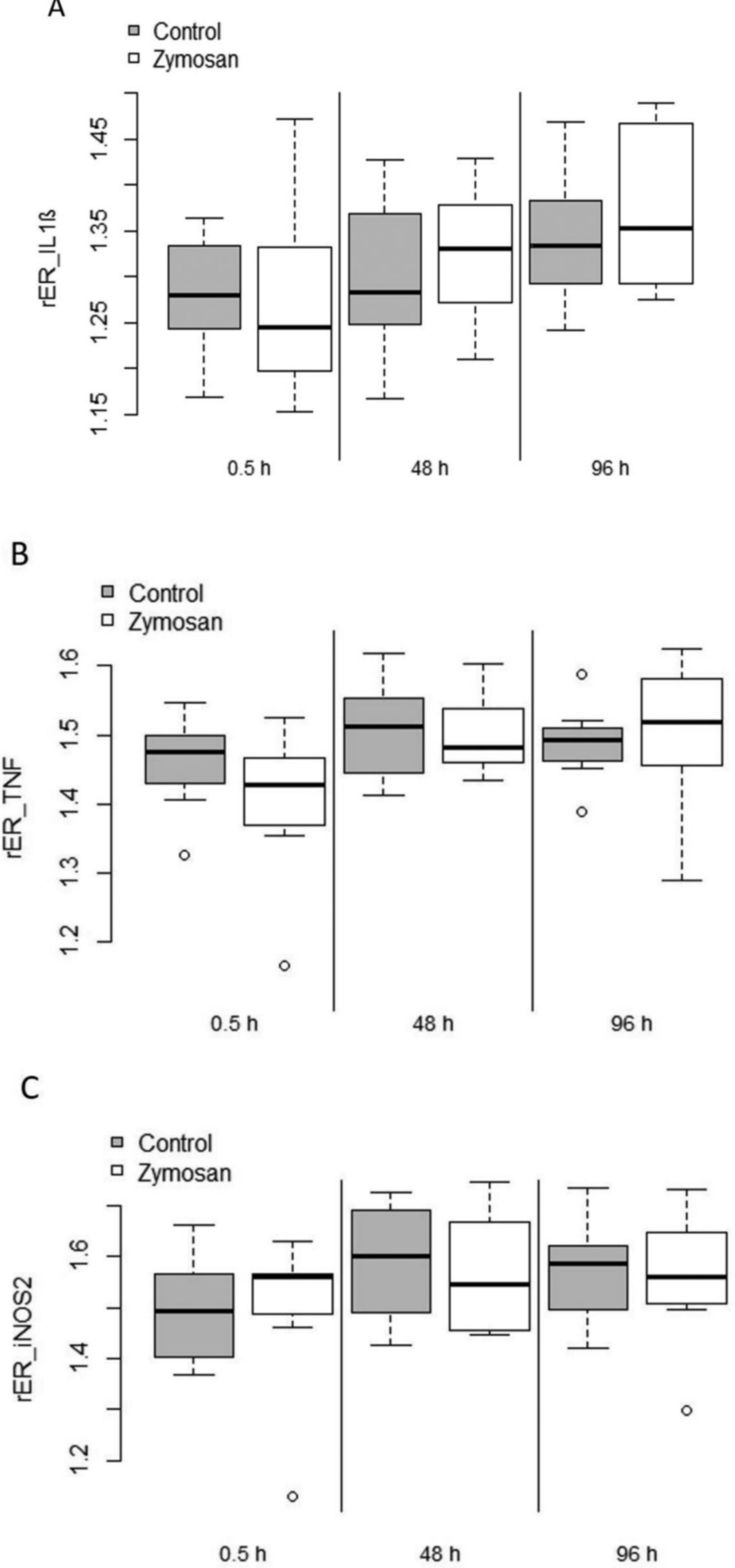


Figure 2.4: Boxplots of relative immune gene expression ratios (rER) of three different genes: IL-1β (A), TNFα (B) and iNOS2 (C), measured at three sampling days for zymosan-treated bats (white) and bats of the control group (grey). rERs are given in relation to the expression of ACTB. Upper and lower borders of boxes indicate first and

third quantiles, respectively, whiskers represent maximum and minimum, solid horizontal line within boxes shows the median. Extreme values are shown as open circles.

Haptoglobin

We detected significant differences in haptoglobin levels (square root transformed) for the relationship of treatment group and sampling day (SS=0.531, F=3.84, p=0.033). Zymosan-treated bats had higher levels at 96 h post-injection compared with bats of the control group on the same day (SE=0.192, t=-3.154, p=0.046, Tukey adjusted) (Fig. 2.5). Forearm and body mass (interaction) had no influence on the haptoglobin levels (SS=0.023, F=0.34, p=0.568).

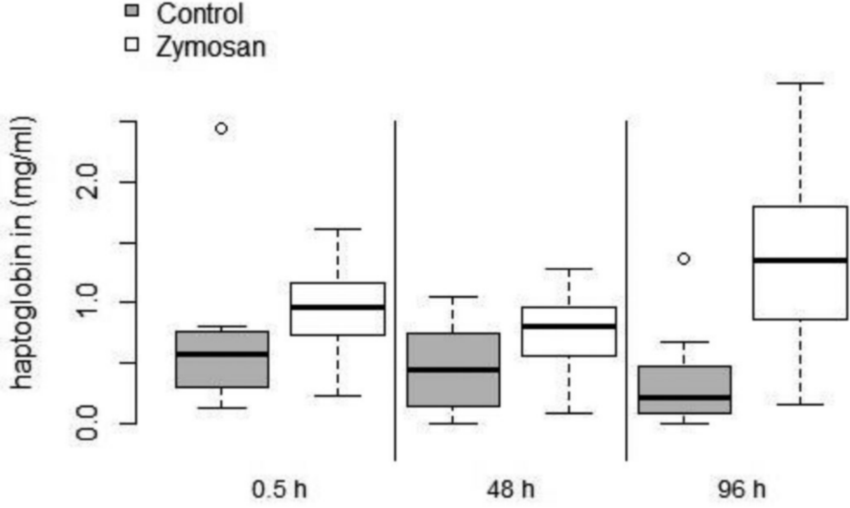
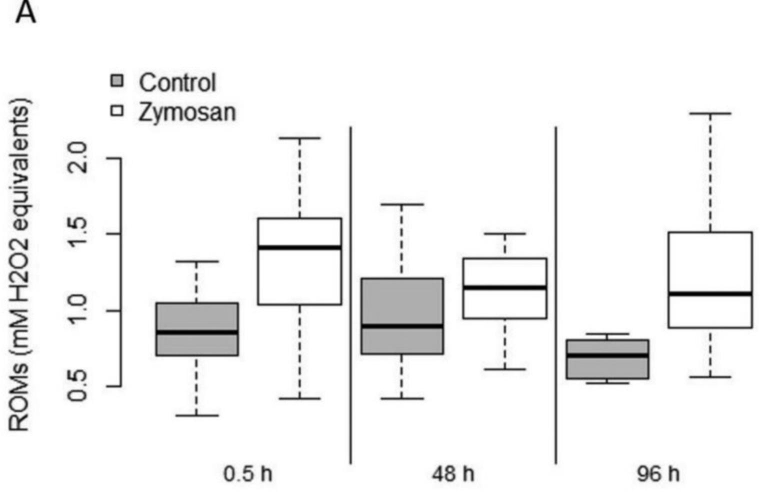


Figure 2.5: Difference in haptoglobin concentration (mg/ml) in the blood of zymosan-treated bats (white) compared with control bats (grey) at three time points of the experiment: at 30 min, 48 h and 96 h after inoculation. Upper and lower borders of boxes indicate first and third quantiles, respectively, whiskers represent maximum and minimum, solid horizontal line within boxes shows the median. Extreme values are shown as open circles.

Reactive oxygen metabolites (ROMs) and plasma non-enzymatic antioxidant barrier (OXY)

We observed higher levels of reactive oxygen metabolites (ROMs) in zymosan-treated bats compared with bats of the control group (SS=1.138, F=7.74, p=0.017), but no difference among sampling days (SS=0.190, F=0.65, p=0.531). Also, the interaction between treatment group and sampling days was not significant for ROMs (SS=0.352, F=1.20, p=0.318) (Fig. 2.6A).

There were also no differences in non-enzymatic antioxidant capacity (OXY), neither between the two treatment groups (SS=273.54, F=0.77, p=0.397), nor the sampling days (SS=653.53, F=0.92, p=0.414) nor the interaction between treatment group and days (SS=127.47, F=0.17, p=0.844) (Fig 2.6B). The interaction of forearm and body mass had no influence on both response varibles in the models (ROM: SS=0.003, F=0.02, p=0.896, OXY: SS=14.68, F=0.041, p=0.840). We observed a strong correlation between haptoglobin and ROM levels in bats of the zymosan-treated group (cor=0.630, t=3.71, p=0.001), which was not seen in bats of the control group (cor=0.216, t=1.04, p=0.312) (Fig. 2.7). One outlier was identified in zymosan-treated bats and tested for its influence on the correlation which turned out as negligible because the correlation remained significant after removing this animal (cor=0.46, t=2.18, p=0.043).



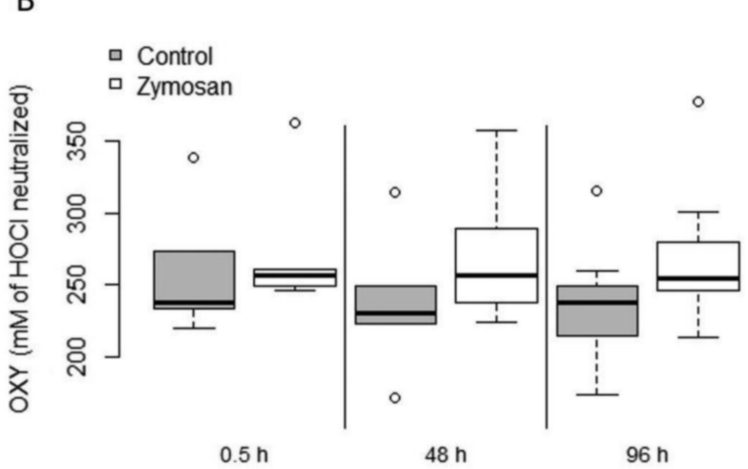


Figure 2.6: Boxplot displaying the concentration of ROMs (A) and OXY (B) in the blood of the zymosan-treated bats (white) and bats of the control group (grey) on the three sampling days. Upper and lower borders of boxes

indicate first and third quantiles, respectively, whiskers represent maximum and minimum, solid horizontal line within boxes shows the median. Extreme values are shown as open circles.

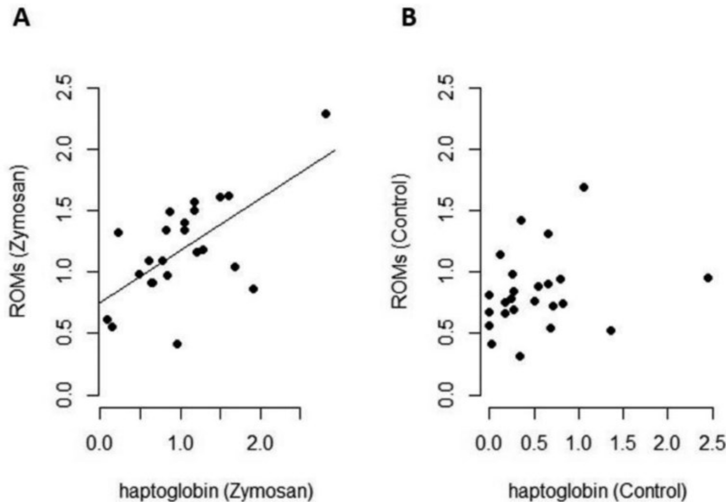


Figure 2.7: Correlations between haptoglobin (mg/ml) and ROM levels (mMol H2O2 equivalents) of zymosan-treated bats (A) and control bats (B). Haptoglobin correlated significantly with dROM in zymosan-treated bats (Pearson's rank correlation). One outlier was identified in zymosan-treated bats and tested for its influence on the correlation which turned out as negligible because the correlation after removing the outlier is still significant (cor=0.46, t=2.18, P=0.043).

Circulating white blood cells (WBC)

In both treatment groups we detected a significant increase in total WBC (SS=1.17E+13, F=3.06, p=0.047), lymphocytes (SS=2.37E+12, F=3.08, p=0.046) and monocytes (SS=1.18E+11, F=4.82, p=0.008) between sampling days. However, neither treatment group affiliation nor body condition of animals had a significant effect on WBC (table 2.3, appendix 7.1.3).

Table 2.3: Comparison of numbers of WBCs between zymosan-treated and control bats (group), between sampling days and interaction of group with sampling days (group:day), and interaction of body mass and forearm length (mass:FA) (linear mixed effect model, ANOVA with Satterthwaite's method, see also appendix. 7.1.3)

response variable	factor	SS	F-value	p-value
total number of WBC	group	6.63E+12	3.47	0.088
	day	1.17E+13	3.06	**0.047**

	group:day	1.17E+13	0.56	0.571
	mass:FA	3.81E+12	1.99	0.174
	group	1.18E+12	3.07	0.104
Lymphocytes	day	2.37E+12	3.08	**0.046**
	group:day	4.15E+11	0.54	0.582
	mass:FA	9.50E+11	2.47	0.121
	group	0.070	0.13	0.721
neutrophils (log)	day	3.193	3.03	0.063
	group:day	0.061	0.06	0.944
	mass:FA	0.000	0.01	0.977
	group	4059.000	0.39	0.545
eosinophils (sqrt)	day	52.000	0.01	0.997
	group:day	57934.000	2.78	0.079
	mass:FA	6797.000	0.65	0.431
	group	0.001	2.49	0.147
basophils (log10)	day	0.002	2.28	0.146
	group:day	0.000	0.73	0.503
	mass:FA	0.000	0.23	0.641
	group	2.59E+10	2.13	0.171
Monocytes	day	1.18E+11	4.82	**0.008**
	group:day	3.99E+10	1.64	0.195
	mass:FA	4.58E+10	3.76	0.072

2.5 Discussion

Using zymosan, a non-infectious fungal antigen, we challenged hibernating bats to induce an immune response and then followed several components of the acute phase response over a period of four days. We did not observe changes in torpor patterns, arousal frequencies or body temperatures between the two experimental groups. However, we noted an increase in

gene expression of IL-1β, iNOS2, and TNFα for both zymosan-treated and control bats. In comparison to control bats, we only detected increasing haptoglobin levels in zymosan-treated bats, indicating an acute phase response. There was no difference in the levels of plasma oxidative status markers or WBC between groups. Also, individuals of both groups lost body mass to a similar extent. Interestingly, the functional response of the innate immune system seemed to be unlinked to torpor behavior or to the regulation of body temperature. Based on all these observations, we conclude that European hibernating bats such as *M. myotis* may have evolved a hibernation-adjusted immunological response to balance the trade-off between tolerance and resistance, and thus between mounting an immune reaction and its energetic costs.

Torpor behavior, body temperatures and fever

During hibernation, immune functions of hypothermic bats are down regulated (reviewed by Bouma et al., 2010a). Our results suggest that European bats overcome this constraint by mounting only selected immune parameters such as haptoglobin during their regular arousals from torpor. Long-term data on body temperatures of hibernating European bats with infections are still lacking and thus it remains unclear if the thermoregulation of bats remains unaffected following a pathogen infection. However, over the short-term period of our experiment, we did not observe a strong effect of zymosan challenge on the torpor behavior of bats. In hibernating golden-mantled ground squirrels (*Spermophilus lateralis*) that were challenged with lipopolysaccharides (LPS), animals responded with fever during the arousal periods (Prendergast et al., 2002). Our zymosan-challenged *M. myotis* bats did not show any signs for a fever response, suggesting that bats seem to be prudent in the use of energy even when dealing with a fungal challenge in the torpid state. Interestingly, *Pd*-infected American bats exhibited fever bursts during arousals from hibernation (Mayberry et al., 2018) and bats surviving the *Pd*-epidemic in North America did not exhibit the 'frequent arousal behavior' of those that had died (Lilley et al., 2016). Thus, their behavioral pattern was more similar to that of European bat species. However, fever responses depend on the type of antigen and the level of infection or dosage (Bastos-Pereira et al., 2018); e.g. zymosan-challenged rats reacted with a fever response, but that response was dose-dependent with the dose being at least 3 mg/kg (Bastos-pereira et al., 2018). Hence, our zymosan dose (0.7 mg/kg) was likely high enough to induce an inflammatory response but might have been too low to induce a fever

response in bats. Additionally, randomly appearing arousals of our bats might have been caused by ultrasonic emissions of the iButtons (Willis et al., 2009). We found slight decreases in body mass in both treatment groups (Fig. 2.1). However, the cost of mounting an immune response may be incurred at the level of torpid metabolic rate, which was not measured (McGuire et al., 2017).

Inflammatory gene expressions

During the acute phase responses, inflammatory genes are usually secreted and the inflammatory cells are activated together with the vascular system (Gruys et al., 2005). We found increases in the expression of all three genes during the course of the experiment (IL-1β, iNOS2 and TNFα), but they were not associated with the zymosan treatment. Thus, these increases might be rather due to the general reactivation of the immune system during the arousals and do not reflect a response to inflammation. Gene expression levels may change within hours (Breen et al., 2000; Fan et al., 1998; Jiménez-Ortega et al., 2009) and hence it could be that we were not able to detect differences between groups due to the time points of blood sampling (Jilma-Stohlawetz et al., 2017). Additionally, the interpretation of the gene expression results is hampered by the fact that we are lacking data on baseline levels. Additionally, measured RIN values suggest some decay of the RNA resulting from a one-year-storage of the RNA extracts. However, because all samples had been collected and extracted simultaneously and RNA extracts were stored at -80°C, a linear and uniform degradation rate of the isolated RNA can be assumed for all samples (Gallego Romero et al., 2014). Although these results have to be viewed cautiously, they fit into the picture of an adjusted, moderate immune response with genes becoming active, but not strongly expressed during arousal. Additionally, our observation confirms a recent study comparing *Pseudogymnoascus destructans* infected *Myotis lucifugus* and *M. myotis* showing an absence of immune gene expression in infected tissue of *M. myotis* (Lilley et al., 2019).

Haptoglobin & oxidative stress

Contrasting the findings from a zymosan-challenge experiment on house sparrows (Coon et al., 2011), we measured higher levels of haptoglobin in zymosan-treated bats compared with control bats during hibernation. Haptoglobin is a hemoglobin-binding protein whose concentration is rapidly increased during acute phase responses in systemic infections (Cray et al., 2009). A study in bears has shown that haptoglobin levels are generally elevated in the

late phase of hibernation (Mominoki et al., 2005), supporting our results for its important immunological function in hibernating mammals. The differential regulation of immunity-related proteins such as haptoglobin may be one adaptation during hibernation that allows mammals to remain in their hypometabolic and hypothermic state, while aiding in the maintenance of immune competence and resistance against infections and diseases (Chow et al., 2013). Acute phase proteins are part of the innate immune system and represent an early-defense, which is immediately activated by inflammation (Cray et al., 2009) and effective for pathogen elimination (Bertaggia et al., 2014; Eaton et al., 1982; Langlois and Delanghe, 1996). Thus, the increased haptoglobin concentration in zymosan-treated bats is reflecting an inflammatory response to the fungal antigen. However, we could not directly show the link between haptoglobin increases and gene expression. Since haptoglobin can be mediated by different immune genes such as interleukin-6-type cytokines and is synergistically enhanced by glucocorticoids (Wang et al., 2001), we might have missed the detection due to our restriction on using only published primers (Kacprzyk et al., 2017).

Besides its function during an acute phase response, haptoglobin also prevents oxidative stress (Bertaggia et al., 2014; Gutteridge, 1987; Schaer et al., 2013; Tseng et al., 2004). We measured higher ROM levels in zymosan-treated bats, but these levels were already higher right from the first sampling and did not show further increases during the following days, which renders the interpretation difficult. It might be that the concentration of ROMs is linked to haptoglobin and haptoglobin inhibited the increases due to its anti-oxidant effect (Sauerwein et al. 2005; van de Crommenacker et al. 2010; Corsetti et al. 2018). It is therefore conceivable that an increase in ROMs took place during the first arousal early in the experiment as a response against the antigen. During the following days any further increase may have been inhibited by haptoglobin. Consistent with this scenario, we found a significant correlation between ROMs and haptoglobin in zymosan-treated bats. However, the strength of the interaction between the inflammatory and some oxidative pathways is generally weak (Costantini et al. 2015; Sebastiano et al. 2018) and we are unsure if ROM concentrations can could really increase during arousal events within a short period of 30 min (first measurement after pathogen challenge in our study in bats).

Circulating WBC

Neutrophils and macrophages are fundamentally important antifungal effector cells (Shoham and Levitz, 2005). During hibernation, when the body temperature is drastically lowered, the concentration of circulating WBC is reduced by up to 90% (Bouma et al., 2010a). However, as shown in ground squirrels, leucocyte levels can be restored during an arousal within 1.5 h (Bouma et al., 2010b). In our experiment, the first blood sample was taken 30 min after the arousal had been induced. Hence, it is possible that the time point of measurement after arousal initiation was too early to detect the full increase in the number of leukocytes. However, we measured increases in numbers of certain leukocyte types during the course of the experiment, suggesting an accumulation of leucocytes with increasing numbers of arousals. Nevertheless, we did not find differences between zymosan-treated and control bats, neither in the total number of WBC, nor in the numbers of specific WBC types. Therefore, we suggest that these increases are a regular response to the arousals (and thus reflect a general boost of "defense status") but are not related to the immune challenge. We argue that during later arousals or even after hibernation, macrophages and neutrophils may even be further elevated in infected bats and that our results may not have covered the full immune response during the five days of the experimental period. Overall, an increase in WBC is generally delayed during an infection compared with acute phase proteins such as haptoglobin (Cray et al., 2009).

2.6 Conclusion

Our study suggests the presence of an adjusted immune function during hibernation as a strategy to retain immune competence while at a state of low metabolic activity. Both zymosan-treated and control bats lost approximately the same amount of body mass and did not differ in torpor patterns over the course of the experiment. However, zymosan bats mounted an acute phase response represented by haptoglobin increases but without increasing arousal frequencies and durations or maximal Tsk during arousal. This contrasts with previous studies suggesting that hibernating mammals have to increase their arousal frequency in order to spend a sufficient amount of time in euthermic conditions or even show fever during arousals to mount an immune response (Bouma et al., 2010a; Carey et al., 2003; Luis and Hudson, 2006; Prendergast et al., 2002; Warnecke et al., 2012). Arousals, fever and

general cellular immune responses are energetically costly as they act systemically. In comparison, the release of haptoglobin and pro-oxidants is much less costly yet still very effective against pathogens as they can be released by immune cells in different tissues. Hence, the adjustment of the immune response during hibernation could be characterized by investing only into those components which are both effective and fast. The efficacy of this immediate response could keep pathogens at bay until they can be fully cleared. The latter may then happen under euthermic conditions during later arousals or after hibernation in spring. From an energy saving point of view, a specialized first line defense based on the innate immune response, including an inflammatory response, is advantageous over a full immune response as long as the pathogen can be confined. In contrast, the activation of immune compounds such as WBC and components of the adaptive immune system as shown in North American bats with white-nose syndrome may require the accelerated consumption of limited energy reserves without the subsequent benefits of pathogen clearance (Moore et al., 2013).

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3 Chapter Two - Immune response of hibernating European bats towards *Pseudogymnoascus destructans* during torpor

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Determinants of defence strategies of a hibernating European bat species towards the fungal pathogen *Pseudogymnoascus destructans*

by

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3.1 Abstract

Pseudogymnoascus destructans (Pd), the causative agent of white-nose syndrome in North America, has decimated bat populations within a decade. The fungus impacts bats during hibernation when physiological functions, including immune responses, are down-regulated. Studies have shown that *Pd* is native to Europe, where it is not associated with mass mortalities. Moreover, genomic and proteomic studies indicated that European bats may have evolved an effective immune defence, which is lacking in North American bats. However, it is still unclear which defence strategy enables European bats to cope with the pathogen. Here, we analysed selected physiological and immunological parameters in torpid, *Pd* infected European greater mouse-eared bats (*Myotis myotis*) showing three different levels of infection (asymptomatic, mild and severe symptoms). From a subset of the studied bats we tracked skin temperatures during one month of hibernation. Contrasting North American bats, arousal patterns remained unaffected by *Pd* infections in *M. myotis*. In general, heavier *M. myotis* aroused more often from hibernation and showed less severe disease symptoms than lean individuals; most likely because heavy bats were capable of reducing the *Pd* load more effectively than lean individuals. In the blood of severely infected bats, we found higher gene expression levels of an inflammatory cytokine (IL-1β), but lower levels of an acute phase protein (haptoglobin), reactive oxygen metabolites (ROMs) and plasma non-enzymatic

antioxidant capacity (OXY) compared to conspecifics with lower levels of infection. We conclude that *M. myotis*, and possibly also other European bat species, tolerate *Pd* infections during torpor by using selected acute phase response parameters at baseline levels, yet without arousing from torpor and without synthesizing additional immune molecules.

3.2 Introduction

Emerging fungal diseases are considered an important threat for wildlife, causing local extirpations of affected animal populations or even the extinction of species. Prominent examples include the decline of amphibian species by chytridiomycosis (O'Hanlon et al., 2018), the emerging threat from the 'snake fungal disease' (Lorch et al., 2016) and the high death toll of North American bats by white-nose syndrome (WNS) (Frick et al., 2015).In the latter case, the cold-loving ascomycete *Pseudogymnoascus destructans* (*Pd*) infects bats during hibernation (Meteyer et al., 2009), a physiological state in which most physiological functions including immune responses are down- regulated (Bouma et al., 2010). The fungus colonizes naked body parts such as wings, ears, and nose and in heavily infected animals, the fungus causes cupping erosions and severe lesions (Cryan et al., 2010). Infected North American bats arouse from hibernation, most likely to resist the infection by cleaning of the superficial fungus and by mounting an innate and adaptive immune response at normothermic temperatures (Field et al., 2015; Hecht-Höger et al., 2020; Lilley et al., 2017; Moore et al., 2013). These resistance responses are accompanied by increased arousal frequencies and lead to prematurely depleted fat stores before the end of the winter period, which ultimately results in death (Verant et al., 2014; Warnecke et al., 2012).

Contrasting to bats in North America, bats infected by *Pd* in Europe do not seem to exhibit abnormal wintering behavior or die because of the *Pd* infection (Fritze and Puechmaille, 2018; Puechmaille et al., 2011; Zukal et al., 2016). Recent molecular studies indicated that *Pd* is native to Eurasia, suggesting a recent introduction to and spread of this pathogen throughout its actual range in North America (Drees et al., 2017; Leopardi et al., 2015; Zukal et al., 2016). Molecular investigations suggest that European bats might have evolved an immunological defence strategy by tolerating low levels of *Pd* infections (Harazim et al., 2018; Hecht-Höger et al., 2020; Lilley et al., 2019). Interestingly, European bats do neither show a cellular immune response (Bandouchova et al., 2018), nor an antibody-

mediated adaptive immune response to the infection (Johnson et al., 2015). Therefore, it is still unknown which immune mechanisms enable European bats to cope with the infections. Moreover, all previous studies compared healthy with colonized bats without taking into the consideration the time elapsed since infection, fungal load or severity of the colonization with the fungus (Bandouchova et al., 2018; Hecht-Höger et al., 2020; but see Harazim et al., 2018). Particularly, immunological studies have not focused on the humoral aspect of the induced and constitutive innate immunity, including the acute phase response (APR) towards *Pd* infections. Yet, such studies would be important because innate immunity represents the first line of defense against pathogen invasions (Tizard, 2008).

During an infection, macrophages recognize and phagocytose the pathogen and release cytokines in order to induce an APR, which includes increase in white blood cell counts (leukocytosis), production of acute phase proteins (APPs), fever, loss in body weight and sickness behavior (Tizard, 2008). Because the production of APPs and other anti-microbial substances does not involve large energetic costs (Nijsten et al., 2000; Skovgaard et al., 2009), these substances may play a central role in the immune defense during hibernation (Fritze et al., 2019; Maniero, 2002), a period in which bats have to be prudent in the use of fat stores. Haptoglobin is an effective APP and it is suggested to be key for the immune defense of hibernating animals (Chow et al., 2013; Mominoki et al., 2005). Although mainly synthesized in the liver, haptoglobin is also a granule protein of macrophages and neutrophils (Theilgaard-Monch, 2006) and the observation of neutrophil infiltration in Pd lesions of bats (Meteyer et al., 2011; Wibbelt et al., 2013b) suggests a participation of haptoglobin in the defence against Pd. Additionally, immune cells release cytotoxic chemicals with pro-oxidant activity during APR, which damage pathogens by oxidative lysis (Costantini, 2014). The release of pro-oxidants might be particularly effective when metabolic functions are suppressed during hibernation.

However, the drawback of releasing pro-oxidants can be an increased oxidative stress, which might damage the host cells and can even constrain immune responses (Costantini and Møller, 2009). Therefore, excess of pro-oxidants needs to be balanced by adequate levels of antioxidants. In North American bats, oxidative stress was identified as a contributor to the high mortality rate of WNS (Moore et al., 2013a). However, it is unknown so far whether *Pd*

infections cause oxidative stress in European bats or if the release of pro- and antioxidants plays a role during an immune defense against *Pd*.

Immune responses are energy-demanding and occur usually at normothermic body conditions while during torpor, immune reactions are suppressed (Bouma et al., 2010; Hildebrand et al., 2005). Regular arousals from torpor are known to be important during hibernation in order to reactivate parts of the immune system to clear accumulated pathogens from the body (Prendergast et al., 2002). However, bats should be thrifty in arousing from hibernation because arousal events are energetically costly (Humphries et al., 2003). Increased arousal frequencies lead to starvation and death in *Pd* infected North American bats (Reeder et al., 2012). North American bats surviving WNS did not show increased arousal patterns (Lilley et al., 2016). For European bats, the link between arousal frequencies and the level of *Pd* infection is still unclear. Therefore, we were interested in studying the interplay between arousal frequencies, innate immunological and oxidative stress parameters and the disease status in European bats, using the greater mouse-eared bat (*Myotis myotis*) as a model.

Specifically, we asked which mechanisms enable hibernating European bats to tolerate or resist *Pd* infections, taking into account the disease severity. By definition, tolerance is the ability to limit the negative impact of an infection on the hosts fitness without directly affecting the pathogen burden (Medzhitov et al., 2012; Schneider and Ayres, 2008). The tolerance of European bats towards *Pd* should therefore be characterized by mechanisms that limit the damage caused by infections without showing energetically costly responses such as increased arousal frequencies or full immune responses. Instead, bats may show hibernation adjusted immune responses as shown in previous work (Fritze et al., 2019) or even show limited immune reactions during torpor to keep pathogens at bay until they can be fully cleared after the hibernation period. Alternatively, resistance is the ability to reduce the pathogen burden (Medzhitov et al., 2012; Schneider and Ayres, 2008). Hence, resistance of bats towards *Pd* should be characterized by responses that directly affect Pd load. In this case, infected individuals are expected to increase their frequency of arousals, during which they reduce fungal load by grooming off the superficial fungus and mount a full immune response to completely clear the pathogen, which would be energetically costly and ultimately would

reduce the fat stores and thus the weight of hibernating bats as observed in North American bats (Warnecke et al., 2012).

We monitored the skin temperature of hibernating bats at different *Pd* infection stages with temperature data loggers and compared body weights to see if the arousal behavior affected the disease severity and body condition. To investigate whether *Pd* infected European bats can show an immune response during torpor, we measured selected immune parameters, both constitutive and induced innate response markers and compared between asymptomatic bats, mildly infected bats (early infection stage of superficial *Pd* colonization) and severely infected bats (late infection stage with lesions caused by *Pd* invasion into the skin). Additionally, we asked if *Pd* infection would affect levels of blood-based markers of oxidative damage and anti-oxidant defenses and discussed whether these markers were associated with the immunity.

3.3 Material and methods

Field work

During the winter months of 2016-2018, we studied greater mouse-eared bats (*Myotis myotis*) at 17 hibernation sites in Germany, in the federal states of Bavaria (Franconia) and Saxony-Anhalt (Harz Mountains) under permits issued by the respective federal animal and ethics committees (permit-nr.: Saxony-Anhalt: 505.6.3-42502-9-019 LIZW; Bavaria: 55.2 2532-2-235) and the conservation authorities (permit-nr.: Harz: Sch3058, Franconia: RMF-SG55.1-8646-2-185-4). In total, 61 bats were included in our study (n=18 in 2016, n=35 in 2017, and n=8 in 2018). Approximately one month before sampling, we attached temperature loggers (iButtons DS1921G, modified by Telemetry Service Dessau, Germany) to a subset of 10 bats without visible infection symptoms by using skin bond latex adhesive (Osto-Bond, Montreal, Canada). The loggers were similar to those loggers reported from North American studies (Reeder et al., 2012) and were used in 3 hibernation sites in the Harz Mountains (n=4 in 2017 and n=6 in 2018). To reduce disturbance, we attached one logger within seconds at each of the selected bats while they were remaining torpid, i.e. without removing or handling the bat.

Assessment of disease severity

Before we sampled bats, we assessed the level of infection visually by using a similar classification scheme as described before (Fritze et al., 2012, Fritze et al., in review) (appendix 7.2.5) and selected individuals that corresponded to different infection levels (no visual fungal infection, first signs or moderately progressed *Pd* colonization and severely progressed *Pd* colonization). Afterwards, we estimated the number of lesions by UV transillumination of wing membranes of the selected bats to assess the damage caused by *Pd*. Lesions were identified by a hand held ultraviolet lamp and visually counted (Turner et al., 2014a) on both wings. We established a white-nose index (WNI) for each bat by classifying the disease severity of bats based on both fungal colonization of bats and the damage shown by lesions. We assigned each of the 61 bats (31 males, 30 females) to one of three categories of infection severity: asymptomatic bats (no visible fungal growth and no lesions detected, n=17 (9 males and 8 females)), bats with mild infection (fungal hyphae on bats and a maximum of one lesion, n= 24 (11 males, 13 females)) and bats with severe infections (fungal growth and between 10 and 300 lesions, n= 20 (11 males, 9 females)).

Sampling procedure

In March, animals were picked by hand from the walls of the hibernacula. We documented visual signs of infection and collected swab samples for molecular verification of our visual classification and further work not included here. We measured body mass with an electronic scale (0.01g accuracy, G&G, Kaarst, Germany) and forearm length with a manual caliper (0.01 mm accuracy). Previous immunological studies on *Pd* infected bats have been conducted on normothermic, aroused bats, e.g. (Field et al., 2015; Lilley et al., 2019; but see Hecht-Höger et al., 2020). Therefore, we aimed to investigate how the immune system is reacting under torpid conditions. To avoid arousal during bleeding, torpid animals were kept under isoflurane anaesthesia. We collected 100 µl of blood from 61 bats with a sterile insulin syringe from the aortic arch (from three bats we were not able to get blood) and mixed the blood with heparin in micro haematocrit tubes (BRAND, Wertheim). Approximately 10 µl of the sample was used for hematology, while the remaining blood was centrifuged to separate plasma and blood pellet (white blood cells, platelets and erythrocytes) and the samples were stored at -80°C until further analysis. All but one bat had been released at the site of capture after sampling was finished. We did not observe fatalities in hibernacula during days following bleeding and

~30 % of the animals were recovered over the winters 2018-2020 during sporadic hibernacula visits (Fritze, unpublished data).

Induced innate humoral immunity

We measured inflammatory cytokine expressions to detect whether an innate immune response is induced by *Pd* infections. We extracted RNA from the blood pellet by using NucleoSpin® (Macherey & Nagel GmbH & Co. KG, Düren) following the manufacturer's instructions. We synthesized cDNA by using the 'RevertAid H Minus First Strand cDNA Synthesis Kit' (ThermoFisher Scientific, Waltham, USA) according to the manufacturer's instructions. For the qPCR, we used bat specific primers for actin Beta (ACTB, housekeeping gene), interleukin 1-Beta (IL-1β), inducible nitric oxide synthase (iNOS2) and tumor necrosis factor (TNF) as described in previous work (Fritze et al., 2019; Kacprzyk et al., 2017) (appendix 7.2.1). The qPCR was carried out on a CFX96 cycler (Bio-Rad, Munich, Germany) using the 'Real Time PCR iQ SYBR Green Supermix' kit (Bio Rad, Hercules, USA) as described in previous work (Fritze et al., 2019). We optimized qPCR conditions prior to all measurement runs by using cDNA that had been reversely transcribed from RNA extracted from an independent *M. myotis* individual. Quality of all qPCR runs was assessed by melt-curve analysis after each run. Runs were considered successful if the PCR products generated a single-peak melting curve well separated from primer-dimers. To assess RNA integrity, RIN values were assigned to the extracted total RNAs on a 2200 TapeStation (Agilent) according to the manufacturer's instructions. The mean of RIN values of RNA samples was 2.3 (SD=2.4) for sampling year 2016, 1.9 (SD=1.2) for 2017 and 2.2 (SD=0.3) for 2018 (full table available in appendix 7.2.2).

Haptoglobin is an APP which is produced and secreted during an induced immune response to infection and trauma (Cray et al., 2009). To measure the concentration of haptoglobin, we followed the standard procedure of the commercial kit "PHASE" TM Haptoglobin Assay (Cat. No. TP-801, Tridelta, Maynooth, Ireland) as previously described (Fritze et al., 2019).

We measured plasma reactive oxygen metabolites (ROMs) and plasma non-enzymatic antioxidant capacity (OXY) which are two markers of oxidative status associated with induced immune responses (Costantini and Møller, 2009). ROMs were determined using the d-ROMs test (Diacron International, Grosseto, Italy) and OXY was quantified using the OXY-adsorbent test (Diacron International) as described in (Fritze et al., 2019).

Constitutive innate humoral immunity

Lysozyme is an antibacterial enzyme active mainly against gram-positive, but also gram-negative bacteria and fungi and is part of the constitutive innate immune system. We measured the lysozyme concentration in plasma following the micro-version of the lyso-plate method described by Costantini et al. (2019).

Bacterial killing activity (BKA) is an integrated measure for the function of the constitutive innate immune system. This method has been used in a wide variety of wildlife species, including bats (Schneeberger et al., 2014). We assessed the BKA of plasma against *Escherichia coli* ATCC 8739 following a previously published protocol for bats (Schneeberger et al., 2014). The only difference compared to the previous assay was that the bat plasma samples were diluted 1:50 in sterile PBS compared with the previous 1:20 dilution.

Statistics

To analyze arousal patterns of our subset of bats carrying temperature data loggers, we identified torpor and arousal events by changes of skin temperature (T_{sk}) recorded at 30-min intervals. Mean temperatures during torpor (henceforth: mean torpor temperature), mean temperatures during arousal (mean arousal temperature) and maximum temperatures during arousals (maximum arousal temperature) were obtained from individual T_{sk} records. Arousals were identified by a T_{sk} increase of at least 10° C (Lilley et al., 2016). Torpor was determined by T_{sk} falling below 10°C for at least 2 hours. Since recording periods differed between individuals, the numbers of arousals were divided by the number of recorded days. To analyze which physiological and immunological parameters are correlated with the different variables of the arousal patterns, we conducted Spearman's rank correlations.

To analyze immunological parameters and compare the levels between disease stages (classified by WNI), we used linear models which were analyzed by ANOVA. For all linear models, response variables were tested for confirmation to normality using a Shapiro-Wilk test. Non-normally distributed data were transformed per square root (sqrt), logarithm 2 (log), Tukey's ladder of Powers (Ttuk) and cube root transformation (Tcub) into normal distributions (for further details see results). Body mass was analyzed by a linear model with WNI, forearm, sex, year and site as covariates. Immune and oxidative response parameters of the blood (rER-values, concentrations of Hp, lysozyme, BKA, ROMs, OXY) were analyzed by linear mixed effect models (lmer) fit by maximum likelihood using Satterthwaite's method (lmerTest package in

R v. 3.5.1, Kuznetsova et al., 2019). As covariates our models, WNI, body mass and forearm length (the latter two in interaction to correct for allometry) and sex were set as fixed effects and site was used as a random effect. Covariates were scaled using the standardize function of 'arm' package (Gelman, 2008) and p values were adjusted for multiple comparisons by using Tukey's method in 'multcomp' package (Hothorn et al., 2019). To find potential interactions between measured parameters, we mapped a correlogram ('corrplot' package, based on Spearman's rank correlations) (Wei et al., 2017).

3.4 Results

Body mass and arousals in relation to disease stages

We found a significant positive correlation between body mass and number of arousals in the 10 bats equipped with temperature data loggers (Fig. 3.1, 3.2A, appendix 7.2.2; rho=0.65, t=-2.8, p=0.043). Bats that aroused more often had fewer lesions (Fig. 3.2B, rho=-0.71, t=2.4, p=0.023).

Among all bats sampled, we did not find a correlation between body mass and number of lesions (Df=58, rho=0.02, t=0.12, p=0.907). This absence of correlation between body mass and disease severity was also confirmed in our linear mixed effect model analysing three disease stages. Asymptomatic bats (n=17) had a lower body mass than mildly infected bats (n=24) (n=24; SE= 0.70, t=2.44, p=0.049, Tukey corrected) but severely infected bats (n=20) did not differ in body mass from asymptomatic bats (SE=0.90, t=1.71, p=0.211) and mildly infected bats (SE=0.82, t=-0.19, p=0.980) (Fig. 3.2C). We did not find differences in body mass across sampling years (SE=1.44, t=-0.55, p=0.584) and sites (SS=44.8, F=0.85, p=0.628).

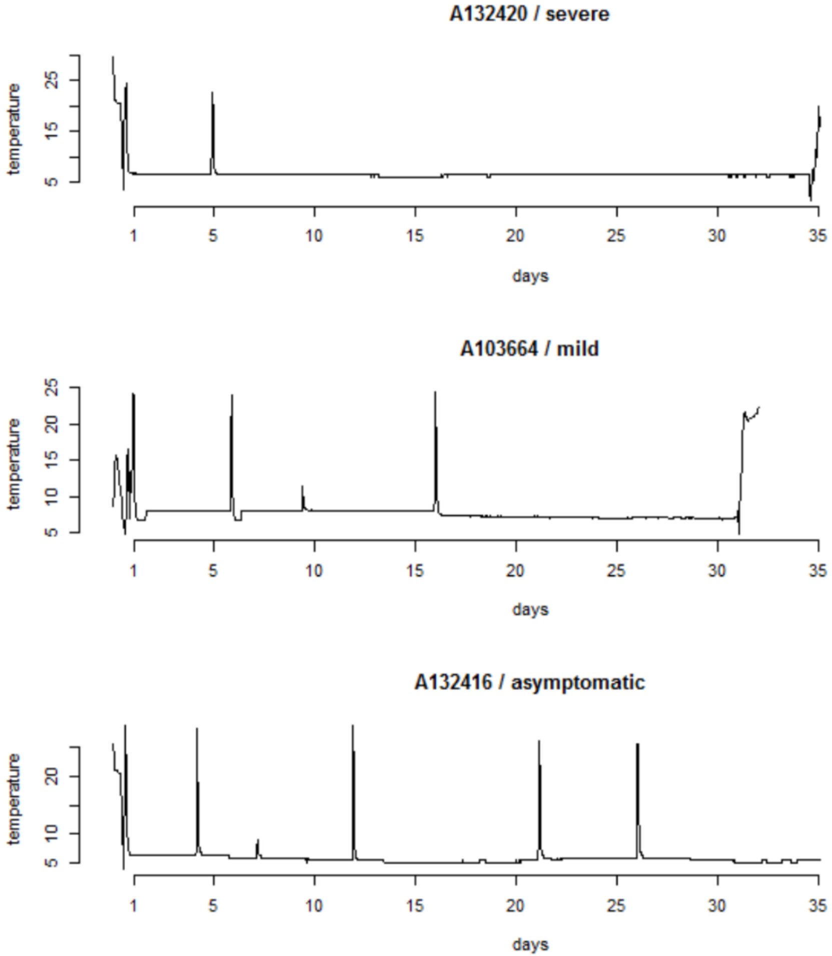

Fig. 3.1: Representative temperature profiles of bats with different white-nose indexes assessed as 'severely' infected (Ring No. A132420), with mild infection (A103664) and asymptomatic bat (A132416). Peaks represent temperature increases >10°C equating arousals from torpor; the first end the last arousals are due to the bat handling, not in the last profile (A132416) the last arousal is missing because the recording time was longer than 35 days.

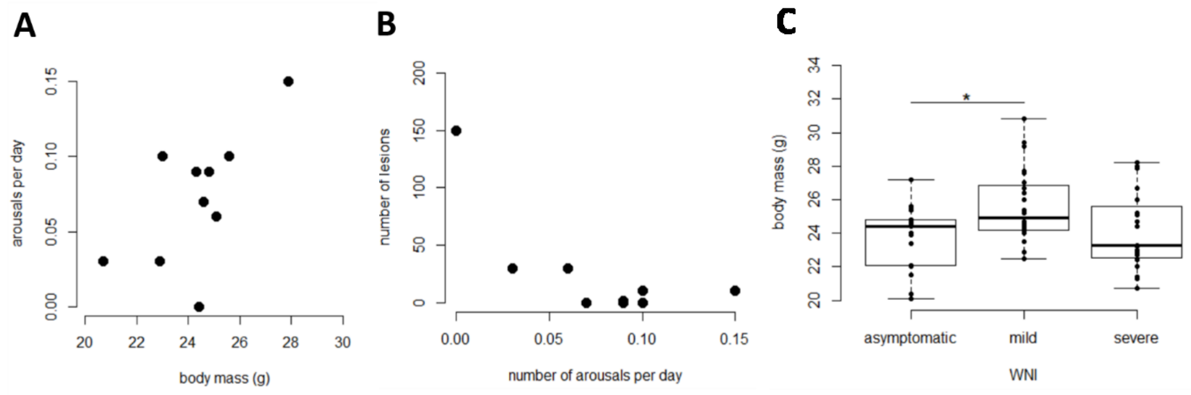

Fig 3.2. Body mass and arousals in relation to disease stages in European greater mouse-eared bats *Myotis myotis*. A) Correlation between body mass (g) and arousal frequency (arousals per day) (rho=0.65, p=0.043, Pearson's product moment correlation) measured in 10 bats equipped with temperature loggers. B) Relationship

between arousals (arousal per day) and lesions caused by *Pseudogymnoascus destructans* (rho=-0.71, p=0.023) measured in 10 bats equipped with temperature loggers. C) Comparison of body mass of bats infected with *Pseudogymnoascus destructans* in different severity stages (WNI). Asterisk indicates statistically significant difference of the linear model (n=61, p=0.049).

Induced innate immunity levels

Severely infected bats showed higher levels of IL-1β gene expression than asymptomatic bats (SE=0.10, t=2.50, p=0.033), while levels of IL-1β expression of mildly infected bats were not significantly different from those of the other two groups (Fig. 3.3 A, SE=0.05, t=1.29, p=0.4). We found a similar pattern for the expression of iNOS2, however, there was only a trend for higher levels of iNOS2 gene expression in severely infected compared with asymptomatic bats (Fig. 3.3 B, SE=0.05, t=1.79, p=0.082); levels of iNOS2 gene expression were not different between mildly infected and the two other categories (Fig. 3.3, SE=0.04, t=0.95, p=0.351). The expression of TNF did not vary between asymptomatic bats and mildly infected (SE=0.04, t=0.95, p=0.353) and severely infected bats (Fig. 3.3 C, SE=0.04, t=1.10, p=0.283). We observed higher haptoglobin (Hp) levels (Tcub transformed) in asymptomatic bats than in severely infected bats (Fig. 3.4A, SE=-0.36, F=-2.96, p= 0.008). Hp levels in mildly infected bats were intermediate and not different from those of asymptomatic (SE=0.12, t=-1.11, p=0.506) and severely infected bats (SE=-0.22, t=-1.92, p=0.132).

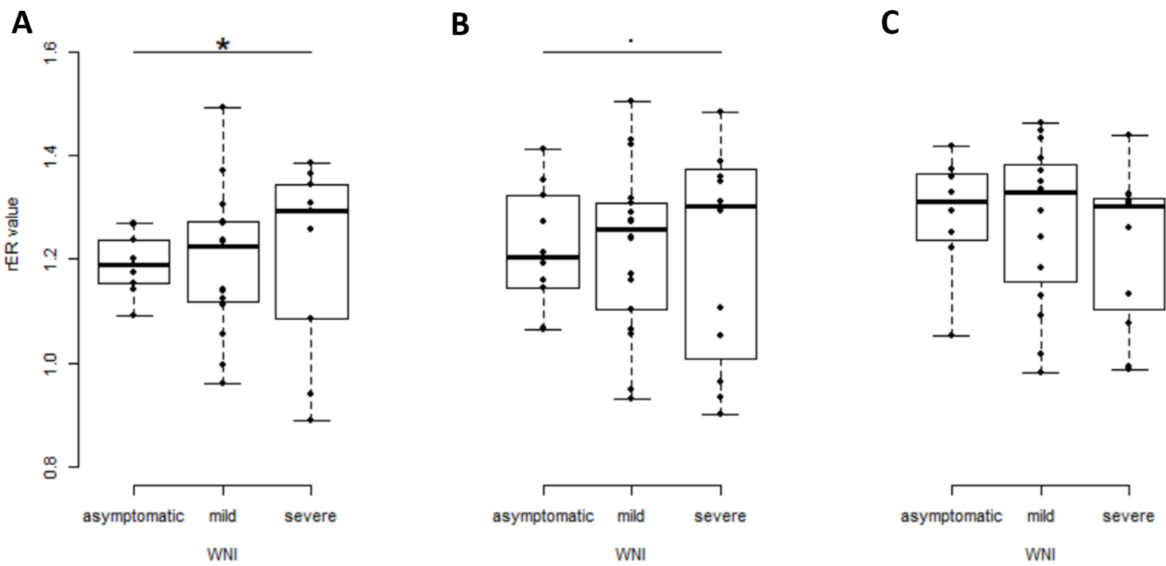

Fig. 3.3. Comparison of gene expression (rER values) of A) IL-1β, B) iNOS2 and C) TNF between asymptomatic, mildly and severely infected bat; asterisk is marking the significant difference of the linear mixed effect models and dots marking a trend in the difference between asymptomatic and severely infected bats.

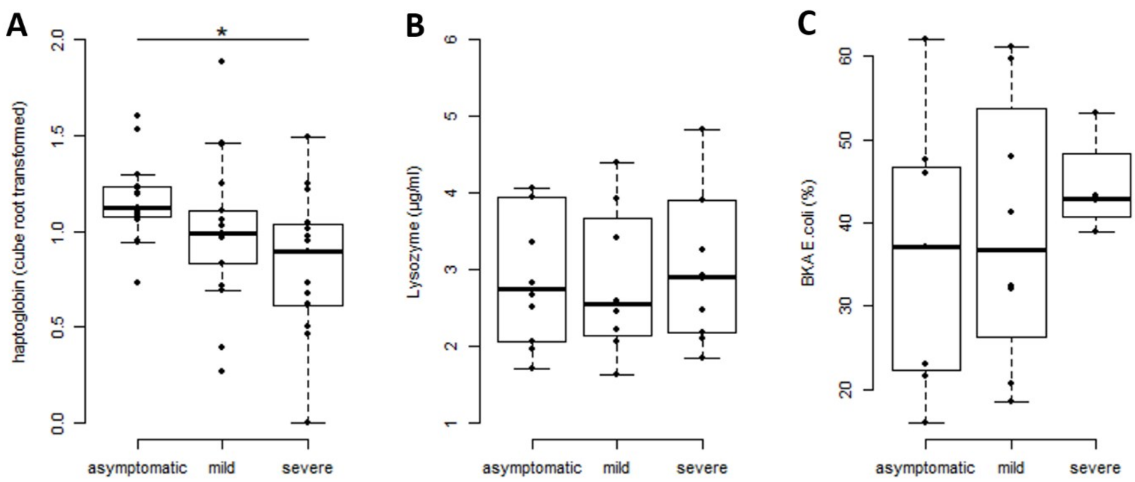

Fig. 3.4. Comparison of innate immune markers of bats infected with *Pseudogymnoascus destructans* in different severity stages (WNI) according to a linear mixed effect models. A) Haptoglobin levels (mg/ml) are significant higher in asymptomatic bats compared to severely infected bats. B) Lysozyme levels between asymptomatic, mildly and severely infected bat; no significant difference (outliers: 11.25, 11.25 and 7.79 were removed from plot and test for its influence on the calculation which turned out as negligible). C) Comparison of BKA levels between asymptomatic, mildly and severely infected bat; no significant difference.

Severely infected bats had lower levels of ROMs (sqrt) than asymptomatic bats (Fig. 3.5A; SE=0.07, t=-2.79, p=0.015). ROM levels of mildly infected bats tended to deviate from those of severely infected bats (SE=0.06, t=-2.2, p=0.070) but not from those of asymptomatic bats (SE=0.06, t=-0.73, p=0.745). For OXY, we documented lower levels in severely infected bats than in asymptomatic bats (Fig. 3.5B, SE=14.47, t=-3.48, p=0.001) and in mildly infected bats (SE=13.03, t=-2.57, p=0.028). Additionally, we found some sex-specific differences. Severely infected males had lower ROM levels than females of the same infection stage (W= 5.5, p= 0.040) and severely infected females tended to have lower OXY levels than severely infected males (W=50, p=0.055).

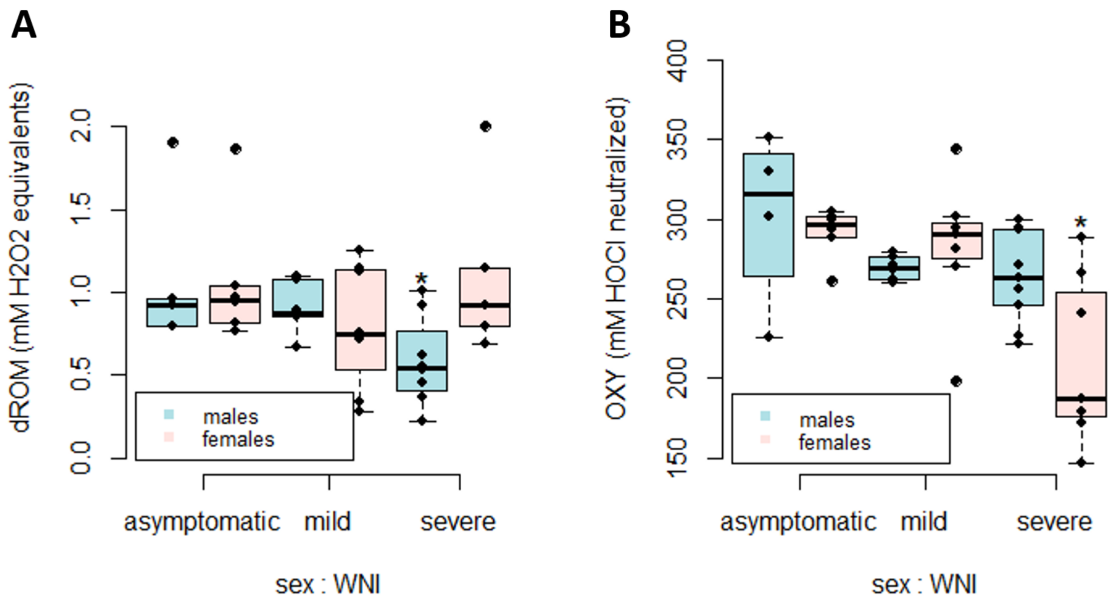

Fig. 3.5: Comparison of oxidative stress markers (A: ROM levels; B: OXY levels) of bats infected with *Pseudogymnoascus destructans* in different severity stages (WNI) according to the linear mixed effect models; blue boxes represent levels of males; red boxes represent levels of females. For both markers we found significant differences between sexes, but only for severely infected bats.

Constitutive innate immunity levels

We did not observe differences in lysozyme levels (Ttuk transformed) in asymptomatic bats compared to mildly infected (SE=0.06, t=0.35, p=0.732) and severely infected bats (SE=0.06, t=0.38, p=0.704, Fig. 3.4B). Also, we did not find differences in BKA between asymptomatic bats and mildly infected (SE=5.88, t=0.02, p=0.984) and severely infected bats (SE=7.39, t=0.17, p=0.864, Fig. 3.4C).

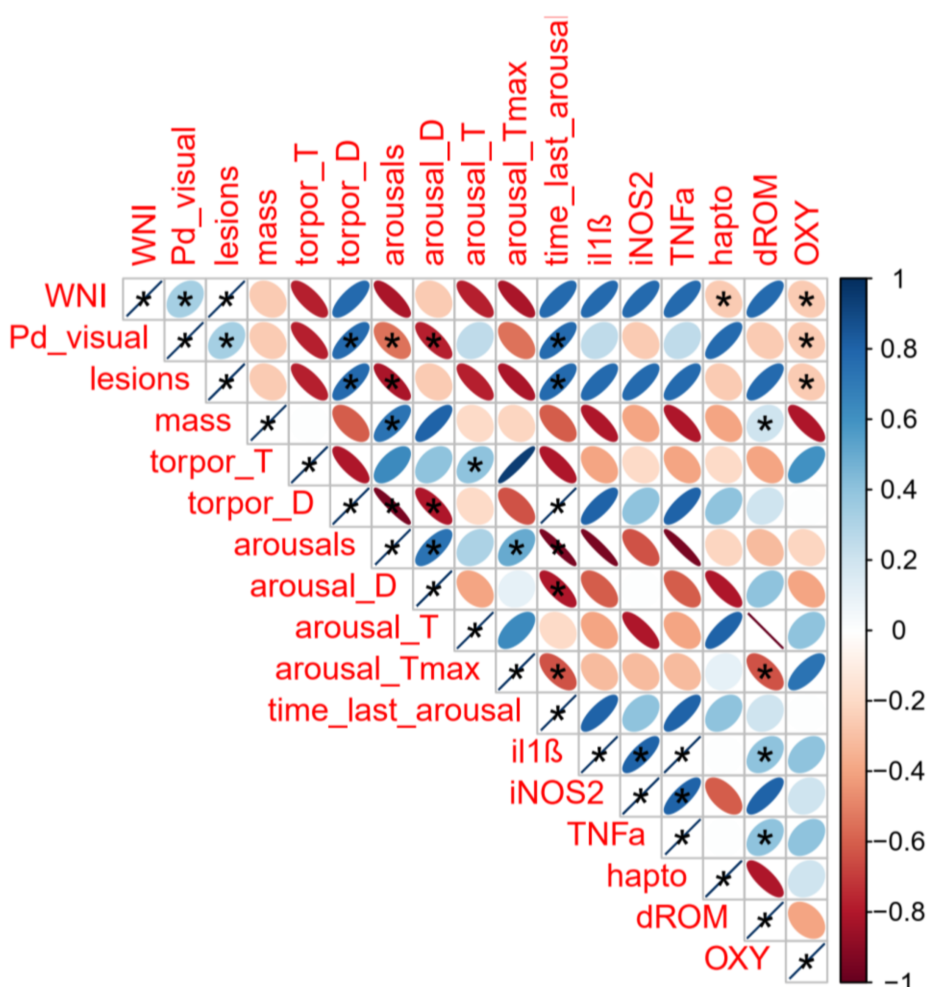

Fig. 3.6: Correlogram of physiological and immunological parameters of our dataset to identify possible interactions. In this method based on Spearman's rank correlations we considered white-nose-index (WNI), visual classification (Pd_visual), number of lesions (lesions), body mass (mass), mean torpor temperature (torpor_T), mean torpor duration (torpor_D), number of arousals (arousals), mean arousal duration (arousal_D), mean arousal temperature (arousal_T), maximum arousal temperature (arousal_Tmax), torpor time (hours) between last arousal and sampling (time_last_arousal), rER values of Il-1β (il1ß), iNOS2, TNF and levels of haptoglobin (hapto), dROM and OXY. Positive correlations are displayed in bluish and negative correlations in reddish colours. Colour intensity and the Ellipses are proportional to the correlation coefficients; on the right of the correlogram, the legend colour shows the correlation coefficients and the corresponding colours, significant correlations are marked by asterisk (for coefficients and p-values see appendix 7.2.4).

3.5 Discussion

In contrast to North American little brown bats (*Myotis lucifugus*), a congeneric species heavily affected by WNS (Reeder et al., 2012; Warnecke et al., 2012), we did not observe any increase in arousal frequencies in European *M. myotis* in response to *Pd* infections. This supports the

hypothesis that European bats tolerate *Pd* infections. Indeed, they do not interrupt their torpor state for mounting an immune response to resist fungal infections. Furthermore, in line with this result, body mass of asymptomatic and mildly infected *M. myotis* were not different compared to severely infected bats as shown in North American bats (Jonasson and Willis, 2011; Verant et al., 2014). Besides our observed 'passive immune response' during torpor which corresponds to tolerance, activation of an immune reaction may take place after hibernation when bats return to normal activity and all components of the immune system are restored (Fuller et al., 2020; Meierhofer et al., 2018; Ohlendorf et al., 2011). Additionally, we found that bats with higher body masses aroused more often and showed less *Pd* infection symptoms indicating that these bats may groom off the fungus more frequently compared to lean individuals. Therefore, our data suggest that bats with higher energy stores are less affected by *Pd* because they have increased arousal frequencies during which they reduce/clear accumulated pathogens as shown for other hibernators (Humphries et al., 2003; Prendergast et al., 2002). In our data, we did not detect that the increased arousal frequency of these bats is causally related to their *Pd* infection status. Therefore, we argue that bats with higher fat store are generally healthier because they spend less time in torpor. On the other hand, lean individuals are more energetically constrained, hence, they have to tolerate the fungal invasion for longer torpor bouts compared to bats with higher fat store.

In our study, we found higher expression of the IL-1β gene in severely infected bats compared to less infected conspecifics. A similar trend was observed for the iNOS2 gene expression. In contrast, severely infected bats had lower plasma haptoglobin (Hp), plasma oxidative damage (ROMs) and non-enzymatic antioxidants (OXY). These results suggest that bats induced an innate immune response towards *Pd* infections by using, but most likely not synthesizing, new inflammatory molecules during torpor. Indeed, if these substances were newly synthetized, we would have expected higher levels for bats with higher infection status. Presumably, torpid bats may only use baseline levels of their immune defences given that the major arm of their immune system are suppressed during torpor (Bouma et al., 2010).

Inflammatory response during torpor

Hibernators regularly interrupt torpor bouts with arousals to restore immune functions, which are down-regulated at lower body temperatures (Bouma et al., 2010; Prendergast et al., 2002). Previous studies on hibernators have shown that genes are expressed at different levels

in torpor (Morin and Storey, 2009). *Pd* infected North American *M. lucifugus* showed elevated levels of inflammatory cytokines during torpor compared to non-infected individuals (Field et al., 2018). Increased immune gene expression in infected North American bats was found, however, both during torpor bouts and arousals, suggesting that an immune response was activated during both physiological stages (Davy et al., 2020; Field et al., 2018). In our work, we lacked timeline data on gene expressions during arousals. However, arousal patterns and the time elapsed between the last arousal and the sampling did not reveal any effects on the gene expression levels (Fig. 6).

We observed decreased levels of Hp and oxidative status markers in severely infected bats, which contrasted with the elevated levels of inflammatory immune gene expressions corresponding to an induced response. Because we studied torpid bats, lower levels of circulating substances in severely infected bats might have been due to an induced utilization of baseline levels of these immune parameters rather than an induced production of these substances (Bouma et al., 2010). Hp is an effective APP against microbial invaders (Eaton et al., 1982), which is immediately activated during inflammation (Cray et al., 2009b) and has an important role during hibernation in bats (Fritze et al., 2019). Early derivatives of oxidative damage (i.e., ROMs) and non-enzymatic antioxidants (OXY) together with the expression of iNOS2 gene, which is also known for its antimicrobial effects (Kuncewicz et al., 2001), show that pro-oxidant reactions are probably associated with the inflammation status in *Pd* infected bats.

Additionally, we found that severely infected males showed lower oxidative damage and higher circulating non-enzymatic antioxidant defenses than severely infected females. However, we did not find sex-specific differences in any other parameters.

Alternatively, lowered levels of immune parameters in the blood of severely infected European bats could be due to the exhaustion of all circulating immune components, which occurs with increasing torpor duration (Bouma et al., 2010). Severely infected bats had longer torpor bouts. However, we neither detected a negative correlation between the measured blood immune parameters and torpor duration nor between the measured blood immune parameters and the number of arousals (Fig. 3.6). In line with our hypothesis, an immune response of selected circulating inflammatory proteins might be functional during torpor, even though this response is slower due to depressed metabolism during hypothermic body

conditions (Carey et al., 2003). Previous work on *M. myotis* has shown a modulated protein expression during torpor in four different proteins such as Kininogen-1 which is also involved in the inflammatory pathway (Hecht et al., 2015).

A mechanistic explanation linking body mass, arousals and defence strategy

Our results confirm tolerance of *M. myotis* towards *Pd* infections indicated by negligible body mass differences among the infection stages as previously reported (Bandouchova et al., 2018; Hecht-Höger et al., 2020). The unaffected torpor patterns are in line with the 'hibernation-optimization hypothesis' which states that hibernators with sufficient energy stores spend less time in torpor and more time at euthermic body temperature to reduce the negative effects of torpor (French, 1985; Gruet and Dufour, 1949; Humphries et al., 2003; Zervanos et al., 2014). Bats with higher energy store can afford more arousals compared to lean individuals. Therefore, body mass (as proxy for energy stored in fat) of hibernating bats seems to directly determine the health status of the bats because increased arousal frequencies are associated with increased grooming events which ultimately reduce *Pd* loads (Brownlee-Bouboulis and Reeder, 2013). In contrast, lean bats showed longer torpor bouts and more severe symptoms because energetically, these bats could not afford multiple inter-bout arousals. Instead, their strategy is to rely on tolerance towards *Pd* infections to save energy (further details in appendix 7.2.6).

Previous studies on North American bats surviving from WNS discuss the occurrence of a combination of tolerance and resistance (active immune response) towards *Pd* infections (Frick et al., 2017). Tolerant North American bats adapted to the disease by an energy saving torpor strategy (less arousals) (Cheng et al., 2019; Frick et al., 2017; Lilley et al., 2016; Moore et al., 2017). Other studies consider bats with reduced *Pd* loads in persistent populations as resistant (Frank et al., 2014; Frank et al., 2019; Langwig et al., 2017). An immunological study shows that North American *M. lucifugus* unsuccessful attempt to resist the infection by up-regulating response pathways including immune cell activation and migration, and inflammatory pathways (Lilley et al., 2019). In contrast, European *M. myotis* has shown tolerance towards *Pd* infection indicated by not activating a transcriptional response (Lilley et al., 2019). Previous work on *M. myotis* challenged by fungal antigen suggest that bats with fungal infections are able to mount hibernation-adjusted immune response during regular arousals with only mild to negligible consequences for the energy budget (Fritze et al., 2019).

which suggests that European bats potentially can mount a resistance response, probably also against *Pd*. However, since we lack data of the immune responses during arousals, the occurrence of a resistance strategy in addition to tolerance remains to be clarified.

3.6 Conclusion

We investigated natural *Pd* infections in European bats and identified defence strategies as a likely reason why European bats can cope with the infection. Our results suggest that the defence strategies of European bats against *Pd* involve tolerance due to a passive immune response during torpor, which adds to previous molecular results on tolerance (Hecht-Höger et al., 2020; Lilley et al., 2019) and contrasts with results from North American bats. First, *M. myotis* did not increase the frequency of their arousal when infection increased, probably to save energy. Since the immune system is down-regulated, inflammatory molecules, such as APPs, are not produced; thus hibernating bats may rely on utilization of circulating baseline levels of APR parameters which can react efficiently to *Pd* infections. Similarly, the decrease in oxidative damage and circulating non-enzymatic antioxidants indicate a downregulation of oxidative processes, possibly to protect tissues and organs against the potential harmful effects of oxidative stress. Finally, we documented that heavier bats aroused more often than lean individuals. This might be because heavier bats (i.e., with higher energy stores) can afford arousing more often to actively clear off the fungus, while bats with lower energy stores have to rely more on their baseline levels of defence molecules in order to save energy (tolerance strategy). We conclude that these mechanisms might explain why bats with higher winter energy budget are healthier and how lean bats can cope with the fungus.

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Pseudogymnoascus destructans infection tolerated in Europe and Palearctic Asia but not in North America. Scientific Reports 6, 19829. https://doi.org/10.1038/srep19829

4 Chapter Three - A visual assessment method to classify disease progressions on hibernating bats infected with *Pseudogymnoascus destructans*

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As

A rapid, in-situ minimally-invasive technique to assess infections with *Pseudogymnoascus destructans* in bats

by

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4.1 Abstract

Emerging infectious diseases may become serious threats to wildlife, a prominent example being the white-nose disease (WND). In case of WND, the cold-loving fungus *Pseudogymnoascus destructans* colonizes bats during hibernation, invades the skin and has already lead to the death of millions of bats in North America. *P. destructans* most likely originated from Europe, where it also causes lesions but without associated mortalities. However, it is still unclear how European bats cope with the fungus. Hence it is important to have tools that precisely characterise disease progression. Because hibernation is a physiological state during which bats are vulnerable to disturbance, in-situ assessments of the clinical status should be carried out minimal-invasively to avoid detrimental impacts on bats. However, currently available disease assessment methods require handling/touching bats and are therefore invasive: i) UV-light trans-illumination of wing membranes to detect lesions and ii) a qPCR-based quantification of fungal material from wing membrane swabs. Since *P. destructans* ('Pd') becomes visible on all furless skin with distinct distribution patterns, we

investigated the use of visible symptoms to assess levels of infections without handling/touching bats. We introduce a technique which we termed ‘Visual Pd-score’ (a visual classification scheme), which can be applied without touching and/or handling the animals. To assess its reliability, we used P. destructans infected greater mouse-eared bats (*Myotis myotis*) to compare the novel method with the two existing golden-standard techniques. Our results show that infection levels obtained from all three techniques are correlated. Importantly, the information carried by the Visual Pd-score is most similar to a composite index combining the information from the qPCR-based and UV-light quantification methods. We conclude that the Visual Pd-score represents a promising index to better characterise disease severity as it is simultaneously representative for fungal colonization and wing damage. We discuss advantages and disadvantages of the applied techniques and conclude that the Visual Pd-score is particularly useful for routine hibernacula counts or large-scale P. destructans-surveillance. In combination with the lesion detection technique the new method is also applicable to immunological studies where both fungal colonization and associated damage have to be investigated, while qPCRs from swabs of all body parts are especially useful if it is necessary to detect cryptic infections, e.g. during the early hibernation period.

4.2 Introduction

Detection of emerging infectious diseases and surveillance of their development are crucial for successful containment efforts because these diseases may represent serious threats to humans, animals and biodiversity in general (Daszak, 2000). To assess the epidemiological characteristics of an outbreak, it is necessary to quickly identify potential infection sources, routes of transmission and pathogenicity of the spreading causative agent. In wildlife species, identification of pathogens and monitoring of disease outbreaks and dynamics is strictly based on field studies which require methods that are compatible with the ecological context (Leendertz et al., 2006; Fritze and Puechmaille, 2018). However, accessing animals is usually associated with capture and handling, both causing potentially stress for the studied animals, which could ultimately influence the disease dynamics and the individuals’ survival. In addition, diagnostic tools for assessing disease severity and impact may cause additional stress depending on the invasiveness of the sampling procedure. While some surveillance methods (e.g. serology, histopathology) require invasively collected samples, other methods such as

molecular or microbiological techniques do not necessarily. In some cases, non-invasively collected samples such as carcasses or faeces may be sufficient. In other cases, visual health check may reveal meaningful information. The choice of method and its level of invasiveness have to be carefully evaluated based on animal welfare, conservation criteria and the specific research question, especially when dealing with endangered species or species already heavily impacted by a disease.

In the case of white-nose disease (WND), the fungus *Pseudogymnoascus destructans* colonizes bats' naked skin when they hibernate in underground sites, ultimately causing the death of millions of bats in North America (Frick et al., 2015). The visible growth of the fungus on the bats' nose (i.e. 'white-nose') is one of the key symptoms originally used to describe the disease (Reeder and Turner, 2008; Veilleux, 2008; Turner and Reeder, 2009). It is also by visually inspecting hibernating bats that the fungus has been originally searched and discovered in Europe (Martinkova et al., 2010; Puechmaille, 2010; Wibbelt et al., 2010; Ohlendorf et al., 2011). Indeed, bats in Europe also get infected by the fungus and show similar disease symptoms; however, there is no detectable mortality associated with *P. destructans* infection in wild bats in Europe, suggesting that the severe impact of the disease on bats in North America is lacking in bats in Eurasia (Puechmaille et al., 2011; Frick et al., 2016; Fritze and Puechmaille, 2018). Since infections are relatively easy to visually identify, visual detection methods have been used to detect presence/absence of *P. destructans* on bats since the early days of the discovery of the disease (also in combination with DNA analysis of the fungus) (Reeder and Turner, 2008; Veilleux, 2008; Turner and Reeder, 2009; Martinkova et al., 2010; Puechmaille, 2010; Wibbelt et al., 2010; Ohlendorf et al., 2011; Puechmaille et al., 2011). At least two studies also proposed scoring systems to visually estimate the quantity/nature of the infection (Fritze et al., 2012; Horáček et al., 2014). However, to assess the dermatological damage, histopathological analysis quickly became the "gold standard" method for the disease diagnostic, for which wing biopsies are taken from the patagium of living, euthanized or freshly dead bats (Meteyer et al., 2009; Pikula et al., 2012; Wibbelt et al., 2013; Zukal et al., 2016). Grading systems have also been proposed for these histopathological criteria (Reeder et al., 2012; Pikula et al., 2017). However, lately less invasive methods became available. For example, recent studies used UV-light transillumination of wing membranes to detect fungal invasions as proposed by Turner et al. (2014). Because this technique allows to count skin lesions, it can be used to assess the progress of the disease by counting the number of lesions

on the wing membranes (Turner et al., 2014; Bandouchova et al., 2015; Pikula et al., 2017). Another approach to assess disease progress is to measure fungal loads on the skin via quantitative PCR techniques (Muller et al., 2013; Langwig et al., 2015). The latter has become particularly popular for European bat species as they do not always show the typical histopathological features described in North American species (Wibbelt et al., 2013). However, these two methods require hibernating bats to be captured, causing unnatural arousals which contributes to fat stores depletion and is thus detrimental for the bats (Thomas et al., 1990; Speakman et al., 1991; Boyles, 2017). Therefore, in the present study, we evaluate a visual scoring of *P. destructans* infections, similar to approaches that have been proposed in the first years of WND research (Fritze et al., 2012; Horáček et al., 2014), in order to promote this technique as an alternative method which is non-disturbing and non-invasive (sensu Pauli et al., 2010).

Previous studies evaluated visual surveys for presence/absence of the fungus in hibernacula and concluded that simple visual surveys, by informed observers, provided reliable evidence of the presence of the fungus, at least in Europe and North America where they were carried out (Horáček et al., 2014; Janicki et al., 2015; McGuire et al., 2016). However, absence of visual sign of *P. destructans* is generally not a sufficient indicator for the absence of the fungus unless surveys are conducted late in the hibernation season and a certain number of individuals are inspected (Janicki et al., 2015; Fritze and Puechmaille, 2018). Additionally, such surveys for presence/absence do not provide information about different infection levels, which led Fritze et al. (2012) and Horáček et al. (2014) to use grading systems to visually record infections, and also injuries supposed to have been caused by the fungus, as five categories. Building on this recording principle, we developed a visual scoring system exclusively based on a visual quantification of fungal growth on all body parts classically infected; this scoring system can be applied i) to detect *P. destructans* presence in hibernating bats and ii) to quantify the level of colonization of *P. destructans* on bats as a measure of disease severity. Such visual scoring represents a least-disturbing, minimal- or non-invasive, instantaneous and low-cost method compared with the standard methods currently applied. Additionally, a low disturbance in-situ technique is urgently needed for large scale and long-term disease surveillance, e.g. during regular hibernacula counts or as standardized method for *P. destructans* infection monitoring and management programs (EUROBATS, 2010; Fritze and Puechmaille, 2018; Bernard et al., 2020).

We compared the quantification accuracy of the new visual scoring system with that of two standard methods: i) counting the number of lesions by UV light transillumination of the wings and ii) quantitative PCR of fungal material obtained by swabbing infected areas of the bat (nose and membranes). We used these measurements to analyse if the Visual Pd-scores correlate with 1) the number of lesions detected by UV-light transillumination, and with 2) the fungal growth as measured by qPCR (independently measured for wing membranes and the muzzle). We used wild greater mouse-eared bats (*Myotis myotis*) as our model, since this Eurasian species is often affected by *P. destructans* when hibernating (Puechmaille et al., 2011; Wibbelt et al., 2013).

4.3 Material and methods

We collected samples from 61 wild *Myotis myotis*. Sampling took place in 2016, 2017 and 2018. We always sampled in March, a month when most fungal infections (and lesions) with *Pseudogymnoascus destructans* are visible on bats in Europe (Puechmaille et al., 2011). All field work was conducted under permissions of the respective local animal and ethics committees (permit no.: Saxony-Anhalt: 505.6.3-42502-9-019 LIZW; Bavaria: 55.2 2532-2-235) and the conservation authorities (permit no.: Harz: Sch3058, Franconia: RMF-SG55.1-8646-2-185-4). Without touching the bats, we assessed the progress of the disease visually by using a classification scheme, hereafter named Visual Pd-score (table 4.1 and supporting material 7.3.4, see also similar method previously used in Fritze et al., 2012 and Horáček et al., 2014). Bats did not arouse as long as we conducted our inspection within few seconds and without coming too close to the bats (> 1 meter), minimizing surveyors body heat and breath reaching the bat (detailed protocol in supporting information 7.3.3). Afterwards, we carefully took the bats from the wall and counted wing membrane lesions by using UV light transillumination as described before (Turner et al., 2014). From a subset of 40 bats, we collected swab samples to assess the fungal load via qPCR. Per bat, we took one swab from the face (muzzle and ears), one swab from each of the wing membranes and one from the tail membrane (swab loads from wing and tail membrane were pooled as 'membrane load' before DNA extraction). We extracted DNA from the swabs with a commercial extraction kit (see below) and measured the amount of *P. destructans* DNA with qPCR (Muller et al., 2013; for details see appendix 7.3.1). We compared the fungal load on the face and wing membranes of a bat and also added both

measurements to obtain a total fungal load for each bat. We then conducted Spearman rank sum correlations and generated a correlation matrix (figure 4.2) to compare all three quantification methods (Visual Pd-score, qPCR, UV light). Additionally, we run a linear model of the total fungal load and lesions (both Tukey's Ladder of power transformed to get normal distributions) and correlated the predicted values of this model with our Visual Pd-score (Spearman rank correlation) to check whether the Visual Pd-score can be used as a proxy for both quantitative fungal load (by qPCR) and number of lesions (by UV light).

Table 4.1: Scoring scheme for the visual assignment of disease progress stages ('Visual Pd-score') of living bats after an infection with *Pseudogymnoascus destructans*. Symptoms describe infected areas (outlined in red on the photos) to assess different stages of disease severity (photo credits: Kaarlis Freibergs, Marcus Fritze, Bernd Ohlendorf). Infected areas considered for the 'Visual Pd-score' are: left/right ear, left/right wing, muzzle, uropatagium. More photo examples available in supporting material 7.3.4.

Progress of disease **[Visual Pd-score]**	**Symptoms**	**photo examples**
Asymptomatic bat [**0**]	➢ No visible fungus on any area of the bat	

First signs of infection [***1***]	➢ Only a single area with infection visible as tiny dot OR ➢ small zone as thin 'powder-like' layer, e.g. on the muzzle, ear, wing, etc.	
Moderately progressed [**2**]	➢ At least one infected area with medium but diffuse infection OR ➢ more than one area with small infection, e.g. tiny dot on one ear AND small area on one wing	
Further progressed [**3**]	➢ At least one area with large infection but without one or both wings nearly entirely infected (covered by fungal growth) like in [4], e.g. whole muzzle; on muzzle and one wing	

Severely progressed [**4**]	➢ At least one wing and one additional area entirely infected (nearly covered by fungal growth)	

Table 4.2: Ranges (minimum, maximum, average and standard deviation) of lesions and *Pd* load on membranes and face (muzzle and ears) with regard to the Visual *Pd* scores.

	Visual *Pd* score	**Average**	**Standard deviation**	**Range (Min-Max)**
Number of Lesions	0	2.1	5	0 - 17
	1	10.2	18.6	0 - 60
	2	50	61.3	0 - 150
	3	52.5	55	0 - 100
	4	150	77.5	100 - 300
fungal load membranes [ng/µl]	0	0.024	0.042	0 - 0.143
	1	0.054	0.094	0 - 0.333
	2	0.194	0.246	0.001 - 0.642
	3	0.279	0.269	0.011 - 0.646
	4	1.084	1.295	0.045 - 3.342
fungal load face [ng/µl]	0	0.02	0.049	0 - 0.181
	1	0.265	0.652	0.001 - 2.385
	2	0.64	0.838	0.008 - 1.872
	3	0.36	0.269	0.018 - 0.585
	4	0.322	0.282	0.003 - 0.738
total fungal load	0	0.044	0.079	0 - 0.276

[ng/µl]	1	0.319	0.742	0.001 - 2.718
	2	0.835	0.965	0.009 - 2.182
	3	0.639	0.517	0.029 - 1.231
	4	1.405	1.304	0.093 - 3.344

4.4 Results

Out of the 41 bats assessed with all three methods, we assigned 14 bats to the Visual Pd-score category 0, 13 bats to category 1, 6 bats to category 2, 4 bats to category 3, and 6 bats to the highest category 4 (Table 4.1). Levels of *P. destructans* infections, measured by all three methods, were variable across the measured individuals (table 4.2, figure 4.2, 4.3 and appendix 7.3.2). The Visual Pd-score was positively correlated with the number of lesions on the membranes (rho=0.65, p<0.001) and with total fungal load (rho=0.63, p<0.001). However, 4 out of 14 bats assigned to Visual Pd-score category 0 showed at least one membrane lesion and all of them carried at least detectable amounts of *P. destructans* DNA. We found a significant correlation between total fungal load and number of membrane lesions (rho=0.42, p=0.004) and between facial fungal load and membrane fungal load (rho=0.74, p<0.001). However, this correlation between facial and membrane fungal load seems to be driven by individuals in the early stages of the disease (strong correlation) while this relationship was weaker in bats at later infection stages (large variance). We observed individuals with a high fungal load on the face but low fungal load on the wings and vice versa (figure 4.3).

Predicted values of the linear model of the total fungal load and membrane lesions were positively correlated with the Visual Pd-score (rho=0.68, p<0.001).

We have also occasionally observed lesions on other parts of the body besides the wing membranes (figure 4.1). However, lesions from other body parts could not be included in the statistical analysis since surveys for such lesions were not examined systematically.

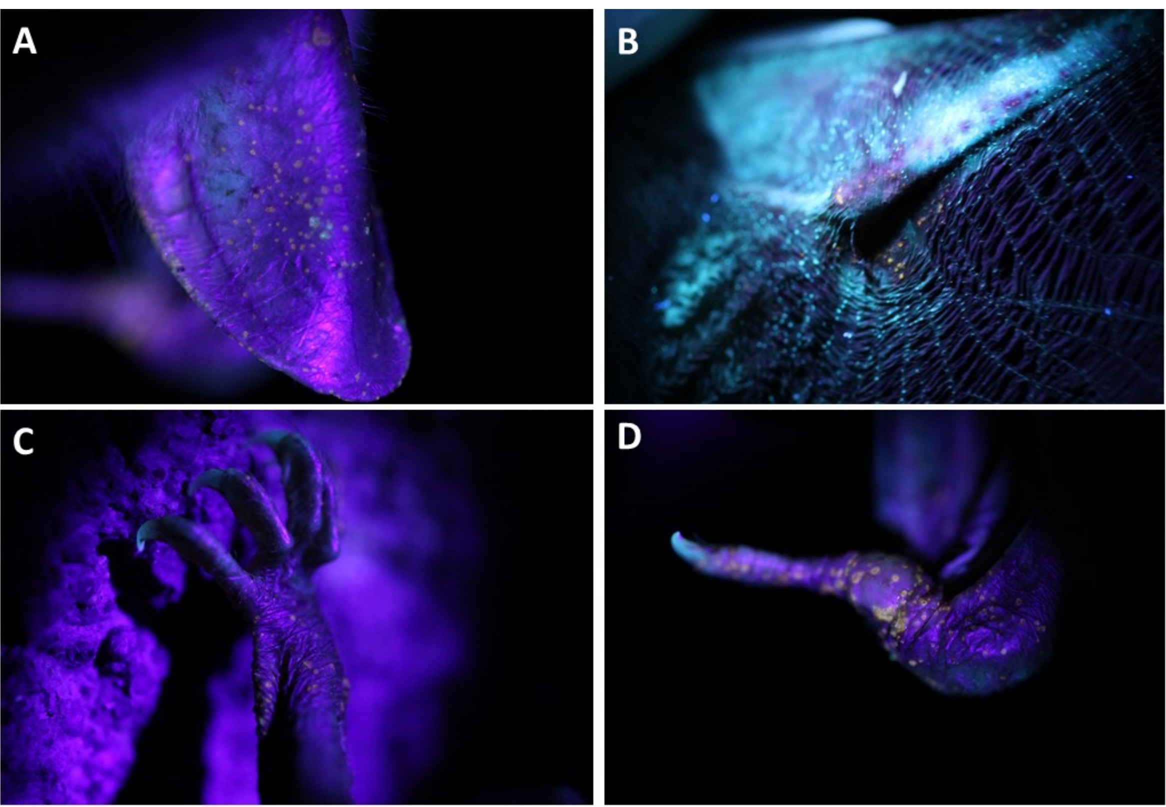

Figure 4.1: Skin lesions caused by *Pseudogymnoascus destructans* on hibernating bats. Lesions are detected by UV light and appear as orange dots on the photos; A) lesions on an ear, B) lesions on an elbow, C) lesions on a foot, D) lesions on a thumb. (photo credits: Sebastien J. Puechmaille)

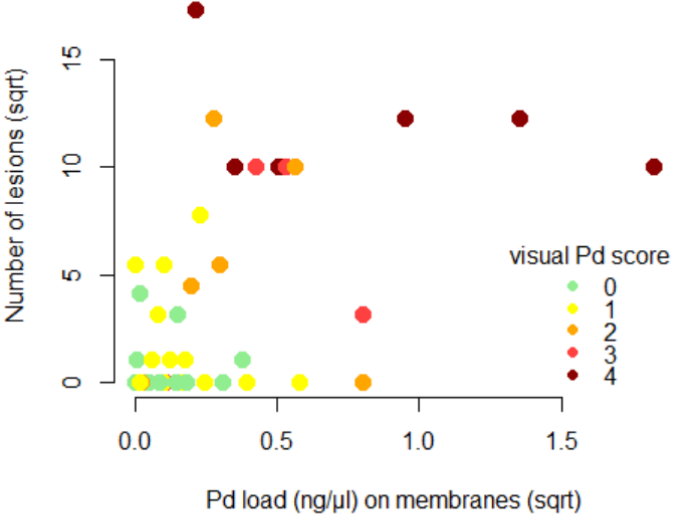

Figure 4.2: Correlation between number of lesions on the membranes and *Pd* load on the membranes (both square root transformed) grouped by Visual *Pd* score categories (colours).

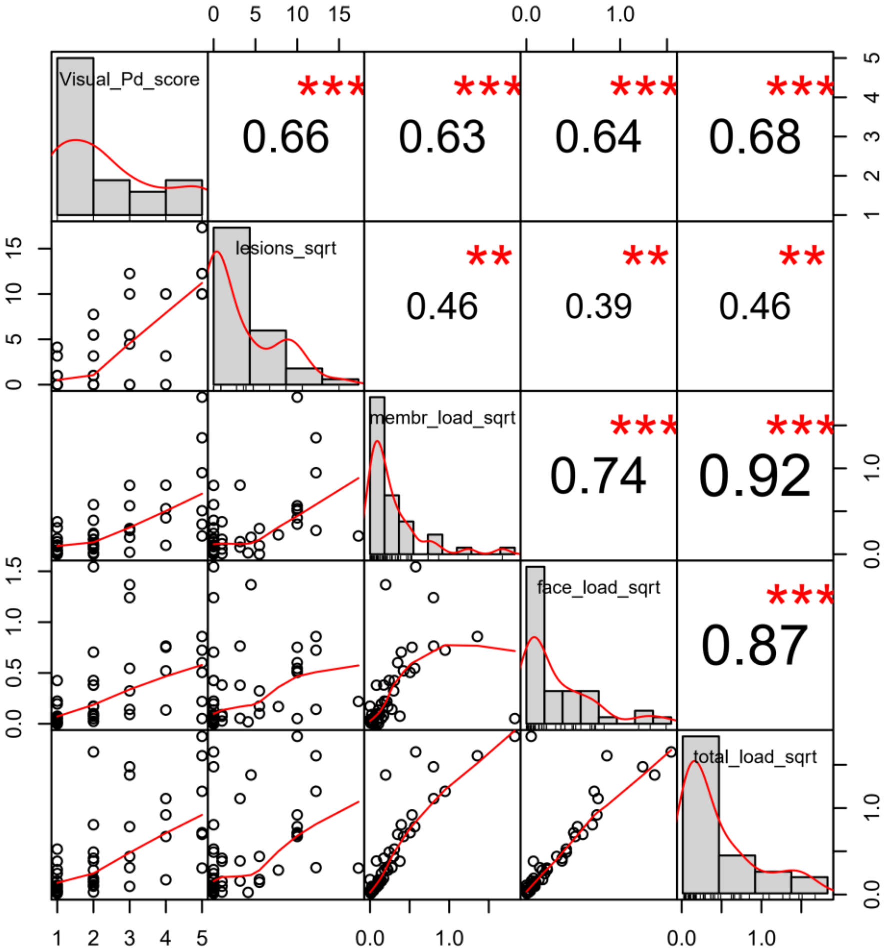

Figure 4.3: Pairwise correlation matrix among Visual *Pd* score, number of lesions on the membranes, fungal load on the membranes (membr_load), fungal load on muzzles and ears (face_load), and total fungal load (membr_load + face_load). Square root tranformed data marked with 'sqrt'. Stars mark the levels of significance, numbers in squares represent the correlation coefficients (Spearman rank correlation, full table provided in appendix 7.3.2), red smoothing lines among the data points indicate directions of the correlations.

Predicted values of the linear model of the total fungal load and membrane lesions were positively correlated with the Visual *Pd* score (rho=0.68, p<0.001).

We have also occasionally observed lesions on other parts of the body besides the wing membranes (figure 4.1). However, lesions from other body parts could not be included in the statistical analysis since surveys for such lesions were not examined systematically.

Table 4.3: Summary of comparison of advantages and disadvantages of three assessment methods of *Pseudogymnoascus destructans* infections.

method	**Visual Pd-score**	**qPCR of wing membrane swabs**	**UV light lesion counts on wing membranes**
price	+ very cheap ± optional: camera for documentation + performed quickly directly in the field	- expensive (sterile DNA-free swabs + chemicals for DNA extraction and qPCR and technician time for DNA extraction & qPCR)	- costs for an appropriate UV lamp ± optional: camera + filters for documentation ± performed directly in the field but time consuming
effort	+ very fast, application directly in the field with limited training/information needed,	- requires a DNA laboratory with qPCR machine - time consuming (field work + laboratory analysis + computer analysis) - often requires permits and expertise for bat handling	+ relatively fast, estimation possible in the field - exact numbers need to be counted on photos - more time consuming compared to Visual Pd-score
Invasiveness	± minimal or non-invasive (see methods and discussion)	- disturbance from hibernation (arousal) - invasive (handling and swabbing of the bat)	- disturbance from hibernation (arousal) - invasive (handling of the bat and stretching the wings over UV light) - fungus + bat shortly exposed to mutagenic UV light
Assessment of *P. destructans* infection status	+ proxy for both fungal colonization and lesions + includes assessment of all body parts + Visual Pd-score >0 is a good proxy for *P. destructans* viability ± information on *P. destructans* growth rate (per bat) when bats are inspected at least twice	± relatively weak correlation with lesions ± indicates amount of fungal colonization on wings, usually not all body parts measured - no information about *P. destructans* viability	± relatively weak correlation with fungal load - no information about quantitative fungal colonization (lesions only occur in late infection stage) - no information about *P. destructans* viability ± quantification for damage but usually only for wings
Accuracy	- imprecise for exact amount of fungal colonization - no detection of cryptic infections + >99% precise about the species ID	- imprecise for exact amount of fungal colonization + detects cryptic infections + >99% precise about the species ID	± allows rough counts of numbers of lesions (hidden lesions or erosions do probably not appear orange and are hence not detected)

4.5 Discussion

We established a visual in-situ assessment protocol (named Visual Pd-score) allowing the assessment of the *P. destructans* infection status of bats without handling and with minimal disturbance. We evaluated this non-invasive scoring system by testing its efficacy and accuracy in comparison with two (invasive) diagnostic methods widely used in WND research: counting the number of wing membrane lesions via UV-light transillumination (Turner et al., 2014) and quantifying *P. destructans* load of infected wing membranes via qPCR (Langwig et al., 2015; Muller et al., 2013). When using our Visual Pd-score, disturbance was minimal and we did not observe arousals, an observation corroborated a subsequent studies showing no or maximal limited effect of census on bat activity at hibernacula, even when monitoring extended to 24-h post-visit (Kilpatrick et al., 2020; Stapelfeldt et al., 2020). However, this required the inspecting surveyor 1) to not handle the focal bat, 2) to keep a minimum distance of >1m to the surveyed bat while avoiding breath and heat to reach the bat, and 3) to switch on headlamp light only for a few seconds, and 4) to step away from the surveyed bat immediately afterwards (see appendix 7.3.3). Therefore, we consider our visual scoring method as a non-invasive approach to assess *P. destructans* infections in hibernating bats as long as the bats do not arouse from hibernation, or minimally-invasive if a minute fraction of bats arouse due to disturbance caused by the overly use of headlamp light, by noise or changes in temperature (Stapelfeldt et al., 2020).

Visual Pd-scoring - a proxy for fungal colonization and lesions

As expected, values of all applied methods were significantly correlated (figure 4.3). These results demonstrate that the Visual Pd-score can be used as a proxy for both the UV method and the qPCR method. Interestingly, the Visual Pd-score was more strongly correlated to each of the standard measures (i.e. the number of membrane lesions, rho=0.66; qPCR membrane load, rho=0.63; qPCR facial load, rho=0.64) than the standard measures were correlated to each other (i.e. number of membrane lesions versus qPCR membrane load (rho=0.46), or qPCR facial loads (rho=0.39). Additionally, the predicted values of the linear model of membrane lesions and total fungal load were significantly correlated with the Visual Pd-score. Hence, results from our study suggest that the Visual Pd-score is a better proxy for the other two methods than these methods are for each other, at least for our dataset on *Myotis myotis*.

Our Visual Pd-scoring includes all body parts, thereby avoiding bias associated with sampling only certain parts as done for the two standard methods (UV light transillumination and qPCR). It has been suggested that damage to the patagium (e.g. wing membranes, tail membranes) may cause more severe physiological disruptions compared to damage to other body parts (e.g. muzzle, feet, ears) (Cryan et al., 2010; Frick et al., 2016; Reeder et al., 2012; Warnecke et al., 2012). Although such differences remain to be confirmed and quantified, we suggest to additionally describe and record the quantity of infection for each body part separately. This information could later be used to calculate a composite index of homeostasis disruption by averaging the score of each body part weighed depending its contribution to physiological disruptions.

Nevertheless, the successful application of the Visual Pd-scoring method may also depend on the visual accessibility of all bat body parts, which in itself depends both on the species (e.g. species size) and the number of individuals in a cluster to be inspected for *P. destructans* presence (Janicki et al. 2015) as well as if the individuals hibernate in crevices. Additionally, the distance to the bats is an important factor that needs to be considered when the Visual Pd-score is used. As a result, to avoid missing some visible fungal growth and consistently evaluating the Visual Pd-score, we recommend inspecting bats from about 1m distance and having a set of reference pictures (see table 4.1 and supporting material 7.3.4) to calibrate surveyors' judgement. In complement and to limit observer bias, the Visual Pd-score can be estimated simultaneously by two observers, and the average of these estimates used.

As demonstrated by the assessment discrepancies between the visual assignments of 'category 0' and the results of the other two methods (figure 4.2), our results confirm that visual surveys are not sufficient to detect incipient *P. destructans* infections because the fungus needs to reach a critical size before it can be detected visually (Janicki et al., 2015; Puechmaille et al., 2011). During early winter phases, the Visual Pd-scoring will inevitably fail to detect *P. destructans* infections. Therefore, if the aim is to verify the presence or absence of *P. destructans* early in the hibernation season or on seemingly healthy bats (at any date), the application of DNA-based approaches is recommended (Gargas et al., 2009; Muller et al., 2013; Puechmaille, 2010). Recognising *P. destructans* from other fungal species growing on bats is critical for the application of the visual score. Indeed, investigations into fungal species growing on bats skin are still scarce (Hubálek et al., 1979; Larcher et al., 2003; Lorch et al.,

2015) and more fungal species, some of which might be visually similar to P. destructans, are most likely awaiting to be discovered. Additionally, the nature of the substrate where the bats are hibernating can complicate the survey when material eroding from the substrate of the hibernacula, such as chalk, may look similar to *P. destructans* infections to an untrained surveyor. However, based on our experience in Eurasian bats species, the rate of correct identification is evaluated to be greater than 99.2% (472 samples genetically confirmed via genotyping (Dool et al., 2020)) after culture out of 476 samples with visual *P. destructans* presence on living bats; n=26 surveyors; Puechmaille, unpublished data). Naturally, visual records from places far away from the currently know species range should be genetically confirmed by mycological standard methods combined with sequencing and/or genotyping (Dool et al., 2020; Gargas et al., 2009; Puechmaille, 2010). The large success in cultivating *P. destructans* from visibly infected bats demonstrates that visual observation of the fungus on a live bat provides strong evidence that the fungus is viable, information that is currently not available via the other two methods. Given that our method does not interfere with the fungal growth, by repeatedly surveying the same individual over time, it is possible to observe the development of fungal growth on bats (Fritze et al., 2012), a unique characteristic of the Visual Pd-score.

Fungal colonization and occurrence of lesions on bats

We confirmed findings from earlier studies that showed qPCR measures of fungal loads to be correlated with the number of lesions in membranes (McGuire et al., 2016; Zukal et al., 2016). However, consistent with the literature (McGuire et al., 2016; Zukal et al., 2016), this correlation is relatively weak and our survey revealed that, for example the individual with the highest number of membrane lesions carried only medium level of *P. destructans* DNA compared to others with less lesions and higher fungal loads. This discrepancy between superficial fungal load and number of membrane lesions might lead to incorrect assessments of the disease status when either of these two methods is applied alone. The broad variation in this relationship might occur because bats arouse from time to time and groom off the fungus (Brownlee-Bouboulis and Reeder, 2013; Puechmaille et al., 2011). Additionally, lesions occur at a later stage of colonization which may lead to the observation of individuals with high fungal loads but no lesions. In North America, initial infections with *P. destructans* are not associated with cupping erosions, pathognomonic lesions for WND diagnosis, and variation in

impact among bat species and populations has also been observed (Langwig et al., 2015; McGuire et al., 2016). Bats with varying degrees of symptoms were also observed among Eurasian bats (Bandouchova et al., 2018; Pikula et al., 2017; Wibbelt et al., 2013). For investigations that aim at assessing disease severity, it is necessary to consider both the fungal growth on bats (Field, 2018) as well as the pathological damage (Pikula et al., 2017), because lesions may only occur in severe infections (McGuire et al., 2016) and because the relationship between fungal growth and pathological damage may vary between geographical regions with different onsets and lengths of hibernation periods (Verant et al., 2012). Additionally, UV light surveys should include other parts than the wing and tail membranes since lesions are also present on ears, elbows, feet and thumbs (figure 4.1).

Additionally, surveying *P. destructans* infection by UV transillumination requires a specific technique and experience. In particular, this approach builds on light with a distinct UV wave length (~360 nm) and intensity or a camera with special filters fitted to lenses. Otherwise, points of infections may not be seen as orange dots by the surveyor. For studies that aim at measuring both infection status and damage (e.g. immunological studies), we recommend combining the Visual Pd-score with additional lesion counts using UV light as recently shown (Fritze et al., 2021). This may replace the histopathology of infected wing areas as a measure of *P. destructans* induced damage (Martínková et al., 2019; Turner et al., 2014).

Quantitative PCR of fungal DNA

Although the qPCR technique is widely used to quantify *P. destructans* load on wing membranes (Bandouchova et al., 2018; Langwig et al., 2015; Martínková et al., 2019; McGuire et al., 2016; Pikula et al., 2017; Zukal et al., 2016), our data show that the established Visual Pd-scoring method may be equally or even superior for quantifying the stage of the disease. Moreover, the visual scoring system is cheap, time efficient, can be performed in-situ. Additionally, swabbing is a relatively imprecise collection method compared with the highly sensitive qPCR technique that is later applied in the lab, often in duplicates or triplicates. Similarly, it has also been shown for the amphibian pathogen *Batrachochytrium dendrobatidis* that data derived from such swab samples may merely indicate the current zoospore shedding or activity rate (Clare et al., 2016). For *P. destructans*, the conidia production for example varies depending on whether the fungus is infected by a Partitivirus or not (Thapa et al., 2016) and is likely to also vary according to the stage of the lifecycle. Therefore, the quantity of DNA

may not represent the skin area affected by the fungus (Clare et al., 2016). Additionally, qPCR DNA load calculations assume that the number of copies of the amplified/target gene is identical across fungal isolates/individuals. Unequal copy numbers between isolates/individuals would inevitably lead to under- or overestimation of prevalence and pathogen load as evidenced in *B. dendrobatidis* (Rebollar et al., 2017). Last but not least, molecular tools only inform us about DNA quantities but not pathogen viability (Fischer et al., 2020), which can be important for understanding the disease dynamics. Taken together, although qPCR produces a precise value for the number of amplified target gene copies, it remains to be investigated for most pathogens how this value relates to the true individual fungal load. Additionally, body parts are usually differentially impacted by fungal growth, as shown in our load comparison between face and wings (figure 4.3) or as shown in previous work, fungal growth may even vary between wings of the same individual (Bandouchova et al., 2018). Thus, sampling only a limited area might be not representative for the whole individual and lead to an inaccurate assessment. Moreover, DNA extractions methods and the choice of qPCR detection threshold also greatly influence the quantification of *P. destructans* (Verant et al., 2016), making comparisons between studies challenging.

4.6 Conclusion

Our visual Pd-scoring method represents a reliable, time and cost-effective minimal-invasive assessment of disease severity of *P. destructans* colonization in European hibernating bats. However, the application of this method, and other existing methods, is dependent on the research question. Each method has advantages and disadvantages (see table 4.3) that need to be carefully considered <for every study. Given its ease of use, low cost and complementarity to existing methods (UV-light, qPCR), we recommend the Visual Pd-score to be used along with qPCR and/or UV-methods, but also independently for routine hibernacula counts as well as for long term and large scale monitoring of *P. destructans* infections (Fritze and Puechmaille, 2018; Janicki et al., 2015). Such surveys should be done as close as possible to the end of the hibernation period because the probability of visually detecting the fungus is higher during late winter (Janicki et al., 2015; Puechmaille et al., 2011).

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5 General Discussion

5.1 Tolerance or resistance - how do European bats respond to fungal infections during hibernation?

In the context of white-nose disease, which has killed millions of bats in North America, I investigated whether European bats that survive despite infections are adapted to the pathogen *Pseudogymnoascus destructans*. Therefore, I investigated physiological and immunological reactions in greater mouse-eared bats (*Myotis myotis*) to find out whether European bats react to the fungal infections with tolerance or resistance responses. I found that *M. myotis* shows different immune responses to fungal infections in comparison to what is known about responses of North American bats. The work presented fills some general knowledge gaps regarding antifungal immune responses of hibernating bats and provides new insights into the field of hibernation immunology.

Since bats are affected by *P. destructans* during hibernation, the question is if responses can occur during torpor or if they are related to arousals from torpor. Thus, in my study of hibernating *M. myotis*, I monitored body temperatures to detect fever or changes in torpor patters in response to infections. I also measured representative parameters of innate immune responses, both during arousals and during torpor, to check how European bats may respond to fungal infections and if such responses can be assigned to tolerance or resistance.

In a first laboratory-based experiment, I used the fungal antigen zymosan to mimic a systemic fungal infection in order to find out how these bats respond to fungal infections in general. In contrast to North American *Myotis lucifugus* infected with *P. destructans*, in *M. myotis* neither arousal frequencies nor body temperatures differed between zymosan-treated and control bats. Consistent with the zymosan experiment, in my field-based approach I neither found

altered torpor patterns or body temperature changes in bats infected with *P. destructans*, as shown in bats in North America (Bernard and McCracken, 2017; Bohn et al., 2016; Mayberry et al., 2018; Reeder et al., 2012; Warnecke et al., 2012; Wilcox et al., 2014). Confirmatory, in both experiments I did not detect body mass changes following the infections. The results of both approaches consistently indicate, that fungus-infected European bats have lower energetic costs in comparison to North American *M. lucifugus*. The latter has been shown to lose their fat store due to increased arousal frequencies (Reeder et al., 2012; Warnecke et al., 2012), which mostly explain the increase in mortality.

In my immunological measurements I found that during arousals, zymosan-inoculated bats increase haptoglobin, an efficient acute phase protein against microbes (Bertaggia et al., 2014; Eaton et al., 1982; Langlois and Delanghe, 1996; Theilgaard-Monch, 2006), but do not mount a full immune response as shown in North American bats infected with *P. destructans* (Field et al., 2018, 2015; Lilley et al., 2019; Moore et al., 2013). I interpret this as a hibernation-adjusted, moderate immune response which is characterized by an increase of only selected immune parameters during regular arousals from torpor. The use of early-defence mechanisms of the innate immune system, here shown by the energetically cheap acute phase response protein haptoglobin, represents an additional energetic optimization of an immune reaction during hibernation. This moderate, selective response of *M. myotis* contrasts with the multiple immune reactions which were shown during arousals of *M. lucifugus* infected with *P. destructans* (Field et al., 2015; Lilley et al., 2019; Moore et al., 2013).

In the blood of torpid *M. myotis* severely infected with *P. destructans* I found higher cytokine gene expressions corresponding to an innate immune response compared to asymptomatic bats. Interestingly, infected bats did not arouse to increase immune response parameters. Instead, bats stayed torpid and concentrations of immune response parameters like haptoglobin, pro-oxidants and anti-oxidants were lower in severely infected bats compared to asymptomatic bats. Thus, I assume that infected bats use baseline levels of immune parameters in the blood to react 'passively' to the pathogen during torpor in order to avoid energy-costly arousals which are likely necessary to synthesize immune proteins since most parts of the immune system are suppressed during torpid conditions (Bouma et al., 2010a).

The results of my studies provide more evidence for previous hypothesises and findings that suggest an immunological tolerance of European bats to *P. destructans* (Bandouchova et al.,

2018; Harazim et al., 2018; Hecht-Höger et al., 2020; Lilley et al., 2019; Zukal et al., 2016a). Here, for the first time, I demonstrated which physiological and immunological mechanisms characterize this tolerance that allows European bats to survive fungal infections during the energy-demanding hibernation period.

Moreover, my investigations have shown that in immunological studies on white nose disease, it is important to compare different stages of disease severity rather than only comparing infected versus non-infected bats. In my field experiment, I demonstrated that physiological responses and consequences as well as immunity levels differed between the stages of disease progression. In this context, I developed an assessment system that allows to distinguish between asymptomatic, mildly infected and severely infected bats. Additionally, I derived a non-invasive method to assess white-nose disease stages that can be used *in-situ* and can replace invasive techniques in many other research approaches. I validated this method which relies on visual quantification of fungal colonization by comparing measurements of invasive gold-standards methods which are usually used in white-nose disease research to quantify *P. destructans* growth on the bats and damage caused by the fungus. This method represents a practicable technique which avoids disturbance and hence reduces detrimental effects on the hibernating bats. Therefore, this 'visual Pd-score' (Pd stands for *P. destructans*) is a valuable method for future research and disease monitoring and can used on a large scale.

5.2 Methodological considerations and comparability

Methodological considerations of sampling designs

Since several approaches to infect European *M. myotis* with *P. destructans* under captive laboratory conditions failed because *M. myotis* did not develop significant disease symptoms (Davy et al., 2020, 2017; Field, 2018; Wibbelt, unpublished), it appeared difficult to design a laboratory-based infection experiment with *P. destructans*. Therefore, in my laboratory experiment, I used the fungal antigen zymosan and successfully mimicked a fungal infection followed by a systemic innate immune response. We used similar dosages according to previous experiments on house sparrows (Coon et al., 2011) and on *M. myotis* in summer (Seltmann et al., in prep.) which elicited significant immune responses, confirming that even

small amounts of antigens are sufficient to induce detectable physiological reactions (Armour et al., 2020).

However, zymosan was inoculated subcutaneously which can be regarded as representative for a systemic response to fungal infections. In comparison, *P. destructans* infects its hosts first locally and growths superficially before it penetrates the skin and causes lesions (Meteyer et al., 2009; Wibbelt et al., 2013). Therefore, immunological comparisons between zymosan-treated and *P. destructans* infected bats can be challenging. Thus, I performed a field experiment on wild-living *M. myotis* naturally infected with *P. destructans* to measure physiological and immunological responses in accordance with and complementary to the zymosan experiment. *P. destructans* infections cause various symptoms and disease grades in bats (Pikula et al., 2017). Comparing those different levels of disease severity or pathogen loads is crucial for immunological studies, especially for pathogens causing local infections such as *P. destructans* (Field, 2018). I thus developed a system to assess different grades of infection; namely categorised as asymptomatic (but exposed to the pathogen), mild and severe infections. My immunological results demonstrate that such categorisation is relevant. Indeed, merging the immunological data of mildly and severely infected bats into one group ('infected'), and comparing those measurements with those from presumably non-infected bats, would have generated a large variance among immune parameters. As a consequence, I might have lost the statistical power to highlight the demonstrated effects, e.g. significant differences only between asymptomatic and severely infected bats.

Alternatively, I could have chosen an approach based on continuous data recorded from lesion counts detected by UV light transillumination (Turner et al., 2014), or from qPCR-measured DNA concentrations sampled with swabs (Martínková et al., 2019). These methods are mostly used in other studies quantifying the pathogen levels (Bandouchova et al., 2018; Langwig et al., 2015; Martínková et al., 2018). When comparing these gold-standard methods and my visual assessment system ('Visual Pd-score), I found that those methods are all correlated. It is worth to notify that the qPCR method considers only selected body parts (mostly one wing or both wings), hence, not sampled infected areas can be missed. The lesion counts quantify the damages, only occurring at a late stage of fungal colonization (Langwig et al., 2015; McGuire et al., 2016). Earlier infection stages are consequently missed. In my studies, I used a combination of methods to visually detect superficial fungal growth on body parts (using the

visual Pd-score) and to quantify skin lesions using UV light. With this combination, namely white-nose index, I distinguished healthy bats (i.e. asymptomatic), mildly infected (i.e. fungal growth but no lesions) and obviously strongly diseased bats (i.e. fungal growth and lesions as severe symptoms). Additionally, I validated visual detections by comparing them with the qPCR method. I could thus confirm that cryptic infections are very difficult to detect visually (Janicki et al., 2015; McGuire et al., 2016). However, I suggest that such very minor infections, in a very early stage of infection, are not sufficient to induce immune responses. This suggestion is also supported by my results.

Comparability of experimental measurements

I measured immune responses during arousals in zymosan-challenged bats and in torpid wild-living bats infected with *P. destructans*. Comparing these results can be challenging because of the different physiological states at which the bats have been sampled. During torpor, most immune system functions are downregulated, whereas during arousals some parts of the immune system are restored (Bouma et al., 2010; Prendergast et al., 2002).

Additionally, zymosan is derived from a different fungal species than *P. destructans*, and unlike the surface of a live microbe it is composed of a limited number of defined components (Underhill, 2003). Zymosan might, therefore, only trigger a general innate immune response while the more specific pathogen *P. destructans* might be recognized by different pattern recognition receptors and might trigger different or additional immunological pathways (Brook and Dobson, 2015). Additionally, zymosan was subcutaneously inoculated and does not increase its pathogenesis because it is a dead, non-infectious material. In comparison, *P. destructans* infects its host locally and uses serine peptidase Destructin-1 as the major extracellular, collagen-degrading endopeptidase to actively invade the skin. Moreover, alterations in fungal cell wall structures have been shown in North American bats during *P. destructans* infections which could allow the fungus to avoid detection by host pattern recognition receptors and antibody responses (Reeder et al., 2017). The immune response towards *P. destructans* could therefore be different and involve other arms of the immune responses compared to responses following the zymosan challenge.

Nevertheless, my results do not show contradicting results between both experiments and consistently lead to the conclusion of an immunological tolerance. I did not detect altered torpor patterns after the zymosan challenge or due to the infection with *P. destructans*.

Furthermore, I found that haptoglobin played an important role in both infection experiments. Haptoglobin synthesis was however delayed by a few days in zymosan-challenged bats suggesting that this protein is only synthesized to a limited extent during arousals. During torpor of bats infected with *P. destructans*, I found significant lower haptoglobin levels in severely infected bats compared to healthy ones, suggesting an involvement of the protein in an immune response. In both experiments, I found tendencies of oxidative stress in infected bats, but this deserves further investigations.

Further, I registered an absence of a cellular immune response in both approaches; I neither detected changes in white blood cell counts or in proportions of different leucocyte types in zymosan-challenged bats nor in wild-living bats naturally infected with *P. destructans* (Czirják et al., unpublished; not included in the paper of chapter 2, but see appendix 7.4). In torpid naturally infected bats, I found an upregulated gene expression of interleukin-1β but I did not detect significant differences in gene expressions between the groups of aroused bats in the zymosan experiment. However, the differences in the immune gene expressions between the torpid and the aroused bats are difficult to interpret because during arousals, bats may generally express immune genes to restore parts of the immune system (Bouma et al., 2010; Prendergast et al., 2002). Thus, I found upregulated immune gene expressions in both zymosan-challenged and control bats while differences between the groups might be masked due to the general upregulation of immune genes induced by the arousals. Alternatively, significant differences of the levels of immune genes in zymosan-challenged compared to control bats might occur delayed, similarly to the delayed increases of haptoglobin.

In summary, in both experiments I consistently found evidence for a hibernation-adjusted antifungal immune response which is characterized by a moderate innate response which is part of the bats' energy saving strategy.

5.3 Physiological and immunological adaptations of European hibernating bats

Recent work on North American bats infected with *P. destructans* support my findings, that body mass - as a proxy for energy store - plays a central role in the interplay between host and pathogen. It has been shown that body mass is the strongest predictor for survival of bats that are exposed to the pathogen (Cheng et al., 2019; Haase et al., 2020). However, in my zymosan

experiment, the course of experiment lasted only five days. Therefore, the duration could have been too short to detect significant changes in the arousal behaviour or body weight. However, I did not detect fever responses during arousals as it was shown in North American bats infected with *P. destructans* (Mayberry et al., 2018).

In my field experiment, I was able to track temperatures of only 10 individuals, thus, the statistical power of observed arousal frequencies is limited. However, the results of both experiments fit plausibly into the overall picture that European bats react to fungal infections with tolerance rather than resistance, as previously suggested (Bandouchova et al., 2018; Harazim et al., 2018; Lilley et al., 2019; Zukal et al., 2016a). Additionally, abnormal winter activities of bats have never been observed in white-nose affected hibernacula across Europe (Puechmaille et al., 2011; Zukal et al., 2016b) and also loss of body masses have never been detected in context with the infections (Bandouchova et al., 2018). My results are also supported by the fact, that presumably adapted North American bats which survived the infections, do not show increased arousal frequencies in comparison to those that die (Lilley et al., 2016). Moreover, it has been shown in hibernating European ground squirrels (*Spermophilus citellus*) that bacterial infection could be a selective force on torpor (Luis and Hudson, 2006). Therefore, I suggest that the absence of altering torpor patterns despite fungal infection is an adaptation in European bats in order to maintain the hypothermic status to save their energy store.

However, future research should focus more on the ultimate costs of the white-nose disease in European bats, e.g. during the period of healing of the infections in spring which has only be investigated on North American bats so far (Fuller et al., 2020; Meierhofer et al., 2018; Meteyer et al., 2011). It would be important to investigate in the physiological costs related to sex or age classes. Insectivorous bats in their first year of life generally deposit less fat prior to hibernation than older bats (Kokurewicz and Speakman, 2006). Thus, taking our results on the relationship between available fat store and affordable arousals, subadults might have less arousals compared to older bats and could be more vulnerable for severe disease symptoms. Additionally, females have to be thriftier with their energy budget compared to males (Jonasson and Willis, 2011). My results on naturally infected bats also indicate that severely infected females suffer more from oxidative stress compared to males. We should therefore estimate yearly survival rates and fitness costs and distinguish between the

aforementioned demographic classes. Moreover, I only used one species in my research. It would be interesting to show how other European bat species respond to the infections. In North America, it has been shown that large species (e.g. *Eptesicus fuscus*) are less often infected and show less severe symptoms compared to the smaller species *M. lucifugus* (Frank et al., 2014). In Europe, the larger species (*M. myotis*) is more often and more severely infected than smaller species like *M. brandtii, M. mystacinus, M. bechsteinii* or others which hibernate in the same microclimate and mostly share the same sites with *M. myotis* (Pikula et al., 2017; Puechmaille et al., 2011b; Wibbelt et al., 2013; Zukal et al., 2016). It could be that those smaller species arouse more frequently than *M. myotis*, thus grooming of the fungus more often. Such hypothesis remains to be verified but could explain why smaller European Myotis species show less symptoms than *M. myotis*.

Alternatively, those small species could show different immunological responses than *M. myotis*. However, a molecular genetic study on white-nose disease did not support the hypothesis that European *Myotis* species may have evolved different responses to the pathogens as no difference in proteins undergoing positive selection was detected among several species (Harazim et al., 2018). The same study suggests similar tolerances in all those species due to historical exposure to *P. destructans* (Harazim et al., 2018). However, besides genetic signatures, it would be worth to investigate in depth the physiological and immunological reactions in several European bat species in order to understand if my observations can be generalized as adaptations and could also explain why European bats show generally milder symptoms compared to North American bats (Pikula et al., 2017; Wibbelt et al., 2013). Such results could further show that European bats generally use tolerance to limit the damage caused by the pathogen and that such adaptation belongs to a general evolutionary process which allows hibernating species to overcome emerging pathogens (Medzhitov et al., 2012; Roy and Kirchner, 2000; Schneider and Ayres, 2008).

In detail, a transcriptomic study has shown that tolerant *M. myotis* did not mount a response that involves the downregulation of inflammatory genes nor the upregulation of anti-inflammatory genes during arousals (Lilley et al., 2019). Nevertheless, in my field experiment I found gene expressions indicating an upregulation of an inflammatory response during torpor. Additionally, concentrations of haptoglobin and pro- and anti-oxidative substances in the blood were lower in infected bats compared to healthy ones. These results suggest an

inflammatory immune reaction in torpor during which baseline levels of immunological substances are used but not synthesized. This finding shows that there are indeed reactions in the torpid bats in response to the fungal infections, although at a limit level. The use of baseline blood concentrations instead of an increase in immune parameters is a new aspect in hibernation immunology and deserves further investigation.

Moreover, the role of haptoglobin which was particularly involved in the antifungal response in both of my experiments, seems to be part of an important adaption. Haptoglobin is mainly synthesized in the liver, but also a granule protein of macrophages and neutrophils (Theilgaard-Monch, 2006). Neutrophils have been observed to be infiltrated in lesions caused by *P. destructans* (Meteyer et al., 2011; Wibbelt et al., 2013), which suggests a participation of neutrophils and haptoglobin in the immune response. However, white blood cells levels are generally low during hibernation (Kuznetsova et al., 2016; Uzenbaeva et al., 2015). My observation of an absence of increases or of a composition changes of white blood cells during arousals of zymosan-challenged bats and also during torpor conditions of bats naturally infected with *P. destructans* (unpublished, but see appendix 7.4) are in line with this general pattern. Nevertheless, remaining concentrations of white blood cells in the blood circulation of torpid bats may also represent a baseline level, which are able to recognize *P. destructans* via their pattern recognition receptors. Hence, white blood cells and especially neutrophils may be involved into the tolerance response but without significant changes in their abundance, e.g. by only recognizing the pathogen and releasing cytokines as well as haptoglobin to induce a moderate inflammatory response. In contrast to the energetically expensive production of white blood cells (Long and Nanthakumar, 2004), production of acute phase proteins and other anti-microbial substances does not involve large energetic costs (Nijsten et al., 2000; Skovgaard et al., 2009). Additionally, white blood cells such as B and T lymphocytes provide specific, but time-lagged protection through antibody production and inducing a cascade of immune responses (adaptive immunity) while neutrophils rapidly protect against microbes without education or much specificity (innate immune response) (Tizard, 2008). Therefore, during hibernation, fast innate immune response mechanisms including efficient acute phase proteins are advantageous compared to slower and energetically more expensive adaptive immune responses. Thus, the involvement of energetically cheap immunity proteins such as haptoglobin rather than production of immune

cells seems to be one adaptation during hibernation that allows mammals to remain in their hypometabolic and hypothermic state (Chow et al., 2013).

The studied example, a fungal infection, shows that tolerance mechanisms are important during hibernation because the animals can keep pathogens at bay without spending too much energy, until they can mount a full immune response after hibernation period when all immunological functions are restored. It is known from other organisms that tolerances can develop through evolutionary processes in which the immune system is 'trained' to use immunoregulatory mechanisms that eliminate or suppress the activation and propagation of immune responses (Billingham et al., n.d.; Soares et al., 2017). Since bats in Europe have been living in sympatry with the fungus, hence, they co-evolved and developed their tolerance strategy to cope with the pathogen in winter (Harazim et al., 2018).

In contrast, organisms that are exposed to recently introduced pathogens initially mount resistance responses which attempt to directly affect invading pathogens (Medzhitov et al., 2012; Schneider and Ayres, 2008). Thus, North American bats which are naïve towards *P. destructans* do not tolerate but try to resist the infections (Langwig et al., 2017; Lilley et al., 2019). This resistance response is characterized by energy-costly reactions like increased arousal frequencies (Reeder et al., 2012; Warnecke et al., 2012) and various immune response which leads to prematurely depleted fat reserves and ultimately to death (Field et al., 2018; Langwig et al., 2017; Moore et al., 2013; Verant et al., 2014).

5.4 Measures and future perspectives

In the following part I will discuss measures that have been considered to combat the white-nose disease in North America and how my studies, as envisioned 'lessons from white-nose disease in Europe', may help to improve such attempts as well as to stimulate further research and monitoring needs.

Treatments and conservation measures

In order to overcome further dramatic bat population declines due to the white-nose disease in North America (Frick et al., 2010), different treatments have been tested to save the bats affected by the fungal pathogen *Pseudogymnoascus destructans*. Different antifungal drugs from human medicine and biocides have shown promising effects to kill *P. destructans*, others

failed due to fungal resistance (Chaturvedi et al., 2011). It was shown that *P. destructans* loose a key enzyme (UVE1) under UV Light that helps repair DNA damage and this susceptibility of *P. destructans* to UV light was seen by the authors as a potential "Achilles' heel" of the fungus to combat white nose syndrome (Palmer et al., 2018). However, UV light as well as ethanol which also kills the fungus in high concentration (Puechmaille et al., 2011) are generally used in the laboratory to kill all kinds of microbes and to destroy DNA, thus, the negative effect on *P. destructans* growth is not surprising but the usability to fight the pandemic remains debatable. Moreover, different substances have shown an inhibition effect on the mycelial and conidiospore growth of *P. destructans*, such as vapour phase mushroom alcohol and vapor phase leaf aldehyde (Padhi et al., 2018, 2016); cold-pressed, terpeneless orange oil, a relatively non-toxic substance which is also used for animal and human skin care (Boire et al., 2016); several essential oils commonly used in food products (Gabriel et al., 2018); different bacteria species from *Pseudomonas* genius (Cheng et al., 2016; Fritze et al., 2012b; Hoyt et al., 2015) and *Rhodococcus rhodochrous* (Cornelison et al., 2014a); and bacterially produced volatile organic compounds (Cornelison et al., 2014b).

All these agents which work quite well in laboratories but have not been established for practical use in the wild because either the toxicity to bats is not clarified or their effects on the cave flora and fauna remains open. Additionally, it seems very ambitious to treat significant numbers of bats in the numerous caves on the North American continent (the approximate current spread of the disease refers to half of the continent), particularly since it is difficult even within one cave to find all bats or to kill all the microscopic spores. Therefore, the applicability of all these methods is questionable, if at all, they can only be used in to a very limited extend or for rehabilitation of bat individuals. Moreover, measures that cannot completely remove the pathogen but help only punctually, may even lengthen natural selection processes and hence delay the occurrence of adapted bat populations. Therefore, I think, it is more important to use conservation measures to stabilize populations of bats that survive the disease in order to assist natural selection of bats which are less susceptible to the pathogen. However, cautious decontamination treatments in caves as well as hygienic measures undertaken by cavers and bat workers can help to avoid or at least delay the further spread of the pathogen and to support the increase of surviving populations that start to tolerate the infections (Shelley et al., 2013; Zhelyazkova et al., 2020).

Indeed, promising observations from white-nose survivors in North America show similar adaptations like European bats that are tolerant like demonstrated in my work. Lilley et al. (2016) has shown that remnant populations of *M. lucifugus* do not show increases in periodic arousals from hibernation compared to bats dying from the white-nose disease (Reeder et al., 2012; Warnecke et al., 2012). Moreover, diseased North American bats with higher body fat store have higher survival rates than bats with lower fat store (Cheng et al., 2019; Haase et al., 2020), which is in line with my results on European *M. myotis*. Recent studies even report that some populations of *M. lucifugus* are beginning to stabilize at reduced population sizes, or showing population increase since previous disease-caused mass mortality events (Dobony and Johnson, 2018; Frank et al., 2019; Langwig et al., 2017). It is suggested that there is an ongoing strong selection of North American bats surviving the fungal infections by tolerant responses towards the pathogen (Cheng et al., 2019; Frank et al., 2019; Frick et al., 2017; Lilley et al., 2019, 2016).

However, immunological adaptations such as demonstrated in my studies are still missing from North American white-nose survivors. To date, there are some studies showing healing processes after hibernation (Fuller et al., 2020; Meierhofer et al., 2018; Pollock et al., 2016). Future research should focus more on immunological responses of North American bats that cope with the infections during winter, e.g. by using similar immune parameters that I used and comparing between vulnerable bats and those which seem to be coping well with the infections in the meantime. Particularly, it would be interesting if such bats use similar tolerance mechanisms as shown in the studies on European bats. As a prospect, it could be that similar to European bats, the relationship between the host and the fungus evolves into a commensal relationship that allows the host to tolerate the infection without disrupting hibernation (Lilley et al., 2019). This tolerance adaptation subsequently resulting from a maladaptive resistance response could to be a general evolutionary mechanism since previous work has shown that resistance traits tend to be polymorphic and tolerance is predicted to be favoured by natural selection in the long-term and become genetically fixed (Roy and Kirchner, 2000).

Therefore, conservation measures that protect roosts, hibernation sites but also foraging habitats are crucial to support the body conditions and the health of the bats and promotes

the population growth of bats with tolerance adaptations. Additionally, population monitoring should be used to control the success of combating the disease.

Open questions, disease surveillance and monitoring

Questions concerning white-nose disease also remain in Europe. For example, no increased mortality has been detected in European hibernacula so far (Fritze and Puechmaille, 2018; Puechmaille et al., 2011b), however, we still lack on data about the costs or even mortality occurring after hibernation. In my work, I have shown that the bats indeed show immune responses although at a limited extend. Thus, there might be still fitness costs due to the immune response which I was unable to detect. Additionally, after hibernation, bats have to survive when food availability is still low in early spring, and at the same time, wound healing of the lesions takes place in this period (Fuller et al., 2020; Meierhofer et al., 2018; Pollock et al., 2016). Moreover, in spring, fertilisation takes place in the females and it is unclear how previous infections affect this process. Therefore, the ultimate costs of white-nose disease in Europe remain unknown so far.

Additionally, white-nose disease was detected European-wide (Fritze and Puechmaille, 2018; Puechmaille et al., 2011b; Zukal et al., 2016b), but long-term disease surveillance including disease severity levels does not take place, e.g. because standardized monitoring schemes are missing.

A useful tool for further research and monitoring of white-nose disease in the wild represents the Visual Pd-score. In addition to previous simple presence/absence reporting of visible infections, my method represents a standardized scheme which even estimates levels of disease severity. Further, in contrast to other gold-standard techniques, the Visual Pd-score can be used to follow progression of the white-nose disease because it does not require bat handling which induces arousals in which bats groom of the fungus (Wilcox et al., 2014). Another big advantage is that this method is non-invasive and thus can be used during routine hibernacula counts, e.g. by bat conservation practitioners. Therefore, it can be easily used to conduct research on a large scale and to establish standardized surveillance and monitoring programs which have been requested by EUROBATS members for years and also in their latest action plan (EUROBATS, 2010; Sylvia et al., 2018).

5.5 Conclusions

Because bats have a high resistance and tolerance to various pathogens and possess a unique immune system (Banerjee et al., 2020, 2017; Irving et al., 2021), it is remarkable that bats in North America are experiencing outstanding high mortality rates due to the fungal pathogen *Pseudogymnoascus destructans*, the causing agent of white-nose disease which occurs during hibernation (Frick et al., 2010). In contrast, European bats seem to cope well with this pathogen with no increased mortality rate reported despite similar high infection rates (Fritze and Puechmaille, 2018; Pikula et al., 2017; Puechmaille et al., 2011). To shed light on the question which mechanisms enable European bats to survive the disease, I studied physiological and immunological responses of European greater mouse-eared bats (*Myotis myotis*) towards fungal infections.

In detail, I focussed on the question whether European bats are immunological adapted because the fungus is native to Europe while in North America it is a novel pathogen (Drees et al., 2017; Leopardi et al., 2015; Puechmaille et al., 2011). In the present work, I investigated whether greater mouse-eared bats (*Myotis myotis*) as representative example of European bats show tolerance or resistance in response to fungal infections.

I found that *M. myotis* tolerate fungal infections by building only moderate innate immune responses during hibernation, a physiological state in which hibernators have to be thrifty with their energy store. In contrast to North American bats, *M. myotis* did neither increase arousal frequencies nor expressed intense immune responses during arousals. Instead, *M. myotis* used selected, energetically cheap innate immune response parameters during both arousals and torpor, probably to keep the pathogen at bay until their immune system can clear it out in spring. Interestingly, I found that the relationship between available energy store (body fat) influences the frequency of regular arousals from torpor in which bats groom of the fungus and also increase selected immune response parameters. Thus, the energy storage of the bats has an influence of the health status during hibernation.

Those observations of tolerance mechanisms in *M. myotis* represent a competent strategy to retain immune competence at a state of low metabolic activity, being crucial during hibernation.

The work also highlights the importance of eco-immunological research, because with this knowledge we learn for future disease outbreaks and we can also derive more efficient and precise measures to fight emerging diseases. By using targeted conservation measures we can support the survival and re-establishment of adapted populations. Additionally, by using science-based, standardized methods which e.g. enable the involvement of bat conservation practitioners, we are able to follow population growths and disease dynamics which ultimately increases the power and action range to combat pandemics.

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7 Appendices

7.1 Appendix Chapter One

Table 7.1.1: Mean time span (duration) from end of torpor state until the temperature had increased by at least 10°C ($\Delta T_{arousal}$) and until reaching the maximum arousal temperature (max $T_{arousal}$) per treatment group.

group	arousal type	level	mean duration (min)
zymosan-treated	artificial	$\Delta T_{arousal}$ >10°C	20.94
control	artificial	$\Delta T_{arousal}$ >10°C	21.56
zymosan-treated	natural	$\Delta T_{arousal}$ >10°C	21.00
control	natural	$\Delta T_{arousal}$ >10°C	21.25
zymosan-treated	artificial	max $T_{arousal}$	36.56
control	artificial	max $T_{arousal}$	35.33
zymosan-treated	natural	max $T_{arousal}$	24.25
control	natural	max $T_{arousal}$	24.63

Table 7.1.2. RIN-values of total RNAs extracted from blood clot samples of 16 *Myotis myotis (Mmyo)*. The RIN-values (RNA integrity values) were measured in order to assess the state of the extracted RNA. The values were determined using the 2200 TapeStation (Agilent) and the RNA ScreenTape. / - no value could be measured. In comparison, fresh *Mmyo*-tissue had a RIN-Value=8

0.5 h after inoculation		48 h after inoculation		96 h after inoculation	
Sample	RIN-value	Sample	RIN-value	Sample	RIN-value
Mmyo1	4.3	Mmyo1	3.3	Mmyo1	3.4
Mmyo2	4	Mmyo2	3.5	Mmyo2	3.3
Mmyo3	/	Mmyo3	3.6	Mmyo3	2.4
Mmyo4	3.3	Mmyo4	4.3	Mmyo4	3.9
Mmyo5	3.5	Mmyo5	4.2	Mmyo5	4.1
Mmyo6	3.9	Mmyo6	4.8	Mmyo6	4.1
Mmyo7	3.7	Mmyo7	3.9	Mmyo7	3.6
Mmyo8	3.9	Mmyo8	3.8	Mmyo8	1
Mmyo9	3.6	Mmyo9	4.2	Mmyo9	4.5
Mmyo10	3.8	Mmyo10	3.6	Mmyo10	4
Mmyo11	3.8	Mmyo11	3.6	Mmyo11	3.1
Mmyo12	4.8	Mmyo12	4.8	Mmyo12	5.5
Mmyo13	5.1	Mmyo13	6.6	Mmyo13	5.9
Mmyo14	3.8	Mmyo14	2.6	Mmyo14	/
Mmyo15	3	Mmyo15	3.3	Mmyo15	3.9
Mmyo16	4.9	Mmyo16	3.8	Mmyo16	3.9

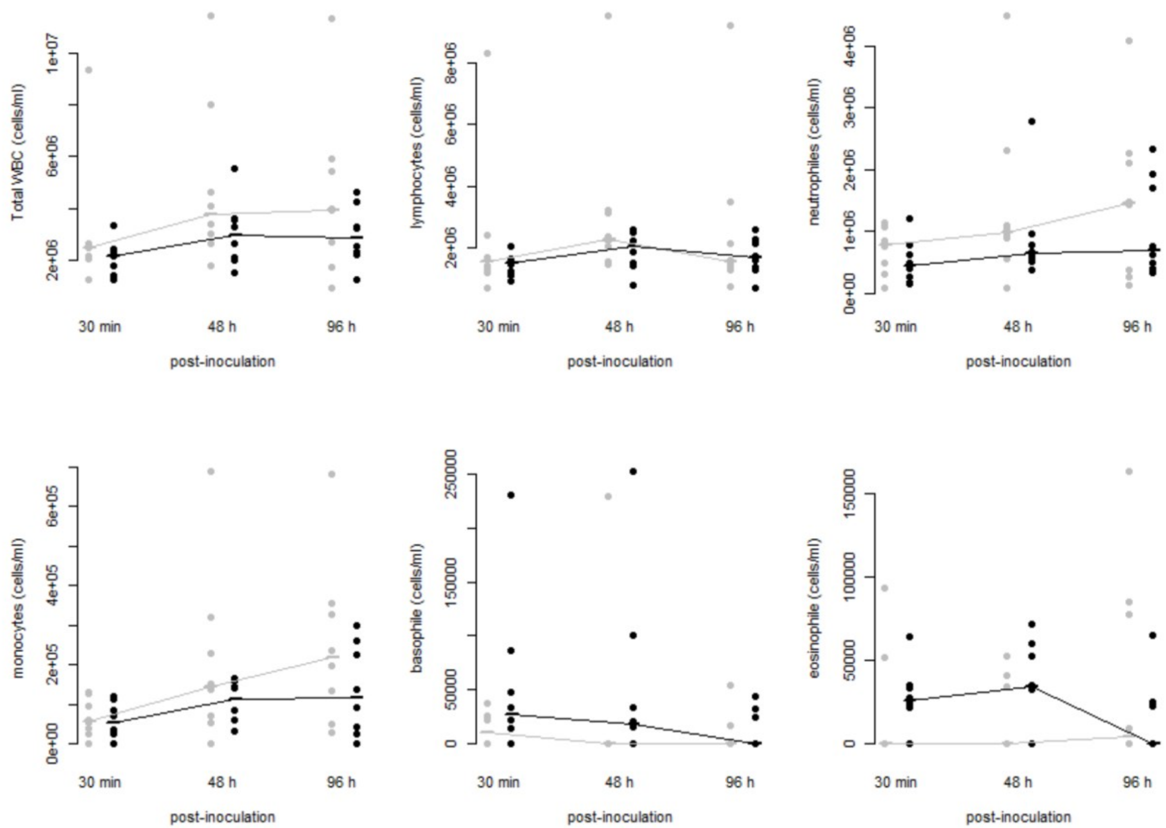


Figure 7.1.3. Concentrations (in cells / µl blood) of different white blood cells (WBC) in zymosan-treated (black) and control bats (grey)

7.2 Appendix Chapter Two

Table 7.2.1: Primer sequences from Kacprzyk et al., 2017 and Fritze et al., 2019 optimized annealing temperatures (T_a) for immune genes of *Myotis myotis*.

Primer	Sequence (5' → 3')	T_a
ACTB	F: AAATGCTTCTAGGCGGACTG	63 °C
	R: AGCCATGCCAATCTCATCTC	
IL1b	F: GAATCCATCGACTGCATGTG	63 °C
	R: GACAGCACCAGGGATTTTTG	
IL10	F: TTCAAGGGTTACCTGGGTTG	Not amplified
	R: TCAGCGTCTTCAGCTTCTCC	
TNFα	F: ATCAACCTCCTCTCTGCCATC	61 °C
	R: ACTGAGCCGATCACCCTTC	
iNOS2	F: CTGTGAGGCGTTCGATGTC	56 °C
	R: GTCCCAGGTCACATTGGTG	

Table 7.2.2: Recording data of 10 individuals carrying iButton temperature loggers; durations in minutes, temperatures in °C.

ring.ID	sex	visual stage	No lesions	WNI	total load	arousals	cold arousals	recording days	arousals per days	mean arousal duration	mean torpor duration	mean arousal temperature	maximum arousal temperature	mean torpor temperature
A137735	w	0	0	asymptomatic	0.002	3	1	29	0.10	300.0	166.5	15.1	22.8	7.3
A137733	m	0	1	mild	0.001	3	0	34	0.09	260.0	200.0	9.9	25	2.7
A132418	w	0	10	severe	0.024	5	0	34	0.15	294.0	131.9	14.1	26.5	6.0
A132420	m	I	30	severe	0.030	1	0	34	0.03	240.0	403.5	14.2	22.5	6.4
A122337	m	IV	150	severe	1.424	0	0	34	0.00	NA	812.5	NA	NA	5.8
A143184	w	II	30	severe	0.194	1	0	34	0.03	210.0	405.3	14.5	21	6.1
A143183	w	I	30	severe	0.020	2	0	34	0.06	285.0	268.5	12.7	21	6.4
A103664	m	I	0	mild	0.240	2	1	30	0.07	240.0	236.0	14.8	24.5	7.6
A109264	m	0	0	asymptomatic	0.059	2	2	22	0.09	315.0	171.0	15.2	22.3	7.9
A137712	m	I	10	severe	0.151	3	0	30	0.10	280.0	176.6	15.0	26.3	7.6

Table 7.2.3: RIN values of the qPCR of the immune gene expressions. Reference samples of two fresh *Myotis myotis* tissues had RIN=6.2 and RIN=6.3. Our measured RIN values were low due to decay of the RNA which may have resulted from the one-hour transport from the field to the freezer and the one-year storage period of RNA extracts at -80°C. However, it is likely that a linear and uniform degradation of isolated RNA applied to all samples because we collected all samples similarly, extracted them simultaneously and the time between collection and thawing/extraction was similar (Gallego Romero et al., 2014). We consider our results robust since two genes related to an inflammatory response were elevated in infected bats, and since two gene expressions showed expected interactions with other parameters such as with ROMs (Fig. 6).

Year	individual	RNA (ng/µl)	RIN value	Average	Variance	SD
2016	3	87.2	0	2.3	5.9	2.4
	4	0.6	1.1			
	5	6.1	6.6			
	6	13.2	5.3			
	7	44.1	2			
	8	19.6	3.7			
	9	0	0			
	10	0	0			
	11	0	0			
	12	138.5	1.2			
	13	83.1	1			
	14	71.7	6.3			
	15	78.8	2			
	16	0	0			
	17	81.9	4.8			
	18	68.8				
2017	1	0.7	1.6	1.9	1.5	1.2
	2	2.6	5			
	3	2.4	0			
	4	4.8	2.5			
	5	2.1	2.3			
	6	2.2	4.1			
	7	2.7	5.3			
	9		2.4			
	11	10	0			
	14	0	1.2			
	15	5.3	0			
	16	8.2	2			
	17	10.7	2.1			
	18	3.1	2.2			
	19	0	1.4			
	22	109	1.8			
	23	19.4	2.1			
	24	29	1.5			
	25	6.6	1.9			
	26	86.6	2.1			
	27	22.8	1.7			
	28	24.5	1.5			
	29	23.3	1.9			
	30	17	2			
	31	28	1.9			
	32	37.3	1.6			
	33	18.2	1.6			
	34	27.2	1.7			
	35	28.1	0			
2018	1	0.4	2	2.2	0.1	0.3
	2	10.4	2.7			
	3	1.3	2.4			
	4	5.5	2.3			
	5	2.1	2.2			
	6	1.8	1.7			
	7	2	2.1			
	8	1.8	2.4			

Table 7.2.4: P-values (lower left half) and spearman rank coefficients (upper right half) from the correlogram (Fig. 6); bold numbers mark significant correlations.

	WNI	wns visual	lesions	Mass	Torpor temp.	Torpor dur.	Arousal s	Arousal dur.	Arousal temp.	max arousal temp.		il1ß	iNOS2	TNFa	hapto	dROM	OXY
WNI		0.33	1.00	-0.26	-0.77	0.77	-0.82	-0.26	-0.77	-0.82	0.77	0.77	0.77	0.77	-0.26	0.77	-0.26
wns_visual	<0.001		0.33	-0.26	-0.77	0.77	-0.54	-0.77	0.26	-0.54	0.77	0.26	-0.26	0.26	0.77	-0.26	-0.26
Lesions	<0.001	<0.001		-0.26	-0.77	0.77	-0.82	-0.26	-0.77	-0.82	0.77	0.77	0.77	0.77	-0.26	0.77	-0.26
Mass	0.774	0.909	0.907		0.00	-0.60	0.74	0.80	-0.20	-0.21	-0.60	-0.80	-0.40	-0.80	-0.40	0.20	-0.80
torpor_T	0.559	0.929	0.671	0.954		-0.80	0.63	0.40	0.40	0.95	-0.80	-0.40	-0.20	-0.40	-0.20	-0.40	0.60
torpor_D	0.212	<0.001	<0.001	0.333	0.645		-0.95	-0.80	-0.20	-0.63	1.00	0.80	0.40	0.80	0.40	0.20	0.00
Arousals	0.358	0.004	0.023	0.043	0.835	0.001		0.74	0.32	0.50	-0.95	-0.95	-0.63	-0.95	-0.21	-0.32	-0.21
arousal_D	0.082	0.004	0.065	0.058	0.430	0.004	0.0203		-0.40	0.11	-0.80	-0.60	0.00	-0.60	-0.80	0.40	-0.40
arousal_T	0.721	0.590	0.945	0.924	<0.001	0.991	0.9787	0.621		0.63	-0.20	-0.40	-0.80	-0.40	0.80	-1.00	0.40
arousal_Tmax	0.783	0.264	0.135	0.391	0.723	0.068	0.0230	0.479	0.815		-0.63	-0.32	-0.32	-0.32	0.11	-0.63	0.74
Time_last_arousal	0.229	0.022	0.042	0.183	0.403	0.006	0.001	0.014	0.599	0.048		0.80	0.40	0.80	0.40	0.20	0.00
il1ß	0.693	0.716	0.363	0.994	0.919	0.600	0.9434	0.914	0.799	0.642	0.592		0.80	1.00	0.00	0.40	0.40
iNOS2	0.880	0.587	0.803	0.787	0.666	0.889	0.6408	0.440	0.981	0.994	0.997	<0.001		0.80	-0.60	0.80	0.20
TNFa	0.328	0.910	0.821	0.920	0.957	0.727	0.9953	0.612	0.675	0.506	0.625	<0.001	<0.001		0.00	0.40	0.40
Hapto	0.042	0.272	0.352	0.328	0.417	0.832	0.5115	0.050	0.325	0.606	0.518	0.499	0.661	0.232		-0.80	0.20
dROM	0.085	0.296	0.575	0.003	0.335	0.481	0.3317	0.841	0.142	0.028	0.27	0.006	0.193	0.001	0.424		-0.40
OXY	0.001	0.002	0.024	0.335	0.771	0.815	0.5300	0.287	0.746	0.192	0.29	0.394	0.997	0.798	0.400	0.852	

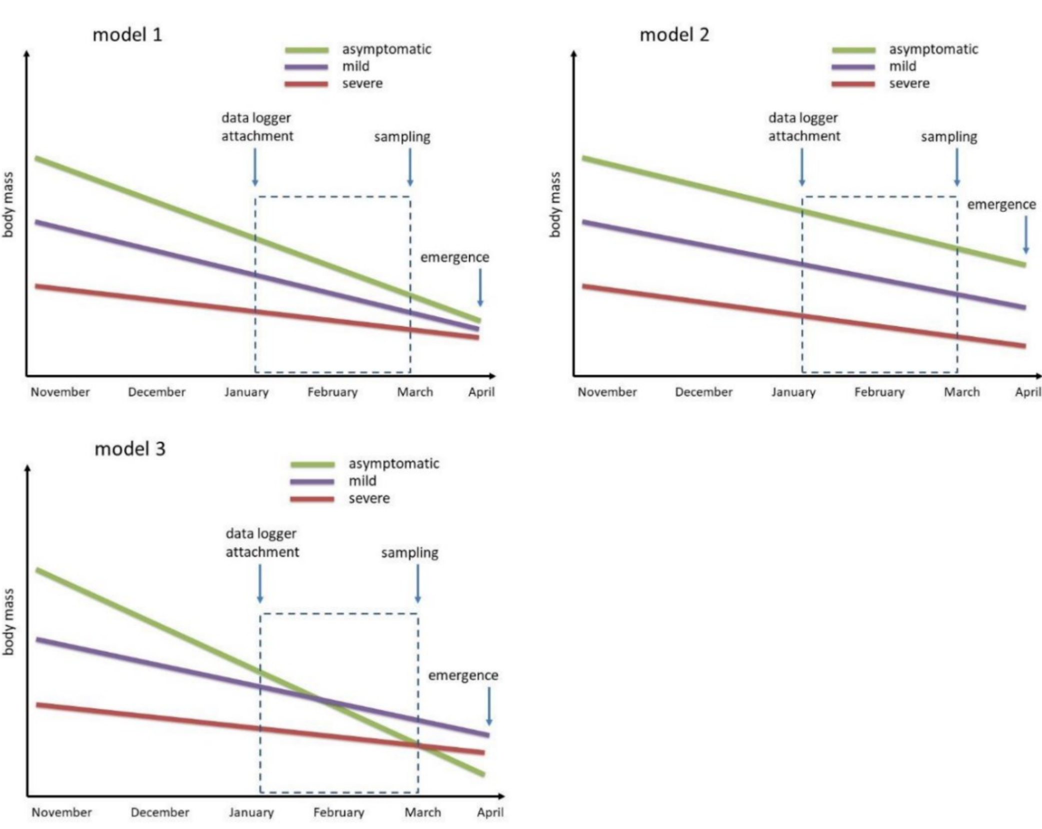
Table 7.2.5: Scheme to classify visual infection stages of *Pseudogymnoascus destructans* on living bats. Symptoms describe infected areas to assess different stages of disease severity (see also (Fritze et al., 2012a)).

Progress of the colonization	Visual white-nose score	Description
Asymptomatic bat	0	Asymptomatic bats - No visible fungus on any area of the bat
First signs of infection	I	First start of an infection - Only single infected area on bat visible - infected area visible as tiny dots (< 0.2 cm) or - infected area visible as thin 'powder-like' layer on the muzzle (< 0.5 cm)
Moderately progressed	II	Moderate infection - infected area clearly visible (> 0.2 cm, < 0.5 cm) or - more than one infected area < 0.2 cm, at least one infection on wing or tail membrane
Further progressed	III	Severe infection whole muzzle colonized by fungus and/or - infection of more than one area of skin or tail membrane
Severely progressed	IV	- Very severe infection - whole bat is severely colonized by fungus - Fungus colonizes all naked areas of the bat, including muzzle, ears, wing/tail membrane - Fungus appears greyish (late stage of colonization)

7.2.6: In our comparative approach, we found differences in body mass between the disease stages of our WNI (Figure 2 in the main text). To illustrate why body mass was slightly higher in mildly infected bats compared with asymptomatic bats we drew a schematic model (Figure below). We hypothesise that the observed link between body mass and infection status is the result from different fat stores prior to hibernation (Figure below). Heavier bats at the start of the hibernation season can arouse more often, leading to higher rates of fat reserves depletion. A direct consequence of the higher number of arousals is the limited or absence of *Pd* development in these (asymptomatic) individuals. The opposite is true for lean individuals. They cannot afford many arousals which leads to a low rate of fat reserve depletion. A direct consequence of the low number of arousals is the high *Pd* colonisation on these individuals. Individuals with an average body mass at the start of the hibernation have an intermediate behaviour and as a result intermediate levels of infection. Our data supports this hypothesis: Bats with higher arousal frequencies ultimately showed fewer lesions compared to bats with fewer arousals (Figure 2 in the main text). Interestingly, there was no mass difference between severely infected and asymptomatic bats while mildly infected individuals remained heaviest. If our hypothesis is correct and all individuals converge towards the same body mass at the end of the hibernation, heavier individuals at the start of hibernation would be expected to be the heaviest throughout the winter and inversely for the lean individuals (=model 2, figure below). This is not exactly what we observe. One possible explanation for this discrepancy is the cost of *Pd* infection post emergence that is not considered in model 2. Upon emergence in spring, asymptomatic individuals will

not have to combat *Pd* and repair the lesions while mild and severely infected ones will have to, requiring further energy and therefore fat reserves. Hence, it could be expected that mildly and severely infected individuals would need extra reserves compared to asymptomatic individuals for post-hibernation emergence (and repairing *Pd* associated damages; model 3). Further studies are necessary to explore the post-hibernation cost of *Pd* infection, possibly by investigating in more details the link between body mass and infection severity toward the end of the hibernation season.

Figure: Theoretical scheme explaining body mass loss in the comparison among asymptomatic (green line), mildly (purple line) and severely (red line) infected bats. The dashed line box indicates the time course of our experiment with data logger attachments on the bats at the beginning of the experiment and the sampling at the end of the experiment. The y-axis on the left represents the body weight. The x-axis represents the timeline of the hibernation season, beginning in November until the emergence from the hibernacula in April. The scheme illustrates that bats with high body masses at the beginning of the hibernation season can (energetically) afford to arouse more often compared to lean bats, with lower fat store. Bats that arouse more often are consequently less infected (mild infected or asymptomatic) compared with bats with less arousals (severely infected).



7.3 Appendix Chapter Three

7.3.1. Details of qPCR measurements to quantify *Pd* loads obtained by bat swabbing:

A quantitative PCR, measuring the number of copies of the IGS, was used as a proxy for quantifying *Pd* load collected on the swabs. *Pd* specific primers and probe for the IGS markers were used (Forward: nu-IGS-0169-59–Gd: 5'– TGC CTC TCC GCC ATT AGT G – 3'; Reverse: nu-IGS-0235-39–Gd: 5' – ACC ACC GGC TCG CTA GGT A – 3'; probe: nu-IGS-0182/0204-Gd: 59–(FAM) CGT TAC AGC TTG CTC GGG CTG CC (BHQ-11) –3') (Muller et al., 2013). First, spore and hyphae loaded swabs were submerged in 150µl H_2O and agitated for 10min. After 5min of centrifugation (16.000 ×g) the swab stick was removed and the spore suspension centrifuged again (10min, 16.000 ×g). The supernatant was discarded and the remaining pellet was suspended in 300µl lysis buffer (NucleoSpin® DNA RapidLyse kit, Macherey & Nagel, Düren, Germany). After addition of 10µl lyticase (3mg/ml) the suspension was incubated for 30min at 30°C, then 10µl proteinase K (20mg/ml) and 10µl DTT (1M) were added, followed by an overnight incubation at 54°C. DNA extraction was then carried out according to manufacturer's recommendation (Macherey & Nagel, Düren, Germany). For the standard dilution curve we extracted DNA from petri dish-cultivated fungal mycelia of *Pd* reference strain CCF3937 to be consistent with material which was previously used for the qPCR technique in Europe (Martinkova et al., 2010), also using the NucleoSpin® DNA RapidLyse kit (Macherey & Nagel). The initial DNA concentration and those of the dilution series were quantified using the Invitrogen™ Quant-iT™ Picogreen™ dsDNA Assay-kit (ThermoFisher, Dreieich, Germany) with the fluorescence being measured on an Infinite 200 Pro M (Tecan, Männedorf, Switzerland). DNA for the dilution series was diluted in five 1:10 dilution steps, from 2.2ng/µl to 0.22 pg/µl.

The qPCR was carried out on a CFX96 cycler (Bio-Rad, Munich, Germany) using the 'Real Time PCR iQ Supermix' (Bio Rad, Feldkirchen, Germany) according to the manufacturer's protocol. Forward and reverse primers (Muller et al., 2013) were used at a final concentration of 0.3 µM each (Zukal et al., 2016a). For amplicon quantification we used a species-specific fluorescently-labeled probe containing a quencher and a minor groove binder. Reaction components were mixed on ice and three replicates were prepared for each dilution step of the reference DNA, bat-derived samples were set-up in duplicates. QPCRs were performed with an initial denaturation step of 95°C for 30s, followed by 40 × {denaturation at 95°C for 20s,

annealing/elongation at 62°C for 1min}. The DNA of the reference *Pd* isolate was used as positive control and to determine DNA concentrations, while H_2O substituted the sample DNA in the no-template controls. The calibration curve for the DNA concentration was generated based on the DNA concentrations of the standard dilution series by plotting quantities of template DNA against qPCR C_t values. The average qPCR efficiency was 1.99 (R2=0.994, slope: -3.319). Cycle threshold values (C_t value) were used to calculate fungal loads in [ng], using the equation: load = $10^{((22.04942 - Ct\ value)/3.34789)}$, which was derived from serial dilutions of a quantified standard of isolate *P. destructans.*

7.3.2 table from the correlation matrix (figure 2). Shown are the correlation coefficients (italic) and the p values between Visual *Pd* score, number of lesions on the membranes ('lesions'), fungal load on wing membranes ('membr_load'), fungal load on the muzzles and ears ('face_load') and total fungal load ('total load'; =membr_load + face_load); square root tranformed data marked with 'sqrt'.

	Visual *Pd* score	lesions (sqrt)	membr_load (sqrt)	face_load (sqrt)	total_load (sqrt)
Visual *Pd* score		*0.66*	*0.63*	*0.64*	*0.68*
lesions (sqrt)	<0.001		*0.46*	*0.39*	*0.46*
membr_load (sqrt)	<0.001	0.002		*0.74*	*0.92*
face_load (sqrt)	<0.001	0.009	<0.001		0.87
total_load (sqrt)	<0.001	0.002	<0.001	<0.001	

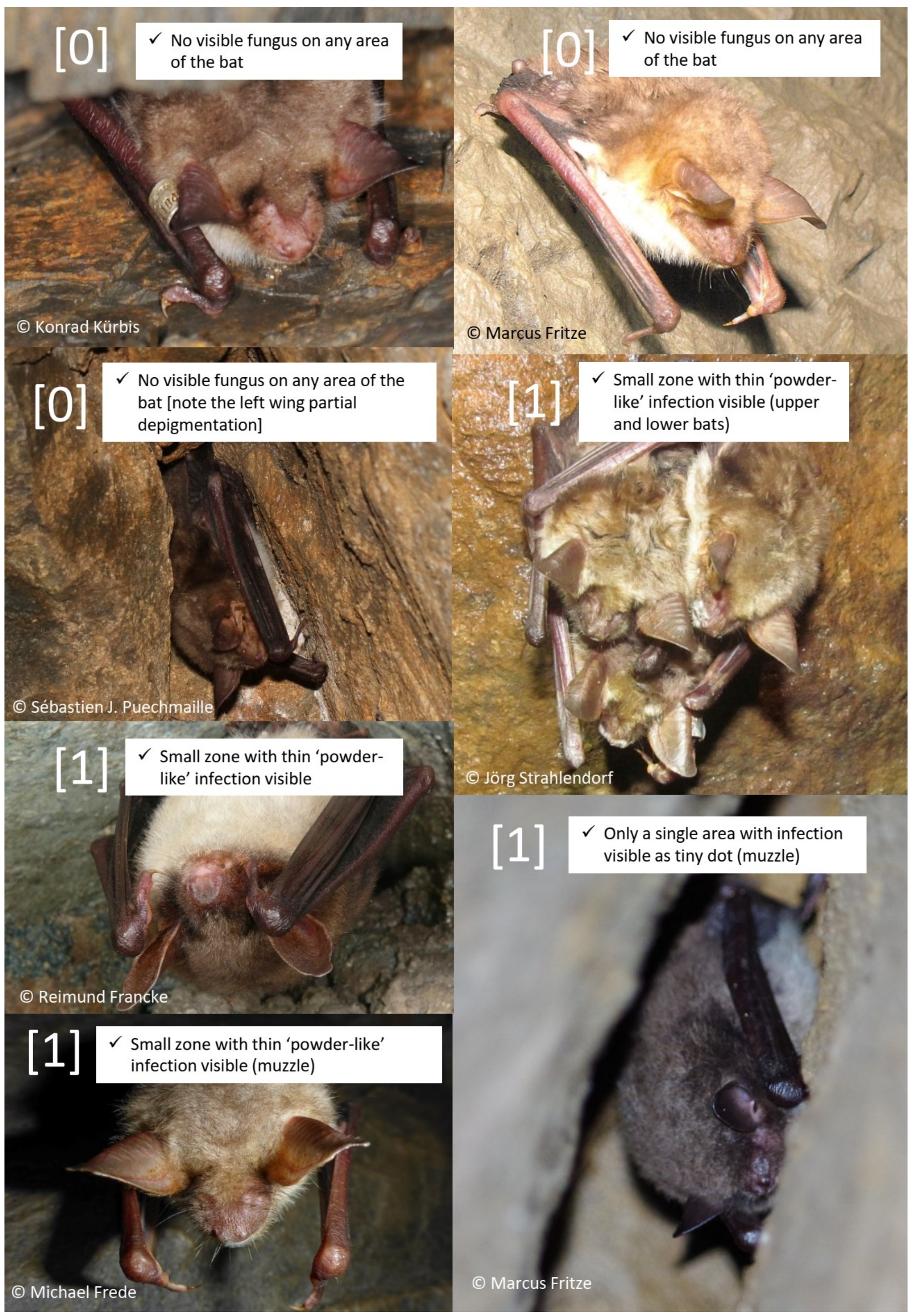
7.3.3. Protocol for assessing infection stages of bats with *Pseudogymnoascus destructans* by using the Visual Pd-score

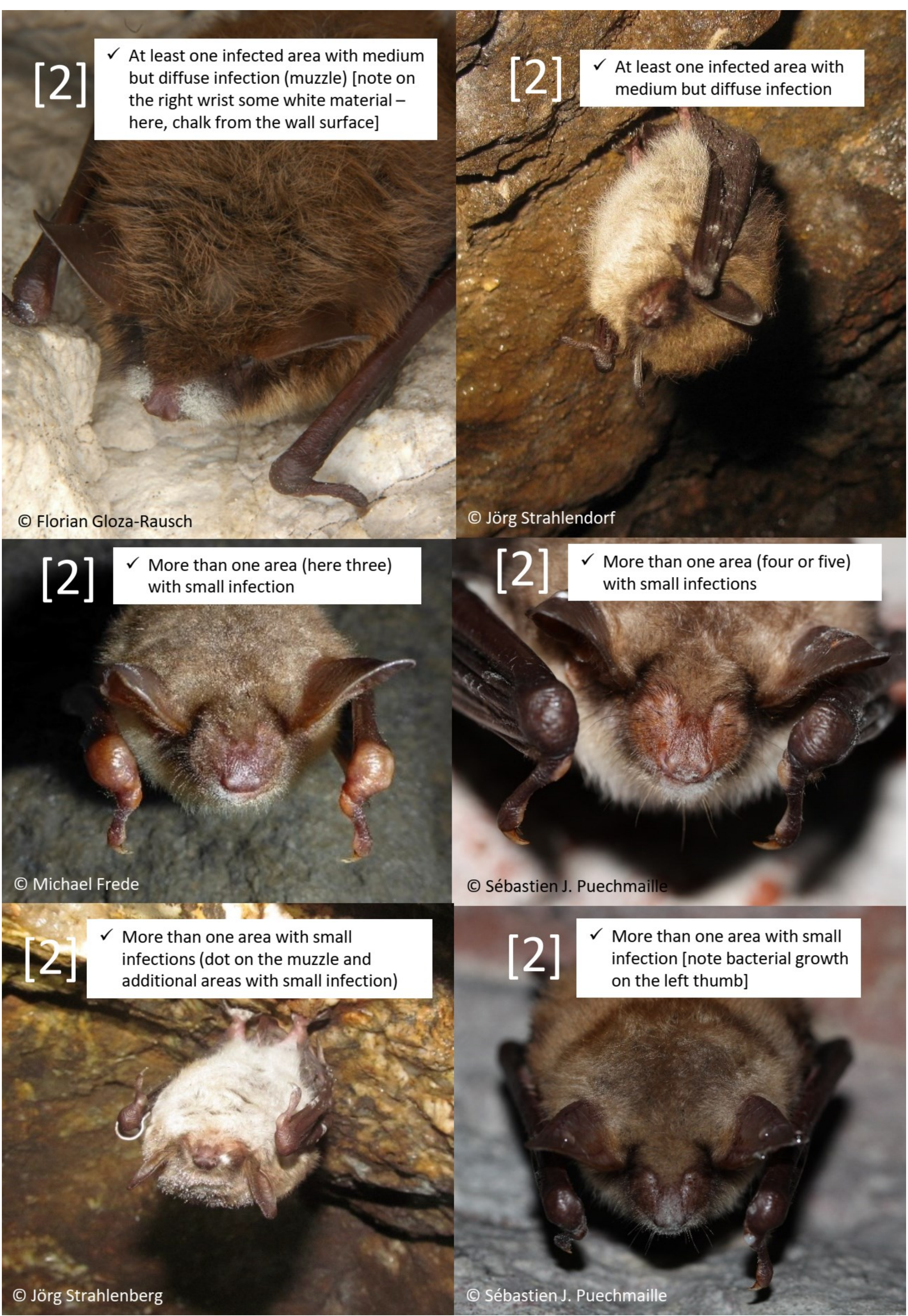
1. **During hibernacula counts, make sure that you avoid disturbances to bats as much as possible, e.g.**
    - **Conduct your survey with a minimal number of persons per hibernation site (the smaller the site, the fewer persons should enter the site)**
    - **Minimize the duration of the survey (try to do the survey quickly and do not spend more time than necessary within the hibernation site)**

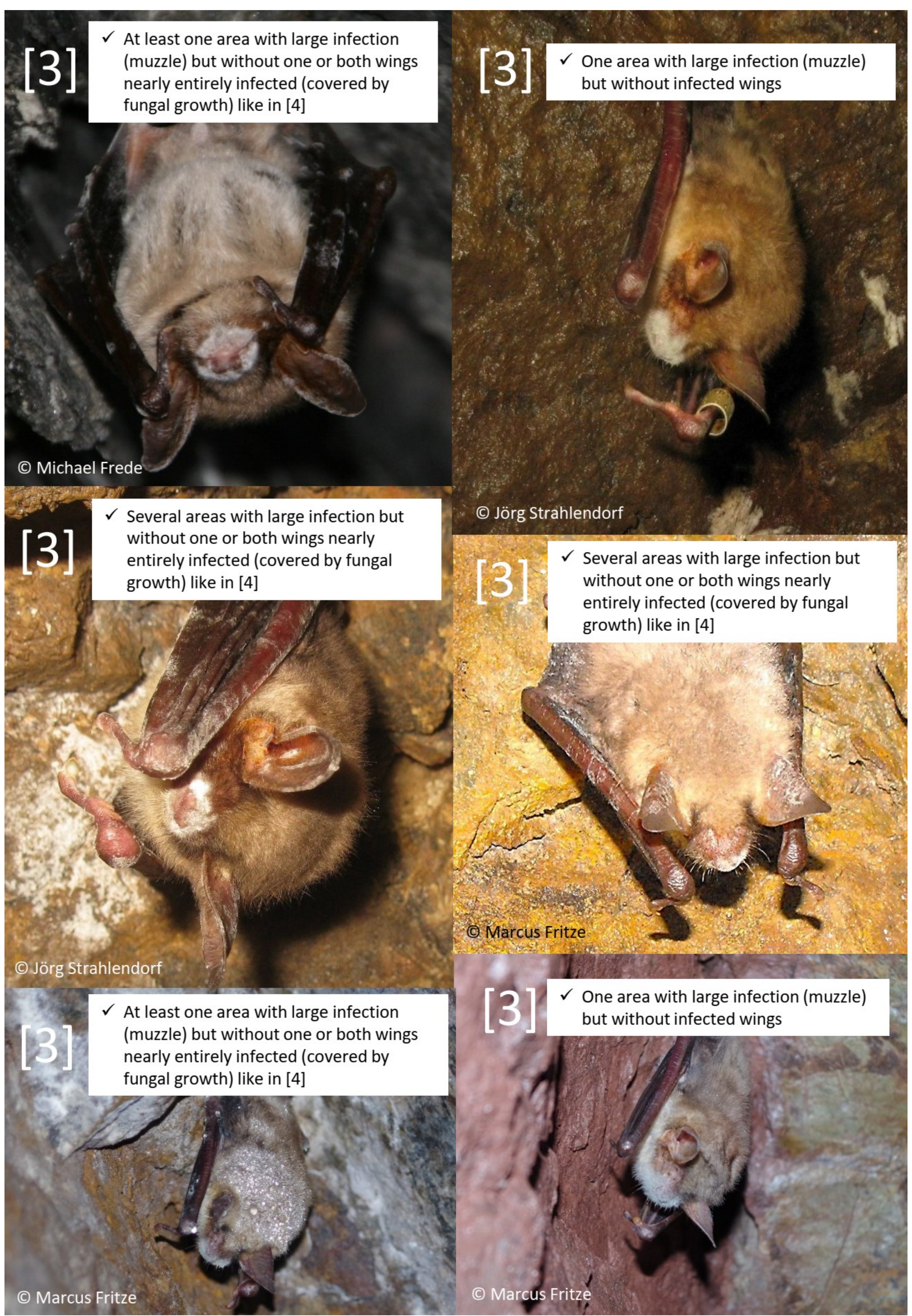
- **Minimize lighting and duration of illumination of the bats (only a few seconds per bat)**
- **Do not stay directly next to or under the bats to avoid changes in temperature near the bats**
- **Be as quiet as possible in the hibernation site**
- **Do not touch the bats**

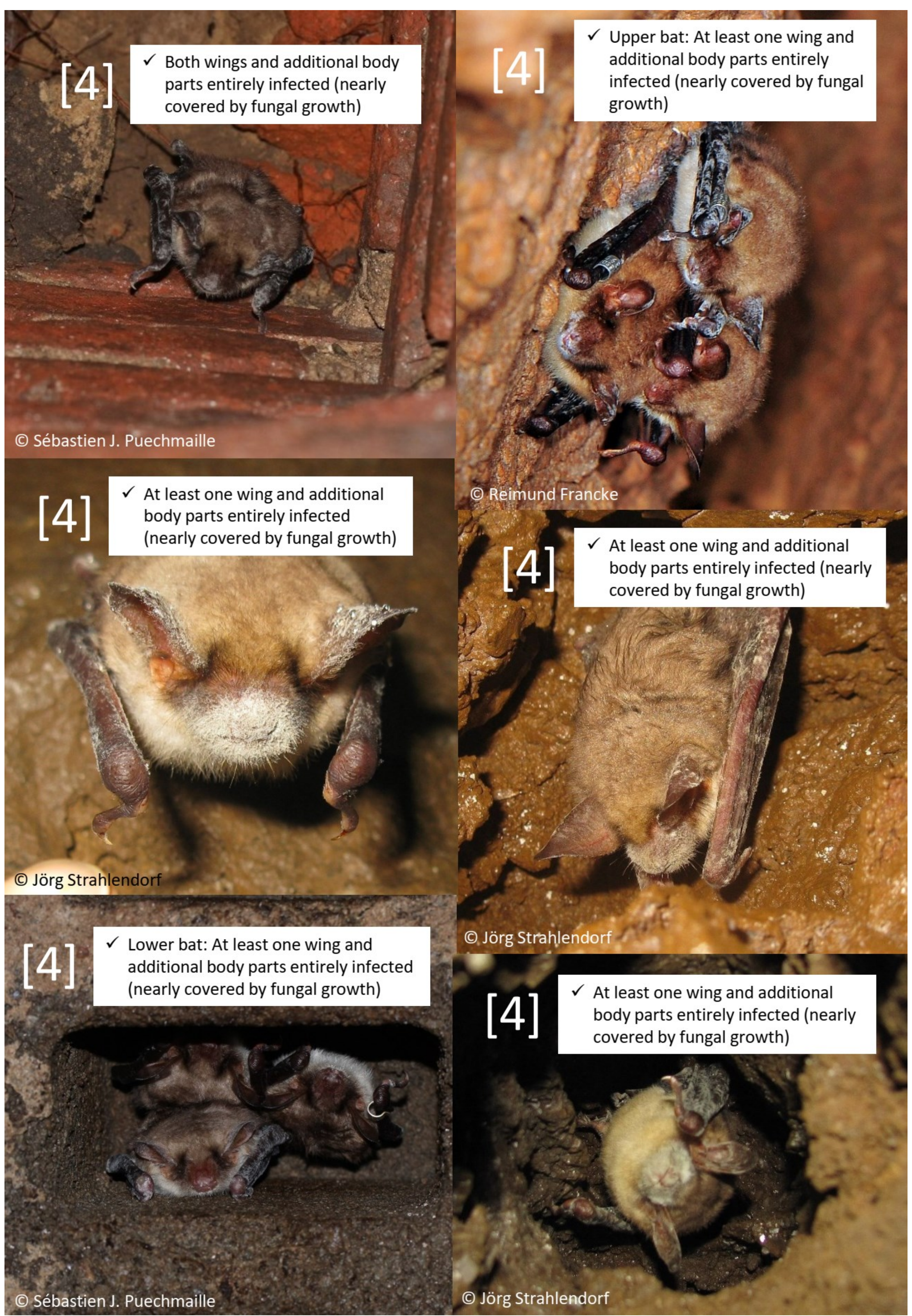
2. **If you see a bat, take a closer look (but do not exceed 1 m distance) to detect infections with *P. destructans*,**
3. **Assesse the progression of the *P. destructans* colonization visually by using the classification scheme (Table 1)**
4. **Change the visual perspective a few times in order to see all body parts of the bat,**
5. **Take a photo if necessary**
6. **Document your assessment, in a protocol, according the classification scheme. It is also recommended to describe which areas are infected and how much.**

7.3.4: Photo examples as a training data set for assessing *P. destructans* infections according to the Visual Pd-score (see table 1).


[0]
✓ No visible fungus on any area of the bat
© Konrad Kürbis
[0]
✓ No visible fungus on any area of the bat
© Marcus Fritze
[0]
✓ No visible fungus on any area of the bat [note the left wing partial depigmentation]
© Sébastien J. Puechmaille
[1]
✓ Small zone with thin 'powder-like' infection visible (upper and lower bats)
© Jörg Strahlendorf
[1]
✓ Small zone with thin 'powder-like' infection visible
© Reimund Francke
[1]
✓ Only a single area with infection visible as tiny dot (muzzle)
[1]
✓ Small zone with thin 'powder-like' infection visible (muzzle)
© Michael Frede
© Marcus Fritze


[2]
✓ At least one infected area with medium but diffuse infection (muzzle) [note on the right wrist some white material – here, chalk from the wall surface]
© Florian Gloza-Rausch
[2]
✓ At least one infected area with medium but diffuse infection
© Jörg Strahlendorf
[2]
✓ More than one area (here three) with small infection
© Michael Frede
[2]
✓ More than one area (four or five) with small infections
© Sébastien J. Puechmaille
[2]
✓ More than one area with small infections (dot on the muzzle and additional areas with small infection)
© Jörg Strahlenberg
[2]
✓ More than one area with small infection [note bacterial growth on the left thumb]
© Sébastien J. Puechmaille


[3]
✓ At least one area with large infection (muzzle) but without one or both wings nearly entirely infected (covered by fungal growth) like in [4]
© Michael Frede
[3]
✓ One area with large infection (muzzle) but without infected wings
© Jörg Strahlendorf
[3]
✓ Several areas with large infection but without one or both wings nearly entirely infected (covered by fungal growth) like in [4]
© Jörg Strahlendorf
[3]
✓ Several areas with large infection but without one or both wings nearly entirely infected (covered by fungal growth) like in [4]
© Marcus Fritze
[3]
✓ At least one area with large infection (muzzle) but without one or both wings nearly entirely infected (covered by fungal growth) like in [4]
© Marcus Fritze
[3]
✓ One area with large infection (muzzle) but without infected wings
© Marcus Fritze


[4]
✓ Both wings and additional body parts entirely infected (nearly covered by fungal growth)
© Sébastien J. Puechmaille
[4]
✓ Upper bat: At least one wing and additional body parts entirely infected (nearly covered by fungal growth)
© Reimund Francke
[4]
✓ At least one wing and additional body parts entirely infected (nearly covered by fungal growth)
© Jörg Strahlendorf
[4]
✓ At least one wing and additional body parts entirely infected (nearly covered by fungal growth)
© Jörg Strahlendorf
[4]
✓ Lower bat: At least one wing and additional body parts entirely infected (nearly covered by fungal growth)
© Sébastien J. Puechmaille
[4]
✓ At least one wing and additional body parts entirely infected (nearly covered by fungal growth)
© Jörg Strahlendorf

7.4 Appendix General Discussion

Leibniz Institute for Zoo and Wildlife Research

Member of the Leibniz Association

Resilience or Resistance - How do European bats cope with White-Nose infections?

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[2]Free University of Berlin

Introduction: The cold-loving fungus *Pseudogymnoascus destructans* (*Pd*), the causing agent of the White-nose syndrome, affects bats during hibernation and causes mass mortalities in North America. In Europe, *Pd* has also been recorded as a widespread pathogen since several decades, but obviously with no recorded mass mortalities. It is suggested that *Pd* is native to Europe and has been brought to North America as a novel pathogen. Hence, our study is investigating if the immune system of European bats reacts to the fungus during hibernation.

Photo: M.Fritze

Results: White-nose infected bats showed no difference in the total amount of white blood cells (all leucocytes), neutrophils, basophils, eosinophils and monocytes. We found significant difference between mild infected and severely infected bats with respect to total numbers of lymphocytes. Body condition index was not different between non-infected and infected bats.

White blood cells (x 1 Mio) per µl

neutrophiles (x 1 Mio) per µl

lymphocytes (x 1 Mio) per µl

p= 0.04

Body Condition Index (BCI)

WNS score

WNS score

Material & Methods:

- Blood samples were collected from 29 bats (*Myotis myotis*) within natural hibernacula.
- White-nose score was determined by visual detection on bats and UV light transillumination of the flight membrane.
- Blood was analyzed by total and differential white blood cell counts (microscopy).
- Body condition index (BCI) was calculated: body mass (g)/forearm length (mm).
- Significance in differences between the infection groups were analysed by ANOVA of linear models with Post-hoc test (Tukey) in R.

Photo: S.J.Puechmaille

Detected lesions under UV light

Photo: M.Fritze

Leucocytes among erythrocytes

WNS score:
0 no visible infections, no lesions
1 mild infection in the face, few lesions (1-20)
2 severe infections with many lesions (> 20)

Discussion: Lymphocytes, neutrophils, basophils, eosinophils and monocytes are subtypes of the white blood cells in the vertebrate immune system. An increase in lymphocytes indicates an immunological response especially by the adaptive part of the immune system, not neccessarely the innate part. No differences in body conditions of non-infected and infected individuals suggest that the European bats fight fungal infections successfully during hibernation.

Conclusions: Lymphocytes play a key role in the immunological defence of *Myotis myotis* against *Pseudogymnoascus destructans*. European bats resist successfully with the White-nose infections by their competent adaptive immune system.

Acknowledgements: We would like to thank Katja Pohle for the instructions in the laboratory work; Janina Radwainski, Bernd Ohlendorf and Carolin Stern for the great help during the field work; Dr. Nicolas Fasel for the helpful comments concerning the statistical analysis.

Further Research: Bat mortality due to White-nose infections in Europe?

Identifying unusual mortality events in bats: a baseline for bat hibernation monitoring and white-nose research
FRITZE, MARCUS & PUECHMAILLE, SEBASTIEN J. 2018
(Mammal Reviews, accepted.)

- 318 hibernacula surveyed in 30 countries
- 206 dead bats were found at 32 sites
- average mortality rate of 0.83 %
- *Pseudogymnosascus destructans (Pd)* detected on bats at 98 sites
- **presence of *Pd* on bats was not a significant predictor of presence or number of dead bats!**

contact: fritze@izw-berlin.de

Treffen der Deutschen Fledermausforscher 2018, 12.01.2018 bis 14.01.2018, Heidesee.

Figure 7.4.1: Poster about preliminary results from white blood cell counts and body conditions from wild-living torpid greater mouse-eared bats infected with *P. destructans.* German Bat Research Meeting (TDFF), Heidesee, 12-14 January 2018.

8 Publications

The following publications are part of this thesis:

Fritze, Marcus, Costantini, D., Fickel, J., Wehner, D., Czirják, G.Á., Voigt, C.C. (2019): Immune response of hibernating European bats to a fungal challenge. Biology Open (2019) 8, bio046078. https://doi.org/10.1242/bio.046078

Fritze, M., Puechmaille, S. J., Costantini, D., Fickel, J., Voigt C.C, Czirják, G.Á. (2021): Determinants of defence strategies of a hibernating European bat species towards the fungal pathogen *Pseudogymnoascus destructans*. Developmental and Comparative Immunology 119 (104017). https://doi.org/10.1016/j.dci.2021.104017

Fritze, M., Puechmaille, S. J., Fickel, J., Czirják, G.Á., Voigt C.C. (n.d.): A rapid, in-situ minimally-invasive technique to assess infections with *Pseudogymnoascus destructans* in bats. Minor revision submitted.

9 Declaration of authorship

I hereby declare that I prepared this thesis independently under the guidance of my supervisors. All direct or indirect sources used are given as references. All contributions of co-authors are acknowledged.

Berlin, 15.02.2021 **Marcus Fritze**