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Markéta Nováková

Souvis genetické informace sekvencí DNA s infekčním onemocněním netopýrů
Consequences of an infectious disease on genetic information in DNA sequences in bats

Bakalářská práce

Vedoucí závěrečné práce: Mgr. Natália Martínková, PhD.

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala a samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 15.5.2015

Markéta Nováková

Poděkování

Ráda bych poděkovala vedoucí práce Natálii Martínkové za věnovaný čas, profesionální vedení a cenné rady a připomínky, bez kterých by tato práce nemohla vzniknout.

Abstrakt

Syndrom bílého nosu je plísňové onemocnění netopýrů způsobující masivní úhyny v severoamerických populacích. Infekční plíseň způsobuje léze na kůži netopýrů, převážně na čenichu, uších a létacích blánách. Infekce plísní je doprovázena vážným narušením metabolismu netopýrů a hibernační fyziologie, které jsou smrtelné pro netopýry v Severní Americe. Evropští netopýři nemoc přežívají ve větším počtu. Protože je plíseň pravděpodobně evropského původu, tato studie předpokládá, že u evropských netopýrů se vyvinuly dědičné obranné mechanismy proti syndromu bílého nosu. Infekce plísní způsobující onemocnění představuje silný selekční tlak, v praktické části jsem proto u 7 genů hledala známky pozitivní selekce pomocí metody maximální věrohodnosti. Zjistila jsem vliv pozitivní selekce u genu pro transglutaminázu 1.

Klíčová slova

syndrom bílého nosu, *Pseudogymnoascus destructans*, pozitivní selekce

Abstract

White-nose syndrome is an emerging fungal disease of bats causing massive die-offs in North American populations. The fungus causes lesions on bat skin, mainly on noses, ears and wing membranes. The infection by the fungus is accompanied by serious disruption of metabolism and hibernation physiology that is lethal to bats in North America. European bats seem to survive the disease in greater numbers. Since the fungus is probably of European origin, this study assumes that bats in Europe have developed inheritable defence mechanisms to the disease. The infection by the fungus serves as a strong selective pressure. We tested sequences of 7 genes for signs of positive selection using maximum likelihood approach. We detected past positive selection in the gene for transglutaminase 1.

Keywords

white-nose syndrome, *Pseudogymnoascus destructans*, positive selection

LIST OF USED ABBREVIATIONS

acad10	acyl-CoA dehydrogenase 10
acp5	acid phosphatase 5
anxa1	annexin A1
aqp3	aquaporin3
bcam	basal cell adhesion molecule
ctnnb1	catenin beta 1
hyal2	hyaluroglucosaminidase 2
ln*L*	Log-likelihood
LRT	likelihood-ratio test
MCMC	Markov chain Monte Carlo
MHC	major histocompatibility complex
MNTD	Mean nearest taxon distance
MPD	Mean pairwise distance
NRI	net relatedness index
NTI	nearest taxon index
ORF	open reading frame
PCR	polymerase chain reaction
Pd	*Pseudogymnoascus destructans*
pxn	paxillin
SEM	scanning electron microscope
tgm1	transglutaminase 1
TLR	toll-like receptors
tlr4	toll-like receptor 4
UV	ultraviolet
WNS	white-nose syndrome

TABLE OF CONTENTS

1 INTRODUCTION

Chiroptera contains more than 1100 species (Simmons, 2005) and is the second largest order of mammals (Teeling & Springer, 2005). Bats represent approximately 20 % of mammal species diversity. They form one of few vertebrate groups that evolved the ability of active flight, aside from birds and extinct *Pterosauria*. These characteristics make bats an outstanding order, which has earned a great interest both of zoologists and public.

In the past decade, North American populations of bats have been facing probably the most lethal and widespread infection of mammals ever. White-nose syndrome, a fungal disease devastating North American bat populations, has first appeared 9 years ago in one cave in Northeast of the United States (Blehert et al., 2009). Ever since it has spread to an enormous area on the North American continent (USFWS, 2015).

Massive die-offs, that accompany the condition of bats, have considerable impact on economies, since bats are active predators of insects. Decreases in bat population numbers cause losses in agriculture (Kunz et al., 2011). White-nose syndrome has appeared in Europe, but with no such disturbing progress and mortality (Martínková et al., 2010).

This thesis aims to review current knowledge about the white-nose syndrome and the fungal infectious agent associated with it. It should consider possible impact of persistent infection by the pathogenic fungus on the genetic information in DNA sequences and focus on possible impact of positive selection and convergence on populations.

In the experimental part, the aim is to determine presence of genetic changes in DNA sequences linked with potential past co-evolution of bats with the fungus. Pressure to adapt to the disease is present in multiple other diseases, thus selective pressure linked with white-nose syndrome should be detectable in the sequences of chosen genes.

2 LITERATURE REVIEW

2.1 White-nose syndrome

White-nose syndrome (WNS) is an infectious disease of insectivorous hibernating bats, which was lethal to more than 5 million bats in the past decade (USFWS, 2015). The first observations of WNS were made in the winter of 2006 in Howes cave in the New York state, USA. The condition was named after visual manifestation of the disease, white fungal conidia on skin and wing membranes of affected bats (Blehert et al., 2009).

Since its discovery 9 years ago, the fungal pathogen has spread to caves and mines, where bats hibernate (hibernacula), across Northeast America (Frick et al., 2010) and mortality has approached 100% in several hibernacula (Turner & Reeder, 2009).A previously unknown pathogen was isolated from bats of several species in North American hibernacula and recently described as a psychrophilic (cold-loving) fungus, *Pseudogymnoascus destructans* (*Pd*) (Gargas et al, 2009; Minnis & Lindner, 2013).

WNS caused mass mortalities have a major impact on ecosystem stability, therefore mass mortality associated with *Pd* causes an ecological disaster. Lacking bats, the main insect predator, has a negative effect on economy. Insect species regulated by bats can cause significant damage in agriculture (Kunz et al., 2011). Considering economic loss caused by bat population decrease (Cleveland et al., 2006), managing WNS in North America becomes more urgent.

The rapid spread of *Pd* is regularly monitored throughout North American hibernacula. In Europe, search for *Pd* presence has become a part of regular yearly screenings in caves and hibernacula in multiple countries.

2.1.1 Geographic distribution of *Pd* and WNS in North America

Infection by *Pd* has spread into populations of bats across the USA. Some bat species, including one of the most common North American bat, *Myotis lucifugus*, may become localy extinct in the next decades (Frick et al., 2010).

As mentioned above, the first record of *Pd* infected bat comes from the Howes Cave, located in Central New York Region, which seems to be the epicentre of the spread of the infection

(Maher et al., 2012). The Howe Caverns, the biggest caves in the Northeast United States, are open to public and, with about 160,000 visitors a year, one of the most popular tourist attractions in the region. This may indicate anthropogenic introduction of the fungus (Foleyet al. 2011; Puechmaille et al., 2011)

According to the U. S. Fish and Wildlife Service, WNS cases have been confirmed in 26 U. S. states as of May 2015, specifically Alabama, Arkansas, Connecticut, Delaware, Georgia, Illinois, Indiana, Iowa, Kentucky, Maryland, Massachusetts, Maine, Michigan, Missouri, New Hampshire, New Jersey, New York, North Carolina, Ohio, Pennsylvania, South Carolina, Tennessee, Vermont, Virginia, West Virginia and Wisconsin (USFWS, 2015)and 5 Canadian provinces, namely New Brunswick, Nova Scotia, Ontario, Prince Edward Island and Quebec (CWHC, 2015.) (Figure 1).

WNS has been confirmed in 7 species in Northeast America: *Eptesicus fuscus*, *Myotis leibii*, *M. lucifugus, Perimyotis subflavus, M. grisescens, M. sodalis, M. septentrionalis* (Table 1) (USFWS, 2015) The last tree mentioned are listed as endangered species in the United States (USFWS, 2015) Additionally to this, infection by *Pd* without symptoms of WNS has been documented in *Corynorhinus*, *Lasionycteris* and *Lasiurus* genera (Bernard et al. 2015).

Table 1 WNS positive and Pd positive species of bats in North America

Species	**Reference**
WNS-positive	
Eptesicus fuscus	(Blehert et al., 2009)
Myotis leibii	(USFWS, 2015)
Myotis grisescens	(USFWS, 2015)
Myotis sodalis	(Meteyer et al., 2009)
Myotis lucifugus	(Blehert et al., 2009)
Myotis septentrionalis	(Blehert et al., 2009)
Perimyotis subflavus	(Blehert et al., 2009)
***Pd*-positive**	
Lasiurus borealis	(Bernard et al., 2015)
Myotis austroriparius	(USFWS, 2015)
Lasionycteris noctivagans	(Bernard et al., 2015)
Corynorhinus rafinesquii	(Bernard et al., 2015)
Corynorhinus townsendii virginianus	(USFWS, 2015)

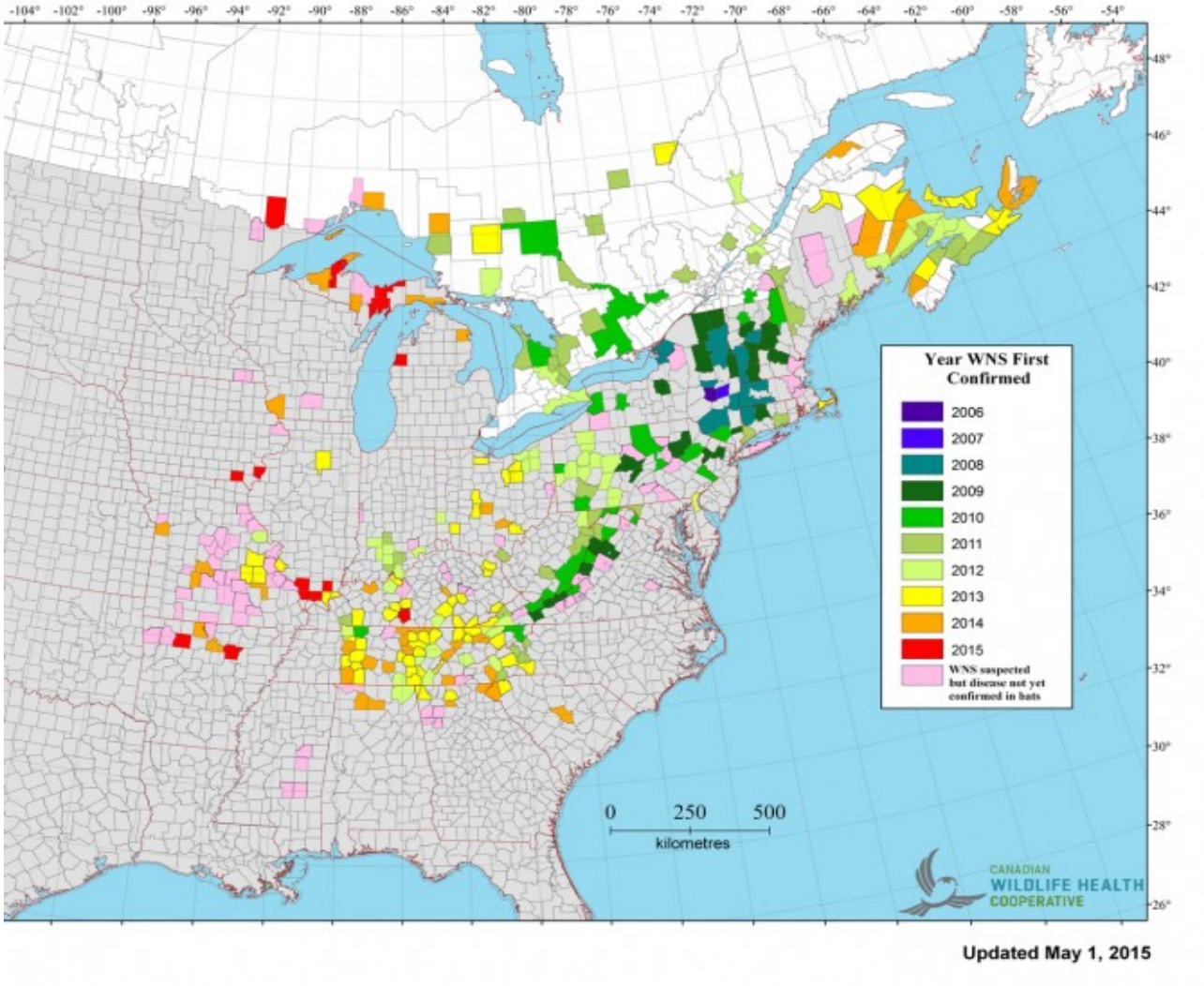

Figure 1 Current distribution of WNS in North America (CWHC, 2015)

2.1.2 Geographic distribution of *Pd* and WNS in Europe

Presence of *Pd* has been found across Europe. First confirmed case of *Pd* infected bat is dated to 2008, when one individual of *M. dasycneme* from Germany was found positive on culture and DNA sequencing (Wibbelt et al., 2010). Bats with confirmed infection by *Pd* were recorded in France (Puechmaille et al., 2010), Portugal (Paiva-Cardoso et al., 2014), Germany, Hungary, Switzerland (Wibbelt et al., 2010), Czech Republic and Slovakia (Martínková et al., 2010), Poland (Sachanowicz et al., 2014)Ukraine, Estonia, Netherlands, Belgium (Puechmaille et al., 2011)Croatia (Pavlinić et al. 2015) and the United Kingdom (Borman and Barlow, 2013) (Table 2). The fungus was also isolated from soil in Spain (Siles et al., 2013). There seems to be no apparent epicentre of the disease spread as in North America.

Apart from the occurrence of *Pd* confirmed by genetic testing, white growth of an unspecified fungus on bat ears and muzzles was described in late 1970s in Estonia, in the early 1980s in Germany (Masing 1984, Feldmann 1984 in Puechmaille et al., 2011), and since the 1990s in France (Christophe Riddeau in personal communication in Puechmaille et al., 2011). There is also photographic evidence of fungal growth on bat wings and skin dated from the mid-1990s from the Czech Republic and Slovakia (Martínková et al., 2010). However, recent studies show, that fungus *Trichophyton redellii* causes superficial infection on bats wings, uropatagium and ears resembling *Pd* infection (Lorch et al., 2015). Therefore, only genetically or histopathologically proven occurrence of WNS can be considered reliable.

Samples of several species in Europe, namely *Barbastella barbastellus, Eptesicus nilssonii, M. bechsteinii, M. brandtii, M. dasycneme, M. daubentonii, M. emarginatus, M. myotis, M. mystacinus, M. nattereri, Plecotus auritu*s and *Rhinolophus hipposideros* also fulfil histopathologic criteria of WNS (Bandouchova et al., 2014; Pikula et al., 2012)

Table 2 Bat species infected by *Pd* in European countries (* species with WNS in Europe)

Species	Country	Reference
*Barbastella barbastellus**	Czech Republic	(Bandouchova et al., 2014; Zukal et al., 2014)
*Eptesicus nilssonii**	Czech Republic	(Zukal et al., 2014)
*Myotis bechsteinii**	Czech Republic	(Zukal et al., 2014)
Myotis blythii	Portugal	(Paiva-Cardoso et al., 2014)
*Myotis brandtii**	Germany	(Wibbelt et al., 2010)
	Estonia	(Puechmaille et al. 2011)
	Czech Republic	(Zukal et al., 2014)
*Myotis dasycneme**	Germany	(Wibbelt et al., 2010)
	Netherlands	(Puechmaille et al. 2011)
	Czech Republic	(Zukal et al., 2014)
*Myotis daubentonii**	Germany	(Wibbelt et al., 2010)
	Netherlands	(Puechmaille et al., 2011)
	United Kingdom	(Borman 2013)
	Poland	(Sachanowicz et al., 2014)
	Czech Republic	(Zukal et al., 2014)
*Myotis emarginatus**	Czech Republic	(Bandouchova et al., 2014; Zukal et al., 2014)
*Myotis myotis**	France	(Puechmaille et al., 2010)
	Germany	(Pikula et al., 2012)
	Hungary	(Puechmaille et al., 2011; Wibbelt et al., 2010)
	Switzerland	(Wibbelt et al., 2010)
	Belgium	(Puechmaille et al. 2011)
	Ukraine	(Bandouchova et al., 2014; Martínková et al., 2010;
	Czech Republic	Pikula et al., 2012; Zukal et al., 2014)
	Slovakia	(Martínková et al., 2010)
	Croatia	(Pavlinić et al., 2014)
	Poland	(Sachanowicz et al., 2014)
*Myotis mystacinus**	Czech Republic	(Martínková et al., 2010)
	Belgium	(Puechmaille et al., 2011)
	Germany	(Puechmaille et al. 2011)
*Myotis nattereri**	Czech Republic	(Bandouchova et al., 2014; Martínková et al., 2010)
Myotis oxygnathus	Hungary	(Wibbelt et al., 2010)
*Plecotus auritus**	Czech Republic	(Bandouchova et al., 2014; Zukal et al., 2014)
*Rhinolophus hipposideros**	Czech Republic	(Zukal et al., 2014)

2.1.3 *Pseudogymnoascus destructans*

White-nose syndrome is an infectious disease caused by a microscopic fungus from phylum Ascomycota, *Pseudogymnoascus destructans* (formerly *Geomyces destructans*) (Blehert et al., 2009; Minnis & Lindner, 2013; Warnecke et al., 2012). The infectious agent has typical morphology (Figure 2), narrow curved conidia, which forms hyphae on bat tissues, particularly wing membranes, noses and ears. The fungal conidia, produced at the end of branched conidiophora, found on bats are typically asymmetrically curved, narrow, 1.5–2 μm wide, 35–90 μm and more long. Conidia are produced as a chain of 2-3 or more spores or solitarily (Puechmaille et al., 2011)

Reproduction of *Pd* is mainly asexual (Gargas et al., 2009), although in Central European hibernacula, both sexual and asexual reproducing forms are present (Palmer et al., 2014). The temperature optimum for the fungus growth is around 4 °C (Chaturvedi et al., 2010; Kubátová et al., 2011; Martínková et al., 2010) which corresponds to approximate temperature in most hibernacula. There is no growth observed at temperature of -10 °C or lower and no growth observed at 24 °C or higher temperature (Chaturvedi et al., 2010). Growth of the fungus on artificial media seems to be fairly slow in general (Gargas et al., 2009).

Besides the growth on bat skin tissue, *Pd* has been found in soil and surfaces of hibernacula (Lindner et al., 2011). It was also experimentally proven that *Pd* is able to survive in cold and non-humid environment for long periods without presence of bats (Hoyt et al., 2014). Presence of *Pd* in places, where bats hibernate and supposedly are not present for several months in summer (Lorch et al., 2013), can also indicate possibility of surfaces serving as a natural reservoir of the fungus (Hoyt et al., 2014).

Considering persistence of *Pd* without bat presence, it is highly important to pay attention to sterilization of caving equipment and laboratory equipment used by researchers in the field (Hoyt et al., 2014) to avoid contamination of another hibernacula. *Pd* spores can be inhibited by heating in hot water or chemical disinfectant cleaning agents containing hydrogen peroxide, ammonium and crystal violet, as well as common detergents (Shelley et al., 2013).

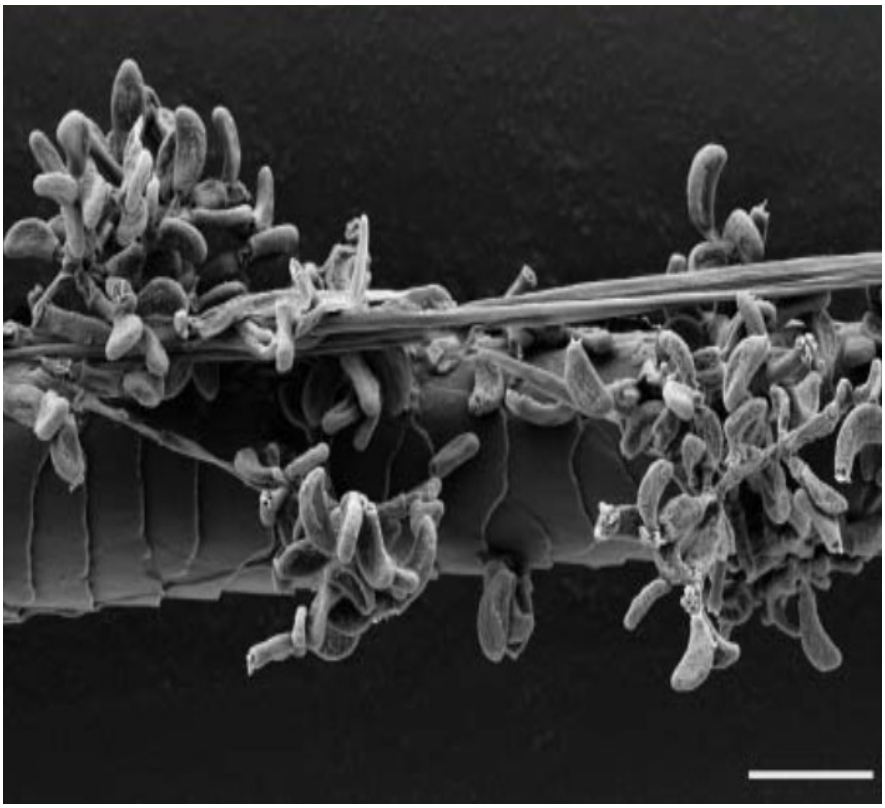

Figure 2 SEM image of a bat hair colonized by *Pd*. Scale bar 10 μm (image from Wibbelt et al., 2010)

2.1.4 White-nose syndrome etiology

In spite of the great interest of researchers after the rapid emergence of WNS, the mechanism of the disease remains unclear. The fungus invades epidermal tissues of bats, forms hyphae in hair follicles and skin and causes lesions (Figure 3), which disrupt ability of normal hibernation and survival during winter season (Reeder et al., 2012; Wilcox et al., 2014). Studies indicate, that the cause of mass mortality may be related to wing damage (Warnecke et al., 2013), weigh loss or changes in metabolism (Cryan et al., 2010). Skin corruption can affect the ability of flight locomotion (Warnecke et al., 2013) and metabolic regulations (Cryan et al., 2010). The most critical impact seems to be on bat wings.

Fungal infection by *Pd* is connected with diversely injured wing membrane, Reichard and Kunz (Reichard & Kunz, 2009) described graduation of damage of the wings starting with depigmentation and spots on membrane, flacking forearm, progressing with necrosis and holes in membrane and reduction of wing tissue along edges. The progressing destruction of bat wings structure may also affect flight locomotion and the ability to fly in general (Voigt, 2013), which is

crucial for suvival at any season. Despite this, wing membrane is capable of quick healing from various injuries (Fuller et al., 2011; Ceballos-Vasquez et al., 2015)

Wings are also said to be an important part of bat metabolism. The wing membrane serves as a cover for more than one half of the body. It is the largest part of tissue exposed to the outer environment. It is needed for hydration regulation and thermoregulation of bat body (Cryan et al., 2010). Corrupted regulation of water balance may be lethal to the animal. Losing water by evaporation during hibernation caused by WNS may be lethal to the animal directly, or may cause more frequent torpor arousals. Evaporative waterloss is correlated with humidity at the hibernation site and most bats choose hibernation sites with higher relative humidity (Willis et al., 2011)

As mentioned above, WNS is a disease of hibernating bats. Bat hibernation also can be corrupted in another profoundly important factor – quality and overall length of torpor. When bats lower metabolic activity and body temperature, any disturbance and arousal require significant amount of energy to raise and keep the body temperature and to restore metabolic functions. Weight loss connected with frequent arousal from torpor seems to have negative effects (Reeder et al., 2012)for survival during hibernation. Various infections in animal body also may impact length of torpor (Carey et al., 2003)

When hibernating, not only the temperature of the body decreases, but hibernation modifies metabolic functions as well. Endocrine system, in particular hormones affecting arousals from hibernation and fat storing preceding hibernation, such as glucocorticoids, leptin and melatonin may affect survival chances during winter season (Willis & Wilcox, 2014).

Hibernation also seems to influence the immune system. It was described that during hibernation, the immune system is unable to respond to bacterial infection. Activity of the complement also seems to be suppressed during hibernation (Moore et al., 2011). Immunity functions are restored during arousals. When immune system recovers, presence of the pathogen may induce intense immune reaction and cause a form of inflammatory syndrome lethal to the bat (Meteyer et al., 2012)

Figure 3 *Myotis lucifugus* affected by WNS (image from USFWS, 2015)

2.1.5 Diagnosis of white-nose syndrome

With the first observations of WNS made in late 1970s in Europe (Masing 1984, Feldmann 1984 and Riddeau in verb in Puechmaille et al., 2011), there were no diagnostic methods used but the observation. No genetic or histopathologic methods were used for observations earlier than 2006, which could have led to mistaking WNS for another similar fungal infections (Lorch et al., 2015). After emergence of WNS in the United States, reliable scientific methods of identification of *Pd* and determination of WNS were developed. The most common methods at presence include histopathologic examination (Meteyer et al., 2009), polymerase chain reaction (PCR) tests (Lindner et al., 2011; Muller et al., 2013)and ultraviolet light transillumination (Turner et al., 2014).

Histopathology is the standard and the most reliable method able to prove presence of skin lesions associated with WNS (Meteyer et al. 2009). However, histopathologic screenings require more specialized training, are more time-consuming and require more tissue samples and thus euthanasia of the bat, hence less invasive methods are preferred.

DNA-based diagnostics as PCR are often used to determine species of the fungus and to distinguish from its relatives. PCR methods target intergenic spacer region of rRNA gene complex (Lorch et al., 2010; Muller et al., 2013; Shuey et al., 2014) and the most advanced

methods are specific to determine *Pd* from closely related species (Shuey et al., 2014).

Unlike histopathology, PCR diagnostics can only prove presence of the fungus. Turner et al. ((Turner et al., 2014) developed a non-lethal method to diagnose WNS based on ultraviolet (UV) light translumination of the wing. This method uses long-wave UV light to determine presence of skin lesions by orange-yellow spots under the UV lamp. There was no similar reaction to common white light. The method does not require any special treatment of the animals and is non-lethal, therefore suitable to be used as the method of common screenings or as a guide for targeted biopsy sampling for histopathology.

Diagnosis of WNS is important for monitoring of the disease and the fungus. Histopathologic reports and genetic information about the fungus may be supportive for better understanding of differences of the disease manifestation in European and North American continents.

2.1.6 Manifestation of the disease in North America and Europe

Presence of *Pd* identical to North American strains was confirmed in various locations throughout Europe. Although the fungus growing on European bats is genetically identical with the pathogen inducing WNS in North American hibernacula, unlike in the North America, there was no mass mortality reported from European hibernation sites (Martínková et al., 2010).

Nevertheless, samples of several species were collected in caves in Central Europe and histopathologic examination have shown pathological changes on muzzles and wing tissues diagnostic for WNS (Bandouchova et al., 2015; Pikula et al., 2012; Turner et al., 2014; Zukal et al., 2014) and confirmed as WNS cases according to criteria used to evaluate histopathology of bats in North America (Meteyer et al., 2009).

Pd and WNS are not specific for any bat family or species (Zukal et al., 2014), but due to ithe fungus cold-loving character, it is limited to hibernating groups of bats. It is known to affect 7 bats species in America from vespertilionid genera of *Myotis, Eptesicus* and *Perimyotis* (Table 1). At present in Europe, *Pd* has been reported from bat family of *Rhinolophidae* (Zukal et al., 2014) and a large and widespread family of *Vespertilionidae* where infection by *Pd* has been reported from genera of *Myotis* (Martínková et al., 2010; Pikula et al., 2012; Zukal et al., 2014), *Eptesicus, Barbastella* and *Plecotus* (Zukal et al., 2014) Not only that the pathogen is present in Europe, but also causes lesions on bat tissues as described in North America. WNS in Europe concerns

especially genus of *Myotis*, but is also present in *Barbastella* and *Plecotus* and Rhinolophus (Bandouchova et al., 2015; Zukal et al., 2014) (Table 2).

Differences in ecology of species suffering from WNS in North America and Europe may explain different lethality of the condition in both localities. European species tend to form small clusters or hibernate solitarily, while American bats hibernate mostly in big aggregations, where physical contact among animals eases contamination by the fungus (Martínková et al., 2010; Wibbelt et al., 2010).

No mass mortalities of WNS in Europe may suggest European origin (Martínková et al., 2010; Puechmaille et al., 2011). This theory is supported by recent study comparing selected genetic markers. North American isolates are of a uniform haplotype, while European isolates show variability of haplotypes throughout the continent (Leopardi et al., 2015).

2.2 Evolution of pathogen and host

From the differences in mortalities and morbidity of WNS it can be assumed that European bats have evolved mechanisms of resistance to the disease. A pathogen, such as a parasitic fungus causing WNS, which evolves together with a host for a long period of time may contribute to changes in genetic information in the host. The ability of adaptation to the changing environment is the basis of evolution (Watt, 2015). Darwin's theory of natural selection (Darwin, 1859) is a central idea of evolutionary genetics, a field explaining for instance modifications in genetic information in order to maintain resistance to the pathogen.

2.2.1 Phylogenetics

Phylogenetics, as a discipline focusing on evolutionary relations between species can provide information about evolutionary history. Adaptation to the new environmental conditions is inevitable for species continuity. In this case, the most interesting mechanisms are positive selection and genetic convergence.

Phylogenetic analyses are based on evolutionary genetics. Phylogenetic trees are graphical representation of gene transfer in time. Frequently used methods used to infer phylogenetic trees include maximum parsimony, distance–matrix methods and probabilistic methods – maximum likelihood (Felsenstein, 1981) and Bayesian inference (Rannala & Yang, 1996)which is used in this study and discussed in further detail.

Bayesian inference of phylogeny

Bayesian phylogenetic inference is a method relying on the Bayes theorem. When adjusted to problems of phylogenetic analysis, the theorem can be expressed as follows:

$$f(\tau,\upsilon,\theta|X)=\frac{f(\tau,\upsilon,\theta)f(X|\tau,\upsilon,\theta)}{f(X)}$$

where τ is topology of the tree, υ is a vector of lengths of branches of the tree, θ is a vector of model parameters and X is the data matrix (Ronquist & Huelsenbeck, 2003).

The Bayesian inference method is based on posterior probabilities of the parameters and the tree given the data. The probabilities are approximated using Markov chain Monte Carlo (MCMC) methods. MCMC are a group of algorithms based on random sampling from possible distribution of probabilities. For phylogenetic application, Metropolis-Hastings algorithm is used for specification of motion. When further step is generated, the algorithm compares it with the preceding step and except the step if posterior probability is higher than in the foregoing (Yang & Rannala, 1997)

Phylogenies are created to reconstruct progress and results of evolutionary processes necessary to react to changing environment. Trees can be used for further analyses including analysis of genetic changes caused by positive selection and genetic convergence related to an infectious disease.

2.2.1 Natural selection

Natural selection is one of the most important mechanisms that change genetic structure of populations, in order to create high functioning complex structures. In the process of biological evolution, there are several mechanisms changing genetic information and increasing or decreasing variability in the population (Lenormand, 2002). New genotypes are produced by mutations, both spontaneous, caused by random errors during DNA replication, and induced by external mutagens.

Natural selection is a process when frequency of an allele either increases or decreases. The individuals with the best fitness are able to reproduce their genetic information in the largest number of copies (Watt, 2015) and increase the frequency of a useful allele in the population. Vice versa, phenotypes with harmful mutations lowering fitness are eliminated from the population in the process of evolution.

Mutations

Natural selection is realized at the level of phenotype. In other words, the adaptive change is to be performed on the function of proteins, but information about protein structure in DNA is the carrier of the inheritable genetic information that can be passed on to another generation. Thus, any changes that are supposed to participate in biological evolution must be encoded in DNA sequences of nucleotides.

Translation of the DNA into proteins is based on a universal genetic code of triplets of nucleotides, codons, translated into amino-acids. Each codon corresponds to one amino-acid, but one amino-acid might be encoded by multiple codons. Some codons indicate beginning of the translation (start codon, also encoding methionine), or its end (stop codons).

Substitutions of a single nucleotide, as the most common type of mutations, may not have impact on the protein structure (synonymous mutations) and not change the open reading frame (ORF), but can also result in changing an amino-acid in chain (missense mutations). Similarly to this, insertions or deletions of whole codon do not change the ORF, but change the overall length of the amino-acid chain and alter protein structure and function. Insertions and deletions (also "indel" mutations) of nucleotides, that are not whole codons change the ORF (frameshift mutations) and generally cause important changes in protein sequence. Other substitutions might result in a stop-codon (nonsense mutations) or encode a different amino-acid (missense mutations). Mutations changing the protein sequence in general (non-synonymous mutations) and more severe alterations of DNA sequence may result in changes in protein structure causing critical malfunction of the protein (Figure 4) (Griffiths et al., 2012).

Frequency of mutations that lower the ability of survival and reproduction in a population is reduced by negative (also referred to as purifying) selection. However, the most common mutations do not affect overall fitness of an individual and therefore are not a subject to selective pressures. So-called neutral mutations may be stabilized or eliminated by other evolutionary processes, as genetic drift, draft or migration (gene flow) of individuals among populations (Chamaryet al., 2006).

Alleles neighbour to the allele under selective pressure may be also fixed by genetic draft. Fixation of an allele in a population (substitution of all the other alleles) may be an effect of genetic drift (Nei et al., 2010), but it is more likely to be a consequence of spread driven by natural selection.

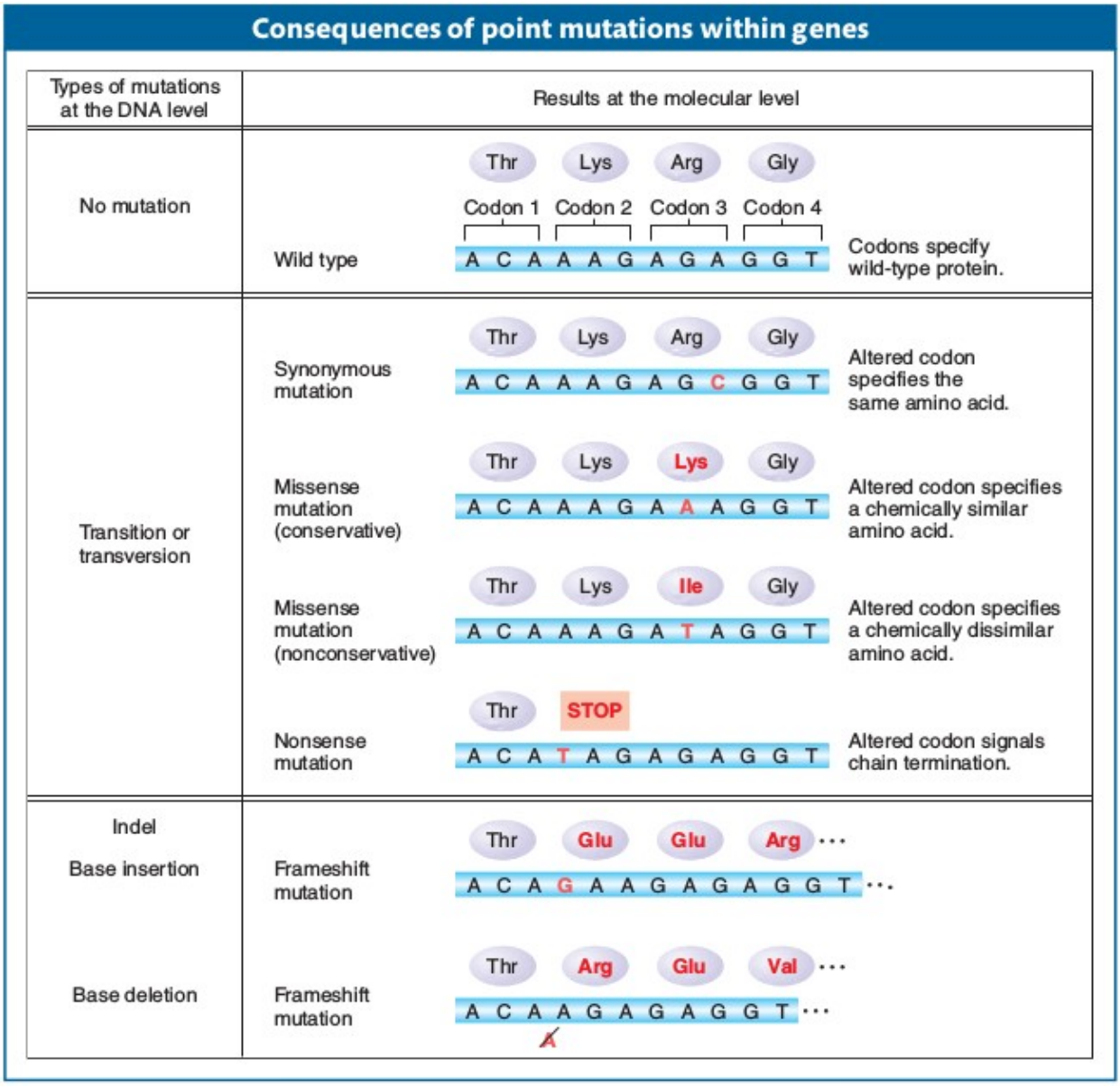

Figure 4 Types of mutations and their consequences on proteins (image from Griffiths et al., 2012)

Positive selection

Mutations that are subject to positive selection are the rarest type of changes in the sequence of DNA. The positive mutations bring a phenotypic advantage to its carrier and facilitate its own distribution into next generations. Past positive selection in sequences can be identified by comparison of the mutation sites and determination of ratio between synonymous and non-synonymous substitutions (Nielsen & Yang, 1998).

Non-synonymous/synonymous rate ratio (ω)

$$\omega = d_N / d_S$$

where d_N represents number of non-synonymous substitutions per site and d_S represents number of synonymous substitutions per site. It is used for identifying selection and specifying its type. Synonymous and non-synonymous substitutions occur with the same rate, ω=1, in case of neutral selection. More often synonymous substitutions prevail, $\omega < 1$, only a few amino-acids change and the process can be described as negative selection. Substitution rate ratio $\omega > 1$ corresponds with positive selection (Sironiet al., 2015). High number of non-synonymous substitutions is a sign of noticeable changes in protein structure and therefore function and activity.

Calculation of ω is not straightforward. The method should consider corrections for multiple substitutions, unequal frequency of codons and different probability of transversions and transitions (Yang & Nielsen, 1998).

2.2.2 Convergent evolution

Convergence is understood as a process, when organisms living under similar conditions and pressures and with the same needs evolve resembling structures, regardless of diverse genetic basis and non-existence of common ancestral basis of the structure. Therefore, functionally same structure evolves in groups that are not monophyletic. In bats, some typical examples of convergent evolution are present – bat wings resembling birds or high-frequency echolocation used as well by whales (Li et al., 2010).

Manifestation of convergence can be apparent on many levels. Morphologic or functional convergence of wings and ability to fly in bats, insects and birds is the basic example of analogous structure. In cases of DNA sequence convergence, mutation applies to the same gene, either different or the same amino-acid site, resulting in proteins with identical function or sequence (Christin et al., 2010)

It is possible to determine past evolution leading to sequence convergence using analysis of phylogenetic trees. The convergent genotype is present non-randomly throughout the tree. Convergent evolution can be determined using nearest taxon index (NTI) and net relatedness index

(NRI) (Webb, 2000).

NTI calculates relatedness to the nearest taxon that shares the investigated trait, using mean nearest taxon distance (MNTD) value for random distribution of the trait ($MNTD_r$) on the tree and observed sample of the trait ($MNTD_s$) on the tree expressed as follows:

$$NTI = -(MNTD_s - MNTD_r) / stdev.MNTD_r$$

Net relatedness index (NRI) considers whole phylogenetic tree. It uses the mean pairwise distance (MPD) parameter, measuring how distant are taxa that share a certain trait, as follows:

$$NRI = -(MPD_s - MPD_r) / stdev.MPD_r$$

Positive values of NTI and NRI occur in species more phylogenetically related than expected, as a sign of phylogenetic clustering in a given group of organisms. If NRI and NTI are equal to 0, distribution of the trait is random. Negative values indicate phylogenetic overdispersion (distance between species) (Santorelli et al., 2014).

2.2.3 Host of an infection as a subject of biological evolution

Infectious diseases are one of the most important causes of massive die-offs in populations. The signs of past or persistent infections are present in genetic information and biogeographic structure of current populations (Quintana-Murci et al., 2007).

Infectious diseases may influence genetic structure by several mechanisms. Intense decrease in population sizes connected with infection cause bottleneck effect, when only individuals with few alleles from the original population survive (O'Brien & Evermann, 1988), therefore genetic variability may lower after mass mortalities connected with WNS in North America. Another mechanism known to influence genetic structure of infected populations is natural selection. Past and present infectious diseases in the population serve as a strong selective pressure. *Pd* is regarded a pathogen of European origin (Leopardi et al., 2015). Signs of evolution together with *Pd* in bats may be detectable in genetic information of European bats. Impact of selective pressure of pathogens is well known in multiple mammalian diseases.

Signs of natural selection as expected in populations of infected organisms are reported from many species, in case of infections such as malaria (Liu et al., 2015), infection by coronaviruses (Vijaykrishna et al., 2007) or HIV infection (Bozek & Lengauer, 2010).

Signs of natural selection may be present in immune system related genes, or genes non-relevant to immunity. As for immune system related genes, influence of natural selection was proven in group of genes necessary for innate immune response, coding receptors of antigens, such as group of toll-like receptors (TLRs) (Areal et al., 2014). Another group of intensely studied immunity related genes under selective pressure include antigen presentation genes, represented by major histocompatibility complex I and II (MHC I and II) proteins (Froeschke & Sommer, 2014).

The example of malaria driven selection also shows, that immune system related genes are not the only subject to the natural selection. (Cagliani & Sironi, 2013). There is a link between resistance to malaria and prevalence of inheritable hemoglobin defect such sickle-cell disease in Africa and thalassemia in Mediterranean areas. When under selection driven by a certain pathogen, a population may involve resistance, which is accompanied by phenotypic disadvantage in other perspectives. Therefore, development of resistance against a pathogen may affect overall fitness of an individual when under different conditions. European bats may have experienced selection on both immune system related and non-related genes.

3 EXPERIMENTS

3.1 Materials and methods

The dataset included sequences from 7 loci of genes, from representatives from families of *Vespertilionidae, Rhinolophidae* and *Miniopteridae.* Genes were selected based on their function in metabolic processes assumed to be relevant in adaptations to WNS (Szolgayová, 2013). DNA sequences used for our study were obtained from previous research and were commercially sequenced on the Pacific Biosciences platform (Jakešová, 2014; Szolgayová, 2013; Vozáriková, 2014).

Table 3 List of genes used for the analysis

Gene	Protein	Sequences	Alignment length
acad10	acyl-CoA dehydrogenase 10	7	258
anxa1	annexin A1	26	192
aqp3	aquaporin3	19	216
bcam	basal cell adhesion molecule	26	570
ctnnb1	catenin beta 1	31	156
hyal2	hyaluroglucosaminidase 2	9	720
tgm1	transglutaminase 1	36	597

DNA sequences were aligned in Geneious 8.0.4 (Kearse et al., 2012). In the same software, sequences were edited. The analysis of positive selection requires only coding sequences of DNA, thus introns were excised using annotated sequence of *M. lucifugus* or *M. brandtii*. Afterwards, multiple sequence alignments format was converted from FASTA alignment format to PHYLIP alignment format using readSeq (Gilbert, 2002).

MrBayes 3.2 (Ronquist et al., 2012) plugin in Geneious 8.0.4. was used for construction of phylogenetic trees. The software uses Bayesian inference discussed above to construct the trees. MCMC run for 1 000 000 generations with 4 heated chains at the temperature of 0.5. First 1000 calculated trees were excluded from the tree construction (burnin=1000). HKY85 substitution model that assumes non-equal nucleotide frequencies and differences in transition/transversion rate, was used for the tree reconstruction. Tree topologies were used in the NEWICK format.

For the analysis of positive selection impact on the DNA sequences, codeml program of the PAML (Yang, 2007) package was used. Control file for the program (Table 4) specified variables for the analysis. Besides the sequence alignment, tree and output files, it specifies what is included in the output file, next it specifies sequence type, in our case codon sequences were used. An important parameter that needs specification is NSsites, parameter specifying site models to be calculated.

The output of codeml provides information about d_N/d_S ratios under different nested models that need to be compared. If the estimated ω was higher that 1 (indicating positive selection), ln$L$ (log-likelihood) values of the estimation were compared by the likelihood ratio test (LRT). χ^2 test was used for estimation of p-values in R.

Table 4 Control file for codeml, , example of the *ctnnt1* gene.

```
seqfile  =  ctnnb1.phy
treefile =  ctnnb1.newick
outfile  =  ctnnb1.txt
             noisy = 0      * 0,1,2,3,9: how much rubbish on the screen
           verbose = 1      * 1: detailed output, 0: concise output
           runmode = 0      * 0: user tree;  1: semi-automatic; 2:automatic
                            * 3: StepwiseAddition; (4,5):PerturbationNNI
           seqtype = 1      * 1:codons; 2:AAs; 3:codons-->AAs
         CodonFreq = 3      * 0:1/61 each, 1:F1X4, 2:F3X4, 3:codon table
             clock = 0      * 0: no clock; 1: clock; 2: local clock
             model = 0      * models for codons:
                            * 0:one, 1:b, 2:2 or more dN/dS ratios for
                            * branches
       NSsites = 0 1 2      * dN/dS among sites. 0:no variation, 1:neutral,
                            * 2:positive 3:discrete;4:freqs;
                            *5:gamma;6:2gamma;7:beta;8:beta&w;9:beta&gamma;
                            * 10:beta&gamma+1;11:beta&normal>1;
                            * 12:0&2normal>1;13:3normal>0
             icode = 0      * 0:standard genetic code; 1:mammalian mt;
         fix_kappa = 0      * 1: kappa fixed, 0: kappa to be estimated
             kappa = 2      * initial or fixed kappa
         fix_omega = 0      * 1: omega or omega_1 fixed, 0: estimate
             omega = 1      * initial or fixed omega, for codons or codon-
                            * transltd AAs
         fix_alpha = 1      * 0: estimate gamma shape parameter; 1: fix it
                            * at alpha
             alpha = .0     * initial or fixed alpha, 0:infinity (constant
                            * rate)
            Malpha = 0      * different alphas for genes
             ncatG = 4      * number of categories NSsites models
             getSE = 0      * 0: dont want them, 1: want S.E.s of estimates
      RateAncestor = 0      * (1/0): rates (alpha>0) or ancestral states
                            * (alpha=0)
       fix_blength = 1      * 0: ignore, -1: random, 1: initial, 2: fixed
            method = 1      * 0: simultaneous; 1: one branch at a time
```

3.2 Results

Table 5.1 Results of positive selection estimation for ***acad10***.

Model	**ln*L***	**Parameters**	$2\times(lnL1-lnL0)$	***p*-value**
Nearly Neutral	-357.949	*p*: 0.953 0.047 ω: 0.000 1.000	0.512	0.774
Positive Selection	-357.693	*p*: 0.986 0.000 0.014 ω: 0.000 1.000 9.181		

In case of the *acad10* gene, estimated ω was higher that 1 at one site, thus LTR test was used (Table 5.1). It did not show any significant difference between the models.

Table 5.2 Results of positive selection estimation for ***anxa1***.

Model	**ln*L***	**Parameters**	$2\times(lnL1-lnL0)$	***p*-value**
Nearly Neutral	-483.477	*p*: 0.556 0.444 ω: 0.000 1.000	3.271	0.195
Positive Selection	-481.842	*p*: 0.571 0.333 0.096 ω: 0.023 1.000 3.652		

Anxa1 gene also showed ω value greater than one in the positive selection model. *p*-value was lower (Table 5.2), than in the previous gene , but not reaching level of significance.

Table 5.3 Results of positive selection estimation for ***aqp3***.

Model	**ln*L***	**Parameters**	$2\times(lnL1-lnL0)$	***p*-value**
Nearly Neutral	-384.773	*p*: 0.939 0.061 ω: 0.000 1.000	0.053	0.974
Positive Selection	-384.747	*p*: 0.948 0.000 0.052 ω: 0.000 1.000 1.402		

Aqp3 gene has ω values slightly higher than 1, but LRT test did not show significant differences between the compared models (Table 5.3).

Table 5.4 Results of positive selection estimation for ***bcam***.

Model	**ln*L***	**Parameters**	$2\times(lnL1-lnL0)$	***p*-value**
Nearly Neutral	-1476.206	*p*: 0.801 0.199 ω: 0.092 1.000	2.172	0.338
Positive Selection	-1475.120	*p*: 0.819 0.174 0.008 ω: 0.105 1.000 9.491		

The ω value in codeml test for *bcam* was higher than one, but the LRT test showed no significant difference between the two chosen models (Table 5.4).

Table 5.5 Results of positive selection estimation for ***ctnnb1***.

Model	**ln*L***	**Parameters**	$2\times(lnL1-lnL0)$	***p*-value**
Nearly Neutral	-320.815	*p*: 0.999 0.001 ω: 0.096 1.000	NA	NA
Positive Selection	-320.815	*p*: 1.000 0.000 0.000 ω: 0.096 1.000 1.000		

The only gene, where ω value was not bigger than 1 for any site was *ctnnb1* in my analysis. LRT test was not preformed for this case (Table 5.5).

Table 5.6 Results of positive selection estimation for ***hyal2***.

Model	**ln*L***	**Parameters**	$2\times(lnL1-lnL0)$	***p*-value**
Nearly Neutral	-1177.677	*p*: 0.947 0.052 ω: 0.044 1.000	1.650	0.438
Positive Selection	-1176.851	*p*: 0.979 0.000 0.021 ω: 0.059 1.000 2.579		

Highest estimation result of ω in case of *hyal2* was slightly bigger than one, but LRT test did not show any significant difference between the models (*p*-value=0.438) (Table 5.6).

Table 5.7 Results of positive selection estimation for ***tgm1***.

Model	**ln*L***	**Parameters**	$2\times(lnL1-lnL0)$	***p*-value**
Nearly Neutral	-1289.404	*p*: 0.875 0.125 ω: 0.048 1.000	17.481	0.001
Positive Selection	-1280.663	*p*: 0.919 0.051 0.031 ω: 0.084 1.000 5.296		

Estimation of ω in case of the *tgm1* gene was higher than one, and the likelihood ratio test showed significant (*p*-value=0.001) difference between the nearly neutral evolution model and the positive selection site model (Table 5.7).

4 DISCUSSION

Recently, WNS has been claimed a disease of European origin (Leopardi et al., 2015) based on genetic uniformity of American population of the fungus. Presence of *Pd* in Europe for a long time probably changes genetic information of the host. Our hypothesis was, that co-existence of bats with the fungus has left consequences on the genetic information in European bats in order to gain resistance to the disease. Sequences of DNA provide interesting information not only about past selection, but also about types of genes that are under selective pressure and therefore important for mechanism of disease resistance.

Mechanisms of pathogenesis of WNS probably include more factors. As discussed above, impact of skin lesions (Warnecke et al., 2013) and water metabolism (Cryan et al., 2010) seem to be the most significant factors affecting mortality of WNS. The analysis performed in this study focused on genes that may improve resistance of bats to WNS. Genes examined in this study were involved in water metabolism, energy metabolism or genes important for epithelial structure and function. In previous analysis, positively selected sites were detected in *ctnnb1* and *pxn* genes (Vozáriková, 2014). Here, partial *ctnnb1* was analysed for a larger number of species and no selection was found.

The *pxn* gene (http://www.genecards.org/cgi-bin/carddisp.pl?gene=PXN) encodes paxillin, a cytoskeletal protein, its specific function in cells is not quite clear. Paxillin sequences were not included in this study. *The ctnnb1* gene (http://www.genecards.org/cgi-bin/carddisp.pl?gene=CTNNB1) encodes catenin β1, a protein with regulatory and signalling function important for cell adhesion and for epithelial cell growth and proliferation. It is an integral part of the Wnt signalling cascade.

Previous study confirmed positive selection in this gene at approximately 1.9 % of sites (p=0.019) (Vozáriková, 2014). No signs of positive selection were found in *ctnnb1* in this study (p=0.000, ω=0.000). Sequence data for both studies were obtained by the same method. Differences in results of positive selection estimation may be explained by using another set of species, where the gene was not under selective pressure. More likely explanation is that another part of coding sequence was used for the analysis. In my analysis, sequence of 156 nucleotides from a single exon was used from total of 16 exons. Vozáriková (2014) studied the complete coding sequence, but she does not provide information on position of sites under selection. The gene region under selection might have not been sampled in this study.

This study found positively selected sites in the *tgm1* gene. The *tgm1* gene (http://www.genecards.org/cgi-bin/carddisp.pl?gene=TGM1) encodes transglutaminase 1, a membrane protein involved in formation of outer layers of skin epithelia. Previous analysis (Vozáriková, 2014) showed ω values to be higher than 1 (ω=2.129 for positive selection site model) and 2.6 % sites under positive selection, but LTR showed no significant difference between used nested models for selection estimation. As in the preceding case, differences may be caused by use of slightly different set of sequences. Our dataset for *tgm1* analysis included 597 nucleotides from 3 exons from total of 15 and 36 species compared to 8 species analysed by Vozáriková (2014).

Both *ctnnb1* and *tmg1* are genes responsible for proper skin function. Diseases associated with mutations in those genes manifest with skin and hair follicles problems. Mutation in *tgm1* known in other mammals and humans leads to ichthyoses, a group of skin disorders characterised by dry and squamouse skin (Russell et al., 1995).

In case of bats, skin disorders are closely related to water balance and energetic metabolism, as skin forms a large exposed part of the body (Cryan et al., 2010). Integrity of bat skin tissues and its resistance is essential for hydration of bat organism. Improved ability to keep water balance and to maintain homeostasis may be one of key mechanisms of defence against the deadly pathogen.

Selective pressure on genes connected with skin is an important factor, but it cannot be considered the sole mechanism of resistance to WNS by European bats. Immune system related genes are another group likely to be under selective pressure in any population facing persistent infectious disease, yet impact of natural selection by WNS on immunity related genes is unclear. Our study included two genes related to immune system, *anxa1* (http://www.genecards.org/cgi-bin/carddisp.pl?gene=ANXA1) and *bcam* (http://www.genecards.org/cgi-bin/carddisp.pl?gene=BCAM). Annexin 1, endoded by *anxa1*, is a membrane binding protein with anti-inflammatory function (inhibitor of phospholipase A2). Basal cell adhesion molecule, a product of *bcam* gene is a protein from immunoglobulin family binding to laminin, competing with integrins. No positive selection was detected neither in *anxa1* nor in *bcam* genes.

Genetic point of view of the immune system under persistent infection can provide information about alterations in coding sequences in bat populations. Besides, immune system of hibernating mammals in general has some specific characteristics (Carey et al., 2003). Their detailed knowledge can be beneficial in research of other vertebrate populations. Therefore, further studies and analyses of signatures of positive selection driven by the *Pd* infection in bats are needed.

5 CONCLUSIONS

In the first part, this study reviews a case of fungal infection, white-nose syndrome, with varying regional impact on bat populations and possible impact of natural selection and convergent evolution on bat DNA sequences.The infectious disease present throughout Europe causes massive mortalities of bats in the United States and Canada. Past co-evolution with the disease has likely increased chances to survive the disease in Europe.

Not only different hibernation ecology, but also different genetic basis and adaptations are relevant factor for bat surviving WNS in Europe. Our data support the theory of genetic adaptation to the disease in European bats causing differences in mortalities.

In the experimental part, positive natural selection was detencted in the *tgm1* gene. In 6 other genes used in this study, significant signs of positive Darwinian selection were not found.

Studies of the emerging disease in bats help to understand mechanisms of other diseases with similar course in various vertebrate populations. Aside from signs of positive selection, further analysis of the DNA sequences from our study may also provide information about genetic convergence in bat species suffering from the disease.

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List of online resources

CWHC - *Canadian Wildlife Health Cooperative*. 2015. Available at http://www.cwhc-rcsf.ca/.

USFWS - *U.S. Fish & Wildlife Service*. 2015. Available at https://www.whitenosesyndrome.org/.

Strains of *Pd* from Spain and United Kingdom

GenBank: HG798544 (Borman and Barlow 2013)
GenBank: KC427035 (Siles et al. 2013)

GeneCards reference of proteins under positive selection

http://www.genecards.org/cgi-bin/carddisp.pl?gene=ANXA1

http://www.genecards.org/cgi-bin/carddisp.pl?gene=BCAM

http://www.genecards.org/cgi-bin/carddisp.pl?gene=CTNNB1

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