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Plasma proteomic profiles differ between European and North American myotid bats colonized by *Pseudogymnoascus destructans*

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Abstract

Emerging fungal diseases have become challenges for wildlife health and conservation. North American hibernating bat species are threatened by the psychrophilic fungus *Pseudogymnoascus destructans* (*Pd*) causing the disease called white-nose syndrome (WNS) with unprecedented mortality rates. The fungus is widespread in North America and Europe, however, disease is not manifested in European bats. Differences in epidemiology and pathology indicate an evolution of resistance or tolerance mechanisms towards *Pd* in European bats. We compared the proteomic profile of blood plasma in healthy and *Pd*-colonized European *Myotis myotis* and North American *Myotis lucifugus* in order to identify pathophysiological changes associated with *Pd* colonization, which might also explain the differences in bat survival. Expression analyses of plasma proteins revealed differences in healthy and *Pd*-colonized *M. lucifugus*, but not in *M. myotis*. We identified differentially expressed proteins for acute phase response, constitutive and adaptive immunity, oxidative stress defence, metabolism and structural proteins of exosomes and desmosomes, suggesting a systemic response against *Pd* in North American *M. lucifugus* but not European *M. myotis*. The differences in plasma proteomic profiles between European and North American bat species colonized by *Pd* suggest European bats have evolved tolerance mechanisms towards *Pd* infection.

KEYWORDS

hibernation, intercontinental differences, *Myotis lucifugus*, *Myotis myotis*, resistance, tolerance, white-nose syndrome

1 | INTRODUCTION

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

WNS is characterized by growth of *Pd* on exposed skin regions of hibernating bats (Lorch et al., 2011; Warnecke et al., 2012). The fungus invades the epidermis and dermis, which leads to distinctive cupping erosions (Cryan, Meteyer, Boyles, & Blehert, 2010; Meteyer et al., 2009). Clinical pathology of *Pd* infection in North American species is associated with abnormal behaviour, a disturbed natural hibernation cycle characterized by increased arousal frequency resulting in premature depletion of fat stores during hibernation (Reeder et al., 2012). Diseased animals show disturbed electrolyte and hydration balance (Cryan et al., 2013; Willis, Menzies, Boyles, & Wojciechowski, 2011), oxidative stress (Moore et al., 2013), chronic respiratory acidosis (Verant et al., 2014), altered complement protein activity (Moore et al., 2011) and fever response (Mayberry, McGuire, & Willis, 2018). *Pd* infection does not produce primary inflammatory cellular infiltration into infected tissues (Meteyer et al., 2009), which would be associated with leukopenia (i.e., low white blood cell counts) during mammalian hibernation (Bouma, Carey, & Kroese, 2010). The expression of inflammatory, wound healing and metabolic genes increases without the recruitment of neutrophils and T cells to the site of invasion (Field et al., 2015). WNS may also elicit an exaggerated inflammatory response during arousal (Meteyer, Barber, & Mandl, 2012), which might further complicate the pathology of the disease. How WNS causes death in bats is not clear (Wibbelt, 2018), but is probably a combination of various physiological disturbances provoked by *Pd* resulting in a multistage progression of the disease (Verant et al., 2014).

In contrast to North America, *Pd* is widely distributed among hibernating bat species in Europe (Puechmaille et al., 2011; Zukal et al., 2014), but without associated clinical signs or mortality (Wibbelt et al., 2013). There are 17 bat species which are colonized by the fungus in Europe, with the greater mouse-eared bat (*Myotis myotis*) being most often infected (Wibbelt, 2018). Recently, four additional species tested positive for *Pd* in Northeast China extending the host and the geographic range of the pathogen (Hoyt et al., 2016). Lesions associated with *Pd* infection in European bats are less pronounced than in North America (Wibbelt et al., 2013). However, deep cutaneous invasion associated with neutrophil infiltration has been observed in some species (Bandouchova et al., 2015;Wibbelt et al., 2013). The differences in epidemiology and pathology of *Pd* infection in European and North American bats suggest that European bats have co-evolved with the fungus developing immunologic and perhaps behavioural defence mechanisms to the fungus (Bandouchova et al., 2018; Lilley et al., 2019; Puechmaille et al., 2011; Wibbelt et al., 2010; Zukal et al., 2016).

Natural and experimental infection studies of the physio- and immunopathological aspects of WNS have been performed on susceptible and resistant North American bat species. However, similar studies are rare for European species. The different approaches and experimental setups used among studies complicate data interpretation and across-study comparisons (Johnson et al., 2015; Moore et al., 2018; Davy et al., 2017, but see Lilley et al., 2019).

Several studies have demonstrated weak correlations between transcriptome and proteome data (e.g., Gygi, Rochon, Franza, & Aebersol, 1999; Lu et al., 2004; Maier, Güell, & Serrano, 2009; Moritz, Mühlhaus, Tenzer, Schulenborg, & Friauf, 2019). The information available on pathophysiological aspects of *Pd* colonization is generally based on transcriptome data (Davy et al., 2017; Field et al., 2015, 2018; Lilley et al., 2019) and it would be reasonable to expect lack of transcriptome and proteome concordance. These methods are both necessary and complementary to understand the underlying molecular changes associated with the various disease states in different hosts. To address the paucity of comparative European and North American *Pd* infection studies and to get further insights on the molecular mechanisms associated with the infection, we compared the plasma proteomic profile of hibernating North American *M. lucifugus* and European *M. myotis*. Blood plasma composition is an important indicator of physiological changes during infection in human (Anderson, Polanski, et al., 2004) and veterinary medicine (Bilić et al., 2018; Ghodasara, Sadowski, Satake, Kopp, & Mills, 2017; Horvatić et al., 2016). Furthermore, proteomic approaches have been used to understand the pathways associated with hibernation in several model and non-model organisms (Grabek, Martin, & Hindle, 2015), including bats (Hecht et al., 2015). We hypothesized that differences between healthy and *Pd*-colonized individual proteomic profiles will vary among species from different continents and reflect patho-physiological changes associated with the colonization by *Pd*.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

Capture, handling and sample collection protocols for this study were reviewed and approved by the animal welfare and ethics committee of the Leibniz Institute for Zoo and Wildlife Research (permit #2011-12-01) and the Macdonald Campus Facility Animals Care Committee, McGill University (protocol #6096), by the corresponding authorities in Bavaria, Germany (permit #55.1-8642.00-9/10) and by the Ministère des Ressources naturelles et de la Faune (permit #2012-03-15-002-00-S-F). All protocols complied with existing guidelines from Germany and Canada.

2.2 | Sample collection

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

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

Although histopathological lesions characteristic of WNS have been described in European species, European bats cannot be considered diseased (Wibbelt, 2018). We assigned collected bats from Europe and North America to healthy and *Pd* colonized groups according to their *Pd* colonization status. Colonization status was defined by the results of analysing the samples collected with adhesive tapes. After collection, the tape samples were transferred to glass slides which were examined under light microscope for characteristic *Pd* conidia. If observed, isolation and mycological confirmation of the fungus was subsequently performed (Wibbelt et al., 2010). In the case of *M. lucifugus*, *Pd* infection was also diagnosed histologically (Meteyer et al., 2009).

2.3 | 2D fluorescence difference gel electrophoresis (2-D DIGE)

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

We performed 2-D DIGE analyses across six SDS gels for each species. Samples were labelled using the G-Dye Refraction-2D labelling kit (NH DyeAGNOSTICS GmbH, Germany) according to the manufacturer's protocol. Briefly, after we determined the total plasma protein concentration using a NanoDrop, samples were diluted to the required concentration of 5 µg protein/µl in labelling buffer (30 mM Tris, 7 M urea, 2 M thiourea, 4% w/v 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate [CHAPS] pH 8.5; final volume = 10 µl). Individual samples were labelled with G-Dye200 or G-Dye300 using dye swaps between the samples. An internal standard (IS) consisting of a pool of all samples was always labelled with G-Dye100. We applied two individual samples (one stained with G-Dye200 and one stained with G-Dye300) and the internal standard on each gel. For this, these three components were diluted in a total volume of 450 µl rehydration buffer (8 M Urea, 1% w/v CHAPS, 13 mM dithiothreitol [DTT], 0.5% v/v Servalyt [SERVA Electrophoresis GmbH, Germany]) and loaded on IPG BlueStrips pH 3–10 NI/24 cm (SERVA Electrophoresis GmbH, Germany) for active (50 V, 15 hr) sample-in-gel rehydration using PROTEAN IEF Cell tray (Bio-Rad, USA). Isoelectric focusing was performed using the following conditions: step 1, 300 V, 150 V/hr rapid; step 2, 600 V, 300 V/h rapid; step 3, 1,500 V, 750 V/hr rapid; step 4, 3,000 V, 48,000 V/hr rapid; step 5, 6000 V, 10,000 V/hr rapid; step 6, 300 V, 5 hr; total 60,700 V/hr.

After the IEF run, the IPG stripes were equilibrated in equilibration buffer (EB: 6 M Urea, 2% SDS, 0.375 M Tris, 20% v/v glycerol) with first 20 mg/ml DTT for 15 min, followed by EB with 25 mg/ml iodoacetamide (IAA) for 15 min. For running the second dimension, stripes were placed on 12.5% SDS gels in 27.5 × 22 cm low fluorescence glass cassettes (NH DyeAGNOSTICS GmbH, Germany) and overlaid with 1% agarose including bromophenol blue. Gel electrophoresis was performed in a SE900 electrophoresis unit (Hoefer Inc., USA) for a minimum of 2,400 V/hr and a maximum of 2,550 V/hr at 80 mA/gel, 100 W and 100 V. Imaging of the gels was performed by fluorescence scanning on a Typhoon 9,400 Imager (GE Healthcare, USA) at excitation/emission wavelengths of 498/524 nm (G-Dye100), 554/575 nm (G-Dye200) and 648/663 nm (G-Dye300).

To evaluate the expression pattern of protein spots separated by 2-D DIGE for healthy and *Pd*-colonized individuals, sample gels were analysed using the Delta2D software (DECODON, Germany). Briefly, an IS G-Dye100 image was designated as the master gel based on the largest number of detectable spots, and then connected to all images by a "sample-in-gel" warping strategy

in the Delta2D software. For warping of gels, we defined matched vectors between distinct protein spots. They were chosen automatically and manually. For expression analysis of protein spots, a fused image of all sample images (G-Dye200 and G-Dye300) was generated and a consensus spot pattern for normalization against the internal standard images was applied. Matched protein spots present in all sample images with a minimum of 1.5-fold change between the healthy and *Pd*-colonized state were statistically analysed using a nonparametric Wilcoxon Rank Sum test (alpha: $p < .05$) with the Delta2D statistic software TMeV (Decodon). Protein spots on the edges and in the upper region of the gels where no adequate protein separation was achieved were excluded from spot normalization and analysis in the Delta2D software.

2.4 | Preparative 2D gel for protein identification

Spot picking on preparative gel was performed by NH DyeAgnostics Services (NH DyeAGNOSTICS GmbH, Germany). Preparative gel separation was performed with pooled samples of all *M. lucifugus* or *M. myotis* individuals. A standardized company protocol was used. According to this, pooled plasma was labelled with G-Dye300 and mixed with unlabelled sample. A total protein concentration of 900 µg was applied per IPG stripe (pI 3–10 NI) and passively rehydrated. We performed isoelectric focusing on an IEF 100 Focusing Unit (Hoefer Inc, USA) and second dimension separation on a SE900 electrophoresis unit (Hoefer Inc., USA) with a 12.5% SDS gel. After 2-D electrophoresis, the gel was stained with Coomassie Brilliant Blue and fixed (40% ethanol, 10% acetic acid). Preparative gel images were matched with sample images in the Delta2D software. According to analysed expression profiles proteins spots with a fold change difference of ≥1.5 and a statistical significance of $p < .05$ between healthy and *Pd*-colonized individuals were picked for protein identification. It was not possible to pick all differentially expressed spots as not all spots were distinguishable on the Coomassie blue stained gel.

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

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

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

We analysed the processed MS data on a MASCOT (mass spectral search algorithm) server (version 2.2.2, Matrix Science Ltd, London) and searched in-house against a combination of NCBI database of 3 *Myotis* species (*M. brandtii*, *M. davidii*, *M. lucifugus*; FASTA files; 87,021 entries). For LC-MS/MS, the mass tolerance of precursor and sequence ions was set to 10 ppm and 0.35 Da, respectively. A maximum of two missed cleavages was allowed. Methionine oxidation and the acrylamide modification of cysteine were used as variable modifications. A protein was accepted as identified if the total MASCOT score was greater than the significance threshold and at least two peptides appeared the first time in the report and were the top ranking peptides. For LC-MS/MS data, the ions score was $-10*\log(p)$, where p is the probability that the observed match is a random event, i.e., individual ions scores > 41 indicate identity or extensive homology ($p < .05$).

3 | RESULTS

In the European bat species *M. myotis*, we observed 157 matched protein spots in all samples based on consensus spot patterns of analysed expression profiles. We did not detect different levels of protein expression between healthy and *Pd*-infected in *M. moytis*. Therefore, proteins were not identified in detail.

In the North American bat species *M. lucifugus*, we also observed 157 matched protein spots, of which eleven protein spots (7%) showed a significant ($p < .05$) differential expression with a minimum of a 1.5-fold difference between healthy and diseased conspecifics. We detected a significant upregulation of eight protein spots and a significant downregulation of three protein spots in WNS-positive *M. lucifugus* compared to healthy conspecifics (Figure 1 and Table 1).

We were able to pick and identify eight out of the 11 proteins with differential expression profiles (MI1, MI2, MI3, MI5, MI8, MI9 upregulated; MI4, MI10 downregulated; Table 1). MS data suggested multiple protein IDs for all protein spots. The top five ranked protein IDs with recurrent protein IDs under the top ranks based on MS peptide scores are shown in Table 1, excluding serum albumin matches.

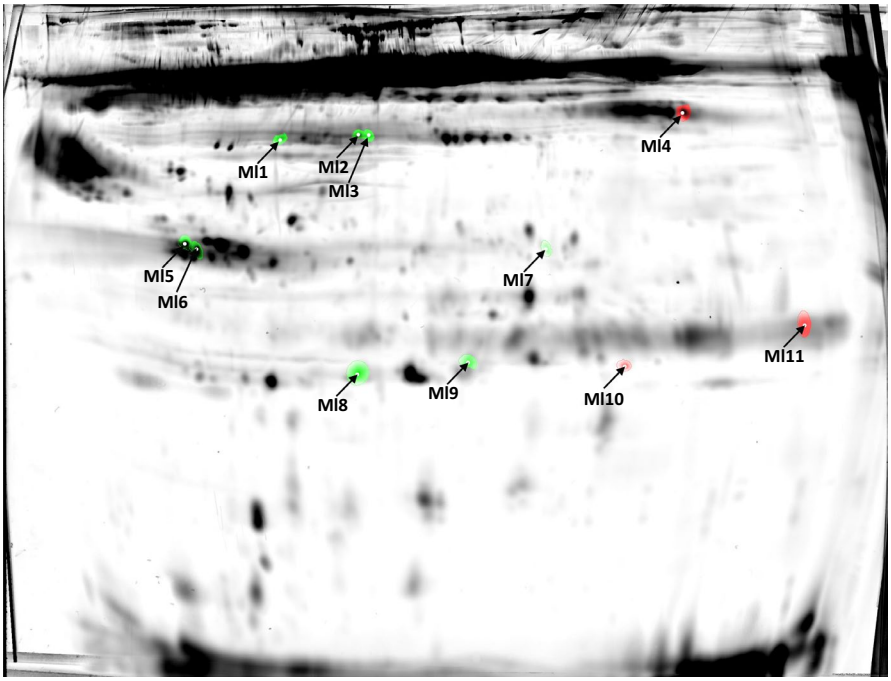

FIGURE 1 *Myotis lucifugus* plasma proteome of healthy and diseased individuals. Fused image of representative images of G-dye labelled *M. lucifugus* plasma samples separated by 2-DIGE. Differentially expressed protein spots ($p < .05$; minimum fold-change 1.5) are displayed in either green for upregulated or red for downregulated proteins in *Pd* infected individuals. Spot numbers correspond to Table 1 [Colour figure can be viewed at wileyonlinelibrary.com]

Protein IDs for upregulated protein spots included proteins involved in acute phase response (Alpha-2-macroglobulin-like [A2M-like], Serotransferrin [TF] and fibrinogen chains alpha [PFα], beta [PFβ], beta isoform X1 [PFβ-X1], gamma isoform X2 [PFγ-X2], complement system [Complement C3-like {C3-like}]), adaptive immunity (immunoglobulin lambda-like polypeptide 5-like [IGLL5]), lipoproteins (Apolipoprotein A-I [Apo-AI], Serum paraoxonase/arylesterase 1 isoform X1 [PON1-X1]), metabolism (Alpha-2,8-sialyltransferase 8F [SIAT8-F], S-adenosylmethionine synthetase isoformtype-2 [MAT2]), oxygen transport (haemoglobin subunit beta-like [Hbβ-like]) and antioxidative defence (6-phosphogluconate dehydrogenase [6PGD], Protein DJ-1 isoform X1 [DJ-1-X1]).

For downregulated protein spots, we found protein IDs involved in acute phase response (A2M-like, PFα, TF), desmosomes (Desmoplakin [DP], junction plakoglobin isoform X1 [JPG-X1]), exosomes (Actin, cytoplasmic 2 [ACT2], Tubulin beta-2B chain-like isoform X1 [TB-β-2B-like-X1], 14-3-3 protein sigma), adaptive immunity (putative V-set and immunoglobulin domain-containing-like protein IGHV4OR15-8-like) and antioxidative defence (α 1-microglobulin/bikunin precursor [AMBP] isoform X1 [AMBP-X1]). Among the top five ranked protein IDs, we identified A2M-like, TF, PF, Apo-AI, Hbβ-like and IGLL5 in more than one protein spot.

4 | DISCUSSION

The comparative expression analysis of plasma proteins between healthy and *Pd*-colonized European *M. myotis* did not reveal different levels of protein expression. This is in agreement with the lack of pathological changes associated with the *Pd*-colonization and the lack of mortality in *M. myotis* from *Pd* infection (Davy et al., 2017; Johnson et al., 2015; Lilley et al., 2019; Wibbelt et al., 2010). However, North American *M. lucifugus* showed significant differences in expression profiles, indicating a different response to *Pd*-infection than observed in *M. myotis*.

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

Pd infection in *M. lucifugus* shows similarities to other fungal skin infections in euthermic animals (Field et al., 2015). Host response against fungi is initiated by the recognition of fungal proteins by epithelial and innate immune cells resulting in the activation of the immune system through the production of inflammatory cytokines (Romani, 2011). In euthermic animals, the production of proinflammatory cytokines activates the acute phase response (APR), a systemic immune reaction characterized by the production of acute phase proteins (APPs), fever, leucocytosis (i.e., increase in white blood cell counts) and the display of sickness behaviour (anorexia, lethargy; Cray, Zaias, & Alman, 2009; Schneeberger, Czirják, & Voigt, 2013). Several aspects of the acute phase response of hibernating mammals have been shown to be constrained by the immunosuppression during the torpid state (but see Fritze et al., 2019), which includes lack of fever (Prendergast, Freeman, Zucker, & Nelson, 2002) and the lack of leucocytosis (Bouma et al., 2010). WNS-positive *M. lucifugus* lack the recruitment of innate immune cells and T cells to

Spot number	Fold change	Protein ID	MS protein score	Accession number
MI1	+1.57	A2M-like	1,277	XP_006084289.1
		TF	849	XP_006083771.1
		PFα	443	XP_006081874.1
		PON1-X1	369	XP_006085046.1
		6PGD	304	XP_006104980.1
MI2	+2.03	A2M-like	1,415	XP_006084289.1
		PFγ-X2	810	XP_006081876.1
		TF	652	XP_006083771.1
		PFα	434	XP_006081874.1
		PFβ	293	XP_006081872.1
MI3	+2.74	PFγ-X2	568	XP_006081876.1
		A2M-like	483	XP_006084289.1
		PFβ-X1	238	XP_006081872.1
		PFα	212	XP_006081874.1
		TF	184	XP_006083771.1
MI4	−3.15	PFα	2,121	XP_006081874.1
		TF	1,138	XP_006083771.1
		A2M-like	172	XP_006084289.1
		IGHV4OR15-8-like	113	XP_006776697.1
		AMBP-X1	76	XP_005880980.1
MI5	+4.39	Apo-AI	2,160	XP_006103027.1
		C3-like	945	XP_006107818.1
		A2M-like	928	XP_006084289.1
		Hb-β-like	742	XP_005880462.1
		IGLL5	652	XP_005871762.1
MI6	+3.37	*No protein identification possible*	/	/
MI7	+5.17	*No protein identification possible*	/	/
MI8	+1.8	Apo-AI	241	XP_006103027.1
		TF	184	XP_006083771.1
		A2M-like	184	XP_006084289.1
		DJ-1-X1	151	XP_006102968.1
		Hb-β-like	90	XP_005880461.1
MI9	+1.57	IGLL5	76	XP_005871755.1
		PFα	67	XP_005860555.1
		A2M-like	63	XP_006084289.1
		SIAT8-F	36	XP_005856790.1
		MAT2	33	XP_005882476.1
MI10	−37.04	DP	978	XP_006105268.1
		JPG-X1	569	XP_005875826.1
		ACT2	508	XP_006753072.1
		TB-β-2B-like-X1	221	XP_005869692.1
		14-3-3 protein sigma	197	XP_005875205.1
MI11	−1.94	*No protein identification possible*	/	/

Note: 6PGD, 6-phosphogluconate dehydrogenase; A2M-like, alpha-2-macroglobulin-like; ACT2, actin, cytoplasmic 2; AMBP-X1, α 1-microglobulin/bikunin precursor (AMBP) isoform X1; Apo-AI, Apolipoprotein A-I; C3-like, complement C3-like; DJ-1-X1, protein DJ-1 isoform X1; DP, desmoplakin; Hbβ-like, haemoglobin subunit beta-like; IGHV4OR15-8-like, putative V-set and immunoglobulin domain-containing-like protein IGHV4OR15-8-like; IGLL5, immunoglobulin lambda-like polypeptide 5-like; JPG-X1, junction plakoglobin Isoform X1; MAT2, S-adenosylmethionine synthetase isoformtype-2; PFα, fibrinogen alpha chain; PFβ, fibrinogen beta chain; PFβ-X1, fibrinogen beta chain isoform X1; PFγ-X2, fibrinogen gamma chain isoform X2; PON1-X1, serum paraoxonase/arylesterase 1 isoform X1; SIAT8-F, alpha-2,8-sialyltransferase 8F; TB-β-2B-like-X1, tubulin beta-2B chain-like isoform X1; TF, serotransferrin.

TABLE 1 Differential protein expression in the *Myotis lucifugus* plasma proteome. Protein spots exhibiting differential expression ($p < .05$; minimum fold-change 1.5) using Delta2D software are shown. Fold change reflects differences in protein spot volume comparing healthy to diseased individuals. Identified proteins via LC-MS/MS are displayed with the total MS protein score based on MASCOT searches on NCBI database. Protein IDs listed are the top 5 ranked protein matches based on the MASCOT score excluding protein matches of serum albumin. Spots MI6, MI7, and MI11 were not distinguishable on the preparative gels, thus could not be picked and identified

the site of *Pd* infection despite the apparent expression of inflammatory cytokines and chemokines (Field et al., 2015). We identified several APPs, such as A2M-like, TF and protein chains of PF (α, β, γ), in the majority of differentially regulated protein spots in *M. lucifugus* plasma. The physiological function of APPs is to reestablish homeostasis, promote healing, kill different pathogens and mitigate damage by reactive oxygen species. They also have important roles in the promotion of the host's adaptive immune response (Cray et al., 2009). Iron-binding glycoprotein TF helps maintain iron homeostasis, which is altered during infections and which influences modulation of innate immune defences and prevention of pathogen survival (Ganz & Nemeth, 2015). It is functionally similar to haptoglobin, another important APP in bats (Costantini, Czirják, Bustamante, Bumrungsri, & Voigt, 2019; Fritze et al., 2019), which has been identified in the wing tissue transcriptome of WNS positive *M. lucifugus* (Field et al., 2015). As a major protease inhibitor, A2M is involved in regulating inflammatory processes by scavenging defensins and inhibiting proteases of host and nonhost origin, which protects inflamed tissues against excessive damage (Rehmann, Ahsan, & Khan, 2013). Furthermore, glycoprotein PF has been described as having regulatory properties during inflammation in different tissues (Davalos & Akassoglou, 2012).

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

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

Activation of the immune system by fungal and bacterial pathogens initiates phagocytosis associated with the production of reactive oxygen species (ROS) (Brown, 2011). Increasing level of ROS during host defence leads to oxidative imbalances in affected tissues and thus an upregulation of oxidative stress markers (Schneeberger et al., 2013). For *Pd* infected North American bats, it was observed that the expression of genes related to oxidative stress changes (Field et al., 2015) and that the total antioxidant defence of animals was compromised (Moore et al., 2013). We found upregulated plasma proteins DJ-1-X1, 6PGD and downregulated AMBP-X1, which are associated with host protection during oxidative stress. LPS-induced inflammatory conditions in mice showed an upregulation of protein DJ-1 in response to ROS (Mitsumoto & Nakagawa, 2001). The pentose phosphate pathway, 6PGD being essential, is known to have protective properties against oxidative damage during infections (Riganti, Gazzano, Polimeni, Aldieri, & Ghigo, 2012). Alpha-1-microglobulin, the derivative of protein AMBP (Olsson, Olofsson, Tapper, & Åkerström, 2008), plays a similar role. Alpha-1-microglobulin also has protective effects against increased levels of extracellular haemoglobin associated with different diseases (Olsson et al., 2012; Rother, Bell, Hillmen, & Gladwin, 2005) and was downregulated in our samples of WNS-affected *M. lucifugus*.

One of the energetic costs of the APR during infection is changed lipid metabolism (Khovidhunkit et al., 2004). The identified upregulated plasma proteins APO-AI and PON-X1 are associated with the high-density lipoprotein complex, which is essential for cholesterol trafficking between peripheral cells and the liver (Tall, 1990). An increased level of lipoproteins in WNS-infected *M. lucifugus* may suggest changed triglyceride metabolism. Gene expression of apolipoproteins has also been found to be increased in infected wing membranes (Field et al., 2015).

The downregulation of exosomal proteins (ACT2, TB-β-2B-like-X1 and 14-3-3) may represent the physical disruption of *Pd*-infected tissues (Cryan et al., 2010; Meteyer et al., 2009) and upregulation of genes involved in wound healing (Field et al., 2015). Exosomes function as mediators for intercellular communication (Théry, Ostrowski, & Segura, 2009) and it has been shown that exosome release can be supressed during wound healing (Zhou et al., 2017).

Other differentially expressed proteins identified in *Pd*-infected *M. lucifugus* plasma included SIAT8-F and MAT2, which are associated with metabolic cycles of oligosaccharides (Wang, Liu, Wu, & Sun, 2016) and the amino acid methionine (Finkelstein & Martin, 2000). Downregulated desmosome proteins included DP and JPG-X1, which are both involved in cell signaling (Johnson, Najor, & Green, 2014). Similarly, genes involved in metabolic and cell signaling pathways were altered at the transcriptome level displaying a changed metabolism most probably associated with disturbance of host homeostasis due to WNS (Field et al., 2015; Verant et al., 2014).

European *M. myotis* in contrast with North American *M. lucifugus* did not exhibit any changes in proteomic profile in response to *Pd* infection. This suggests that *M. myotis* tolerates *Pd* colonization. Tolerance in a host-pathogen system is context dependent and influenced by both host and pathogen factors (King & Li, 2018; Mandl, Schneider, Schneider, & Baker, 2018). *Pd* in Europe and North America are genetically very similar (Drees et al., 2017), suggesting that *Pd* was recently introduced to North America (Leopardi, Blake, & Puechmaille, 2015). Therefore, pathogen factors probably play little role in the differences observed in

host mortality and host response between North American and European bats. The lack of response of *M. myotis* to *Pd* probably represents a long-term co-existence of pathogen and host, which has resulted in a balance that yields low rates of host mortality. The change in proteomic profile of North American *M. lucifugus* and extremely high mortality rates suggests this balance has not been reached in North American bats.

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AUTHOR CONTRIBUTIONS

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

DATA AVAILABILITY STATEMENT

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD018142.

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