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SHORT COMMUNICATION

Interruption to cutaneous gas exchange is not a likely mechanism of WNS-associated death in bats

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ABSTRACT

Pseudogymnoascus destructans is the causative fungal agent of white-nose syndrome (WNS), an emerging fungal-borne epizootic. WNS is responsible for a catastrophic decline of hibernating bats in North America, yet we have limited understanding of the physiological interactions between pathogen and host. *Pseudogymnoascus destructans* severely damages wings and tail membranes, by causing dryness that leads to whole sections crumbling off. Four possible mechanisms have been proposed by which infection could lead to dehydration; in this study, we tested one: *P. destructans* infection could cause disruption to passive gas-exchange pathways across the wing membranes, thereby causing a compensatory increase in water-intensive pulmonary respiration. We hypothesized that total evaporative water loss would be greater when passive gas exchange was inhibited. We found that bats did not lose more water when passive pathways were blocked. This study provides evidence against the proposed proximal mechanism that disruption to passive gas exchange causes dehydration and death to WNS-infected bats.

KEY WORDS: Dehydration, White-nose syndrome, Gas exchange, *Pseudogymnoascus destructans*

INTRODUCTION

The catastrophic decline of North America's hibernating bat population caused by white-nose syndrome (WNS) began nearly a decade ago, yet large gaps still remain in our understanding of its physiological effects. The causative agent is a fungus, *Pseudogymnoascus destructans* (*Pd*) (Lorch et al., 2011; Warnecke et al., 2012), but we do not know how an infection with *Pd* leads to mortality in hibernating bats. Researchers have formulated several hypotheses, with most including either dehydration or starvation as a proposed proximal cause of death (Boyles and Willis, 2010; Cryan et al., 2010; Reeder et al., 2012).

Accumulating evidence suggests that dehydration is an important part of the pathogenesis of WNS. Hydrophilic fungal pathogens like *Pd* are known to cause desiccation and dehydration of other hosts (e.g. the effect of *Metarhizium anisopliae* on American dog ticks) (Yoder et al., 2008). *Pd* causes visually severe damage to skin elasticity that could be caused by dehydration; wing membranes appear dry and crumble to the touch (Reichard and Kunz, 2009). Upon closer inspection, *Pd* invades tissues and blood vessels to such extent that it may disrupt physiological mechanisms of wing homeostasis (Cryan et al., 2010; Warnecke et al., 2012). Furthermore, *Pd*-infected bats show signs of dehydration in blood analyses (e.g. electrolyte depletion, hypovolaemia and metabolic acidosis) (Cryan et al., 2013; Verant et al., 2014; Warnecke et al., 2012, 2013). Dehydration has been widely accepted by the scientific community as a plausible ultimate mechanism or component of other mechanisms leading to WNS-related death.

Cryan et al. (2010) suggest that damage to wing membranes by *Pd* causes physiological changes, leading to dehydration and ultimately death. They proposed four possible mechanisms by which infection could lead to dehydration. Specifically, *Pd* infection could cause: (1) loss of dermal integrity, leading to increased cutaneous evaporative water loss (CEWL); (2) increased surface area and wicking of water away from the bat; (3) frequent arousals for thermoregulatory or circulatory reasons leading to more time spent euthermic, when water loss is high; and (4) disruption to passive gas-exchange pathways across the wing membranes, thereby causing a compensatory increase in water-intensive pulmonary respiration.

Here, we test the hypothesis that disruption of passive gas-exchange pathways across the exposed wing and tail membranes alters the relative use of cutaneous and pulmonary respiration (hypothesis 4 above). By limiting the pathway for passive gas exchange, *Pd* may cause an increase in pulmonary gas exchange (either active or passive; *sensu* Szewczak and Jackson, 1992) and elevate water loss to the environment. During hibernation, when O_2 requirements (and CO_2 production) are low, healthy bats may exchange gases passively through their thin, highly vascularized wing membranes (Herreid et al., 1968; Herreid and Schmidt-Nielsen, 1966; Minnaar et al., 2014); however, the proportion of O_2 obtained this way is debated. Cutaneous respiration should be advantageous over pulmonary respiration for conserving energy during winter hibernation, when resources are scarce. Furthermore, cutaneous respiration could also save water, because pulmonary respiration is more water costly than gas exchange through the skin (Herreid and Schmidt-Nielsen, 1966; Muñoz-Garcia et al., 2012; Thomas and Cloutier, 1992; Thomas and Geiser, 1997).

Our goal was to determine whether inhibition of cutaneous respiration increases total evaporative water loss (TEWL). We measured TEWL in whole-body respiration chambers over a range of ambient temperatures (T_a), and then applied a treatment of non-breathable, non-toxic oil to the bats' wings and tail membranes and repeated the measurements. We predicted that torpid bats would lose more water when passive gas exchange was inhibited. We also measured oxygen consumption ($\dot{V}_{O_2}$) and carbon dioxide production ($\dot{V}_{CO_2}$), because inhibition of passive gas exchange could, in theory, lead to more energetically costly active respiration (e.g. because of increased muscle function necessary for pulmonary respiration). We chose to use animals not visibly affected by WNS and an experimental manipulation because there may be multiple, inextricably linked causes of dehydration due to *Pd* infection (Cryan et al., 2010). Using healthy animals allows us to isolate a single mechanism of interest, which would be difficult with WNS-infected bats.

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Received 19 February 2015; Accepted 23 April 2015

The Journal of Experimental Biology

The Journal of Experimental Biology (2015) 218, 1986-1989 doi:10.1242/jeb.118950

List of symbols and abbreviations

CEWL	cutaneous evaporative water loss
$F_{E_{CO_2}}$	fractional concentration of carbon dioxide produced
$F_{E_{O_2}}$	fractional concentration of oxygen consumed
MR	metabolic rate
Pd	*Pseudogymnoascus destructans*
RER	respiratory exchange rate
RH	relative humidity
RQ	respiratory quotient
T_a	ambient temperature
TEWL	total evaporative water loss
$\dot{V}_{CO_2}$	volume of carbon dioxide produced
$\dot{V}_{O_2}$	volume of oxygen consumed
WNS	white-nose syndrome
WVD	water vapor density
WVP	water vapor pressure

RESULTS AND DISCUSSION

Since discovering the emergent fungal pathogen that causes WNS in North American bats, speculation about ultimate and proximal mechanisms of death associated with *Pd* infection has circulated among the scientific community. Our objective was to test one hypothesis, that disruption to passive gas exchange across wing and tail membranes during torpor increases TEWL because bats will compensate for lack of gas exchange by active respiration. Overall, application of oil to wing and tail membranes did not significantly affect torpid TEWL ($F_{1,93}$=0.04, P=0.8362) (Fig. 1), nor was there a T_a×treatment interaction ($F_{4,93}$=0.24, P=0.9174). This suggests that inhibition of cutaneous respiration does not force hibernating bats to substantially change the routes of oxygen uptake and carbon dioxide removal enough to increase water loss (at least within the resolution of our analytical equipment). Furthermore, we would expect TEWL to increase more with increasing metabolic rate (MR) in the treatment group if our proposed hypothesis was true. However, the slope of the relationship between MR and TEWL was not different between the two treatment groups (ANCOVA; P=0.7522). There was not a subject effect on torpid TEWL ($F_{16,93}$=2.39, P=0.4734). As expected, torpid TEWL significantly increased with rising T_a ($F_{4,93}$=12.44, P<0.0001). Across groups, water loss was lowest at 10°C. At 10°C, treatment trial TEWL was 0.0916±0.0401 mg min^{-1} and control trial TEWL was 0.0439 ±0.0484 mg min^{-1}, but the difference between trials was not significant (t_{93}=−0.63, P=0.5276). Across treatments, TEWL at 20°C was 0.4397±0.2101 mg min^{-1}, which was significantly higher than TEWL below 20°C (all P<0.0001). Our experimental design gives an indirect assessment of relative changes in pulmonary and cutaneous gas exchange in response to WNS and provides evidence against the hypothesis that disrupted passive gas exchange is a cause of dehydration, and ultimately death, in *Pd*-infected big brown bats. However, dehydration is a plausible cause of death, so other susceptible species and mechanisms should be explored (Verant et al., 2014; Willis et al., 2011).

As with TEWL, calculated metabolic rates did not significantly differ between control and treatment trials (t_{97}=−0.68, P=0.4999), nor was there a significant T_a×treatment interaction ($F_{4,97}$=0.95, P=0.4396). There are two possible explanations for this result. First, it may indicate that torpid bats do not use cutaneous pathways of gas exchange; however, this would be contrary to previous studies indicating that they do (Herreid and Schmidt-Nielsen, 1966; Makanya and Mortola, 2007; Minnaar et al., 2014; Muñoz-Garcia et al., 2012), including one study on big brown bats (Herreid et al., 1968). Perhaps big brown bats are unique in the relative contribution of passive gas exchange to whole body respiration. This may be why *Pd* is less virulent to big brown bats than other species (Frank et al., 2014). Second, our result may suggest that hibernating bats can compensate for a loss of cutaneous gas exchange with active respiration, but if so, the lack of increase in metabolic rates suggests that pulmonary respiration is energetically negligible for hibernating bats. As expected, T_a significantly affected torpid MR ($F_{4,97}$=8.19, P<0.0001) (Fig. 2). Because these data were highly variable, there was a significant subject effect ($F_{16,97}$=1.89, P=0.0308). Bats minimized MR at 5°C in both control and treatment trials (1.313±0.419 mW and 0.926±0.465 mW, respectively); however, these values were not significantly different

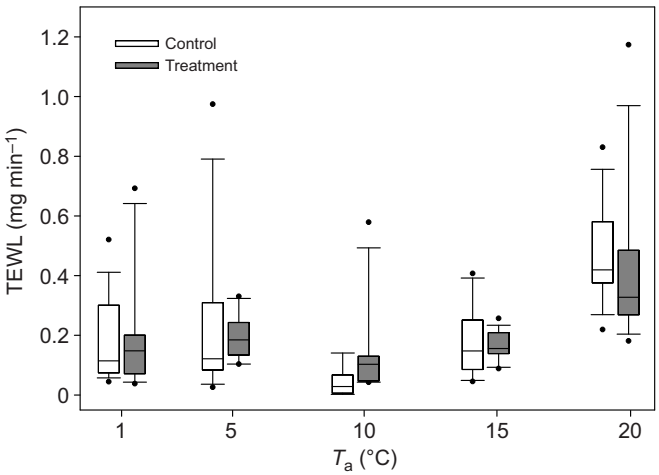

Fig. 1. Total evaporative water loss of torpid *Eptesicus fuscus* exposed to a range of T_a with or without oil applied to wing and tail membranes. Oil treatment at 1°C, 5°C, 10°C, 15°C and 20°C did not significantly affect TEWL ($F_{1,93}$=0.04, P=0.8362). At 1°C and 5°C, RH of incurrent air was 0%; whereas, at T_a>5°C, RH of incurrent air was 50%.

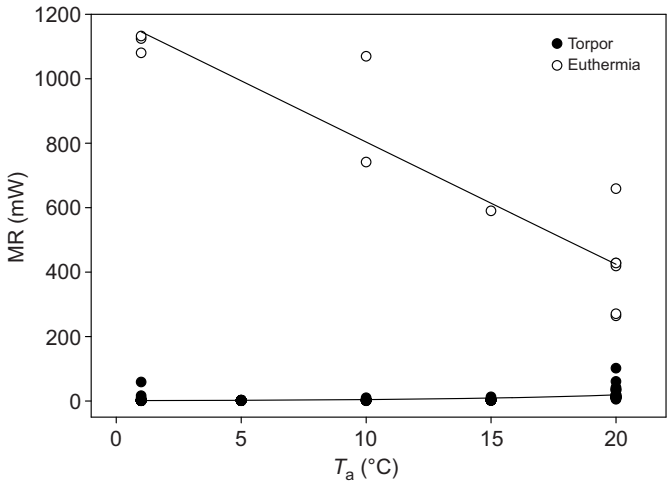

Fig. 2. Metabolic rate of *Eptesicus fuscus* exposed to a range of T_a. As expected, T_a (1°C, 5°C, 10°C, 15°C and 20°C) significantly affected MR ($F_{4,97}$=8.19, P<0.0001), with MR increasing as T_a increased for bats maintaining torpor (filled circles) (y=0.1467^x, r^2=0.8368) and MR decreasing as T_a increased for euthermic bats (open circles) (y=−37.9870x+1183.755, r^2=0.8539). There was no significant difference between control (without oil) and treatment (with oil) groups ($F_{1,93}$=0.46, P=0.4999); therefore, they are represented together here.

(t_{97}=−0.37, P=0.7137). At 20°C, bats torpid MR across treatments was 22.392±21.136 mW, which was significantly higher than MR below 20°C (all P<0.007).

Our study is the first to empirically test one of the hypotheses proposed by Cryan et al. (2010), but we strongly suggest that it should not be the last. It appears that dehydration does not result from disruption to passive gas exchange during torpor in big brown bats. However, we hope researchers continue to test this mechanism on other species as well as other proposed mechanisms related to the dehydration hypothesis. We cannot develop efficient and effective management strategies without a mechanistic understanding of the physiological pathways affected by WNS. Future experimental studies like the one we report here are fundamental to developing this understanding.

MATERIALS AND METHODS

Animal care protocol

We captured 17 (10 male, 7 female) big brown bats (*Eptesicus fuscus* Palisot de Beauvois 1796) in November 2013 from Blackball Mines in North Utica, Illinois, USA (41°34′N, 89°04′W) and transported bats to Southern Illinois University (SIU). We kept bats communally in a mesh cage inside an incubator (1.1 m^3; BatCave, Geneva Scientific, Fontana, WI, USA), specially designed to simulate cave-like conditions (T_a=5°C and RH=85%). We provided mealworms and water *ad libitum*, and periodically hand-fed mealworms to bats. Bats did not show outward signs of WNS, but infection was possible since WNS was confirmed in Blackball Mine in February 2013. All procedures were approved by SIU's Institutional Animal Care and Use Committee and Illinois Department of Natural Resources (SIU IACUC 12-040, IDNR permit NH13.5526).

Experimental protocol

We used open-flow respirometry to calculate TEWL (water vapor density, WVD, mg min^{-1}), $\dot{V}_{O_2}$ (ml min^{-1}) and $\dot{V}_{CO_2}$ (ml min^{-1}) and therefore respiratory exchange ratio (RER) and metabolic rate (MR, mW), over a range of T_a (1, 5, 10, 15 and 20°C). We withheld food for a minimum of 12 h prior to trials. We placed bats individually in 375 ml glass respirometry chambers and used 10 ml of paraffin oil covered by a wire platform to trap urine and feces. To allow bats time to acclimatize, we exposed them to each new T_a for at least 1 h before recording respirometry data. We conducted measurements inside an environmental chamber (BINDER Inc. BD series, Bohemia, NY, USA), equipped with an internal circulating fan that regulated T_a within the incubator at ±0.1°C accuracy.

We regulated flow rates between 150 and 550 ml min^{-1} (Flowbar-8; Sable Systems Int.) to prevent chamber O_2 concentrations from dropping below 20.90%. We scrubbed incurrent air of CO_2 with regenerating soda lime. We collected metabolic data for up to seven bats at a time and used an eight-channel multiplexer (RM-8 Gas Flow Multiplexer, Sable Systems Int.) to switch between chambers. The multiplexer selected a single chamber from which subsampled gas and water measurements were taken for 1 h with a sample rate of 1 min. Before switching to the next bat chamber, baseline air was subsampled from an empty chamber for 30 min, sampling every 1 min.

The fractional concentrations of water vapor pressure (WVP, kPa), oxygen (F_eO_2) and carbon dioxide (F_eCO_2) of the excurrent air were measured with RH-300, Oxzilla II, and CA-10a analyzers, respectively (Sable Systems International, Las Vegas, NV, USA). Prior to experiments, gas analyzers were calibrated using dry high-purity nitrogen gas and spanned with trace gases of oxygen and carbon dioxide. The RH-300s were spanned with saturated and dried air generated with a dew point generator (DG-4, Sable Systems International).

During measurements at high T_a (10–20°C) the dew point generator maintained the incurrent airstream at 50% relative humidity (RH). We recorded RH of the incurrent airstream and barometric pressure (kPa) in the room to calculate incurrent WVP (eqn 14.4, Lighton, 2008). While it would have been ideal to maintain high RH in all trials, the DG-4 was unable to maintain constant RH at T_a<10°C. To avoid unnecessary error of incurrent air stream RH, we dried incurrent air for trials at 1°C and 5°C using indicating chemical desiccants (DRIERITE Co. LTD, Xenia, OH, USA), therefore, WVP was 0 kPa. At low T_a, WVP is minuscule; WVP of air with 50% RH at 1°C is 0.329 kPa and 0.435 kPa at 5°C. Regardless, using dry and wet air at different T_a does not affect this study's purpose, as we were interested in comparing TEWL of bats between trials at each T_a. That is, bats measured with oil and without oil in the same conditions are directly comparable, so RH differences between T_a are inconsequential to our analysis.

In control trials, we exposed bats to the range of T_a (1, 5, 10, 15 and 20°C) under normal conditions. In the treatment trials, we exposed the same bats from our control group to the same T_a, but with non-toxic vegetable oil applied to furless skin on both sides of their wings and tail. In theory, the oil should non-invasively retard cutaneous respiration (Darmstadt et al., 2002). To ensure bats did not lick off the oil, we placed bats inside wire cylinders to limit movement. Within these cylinders, bats roosted, but could not flex their wings enough to groom themselves. To minimize potential bias, we used wire cylinders for both trials.

Data analysis

We calculated WVD, $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ using the most level 10 min samples from 1 h recordings at each T_a. Gas and water were corrected for dilution of water effect (when necessary) and standard temperature and pressure (eqn 4.10, Lighton, 2008), using the *post hoc* analysis program Expedata (Sable Systems Int.). For recordings where $\dot{V}_{O_2}$ was stable, we calculated RER ($\dot{V}_{O_2}/\dot{V}_{CO_2}$) Average RER for bats in this study was 0.79, indicating they used a mix of fat and proteins to fuel metabolism, which is reasonable. When possible, we used values of $\dot{V}_{O_2}$ to verify $\dot{V}_{CO_2}$ (eqn 10.8, Lighton, 2008). Some $\dot{V}_{O_2}$ recordings at low flow rates were unstable, in which case we used the estimated RQ (0.79) to calculate $\dot{V}_{CO_2}$ (eqn 10.5, Lighton, 2008). Using energy equivalents from the literature (Brody, 1945), we calculated MR by converting $\dot{V}_{CO_2}$ (ml min^{-1}) to energy (kJ l^{-1}) to power (mW). At T_a 1–20°C (i.e. temperatures clearly below the thermal neutral zone; Willis et al., 2005), we identified bats that used torpor based on their MR. Bats with MR >250 mW (~15 mW g^{-1}) were considered euthermic and were excluded from the analysis.

Statistical analyses

We recorded bats' mass before and after sampling to the nearest 0.25 g using a suspension scale (Pesola). The average initial mass for control trials was 17.2 g (N=15) and for treatment trials was 16.7 g (N=15). Between trials, there was no difference in initial mass (paired t-test; t=1.97, d.f.=14, P=0.069), so we did not include mass effect in the model.

We compared MR, specific gas exchange ($\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$) and TEWL using repeated-measures analyses of covariance (RM-ANCOVA) in PROC MIXED of SAS (SAS Institute Inc., Cary, NC, USA). We considered T_a and treatment (control or oil) as fixed effects, and individual and sex as random effects in all models. When comparing TEWL, we included MR as a predictor variable to account for its effects on TEWL. In all models, we first determined the most appropriate covariance structure (compound symmetry) for the data based on Akaike's Information Criterion (AIC). All values are presented as means±s.d.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Both authors contributed to all aspects of this project and manuscript preparation.

Funding

This work was funded by Southern Illinois University's Cooperative Wildlife Research Laboratory and Department of Zoology and Southern Illinois University's Graduate and Professional Student Council.

References

Boyles, J. G. and Willis, C. K. R. (2010). Could localized warm areas inside cold caves reduce mortality of hibernating bats affected by white-nose syndrome? *Front. Ecol. Environ.* **8**, 92-98.

Brody, S. (1945). *Bioenergetics and Growth*. New York: Reinhold.
Cryan, P. M., Meteyer, C. U., Boyles, J. G. and Blehert, D. S. (2010). Wing pathology of white-nose syndrome in bats suggests life-threatening disruption of physiology. *BMC Biol.* **8**, 135.
Cryan, P. M., Meteyer, C. U., Blehert, D. S., Lorch, J. M., Reeder, D. M., Turner, G. G., Webb, J., Behr, M., Verant, M., Russell, R. E. et al. (2013). Electrolyte depletion in white-nose syndrome bats. *J. Wildl. Dis.* **49**, 398-402.
Darmstadt, G. L., Mao-Qiang, M., Chi, E., Saha, S. K., Ziboh, V. A., Black, R. E., Santosham, M. and Elias, P. M. (2002). Impact of topical oils on the skin barrier: possible implications for neonatal health in developing countries. *Acta Paediatr.* **91**, 546-554.
Frank, C. L., Michalski, A., McDonough, A. A., Rahimian, M., Rudd, R. J. and Herzog, C. (2014). The resistance of a North American bat species (*Eptesicus fuscus*) to White-nose Syndrome (WNS). *PLoS ONE* **9**, e113958.
Herreid, C. F. and Schmidt-Nielsen, K. (1966). Oxygen consumption, temperature, and water loss in bats from different environments. *Am. J. Physiol.* **211**, 1108-1112.
Herreid, C., Bretz, W. and Schmidt-Nielsen, K. (1968). Cutaneous gas exchange in bats. *Am. J. Physiol.* **215**, 506-508.
Lighton, J. R. (2008). *Measuring Metabolic Rates: A Manual for Scientists: A Manual for Scientists*. USA: Oxford University Press.
Lorch, J. M., Meteyer, C. U., Behr, M. J., Boyles, J. G., Cryan, P. M., Hicks, A. C., Ballmann, A. E., Coleman, J. T. H., Redell, D. N., Reeder, D. M. et al. (2011). Experimental infection of bats with Geomyces destructans causes white-nose syndrome. *Nature* **480**, 376-378.
Makanya, A. N. and Mortola, J. P. (2007). The structural design of the bat wing web and its possible role in gas exchange. *J. Anat.* **211**, 687-697.
Minnaar, I. A., Bennett, N. C., Chimimba, C. T. and McKechnie, A. E. (2014). Partitioning of evaporative water loss into respiratory and cutaneous pathways in Wahlberg's epauletted fruit bats (*Epomophorus wahlbergi*). *Physiol. Biochem. Zool.* **87**, 475-485.
Muñoz-Garcia, A., Ben-Hamo, M., Pinshow, B., Williams, J. B. and Korine, C. (2012). The relationship between cutaneous water loss and thermoregulatory state in Kuhl's Pipistrelle, *Pipistrellus kuhlii*, a Vespertillionid bat. *Physiol. Biochem. Zool.* **85**, 516-525.
Reeder, D. M., Frank, C. L., Turner, G. G., Meteyer, C. U., Kurta, A., Britzke, E. R., Vodzak, M. E., Darling, S. R., Stihler, C. W., Hicks, A. C. et al. (2012). Frequent arousal from hibernation linked to severity of infection and mortality in bats with white-nose syndrome. *PLos ONE* **7**, e38920.
Reichard, J. D. and Kunz, T. H. (2009). White-nose syndrome inflicts lasting injuries to the wings of little brown myotis (*Myotis lucifugus*). *Acta Chiropterol.* **11**, 457-464.
Szewczak, J. M. and Jackson, D. C. (1992). Apneic oxygen uptake in the torpid bat, *Eptesicus fuscus*. *J. Exp. Biol.* **173**, 217-228.
Thomas, D. W. and Cloutier, D. (1992). Evaporative water loss by hibernating little brown bats, *Myotis lucifugus*. *Physiol. Zool.*, 443-456.
Thomas, D. W. and Geiser, F. (1997). Periodic arousals in hibernating mammals: is evaporative water loss involved? *Funct. Ecol.* **11**, 585-591.
Verant, M. L., Meteyer, C. U., Speakman, J. R., Cryan, P. M., Lorch, J. M. and Blehert, D. S. (2014). White-nose syndrome initiates a cascade of physiologic disturbances in the hibernating bat host. *BMC Physiol.* **14**, 227.
Warnecke, L., Turner, J. M., Bollinger, T. K., Lorch, J. M., Misra, V., Cryan, P. M., Wibbelt, G., Blehert, D. S. and Willis, C. K. R. (2012). Inoculation of bats with European *Geomyces destructans* supports the novel pathogen hypothesis for the origin of white-nose syndrome. *Proc. Natl. Acad. Sci. USA* **109**, 6999-7003.
Warnecke, L., Turner, J. M., Bollinger, T. K., Misra, V., Cryan, P. M., Blehert, D. S., Wibbelt, G. and Willis, C. K. R. (2013). Pathophysiology of white-nose syndrome in bats: a mechanistic model linking wing damage to mortality. *Biol. Lett.* **9**, 20130177.
Willis, C. K. R., Lane, J. E., Liknes, E. T., Swanson, D. L. and Brigham, R. M. (2005). Thermal energetics of female big brown bats (*Eptesicus fuscus*). *Can. J. Zool.* **83**, 871-879.
Willis, C. K. R., Menzies, A. K., Boyles, J. G. and Wojciechowski, M. S. (2011). Evaporative water loss is a plausible explanation for mortality of bats from white-nose syndrome. *Integr. Comp. Biol.* **51**, 364-373.
Yoder, J. A., Benoit, J. B., Denlinger, D. L., Tank, J. L. and Zettler, L. W. (2008). An endosymbiotic conidial fungus, *Scopulariopsis brevicaulis*, protects the American dog tick, *Dermacentor variabilis*, from desiccation imposed by an entomopathogenic fungus. *J. Invertebr. Pathol.* **97**, 119-127.