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Huddling reduces evaporative water loss in torpid Natterer's bats, *Myotis nattereri*

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ABSTRACT

Periodic arousals during hibernation consume most of the winter energy budget for hibernating mammals. Evaporative water loss (EWL) is thought to affect the frequency of arousals and thus energy balance, and might have dramatic implications for over-winter survival and fitness. We hypothesized that huddling affects EWL and energy expenditure in torpid mammals. We tested this hypothesis using bats as a model and predicted that, during torpor, EWL and energy expenditure of huddling individuals would be lower than in individuals that are not in a huddle. We measured EWL and metabolic rate of torpid *Myotis nattereri* (Kuhl, 1817) huddling in groups or roosting individually. Evaporative water loss in huddling individual bats was almost 30% lower than in solitary animals ($P = 0.03$), even after correcting for the effects of metabolic rate. Our results suggest that conservation of water is a substantial benefit underlying huddling by bats during hibernation. Ultimately, huddling could reduce the total cost of hibernation by reducing the number of expensive periodic arousals from torpor caused by the need to supplement water.

1. Introduction

Endothermy allows mammals and birds to remain active at low ambient temperatures (T_a), but comes at the cost of high energy requirements and limited ability to cope with resource scarcity. As a result, many species use either bouts of torpor (periods of reduced metabolic rate (MR) and body temperature (T_b)) or hibernation (*i.e.* repeated, multi-day torpor bouts during winter), or both, when T_a falls and resources are inadequate to maintain a balanced energy budget (Geiser, 2004). Despite its energetic benefits, torpor is also associated with ecological and physiological costs (Humphries et al., 2003) such as increased risk of predation (Radzicki et al., 1999; but see Stawski and Geiser, 2010), accumulation of sleep debt (Daan et al., 1991), or reduced synaptic function (Strijkstra et al., 2003). As a result, heterothermic animals cannot maintain low T_b indefinitely, and all hibernators periodically rewarm to their normothermic T_b during the winter (French, 1985, 2000). These arousals consume up to 90% of the total energy used during hibernation (Thomas et al., 1990; Karpovich et al., 2009), but the reasons for arousals to take place are still not fully understood. A number of non-mutually exclusive hypotheses have been proposed (Daan et al., 1991; Prendergast et al., 2002; Malan, 2010; Hope and Jones, 2012; Czenze et al., 2013), one of which relates frequency of arousals to rates of water loss during hibernation (Thomas and Geiser, 1997; Ben-Hamo et al., 2013).

Heterothermy influences much of the ecology and physiology of hibernating animals including timing of reproduction (King et al., 1991; Bieber et al., 2012), lifespan (Wilkinson and South, 2002; Turbill et al., 2011), habitat selection (Willis and Brigham, 2005; Olson and Barclay, 2013) and social behavior (Arnold, 1993; Blumstein et al., 2004; Willis and Brigham, 2007). The social or communal lives of many heterothermic animals are shaped by group thermoregulation and huddling (Arnold, 1993; Willis and Brigham, 2004) and, thus, understanding proximate benefits of huddling is important for identifying selective pressures shaping sociality in these species. There are several hypotheses to explain why heterothermic animals might benefit from huddling during hibernation (reviewed by Gilbert et al., 2010). Huddling can reduce heat loss by reducing individual surface area exposed to ambient air (Pinshow et al., 1976; Gilbert et al., 2010) or by reducing the difference between T_b and T_a as metabolic heat from several hibernating individuals warms the local microclimate (Arnold et al., 1991). For hibernating animals in cold microclimates, huddles might serve as a buffer against fluctuations of T_a, especially if T_a falls below the minimum set-point for T_b regulation, which might result in arousal from hibernation. Huddling may also minimize heat loss during energetically costly arousals and subsequent periods of normothermy (Arnold, 1993; Ruf and Arnold, 2000; Boyles et al., 2008). Boyles et al. (2008) tested these two hypotheses and found strong support for the arousal hypothesis based on differences in the sizes of clusters at different T_a's. However, they did not consider a third hypothesis, namely that huddling could be additionally beneficial by reducing evaporative water loss (EWL).

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During torpor animals lose water due to EWL, albeit at a low rate (but see: Marom et al., 2006; Muñoz-Garcia et al., 2012), and must arouse periodically to restore water balance (Thomas and Geiser, 1997). Thus, reduced water loss during torpor could result in dramatic indirect energy savings, and improved survival and fitness, by reducing arousal frequency and over-winter energetic costs (Thomas and Cloutier, 1992; Thomas and Geiser, 1997).

Temperate-zone bats provide a good opportunity to test hypotheses about relationships between huddling behavior, EWL and hibernation energetics. Bats use both short-term torpor during the active season and hibernation in winter (Altringham, 2011), and many species during hibernation form large aggregations of tightly clustered individuals with considerable variation in huddling within and among species (Henshaw and Folk, 1966; Clawson et al., 1980; Boyles et al., 2008; Boratyński et al., 2012). Evaporative water loss both during normothermy and torpor tends to be higher in bats than in non-volant mammals of the same size (Chew and White, 1960; Webb, 1995; Muñoz-Garcia et al., 2012) which could be part of the explanation as to why most bats hibernate in humid roosts (Thomas and Cloutier, 1992; Altringham, 2011; but see: Twente, 1955) and why the tendency to huddle in hibernating *Myotis myotis* increases with decreasing humidity (Boratyński et al., 2012). Evaporative water loss increases with increasing water vapor pressure deficit (Webb et al., 1995) and this appears to affect use of torpor with potential implications for survival (Thomas and Cloutier, 1992; Thomas and Geiser, 1997; Willis et al., 2011). For example, recently, Ben-Hamo et al. (2013) observed that higher total EWL was correlated with shorter torpor bouts in desert-dwelling *Pipistrelus kuhlii*. If EWL during torpor influences arousal frequency during hibernation then natural selection should favor behavioral and physiological adaptations for water conservation. To the best of our knowledge, this hypothesis has not been tested experimentally.

We, therefore, tested the hypothesis that huddling provides benefits to torpid animals in terms of both energy and water savings. We predicted that torpid bats in a huddle would have lower EWL than solitary individuals and that this difference would be more pronounced in dry than in humid air. We also predicted that bats in a huddle would be torpid at a lower energy cost than solitary bats. We tested these predictions using Natterer's bats (*Myotis nattereri*, Kuhl 1817) that, in nature, often hibernate in huddles, usually smaller than 20 individuals, and can be readily captured at our study sites.

2. Materials and methods

2.1. Animals and housing

All experimental procedures were approved by the Regional Directorate for Environmental Protection (permit # WPN.6401.6.29.2012.MO) and by the Local Committee for Ethics in Animal Research in Bydgoszcz, Poland (decision # 03/2013). In January 2013 we collected 20 male *M. nattereri* from hibernacula located in 19th century military fortifications in Toruń, central Poland (53°00′ N, 18°35′ E). Immediately after capture, the bats were placed in individual cotton bags and transported ~10 km by car to our laboratory at Nicolaus Copernicus University. Once in the laboratory, we implanted a LifeChip passive transponder tag (PIT-tags, Destron Fearing, South St. Paul, MN, USA) under the skin of each bat. The bats were acclimated to laboratory conditions for two weeks prior to measurements. During acclimation and between metabolic measurements bats were kept indoors in two identical flight cages (2 × 1.5 × 1.5 m), in a dimly lit animal holding room at constant T_a (~15 °C) and on a winter (*i.e.*, ~9 h light) photoperiod. Bats were trained to feed on their own. Food (mealworms) and water were provided *ad libitum*. Following Barnard (1995), every four days we supplemented their diet with vitamins (solBiosupervit, Biofaktor, Skierniewice, Poland) added to the water.

2.2. Respirometry

Metabolic rate was measured using indirect calorimetry, and total EWL (*i.e.*, cutaneous plus evaporative) was determined based on the difference in water vapor density between the incurrent and excurrent airstreams. We used two open-flow respirometry systems set up in parallel, one to collect data from solitary bats and the second to collect data from huddling bats (Fig. 1). Compressed outside air was dried and scrubbed of CO_2 with a PureGas Generator (PCDA1120-132; Puregas, Westminster, CO, USA) and then air pressure was reduced to atmospheric pressure. We humidified incurrent air (water vapor pressure (WVP) ~0.6 kPa H_2O) using a bubbler made from a 500 ml volumetric flask filled with distilled water with an aquarium stone mounted on the inlet tubing, which was kept in a temperature-controlled cabinet. We saturated the airstream in the bubbler at 6 °C and then cooled it to ~1 °C in a custom-built condenser submerged in a temperature-controlled ethylene glycol bath (Fisherbrand, FBC 635) to provide a saturated airstream at a precisely known dew point (for the detailed description of the method see Marom et al., 2006 and Lighton, 2008). To provide dry incurrent air (WVP ~0.005 kPa H_2O) to the bats, we plumbed an alternative airstream which bypassed the bubbler and condenser. We used precision needle valves upstream of the mass-flow meter (FlowBar 4, Sable Systems International (SSI), Las Vegas NV, USA) to regulate flow separately for three separate airstreams: a baseline, the single animal chamber and the group chamber. Flow rates were controlled and measured with a mass-flow meter (FlowBar-4, SSI) upstream of the respirometry chambers at a rate between 150 and 600 ml min^{-1} for the solitary bat chamber and between 600 and 1200 ml min^{-1} for the group chamber.

Throughout each experimental trial, bats were sealed in glass respirometry chambers (volume: 850 ml for the group, 400 ml for the solitary bat), lined with galvanized steel mesh to allow individuals or groups to hang comfortably. To prevent evaporation of water from excreta we covered the bottom of each chamber with a ~1 cm deep layer of paraffin oil. Bats were protected from the paraffin oil by a steel mesh floor. To ensure adequate mixing of the air in the chambers, the inlet tube was positioned at the bottom of the chamber just below the mesh floor but above the layer of paraffin oil, and the outlet tube was positioned at the top of the respirometry chamber.

The microenvironment experienced by the animals was always more humid than the dry or humidified incurrent air we supplied because air in the chambers consisted of a mixture of the fresh air we supplied and humidified air exhaled by the animals. Thus we were able to precisely control WVP in the respirometry chambers with fine adjustments of the incurrent flow rate. The incurrent air mixed in the chambers with air exhaled by animals so that WVP in the excurrent air (*i.e.*, the microclimate experienced by the animals) averaged 0.29 ± 0.06 kPa for the dry air treatment and 0.71 ± 0.04 kPa for the humid air treatment. We did not expose bats to higher humidity because this led to condensation in excurrent tubing, which would have compromised our measurements of excurrent WVP. Nevertheless, these humid air conditions lie within the range of WVP at which *M. nattereri* hibernate (WVP between 0.58 and 1.00 kPa at T_a between 1.5 and 7.0 °C; Lesiński, 1986).

Excurrent air from each of the two animal chambers was subsampled at ~75 ml min^{-1} and then WVP was measured for each airstream with a RH-300 humidity analyzer (SSI). Each airstream was then dried by being passed through a column of magnesium perchlorate (Anhydrone, J.T. Baker, Phillipsburg, NJ, USA) and CO_2 concentration was measured with one of two gas analyzers, FoxBox-C or CA-10 (SSI). Both of these analyzers rely on an identical mechanism/gas sensor (*i.e.*, a dual wavelength infrared sensor). We calibrated each humidity analyzer every week during the experiment using the two-point calibration method described in detail by Marom et al. (2006). Similarly, both CO_2 analyzers were also calibrated weekly using two-point calibration with pure N_2 (AirProducts; Warsaw, Poland) running though the

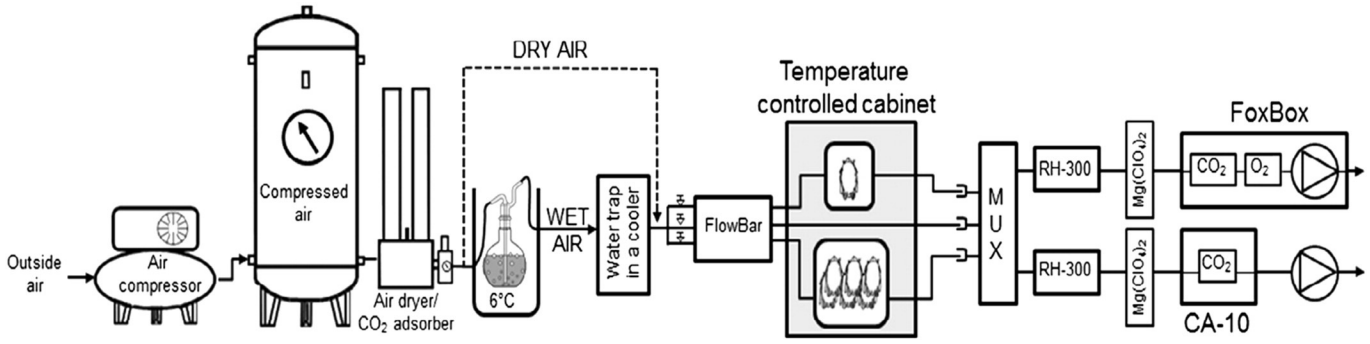

Fig. 1. Diagram of the respirometry systems used in the studies of metabolic rate and evaporative water loss in solitary and huddling *Myotis nattereri*. FlowBar — multichannel mass flow meter, MUX — multiplexer, RH-300 — humidity analyzer, FoxBox and CA-10 — CO_2 analyzers. The incurrent air stream was either humidified (humid air treatment) to water vapor pressure ~0.6 kPa H_2O using a bubbler made from a 500 ml volumetric flask filled with distilled water with an aquarium stone mounted on the inlet tubing, or bypassed the humidifier, and dry air was directed to the respirometry chambers (dry air treatment). See text for the detailed description of the system.

analyzer for at least 5 min, followed by a precision calibration mixture of 1% CO_2 (±0.002%) in N_2 (AirProducts; Warsaw, Poland) for at least an additional 5 min.

We used ExpeData (v. 1.43, SSI) for all recordings and a computer-controlled multiplexer (MUX, SSI) to automate baseline recording from a reference airstream. Sampling occurred at a rate of 1 Hz, with 5 min of reference gas read every 30 min. Temperature in the respirometry chambers was measured with thermistor probes calibrated against a precise mercury-in-glass thermometer connected to an analog-to-digital interface (UI-2, SSI) controlled by ExpeData.

2.3. Experimental protocol

We had access to two flight cages so we randomly assigned bats to one of two groups for housing. Each day, out of all animals we selected a list of candidate bats to be assigned to the group of five or six individuals, or to the solitary chamber, and then randomly selected from the list of candidates. The list ensured that each individual underwent the solitary trial only once and that no bats were run on consecutive nights (*i.e.*, individuals always had at least one full day between metabolic trials).

Before and after metabolic measurements bats were weighed with an electronic balance to ±0.1 g (Scout Pro 402, Brooklyn, NY, USA). Respirometry chambers were placed in a dark, temperature-controlled cabinet (MIR 153, Sanyo, Japan) throughout the recording of gas exchange variables. Experiments began at the time corresponding to natural dusk (~17:00, UTC + 1) to ensure that bats would be torpid the following morning. All experiments lasted for ~23 h. We set T_a ~20 °C for the first 3 h of the recording to allow bats to acclimate to the chambers and then, over 3 h, gradually reduced T_a to ~6 °C. All experiments commenced using dry incurrent air, which prevented condensation of water vapor inside the chambers and the excurrent tubing. After several hours we gradually decreased T_a to the final 6 °C, which reflects the temperature at which *M. nattereri* often hibernate in nature (Webb et al., 1996). Within 3 h of the beginning of recording, we switched to the humid airstream. Bats remained in these conditions for ~10 h overnight. The next morning (~07:00), once bats were in deep ($\dot{V}$ CO_2 < 0.01 ml min^{-1}, visible pattern of intermittent ventilation; Szewczak and Jackson, 1992), steady-state torpor, the air stream was switched back to dry air and EWL and respiratory gas exchange were measured until ~12:00. We then switched back to humid air for a final series of measurements until ~16:00.

The door of the temperature-controlled cabinet was equipped with a small (15 by 15 cm) window which we covered with a cardboard flap during experiments. We visually confirmed that bats were huddling (*i.e.*, all bats in contact with each other) every few hours throughout respirometry trials and at the end of each trial by quickly lifting the flap to directly observe the bats. This eliminated the need to open the door of the cabinet which could have disturbed the bats. There was never any change in the metabolic trace following these observation periods. We excluded trials from the analysis when bats in the group were not huddling (*i.e.*, not in direct physical contact with each other). Occasionally one or more bats in the group chamber did not enter or did not remain torpid throughout the recording session. This was readily obvious from the metabolic trace and we also excluded these trials from the analysis. We confirmed that all bats were in deep torpor at the end of their trials by measuring rectal T_b using a thermometer (TN408LC, ZyTemp, Taiwan) with a type-K thermocouple (0.6 mm in diameter) calibrated against a precise mercury-in-glass thermometer. Twenty-four measurements of EWL and torpor metabolic rate (*t*MR) met these criteria: 10 for clusters (5 in dry and 5 in humid air) and 14 for solitary bats (7 in dry and 7 in humid air).

2.4. Data analysis

We automated the calculations for $\dot{V}$ CO_2 and EWL using Expedata. $\dot{V}$ CO_2 was calculated following equation 10.4 and EWL following equation 10.9 from Lighton (2008). We used data recorded only during periods when we knew that bats were in deep steady-state torpor for more than 10 h. For each of the 30 min sampling sessions, we programmed Expedata to discard the first minute of data and then calculate the average of the following 29 min of values collected at 1 Hz. This period was long enough to capture several episodes of intermittent ventilation in both, solitary and huddling bats (see Szewczak and Jackson, 1992 for a description of this ventilation pattern in torpid bats). For analysis, we chose episodes of minimum stable EWL and concurrent $\dot{V}$ CO_2 for both chambers – the one containing a huddle of bats and the one containing an individual – during a period when the WVPs of the excurrent airstreams of both chambers were stable and similar (see Results). Fulfilling this second criterion was essential to ensure that we could compare EWL of clustered and solitary bats because the rate of EWL is negatively correlated with humidity of the air surrounding an animal (Procter and Studier, 1970; Thomas and Cloutier, 1992). Bats were not fed for ~24 h before data collection began at 07:00 to ensure they were post-absorptive during recording. Thus, assuming that the bats were post-absorptive and fat was their major source of energy, we calculated MR (mW) from $\dot{V}$ CO_2 using the energetic equivalent of CO_2 production assuming RQ = 0.7 (28.008 J ml CO_2^{-1}; Gessaman, 1987).

The setup of the gas exchange measurement system did not allow us to measure gas exchange and EWL of each individual within a group, so we calculated mean values by dividing the *t*MR and EWL of the huddle by the number of bats in the huddle. For analyses where body mass (m_b) was used as a covariate, we also divided the total m_b of all bats in the group by the number of individuals.

There was no difference in the WVP of the air to which solitary or huddling bats were exposed in either dry ($P = 0.97$) or humid air ($P = 0.17$; see Results) so we treated humidity as a categorical variable with two levels: dry and humid. We also found no differences in *t*MR (calculated per individual bat) between groups of five *vs.* six individuals (Kruskal–Wallis; dry air: $H = 0.00$, $N = 5$, $P = 1$; humid air: $H = 0.33$, $N = 5$, $P = 0.56$) and EWL (Kruskal–Wallis, for dry air: $H = 1.33$, $N = 5$, $P = 0.25$; for humid air: $H = 0.33$, $N = 5$, $P = 0.56$) so we also treated group size as a categorical variable with two levels: solitary and group.

We tested all data distributions for normality prior to analysis using the Shapiro–Wilk test. To achieve normality, m_b and EWL were natural-log transformed. Since the distribution of T_a differed from normal, and transformations did not correct it, T_a inside solitary *vs.* group chambers was compared using a Mann–Whitney *U* test. The distribution of WVP inside solitary *vs.* group chambers did not differ from normal and thus was compared using Student's *t*-test. We compared *t*MR of solitary and clustered bats using the general linear model (GLM) procedure in IBM SPSS v.21 (IBM Corp., 2012) with T_a and ln m_b as covariates, humidity and group size as fixed factors and the interaction between humidity and group size. We also used the GLM to compare ln-EWL of solitary *vs.* clustered bats in humid and dry air with *t*MR and ln-m_b as covariates, humidity and group size as fixed factors and the interaction between them. Ambient temperature and *t*MR were correlated (Pearson's $r > 0.7$), so we excluded T_a from the initial GLM for EWL because *t*MR likely has a stronger direct influence on EWL. We assumed so because respiratory gas exchange, and thus *t*MR likely affect respiratory EWL (*e.g.* Williams and Tieleman, 2000; Muñoz-Garcia et al., 2012). We confirmed that residuals for both *t*MR and EWL models were normally distributed. To report the effects of all independent variables and the interactions, we decided not to simplify the models.

Data for T_a, WVP and m_b are reported as the mean ± SD. We report EWL and *t*MR, and their upper and lower 95% confidence intervals (CI), as estimated marginal means after controlling for effects of covariates. Estimated marginal means for EWL and its 95% CI were calculated based on ln-transformed EWL values so we report back-transformed values. For clarity of presentation data in the figures are plotted without transformation or adjustment for covariates in the models. We assessed significance of the $P \leq 0.05$ level. For statistical results that were marginally significant but potentially significant biologically we calculated Cohen's effect size *d* (Cohen, 1988; Becker, 2000).

3. Results

On average, T_a tended to be higher by ~0.7 °C in the chamber containing a huddle of bats (7.09 ± 0.11 °C, range: 6.91–7.27 °C) than in the chamber with a single bat (6.37 ± 0.43, range: 6.10–7.34 °C), both in dry ($U = 5$, z = -1.949, $P = 0.051$) and humid air ($U = 5$, $z = -1.949$, $P = 0.051$). Although the difference in T_a did not quite reach our criterion for significance, the effect size was large (Cohen's $d = 2.29$). In dry air ambient WVP did not differ between the chamber with huddling bats (WVP = 0.31 ± 0.09 kPa) and individuals (WVP = 0.31 ± 0.08 kPa; $t = 0.04$, df = 8.20, $P = 0.97$) and this pattern was consistent in humid air (huddling bats: 0.72 ± 0.02 kPa, and solitary bats: 0.69 ± 0.04 kPa; $t = 1.50$, df = 8.97, $P = 0.17$).

There was no difference in mean m_b of bats measured solitarily (7.42 ± 0.41 g) or in groups (7.14 ± 0.43 g, $t = 1.55$, df = 9.89, $P = 0.15$). To account for the difference in T_a experienced by huddling *vs.* solitary bats and for possible effects of m_b we compared estimated marginal means of *t*MR for solitary and huddling bats at $T_a = 6.67$ °C and $m_b = 7.30$ g. There were no differences in *t*MR between bats measured in humid *vs.* dry air (Table 1) or between huddling bats and individuals (Table 1, Fig. 2). The absence of this effect was not related to ambient humidity as there was no interaction between solitary *vs.* huddling and dry *vs.* humid air (Table 1). In huddling bats, *t*MR of an average individual equaled 3.26 mW (95% CI: 2.59–3.93 mW) while *t*MR of solitary bats averaged 3.28 mW (95% CI: 2.76–3.80 mW) both in dry and humid air (Table 1, Fig. 2). Respiratory gas exchange likely affected respiratory EWL (*e.g.* Williams and Tieleman, 2000) so it was necessary to include *t*MR as a covariate in the EWL analysis. After controlling for *t*MR, huddling clearly affected EWL as did ambient humidity with no interaction between these terms (Table 2). Therefore, we compared EWL separately for solitary and huddling bats in wet *vs.* dry air. After accounting for *t*MR (*i.e.*, 3.27 mW) and ln-transformed m_b (*i.e.*, at 7.30 g), EWL of an average individual in a huddle (0.23 mg H_2O min^{-1}, 95% CI: 0.19–0.27 mg H_2O min^{-1}) was ~27% lower than that of a solitary bat (0.30 mg H_2O min^{-1}, 95% CI: 0.26–0.34 mg H_2O min^{-1}) both in dry and humid air (Table 2, Fig. 3). On average, EWL of bats in humid air (0.16 mg H_2O min^{-1}, 95% CI: 0.14–0.18 mg H_2O min^{-1}) was ~64% lower than of bats measured in dry air (0.43 mg H_2O min^{-1}, 95% CI: 0.38–0.50 mg H_2O min^{-1}; Table 2).

Table 1
Results of the general linear model explaining variation in torpid metabolic rate in solitary *Myotis nattereri* or huddling individuals in dry or in humid air. ln-m_b — ln-transformed body mass, T_a — ambient temperature.

Source of variation	df	F	*P*
Intercept	1	0.537	0.473
'Solitary or huddling'	1	0.002	0.968
'Dry or humid air'	1	0.320	0.579
ln-m_b	1	0.380	0.545
T_a	1	1.323	0.265
'Solitary or huddling' × 'dry or humid air'	1	0.010	0.920
Error	18		

Adjusted $r^2 = -0.032$.

4. Discussion

In his study of huddling in slugs (*Limax pseudoflavus*), Cook (1981) argued that the primary function of huddles is conservation of water. Our results are consistent with this hypothesis for bats, at least during bouts of deep torpor. As hypothesized by Thomas and Cloutier (1992), we found that huddling reduced EWL during torpor by almost 30%. We also found that huddling had no detectable effect on *t*MR. Taken together these results support the hypothesis that reduced EWL may be an important incentive to huddle in hibernating bats.

Field data are consistent with our findings that huddling is important for reducing EWL. Boratyński et al. (2012) found that under natural conditions hibernating bats were more likely to huddle in low humidity microclimates, and suggested that hibernating bats huddle to reduce EWL. The few studies in which the consequences of huddling have been analyzed focused on normothermic mammals and provide equivocal results. On the one hand Studier (1970) found that normothermic *Myotis thysanodes* and *Myotis velifer* in huddles had lower EWL than solitary bats. On the other hand, average EWL per bat in a huddle of six normothermic, pregnant *Myotis lucifugus* was higher than EWL of solitary pregnant females (Procter and Studier, 1970). In normothermic naked mole rats *Heterocephalus glaber*, a reduction of EWL was positively correlated with huddle size (Withers and Jarvis, 1980; Yahav and Buffenstein, 1991). Of course, normothermic animals are defending high T_b while, during torpor T_b approximates T_a which means the effect of huddling on EWL may differ between these thermal states. Nevertheless, our results complement these data from normothermic mammals and highlight the importance of huddling for water economy during torpor.

The benefits of huddling likely result from reduction of the surface area of exposed skin. Total EWL of bats is much higher than that of other, similar-sized mammals likely because of the flight membranes (Webb, 1995). Even with wings folded at rest, the exposed skin surface of bats is still considerably larger than that of most other mammals. In non-volant mammals EWL is primarily respiratory (Withers, 1992) while in birds ~50–70% of EWL results from cutaneous evaporation (Wolf and Walsberg, 1996; McKechnie and Wolf, 2004; Muñoz-Garcia

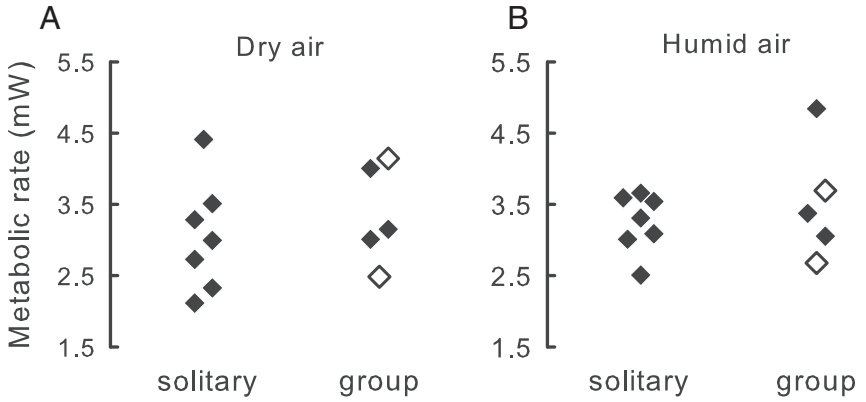

Fig. 2. Metabolic rate of torpid solitary and huddling *Myotis nattereri* measured in dry (A) or humid (B) air at ambient temperatures between 6.10 and 7.34 °C (for details see text). Groups consisted of five (white diamonds) or six (black diamonds) bats. Data for huddling bats were calculated by dividing the measurements by the number of bats in the huddles. All data are presented untransformed, without adjusting for body mass or ambient temperature.

and Williams, 2005). Muñoz-Garcia et al. (2012) reported that in a desert bat, *Pipistrellus kuhlii*, both in shallow torpor and in normothermy cutaneous evaporation accounted for ~70% of EWL. If these high rates of cutaneous EWL also apply to mesic species (but see Kurta et al., 1989 for the discussion of the effect of laboratory conditions on EWL), one may infer that the reduced surface area of exposed skin in huddling bats (Canals et al., 1997) led to a considerable reduction of cutaneous EWL for *M. nattereri* (Table 2, Fig. 3).

Contrary to our prediction, huddling did not ameliorate the effects of ambient humidity on EWL. It is possible that huddling would have had a more pronounced effect on EWL across a larger range of humidity conditions. Unfortunately, the accumulation of condensation inside the gas exchange system did not allow us to expose bats to higher WVP inside metabolic chambers (see: Material and methods). Nevertheless, in both huddling and solitary bats, EWL per individual was consistently higher in dry air than in humid air and we found no interaction between ambient humidity and huddling. This suggests that, at least up to huddle sizes of five or six individuals, huddling does not obviate the need for bats to carefully select microclimates. Huddles of bats still face high rates of EWL that could be biologically significant even in moderately dry conditions.

Also, contrary to our prediction, and some previous studies on bats, we detected no effect of huddling on *t*MR (Table 1, Fig. 2). Brown (1999) found that MR of normothermic *Miniopterus schreibersii* decreased with increasing number of individuals in a huddle at T_a's between 5 and 30 °C and Roverud and Chappell (1991) found that MR at T_a = 10 °C was negatively correlated with huddle size in *Noctilio albiventris*. The only study that we are aware of, which analyzed an effect of huddling on the metabolism of torpid bats, reported that mass-specific O_2 consumption did not differ between huddling bats and those roosting alone (Brown, 1999). However, indirect evidence suggests that huddling bats are able to maintain a lower T_b than solitary individuals (Twente, 1955; Funakoshi and Uchida, 1978; but see also Kurta and Fujita, 1988) suggesting that huddling may indeed lead to lower energy expenditure during deep torpor. Nevertheless, not all mammals reduce *t*MR during huddling. *Phodopus sungorus* in huddles of four individuals maintained higher T_b during torpor than did solitary individuals (Jefimow et al., 2011); this would have increased the metabolic rate. A methodological explanation for the absence of an effect of huddling on *t*MR in our study could be that some individuals in the group chamber had not reached deep, steady-state torpor during the period of recording that we sampled for analysis. We reject this possibility for several reasons. First, the duration of our recordings was more than adequate to allow all bats to reach deep torpor. Second, when we removed bats from the chambers we confirmed that all huddling and solitary individuals were in deep torpor based on their T_b. Third, close inspection of metabolic recordings revealed stable traces for both solitary bats and groups during the time window we sampled. Any trials that did not meet these criteria were excluded from analysis.

A more likely explanation is that greater cumulative heat production by several bats in the group chamber affected the chamber microenvironment, and thus *t*MR. Huddling animals can elevate T_a in their immediate microenvironment (Davis, 1970; Contreras, 1984) and in deep torpor under natural conditions bats are able to maintain T_b which is higher by ~1 °C than the temperature of the wall on which they roost (Twente, 1955; Funakoshi and Uchida, 1978, but see: Kurta, 1985). In our experiment T_a tended to be slightly higher (~0.7 °C) in the chambers with huddling bats in our study. This, in turn, could have increased *t*MR of huddling bats *via* simple Q_{10} effects. Data are not available for *M. nattereri* but a T_a difference of 0.7 °C could increase the average *t*MR by ~10% for similar-sized *M. lucifugus* (Hock, 1951).

Despite the slightly higher T_a, *t*MR of huddling bats was virtually identical to that of solitary individuals in our study (Table 1, Fig. 2). This suggests that huddling did result in energy savings and that, if a huddle of bats had experienced exactly the same T_a as a solitary individual, their *t*MR would have been lower. Interestingly, under natural conditions hibernating bats tend to form huddles at lower T_a's than those selected for hibernation by solitary conspecifics (Henshaw and Folk, 1966; McNab, 1974; Clawson et al., 1980; Brown and Bernard, 1994; Boyles et al., 2008; Boratyński et al., 2012). Our results suggest that this pattern could allow huddling bats to compensate for potentially increased cumulative heat production and local warming during torpor by selecting slightly colder microclimates to reduce *t*MR. Although huddling may have resulted in some energy savings during torpor, our findings support the hypothesis that reduced EWL is even more important than direct energetic savings as a motivation for huddling by torpid bats. Even assuming that a slightly colder local T_a would have allowed bats in the group chamber to reduce *t*MR relative to solitary individuals, this effect would have been smaller than the 30% reduction in EWL that we observed.

Table 2
Results of the general linear model explaining variation in evaporative water loss (ln-transformed) for torpid solitary *Myotis nattereri* or huddling individuals in dry or humid air. *t*MR — torpor metabolic rate, ln-m_b — ln-transformed body mass.

Source of variation	df	F	*P*
Intercept	1	0.976	0.336
'Solitary or huddling'	1	5.604	0.029
'Dry or humid air'	1	109.805	0.000
ln-m_b	1	2.607	0.124
*t*MR	1	0.465	0.504
'Solitary or huddling' × 'dry or humid air'	1	0.107	0.747
Error	18		

Adjusted r^2 = 0.831.

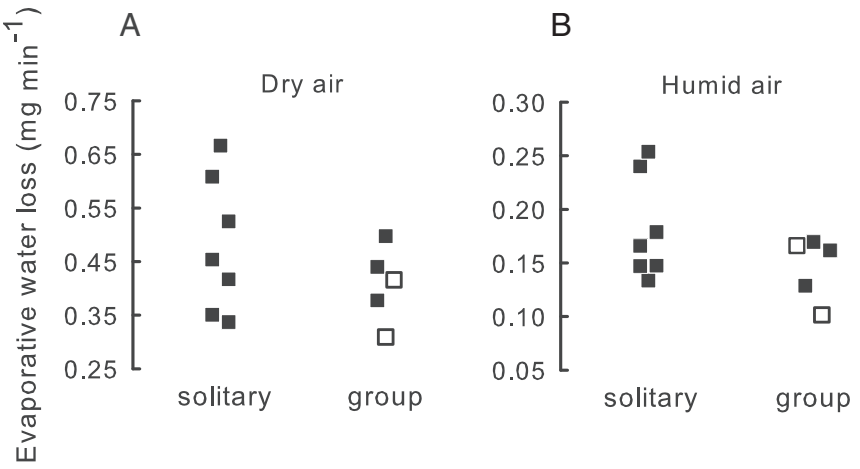

Fig. 3. Evaporative water loss during torpor in solitarily and huddling *Myotis nattereri* in dry (A) or in humid (B) air at ambient temperatures between 6.10 and 7.34 °C (see text for details). Huddles of five bats are indicated with white squares and huddles of six bats with black squares. Data for huddling bats were calculated by dividing the measurements by the number of bats in the huddle. All data were presented untransformed, without adjusting for body mass or metabolic rate. Note different scales on each plot.

This leads to an alternative (although not mutually exclusive) explanation for the above-described negative correlations between huddling tendency and T_a in the wild (*e.g.*, Boyles et al., 2008; Boratyński et al., 2012). Bats may have a greater tendency to huddle in cold environments because these environments are characterized by lower WVP with consequent greater EWL. By selecting colder environments, huddling bats may reduce energetic costs, while concomitantly compensating for the increased water loss that would occur in a drier environment.

Empirical evidence supports the hypothesis that periodic arousals during hibernation are linked to the need to restore water balance (Fisher and Manery, 1967; Thomas and Cloutier, 1992; Thomas and Geiser, 1997; Ben-Hamo et al., 2013). Most recently, research on white-nose syndrome (WNS) in hibernating North American bats has supported this link since clinical signs of dehydration were found in bats inoculated with the fungal pathogen *Pseudogymnascus destructans* potentially due to increased fluid loss across fungal lesions, as well as increased arousal frequency leading to starvation (Cryan et al., 2010; Warnecke et al., 2012, 2013). This link between EWL and periodic arousals suggests that huddling could result in significant indirect energetic benefits by prolonging torpor bouts (Thomas and Geiser, 1997; Ben-Hamo et al., 2013). This in turn may strongly affect bat over-winter energy expenditure and, therefore, survival.

Our data suggest that reduced EWL in huddling bats resulted from a reduction in skin surface exposed to ambient conditions rather than by depression of respiratory gas exchange, because of the absence of a huddling effect on *t*MR. Based on our results, a substantial benefit underlying huddling by bats during hibernation is the conservation of water during torpor bouts. Ultimately, huddling could reduce the total cost of hibernation by reducing the number of expensive periodic arousals from torpor caused by the need to restore water balance.

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Author contributions

M.S.W., J.S.B., C.K.R.W. and M.J. designed the study, collected and analyzed data and wrote the manuscript.

Abbreviations

CI	confidence interval
EWL	evaporative water loss
GLM	general linear model
m_b	body mass
MR	metabolic rate
Q_{10}	Q_{10} temperature coefficient
RH	relative humidity
RQ	respiratory quotient
T_a	ambient temperature
T_b	body temperature
*t*MR	metabolic rate in torpor
$\dot{V}\ CO_2$	rate of CO_2 production
WVP	water vapor pressure

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Appendix A. Supplementary data

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