

## RESEARCH ARTICLE

# Waking to drink: rates of evaporative water loss determine arousal frequency in hibernating bats

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### SUMMARY

**Bats hibernate to cope with low ambient temperatures ( $T_a$ ) and low food availability during winter. However, hibernation is frequently interrupted by arousals, when bats increase body temperature ( $T_b$ ) and metabolic rate (MR) to normothermic levels. Arousals account for more than 85% of a bat's winter energy expenditure. This has been associated with variation in  $T_b$ ,  $T_a$  or both, leading to a single testable prediction, i.e. that torpor bout length (TBL) is negatively correlated with  $T_a$  and  $T_b$ .  $T_a$  and  $T_b$  were both found to be correlated with TBL, but correlations alone cannot establish a causal link between arousal and  $T_b$  or  $T_a$ . Because hydration state has also been implicated in arousals from hibernation, we hypothesized that water loss during hibernation creates the need in bats to arouse to drink. We measured TBL of bats (*Pipistrellus kuhlii*) at the same  $T_a$  but under different conditions of humidity, and found an inverse relationship between TBL and total evaporative water loss, independent of metabolic rate, which directly supports the hypothesis that hydration state is a cue to arousal in bats.**

Key words: water balance, hibernation, *Pipistrellus kuhlii*, torpor bout length.

Received 1 August 2012; Accepted 23 October 2012

### INTRODUCTION

Hibernation is an adaptive strategy in bats that facilitates coping with low ambient temperatures ( $T_a$ ) and scarce food during winter (Geiser, 2004). The over-winter survival of hibernating bats depends on the quantity of energy that animals store prior to hibernation, the rate of depletion of these reserves and the duration of winter (Humphries et al., 2002). The decline in metabolic rate (MR) and body temperature ( $T_b$ ) of a bat during hibernation enables a significant reduction of usage of nutrients and water, enhancing the probability of survival (Geiser and Koertner, 2010). Hibernation is not a constant state of reduced  $T_b$  and MR; rather, it comprises bouts of torpor interspersed with periods of arousal, when the animal returns to its normothermic  $T_b$  and MR (French, 1985). A single torpor bout can last as long as 350 h in bats, but periods of arousal rarely exceed 24 h (Geiser and Ruf, 1995). Although bats arouse for only 5–10% of the time they are hibernating, arousals can account for over 85% of a hibernating bat's energy expenditure (Wang, 1978; Thomas et al., 1990; Geiser and Ruf, 1995; Dunbar and Tomasi, 2006; Jonasson and Willis, 2012). Because arousals involve such high energetic costs, one might assume their occurrence to be obligatory and adaptive. Presumably, critical processes or functions that must be periodically restored at normothermic  $T_b$  for the organism's survival necessitate these arousals. However, mechanistic explanations as to why bats awaken during hibernation and how arousals are triggered remain controversial.

Many different hypotheses attempting to explain periodic arousals have been proposed (Strumwasser, 1959; Galster and Morrison, 1970; Baumber et al., 1971; Daan et al., 1991; Thomas and Cloutier, 1992; Németh et al., 2010), as reviewed by Thomas and Geiser (Thomas and Geiser, 1997). One hypothesis proposes that during

hibernation there is an accumulation of metabolic wastes, such as ketone bodies, and a need to replenish energy-rich substrates (Galster and Morrison, 1970; Baumber et al., 1971). Another suggests that bats arouse to restore body water and electrolyte balance (Thomas and Cloutier, 1992; Thomas and Geiser, 1997; Németh et al., 2010). All these hypotheses relate arousals to processes – such as metabolism and water loss – that vary in the same direction with  $T_b$  and  $T_a$ . Consequently, they give rise to the same prediction, namely that torpor bout length (TBL) is negatively correlated with  $T_a$  and  $T_b$ . Although changes in  $T_a$  and  $T_b$  affect TBL and the frequency of arousals (e.g. Twente et al., 1985; Geiser and Kenagy, 1988), this correlation alone cannot establish a causal link between arousal and the proposed processes, and, in addition, it introduces a difficulty in distinguishing between the hypotheses. Here, we tested the 'water balance' hypothesis, first proposed by Fisher and Manery (Fisher and Manery, 1967), which asserts that hibernating animals continuously lose water through evaporation while hibernating, and the ensuing dehydration initiates arousals, during which the animals rehydrate by drinking (Thomas and Cloutier, 1992; Thomas and Geiser, 1997).

During torpor, all water losses are assumed to be evaporative, because torpid animals are not known to urinate or to defecate (Nelson, 1989). Moreover, because of the decrease in MR during torpor, the rate of respiratory water loss declines, leaving cutaneous water loss (CWL) as the main avenue of water loss during hibernation (Herreid and Schmidt-Nielsen, 1966; Morris et al., 1994; Hosken and Withers, 1997; Hosken and Withers, 1999). The rate of CWL is directly proportional to the difference in water vapor density ( $e_w$ ) between the skin surface ( $e_s$ ) and the adjacent ambient air ( $e_a$ ),  $\Delta e_{s-a}$ . An increase in  $\Delta e_{s-a}$ , or a reduction in the effective

boundary layer at the body surface, will increase the rate of CWL (Schmidt-Nielsen, 1997). The  $T_b$  of hibernating animals generally closely follows  $T_a$  (Schmidt-Nielsen, 1997; Geiser, 2004), and thus a decrease in  $T_b$  will reduce  $e_w$  at the skin surface, resulting in lower  $\Delta e_{s-a}$  and low CWL.

We tested the water balance hypothesis in groups of a small bat, Kuhl's pipistrelle (*Pipistrellus kuhlii* Kuhl 1817), that were all maintained at constant  $T_a$  and constant absolute humidity. We assumed that at constant  $T_a$ , bats have similar  $T_b$  and MR values, and that water vapor density at the skin surface is saturated at skin temperature. We manipulated  $\Delta e_{s-a}$  by changing  $e_a$ , and measured water loss in Kuhl's pipistrelles in both dry and humid air at constant  $T_a$ . According to the water balance hypothesis, we predicted that: (1)  $e_a$  is independent of MR; (2) the rate of total evaporative water loss (TEWL) is higher when bats are measured in dry conditions because of higher  $\Delta e_{s-a}$ ; and (3) the rate of TEWL and TBL of the bats during hibernation are negatively correlated.

## MATERIALS AND METHODS

### Animals

We captured 25 adult, non-reproductive female *P. kuhlii* with mist nets in July and August 2010 and 2011 at Midreshet Ben-Gurion, Israel (30°52'N, 34°46'E) (Korine and Pinshow, 2004). During September 2010 and 2011, we kept bats in an outdoor flight cage (2.5×2.5×3 m), and provided them with mealworms (*Tenebrio molitor* larvae) and water with a vitamin supplement, *ad libitum*.

This research was performed under permit 37066 from the Israel Nature and National Parks Protection Authority and permit IL-71-12-2010 from the Ben-Gurion University committee for the ethical care and use of animals in experiments.

### Induction of hibernation

To induce hibernation, we moved the bats into a temperature-controlled room where we decreased  $T_a$  by 1.5°C day<sup>-1</sup> for 15 days, until a stable  $T_a$  of 9.9±1.4°C (mean ± s.d.) was reached. Throughout the experiment, we maintained a light regime following the natural photoperiod. We provided each bat 2.5 g of mealworms per day, which is almost double their daily *ad libitum* food intake when not preparing for hibernation (1.36±0.12 g; M.B.H., personal observations). The decrease in  $T_a$  and day length, and the increase in available food, induced hibernation in all the bats. Once body mass ( $m_b$ ) of the bats stabilized (7.28±0.66 g;  $F_{1,99}=0.46$ ,  $P=0.50$ ), we moved them to an opaque plastic box (67.5×46×31.5 cm, model 17305368, Keter Plastics, Carmiel, Israel); its inner walls were covered with plastic mesh to allow the bats to perch. The box had an inlet port through which air was pumped at 150 cm<sup>3</sup> min<sup>-1</sup>. Absolute humidity of the air inside the box was 5.03±0.09 g H<sub>2</sub>O m<sup>-3</sup> for the duration of the experiment.

### Measurement of metabolic rate and TEWL

We measured CO<sub>2</sub> production ( $\dot{V}_{CO_2}$ ) and TEWL of bats using an open-flow respirometry system. For each set of measurements, four or five bats were fasted for 4 days and measured simultaneously, each in a 350 cm<sup>3</sup> plastic metabolic chamber (HPL9314, Lock&Lock, Hana Cobi, Korea); the chambers were all placed in a temperature-controlled cabinet (Precision 815, Thermo Scientific, Cleveland, OH, USA) in which the temperature was either 4.8±1.2°C or 10.1±0.3°C. The duration of the measurement runs varied between 4 and 16 days, because we did not finish a run until the last bat arose. Therefore, the other three or four bats of the group may have entered several torpor bouts of varying length. We recorded the number and duration of torpor bouts in all the bats.

One group of bats ( $N=16$ ) was measured twice, once exposed to CO<sub>2</sub>-free dry air [0.58±0.05 g H<sub>2</sub>O m<sup>-3</sup>, relative humidity (RH)=6%] and once exposed to CO<sub>2</sub>-free humid air (6.7±0.7 g H<sub>2</sub>O m<sup>-3</sup>, RH=65%), at a  $T_a$  of 10.1±0.3°C, the preferred minimum temperature for *P. kuhlii* (Muñoz-Garcia et al., 2012). To obtain this level of humidity, air was bubbled through an aquarium stone in a container of water maintained at 5.9±0.22°C in a temperature-controlled bath (Thermo-Haake V26, Karlsruhe, Germany). Another group of bats ( $N=9$ ) was measured only in dry air (0.41±0.30 g H<sub>2</sub>O m<sup>-3</sup>, RH=6%) at a  $T_a$  of 4.8±1.2°C. We did not measure bats in humid air at this  $T_a$  because, at saturation, absolute humidity is not substantially higher than in dry air, and it is problematic to measure TEWL when the air is saturated at such low  $T_a$ . For all measurements, air flowed through the metabolic chambers at a rate of 100 cm<sup>3</sup> min<sup>-1</sup>, governed by an eight-channel gas controller and monitor (G245, Qubit Systems, Kingston, ON, Canada). The air exited to an eight-channel gas multiplexer (G244, Qubit Systems) that selected channels sequentially for sampling. Air samples passed through a dew-point meter (RH-100, Sable Systems International, Las Vegas, NV, USA) and a CO<sub>2</sub> analyzer (S158, Qubit Systems). During measurements, we recorded the difference in fractional concentration of CO<sub>2</sub> between excurrent air and reference atmospheric air, and dew-point temperature. We measured the  $T_a$  of incurrent air at the dew-point meter with a calibrated, 36-gauge type-T thermocouple, and barometric pressure with a mercury barometer (model 230-7420, Nova Lynx, Auburn, CA, USA).

We used  $\dot{V}_{CO_2}$  instead of oxygen consumption ( $\dot{V}_{O_2}$ ) to calculate MR because  $\dot{V}_{O_2}$  in torpid bats was so low that measurement errors were unacceptable. We assumed that bats oxidized only fat during hibernation and therefore used 0.71 as the respiratory exchange ratio to convert  $\dot{V}_{CO_2}$  into  $\dot{V}_{O_2}$ , and that to mW using 19.66 J ml<sup>-1</sup> O<sub>2</sub> (Schmidt-Nielsen, 1997).

TEWL of bats was calculated following Williams and Tieleman (Williams and Tieleman, 2000). At the beginning and the end of measurements, we weighed the bats to ±0.01 g with a portable electronic balance (Scout SP202, Ohaus, NJ, USA) and measured rectal temperature ( $T_b$ ) using a 36-gauge type-T thermocouple coated with lacquer. We considered bats to be hibernating when their torpor bout was longer than 24 h. Torpor bout length (TBL) was calculated as the time from when a bat reduced its MR by more than 50% below its normothermic level (Muñoz-Garcia et al., 2012) until MR returned to the normothermic level. We calculated TBL of the first torpor bout (>24 h) of each run for each bat. We measured groups of bats simultaneously (see above), and during each run we recorded the number of arousals for each bat. These data were standardized by dividing the number of arousals by the duration of the run in days.

### Statistical analysis

We tested data for normality with the Shapiro-Wilk test and for homogeneity of variance using Bartlett's test. Whenever necessary, we used logarithmic transformation of the data to comply with the assumptions of parametric tests. We compared initial  $m_b$  and TBL of bats from different treatment groups using two-way ANOVA with humidity and  $T_a$  as fixed factors. To compare differences in MR and TEWL of bats from different humidity groups we used analysis of covariance (ANCOVA) with  $T_b$  as the covariate. We tested the effect of MR and TEWL on TBL by multiple linear regression, and excluded predictors by backward elimination. When only one predictor was found significant, we used linear regression. Finally, we used linear regression to assess the relationship between (1) number of arousals and TEWL; (2)  $m_b$  loss and TEWL; and (3)

$m_b$  loss and TBL. Data are reported as means  $\pm$  1 s.d., and null hypotheses were rejected at  $\alpha=0.05$ . All statistical tests were performed using the program R (version 2.12.2; 'R & R', Statistics Department, University of Auckland).

## RESULTS

Mean  $m_b$  at the beginning of each run of measurements did not differ between bats exposed to different conditions of air humidity ( $F_{1,36}=0.07$ ,  $P=0.79$ ), or between different  $T_a$  ( $F_{1,36}=0.02$ ,  $P=0.90$ ; dry at 5°C,  $m_b=6.41\pm 1.04$  g; dry at 10°C,  $m_b=6.45\pm 0.88$  g; humid,  $m_b=6.51\pm 0.65$  g). We therefore excluded  $m_b$  from further analyses.

### The effect of humidity

The mean rate of TEWL of bats measured in dry air ( $82.9\pm 39.4$  mg day<sup>-1</sup>) was significantly higher than that in humid air ( $47.2\pm 24.6$  mg day<sup>-1</sup>;  $F_{1,35}=11.87$ ,  $P=0.002$ ). This difference was not affected by the relationship between TEWL and  $T_b$  ( $F_{1,35}=3.95$ ,  $P=0.06$ ). The mean rate of TEWL of bats measured in dry air at 10°C ( $99.4\pm 52.1$  mg day<sup>-1</sup>) was significantly higher than that measured at 5°C ( $55.5\pm 17.4$  mg day<sup>-1</sup>;  $t_{22}=3.00$ ,  $P=0.008$ ). However, MR of bats did not differ significantly between humidity conditions ( $F_{1,35}=0.23$ ,  $P=0.63$ ; dry, MR=5.00 $\pm$ 2.52 mW; humid, MR=4.25 $\pm$ 1.13 mW), and variation in  $T_b$  did not significantly affect MR within this range ( $F_{1,35}=0.64$ ,  $P=0.43$ ). Mean MR of bats measured in dry air at 10°C (5.30 $\pm$ 1.77 mW) did not differ from that at 5°C (4.49 $\pm$ 3.50 mW;  $t_{22}=0.65$ ,  $P=0.53$ ).

### Torpor bout length

When we combined data for the two humidity regimes, TEWL of bats was negatively correlated with TBL ( $F_{1,38}=3.79$ ,  $P=0.05$ ; Fig. 1A), whereas MR was not ( $F_{1,38}=0.002$ ,  $P=0.96$ ). When we restricted analyses to bats in dry air, TBL was negatively correlated with TEWL of bats ( $F_{1,22}=5.26$ ,  $P=0.03$ ,  $R^2=0.19$ ; Fig. 1B), but not with MR ( $F_{1,22}=0.02$ ,  $P=0.89$ ). However, TBL and TEWL of bats measured in humid air were not correlated ( $F_{1,13}=2.70$ ,  $P=0.12$ ), and neither were TBL and MR ( $F_{1,13}=0.30$ ,  $P=0.59$ ).

TBL did not differ between bats exposed to different air humidity conditions ( $F_{1,37}=1.14$ ,  $P=0.29$ ), but it did differ with  $T_a$  ( $F_{1,37}=11.72$ ,  $P=0.002$ ; dry at 5°C, TBL=7.89 $\pm$ 3.30 g; dry at 10°C, TBL=3.84 $\pm$ 2.18 g; humid, TBL=4.31 $\pm$ 3.16 g).

### Number of arousals

We found that the number of arousals was positively correlated with TEWL of bats measured in dry air ( $F_{1,22}=4.41$ ,  $P=0.05$ ,  $R^2=0.17$ ; Fig. 2), but not in humid air ( $F_{1,13}=2.53$ ,  $P=0.14$ ).

### Body mass loss

The percentages of  $m_b$  lost per day during hibernation and TEWL were positively correlated ( $F_{1,37}=41.35$ ,  $P<0.001$ ,  $R^2=0.53$ ; Fig. 3A), and this relationship was independent of air humidity ( $F_{1,35}=1.52$ ,  $P=0.23$ ). When we tested the association between the percentage of  $m_b$  lost per day during hibernation and TBL, with humidity as a covariate, we found that the interaction term TBL $\times$ humidity was significant ( $F_{1,35}=5.17$ ,  $P=0.03$ ), and we found a negative correlation between the percentage of  $m_b$  lost per day and TBL in bats measured in dry air ( $F_{1,22}=31.42$ ,  $P<0.001$ ,  $R^2=0.59$ ; Fig. 3B), but not in humid air.

## DISCUSSION

We found that TBL was inversely related to the rate of TEWL of bats measured in dry and humid air conditions (Fig. 1A), but TBL was independent of MR, a result that supports the hypothesis that

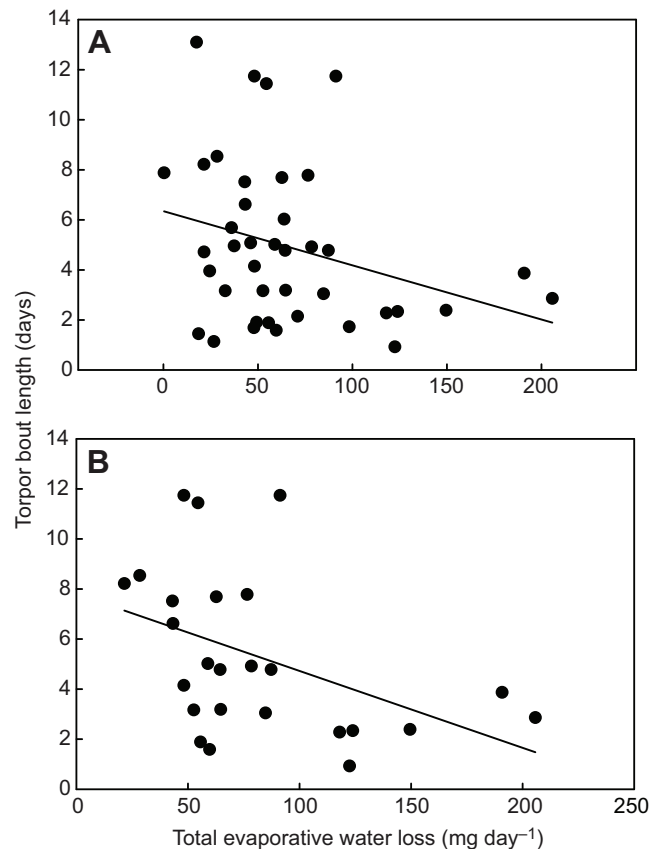


Fig. 1. Relationship between torpor bout length (TBL) and total evaporative water loss (TEWL) in Kuhl's pipistrelle measured while (A) torpid in either dry or humid air conditions (TBL=6.35–0.02 $\times$ TEWL,  $R^2=0.09$ ,  $P=0.05$ ) and (B) torpid in dry air ( $T_a=5^\circ\text{C}$ : 0.41 $\pm$ 0.30 g H<sub>2</sub>O m<sup>-3</sup>, RH=6%;  $T_a=10^\circ\text{C}$ : 0.58 $\pm$ 0.05 g H<sub>2</sub>O m<sup>-3</sup>, RH=6%; TBL=1.99–0.006 $\times$ TEWL,  $R^2=0.20$ ,  $P=0.02$ ).

TEWL influences TBL in bats. However, there was no significant difference in TBL of the bats between the two different conditions of air humidity. This implies that the relationship between TBL and TEWL results from variation in rates of TEWL among individuals, rather than variation within individuals measured under conditions of different air humidity. In addition, because some bats were measured under both the conditions of air humidity, data points of the regression between TBL and TEWL were not independent. Hence, we partitioned our data into two separate regimes of air humidity. We found further support for our prediction in bats measured in dry air, where TBL was inversely related to the rate of TEWL (Fig. 1B) and was independent of MR. Furthermore, we found that the number of arousals per day was positively correlated with rates of TEWL in bats measured in dry air (Fig. 2). However, we did not find such relationships in bats measured in humid air.

In the past, many of the hypotheses proposed to explain periodic arousals of bats from torpor related the phenomenon to processes correlated with MR (Galster and Morrison, 1970; Baumber et al., 1971). As we found no correlation between MR and TBL, regardless of ambient humidity, we could test the relationship between TBL and TEWL while keeping other confounding variables such as  $T_a$  and MR constant. Under these postulates, our results directly support the water balance hypothesis first suggested by Fisher and Manery (Fisher and Manery, 1967). In contrast, until now, evidence supporting the water balance hypothesis has been indirect or theoretical. For example, Thomas and Cloutier (Thomas and

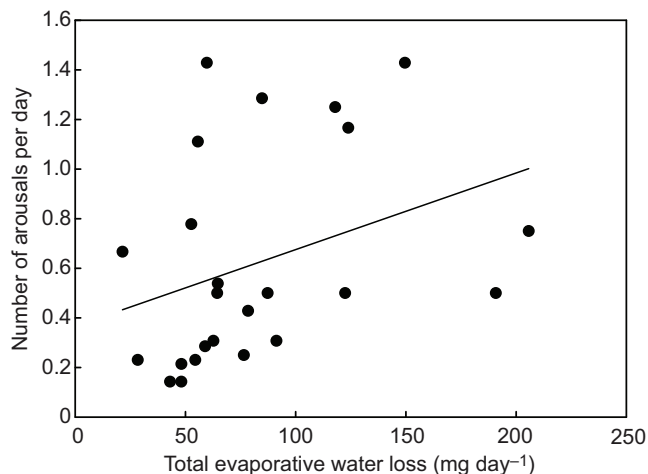


Fig. 2. Relationship between number of arousals per day of hibernation and total evaporative water loss (TEWL) in Kuhl's pipistrelle measured while torpid in dry air ( $T_a=5^\circ\text{C}$ :  $0.41\pm 0.30\text{ g H}_2\text{O m}^{-3}$ , RH=6%;  $T_a=10^\circ\text{C}$ :  $0.58\pm 0.05\text{ g H}_2\text{O m}^{-3}$ , RH=6%). Number of arousals= $-1.23+0.006\times\text{TEWL}$  ( $R^2=0.17$ ,  $P=0.05$ ).

Cloutier, 1992) found that metabolic water production could not cover evaporative water losses of hibernating little brown bats, *Myotis lucifugus*. Others have reported that bats drink opportunistically without moving far from their hibernacula, and some species were observed to drink standing water available in their caves (Twente, 1955; Speakman and Racey, 1989; Boyles et al., 2006). In addition, the theoretical model of Thomas and Geiser (Thomas and Geiser, 1997) predicted the duration of torpor bouts to be based on the rates of TEWL, and they suggested that arousal frequency and rates of TEWL were inversely related (Park et al., 2000), a hypothesis supported by our data. Only recently was it shown that treating hibernating ground squirrels *Spermophilus citellus* with a diuretic agent resulted in a higher frequency of arousals (Németh et al., 2010), thus providing empirical evidence for the possible causal link between water loss and arousal.

We also found that loss of  $m_b$  in hibernating Kuhl's pipistrelles measured in dry air was inversely related to TBL (Fig. 3B), implying that bats that arouse more frequently also lose  $m_b$  at an increased rate. Davis and Hitchcock (Davis and Hitchcock, 1965) suggested that bats that begin hibernation with large fat deposits are more likely to survive the winter than those with smaller reserves. However, if the activity of bats during hibernation varies among individuals because of differences in arousal frequency, then the survival of individuals carrying small fat stores at the onset of hibernation will not necessarily be compromised at its end. In support of this idea, Speakman and Racey (Speakman and Racey, 1989) found no correlation between  $m_b$  at the start of hibernation and  $m_b$  at its end in the greater horseshoe bat, *Rhinolophus ferrumequinum*, or the common pipistrelle, *P. pipistrellus*. According to the water balance hypothesis, bats that have lower rates of TEWL during hibernation arouse less often, thereby substantially reducing energy expenditure and fat depletion during winter. Our data support this hypothesis and suggest that TEWL is an important determinant of TBL and, as a result, also determines the rate of  $m_b$  loss during winter.

In conclusion, TBL and TEWL were significantly related in hibernating Kuhl's pipistrelles, independent of MR and/or  $T_a$ , supporting the water balance hypothesis, while distinguishing it from other hypotheses that relate processes correlated with MR,  $T_a$  or both. We also found that arousal frequency during hibernation was

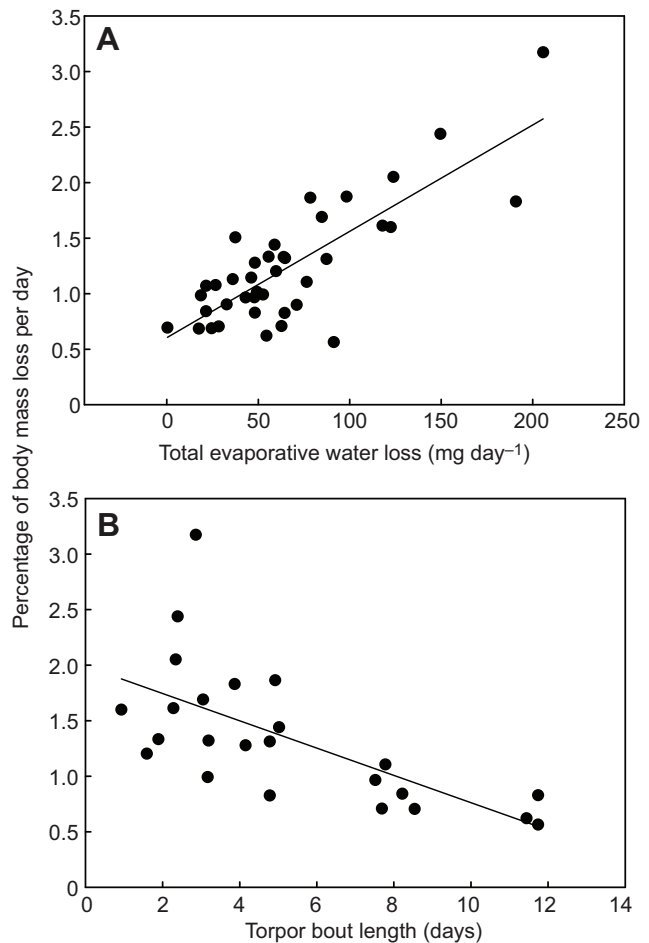


Fig. 3. Relationship between body mass ( $m_b$ ) loss per day (expressed as % of initial mass) and (A) total evaporative water loss (TEWL) in Kuhl's pipistrelle measured while torpid in either dry or humid air conditions ( $m_b$  loss= $-0.29+0.006\times\text{TEWL}$ ,  $R^2=0.53$ ,  $P<0.001$ ) and (B) torpor bout length (TBL) in Kuhl's pipistrelle measured while torpid in dry air ( $T_a=5^\circ\text{C}$ :  $0.41\pm 0.30\text{ g H}_2\text{O m}^{-3}$ , RH=6%;  $T_a=10^\circ\text{C}$ :  $0.58\pm 0.05\text{ g H}_2\text{O m}^{-3}$ , RH=6%;  $m_b$  loss= $0.74-0.10\times\text{TBL}$ ,  $R^2=0.59$ ,  $P<0.001$ ).

positively related with the amount of  $m_b$  lost during this period, suggesting that bats with higher rates of TEWL that arose more frequently lost more  $m_b$  and thus ended hibernation with smaller amounts of fat reserves. These results demonstrate the importance of TEWL in over-winter survival of bats. Most, if not all, temperate zone bats from both suborders, the Yangochiroptera and Yinpterochiroptera, are hibernators (Altringham, 2011). Therefore, because of the similarities in the ecological and behavioral characteristics of these bats, the possibility exists that what we found in *P. kuhlii*, a vespertilionid bat, may occur in other hibernating bat species of different families.

#### ACKNOWLEDGEMENTS

We thank Paloma Larrain for her laboratory and field assistance and all the laboratory members who were quiet and patient while the bats were in torpor close by. This is paper number 782 of the Mitrani Department of Desert Ecology.

#### FUNDING

The research was supported by the US–Israel Binational National Science Foundation [grant no. 2008469, 2008 to C.K. and J.B.W.]. A.M.G. was a Blaustein Postdoctoral Fellow during the study and M.B.H. was the recipient of a Negev Fellowship.

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