1 **Open-flow respirometry under field conditions: how does the airflow through the nest**

2 **influence our results?**

3 Julia Nowack[1,2], Veronika Dill[1,3], Kathrin H. Dausmann[1]

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5 [1]Institute for Zoology, Animal Ecology and Conservation, University Hamburg, Martin-
6 Luther-King-Platz 3, 20146 Hamburg, Germany
7

8 [2]School of Biological and Environmental Sciences, Liverpool John Moores University, Byrom
9 Street, L3 3AF Liverpool, UK (permanent address)

10

11 [3] Federal Research Institute for Animal Health, Südufer 10, 17493 Greifswald - Insel Riems,
12 Germany (present address)

13

14 Corresponding author: Julia Nowack, J.Nowack@LJMU.ac.uk; https://orcid.org/0000-0002-
15 4512-5160; postal address: School of Biological and Environmental Sciences, Liverpool John
16 Moores University, James Parsons Building, Byrom Street, L3 3AF Liverpool, UK
17

18 **Abstract**

19 Open-flow respirometry is a common method to measure oxygen-uptake as a proxy of energy

20 expenditure of organisms in real-time. Although most often used in the laboratory it has seen

21 increasing application under field conditions. Air is drawn or pushed through a metabolic

22 chamber or the nest with the animal, and the O_2 depletion and/or CO_2 accumulation in the air

23 is analysed to calculate metabolic rate and energy expenditure. Under field conditions, animals

24 are often measured within the microclimate of their nest and in contrast to laboratory work, the

25 temperature of the air entering the nest cannot be controlled. Thus, the aim of our study was to

26 determine the explanatory power of respirometry in a set-up mimicking field conditions. We

27 measured O_2 consumption of 14 laboratory mice (*Mus musculus*) using three different flow

28 rates [50 $L*h^{-1}$ (834 $mL*h^{-1}$), 60 $L*h^{-1}$ (1000 $mL*h^{-1}$) and 70 $L*h^{-1}$ (1167 $mL*h^{-1}$)] and two

29 different temperatures of the inflowing air; either the same as the temperature inside the

30 metabolic chamber (no temperature differential; 20 °C), or cooler (temperature differential of

31 10 °C). Our results show that the energy expenditure of the mice did not change significantly

32 in relation to a cooler airflow, nor was it affected by different flow rates, despite a slight, but

33 significant decrease of about 1.5 °C in chamber temperature with the cooler airflow. Our study

34 emphasises the validity of the results obtained by open-flow respirometry when investigating

35 energy budgets and physiological responses of animals to ambient conditions. Nevertheless,

36 subtle changes in chamber temperature in response to changes in the temperature and flow rate

37 of the air pulled or pushed through the system were detectable. Thus, constant airflow during

38 open-flow respirometry and consequent changes in nest/chamber temperature should be

39 measured.

40

41 **Key words:** microclimate; energy expenditure; metabolic rate; *Mus musculus*

42

1. Introduction

Energy is one of the most essential currencies of life and features in virtually all life processes (Tomlinson et al. 2013). Aerobic metabolism, the motor of the energetic machinery, has thus aptly and famously been called "the fire of life" (Kleiber 1961). Measuring energy expenditure provides an understanding of how animals budget their energy flows and can provide insights into the proximate and ultimate reasons of animal behavior (Kleiber 1961). One of the most common methods to indirectly determine energy expenditure in aerobic organisms is open-flow respirometry (also termed open-circuit, flow-through respirometry or indirect calorimetry), which allows quantifying oxygen consumption and/or carbon dioxide production of organisms as a proxy of metabolic rate (MR) in real time to yield information on dynamic patterns of MR. It is an indispensable tool in many areas of science (Lighton 2008). In this method the animal is placed in a metabolic chamber, which is connected to a gas analyser with airtight tubes and air is either pushed or pulled through the metabolic chamber.

Open-flow respirometry is an accurate and non-invasive to minimal-invasive method and has been used in many studies on animal energetics (for a compilation of a small fraction of these see the bibliography of Lighton 2008; but for particalar examples see: marsupials: Nowack et al. 2016; birds: McNab and Weston, 2018; mammals: Geiser et al. 2019) and it can also be used for aquatic animals in (e.g. fish: Clark et al. 2013, Payne et al. 2015; aquatic turtles: Enstipp et al. 2011). With the advent of smaller electronic components, open-flow respirometry has also increasingly been taken to the field, to investigate energy budgets on free-ranging animals, often using natural sleeping sites (burrows, tree hollows, nest boxes) as metabolic chambers (Bartholomew and Lighton 1986; Arnold et al. 1991; Lighton 1996; Lighton and Duncan 2002; Dausmann et al. 2009; Pretzlaff et al. 2010; Rödel et al. 2012; Berg et al. 2017, Langer et al. 2018; Reher et al. 2018). Free-ranging animals are usually exposed to a range of ambient temperatures; however, insulation of nests allows animals to establish a comparatively

68 stable microclimate that can deviate quite substantially from ambient conditions (e.g.

69 Lovegrove et al. 1991, Schmid 1998). In the case of an endothermic animal, this would serve

70 to reduce energy expenditure if this microclimate is closer to the thermal neutral zone (TNZ)

71 of the species (reviewed in Gilbert et al. 2010).

72   Flow rates through the metabolic chamber are usually maintained to constantly

73 replenish the O_2 depleted by the animal (usually maintaining less than a 1% O_2 difference

74 between incurrent and excurrent air). They thus vary according to the energy expenditure of

75 the specific animal species (and individual) being investigated, but also accordingly to the size

76 of the metabolic chamber, the equipment being used and the desired temporal resolution of the

77 measurement (McNab, 2006; Lighton and Halsey, 2011). So far little attention has been paid

78 to the effect of the constant airflow of potentially colder or warmer ambient air through such

79 nests during respirometry on the microclimate within and ultimately the energy expenditure

80 itself. This raises the question of whether results obtained with this method in the field might

81 be skewed. This could be critical for endothermic species, which largely use endogenously

82 generated heat to maintain the body at a metabolically favourable temperature and adapt MR

83 accordingly, depending on the extent of the differential between ambient temperature and

84 preferred body temperature.

85   While this problem can be solved in the laboratory by having a larger coil of tubing of

86 the incurrent air inside a temperature control cabinet to ensure that it is at cabinet temperature

87 by the time it enters the chamber (e.g. see Cheviron et al. 2013), there are limited to no options

88 of controlling the air temperature during measurements with open-flow respiratory in the field.

89 The aim of our study was therefore to validate whether the results obtained by open-flow

90 respirometry as a measure of energy expenditure are affected by a temperature differential

91 between ambient (and thus incurrent) air and the immediate environment of an animal (e. g.,

92 in a nest). We thus evaluated the effects of flowrate (i.e., faster convective heat exchange and

93 a potential disturbance of the fur insulation of animals) and the temperature of air flow on the

94 microclimate of the metabolic chamber or nest, and the energy expenditure of the animal

95 measured in a laboratory set-up mimicking field conditions.

96

97 **2. Material and Methods**

98 *2.1 Model species and housing conditions*

99 The experiments were conducted with 14 young adult (~ 6 weeks old) mice (*Mus musculus;* 8

100 females, 6 males). Mice were chosen as a model for small mammalian species as they are easy

101 to obtain and maintain. Throughout the study, the animals were housed individually at an

102 ambient temperature of 22 °C under a L:D cycle of 12 h:12 h, provided with water and fed *ad*

103 *libitum* using standard animal lab chow. The cages (260 x 260 x 140 mm) were equipped with

104 wood shavings, nesting material and terracotta plant pots with a small entrance hole (diameter:

105 90 mm) placed upside down to serve as a nest.

106

107 *2.2 Pre-experiment: Assessment of microclimate differentials*

108 To estimate naturally occurring nest temperatures and temperature differentials, the nest

109 temperatures of a subset of eight of the fourteen mice were determined by mounting

110 temperature loggers (Hygrochron iButtons/DS1923, Dallas Semiconductor, USA;

111 programmed to log every 10 min, accuracy $\pm$ 0.0625 °C) inside the plant pots but above the

112 animals to avoid any body contact. All temperature loggers were calibrated against a mercury

113 thermometer in a water bath in steps of 3 °C ranging from 1 to 40 °C prior to measurements.

114 Animals were then placed inside their usual cages (which included the plant pots) with

115 commercially available hamster wool in a climate chamber (WK 21', Firma Weiss

116 Umwelttechnik GmbH, Germany) set at 10 °C (temperature climate chamber: T_a) and with the

117 regular photoperiod (12 h:12 h) for 22 h. This temperature was chosen to thermally challenge

118   the mice, without jeopardizing their survival. One mouse did not use the plant pot as a nest and

119   was excluded from the analyses, reducing the sample size to N=7. To estimate the temperature

120   differential between T_a and the temperature an individual was experiencing in the nest, we used

121   the 20 highest nest temperature measurements to ensure that only data with mice present in the

122   nest were used in the analysis.

123   We found that the average nest temperature at a T_a of 10 °C varied between 16.9 °C

124   and 20.6 °C for the seven mice and mean nest temperature was 18.6 ± 1.4 °C (N = 7). Thus,

125   mice established a differential of almost 10 °C between nest temperature and T_a. The

126   information about the naturally established temperature differential between ambient and nest

127   temperature was used as the basis of our main study.

128

129   *2.3 Experiment: Energy expenditure at differing flow rates and temperature differentials*

130   During the experiments, all fourteen individuals were transferred into individual airtight

131   polythene boxes of 1.5 L volume (170 mm x 170 mm x 83 mm), with an air-inlet and outlet, to

132   serve as metabolic chambers. The polythene containers were equipped with wood shavings,

133   but no nesting material or nest structure (i.e. also no terracotta pot) to prevent nest constructions

134   and allow unimpaired airflow through the box, as the nest temperature conditions were already

135   mimicked by the relevant temperature (see above) in the temperature cabinet. A slice of apple

136   (~ 15 g fresh mass) was also provided in the chamber.

137   All experiments were conducted separately, i.e. with one animal at a time, during the

138   resting period of the mice (1000 h - 1400 h) to keep effects of activity to a minimum.

139   Measurements were performed in a randomized order to counteract any potential circadian

140   effects. Animals were weighed to an accuracy of 0.5 g (Cubis Precision Balance, Satorius,

141   Göttingen, Germany) and placed in individual polythene boxes inside a climate cabinet (WTB,

142   Binder Labortechnik GmbH, Germany; Fig. 1) maintained at a constant temperature reflecting

143   the conditions in the nest when the temperature is 10 °C [mean temperature in the climate

144   cabinet (T_c): 20.7 ± 0.5 °C]. It has to be noted that this temperature was below the TNZ of

145   mice, which has a lower critical temperature of 26 to 28 °C for mice >25 g (Speakman and

146   Keijer 2013). The climate cabinet was positioned within a large climate chamber (Fig. 1),

147   which was either maintained at T_a of about 20 °C (19.4 ± 0.5 °C) or 10 °C (10.7 ± 0.3 °C),

148   enabling the temperature of the airflow (a) at the same temperature as in the metabolic chamber

149   (Fig. 1a) and (b) with a 10 °C differential (Fig. 1b). Flow rate was either 50 L*h^{-1} (830 ml min^{-1}

150   ), 60 L*h^{-1} (1000 ml min^{-1}) or 70 L*h^{-1} (1170 ml min^{-1}), to reflect flowrates routinely used for

151   small mammals to keep depletion of O_2 concentration in the metabolic chamber below 1%, and

152   monitored continuously. Oxygen consumption of each individual was thus measured under six

153   different conditions: with three different flow rates and two different air flow temperatures

154   (T_{flow}) in random order. Measurements lasted for 4 h at each of the two temperatures. The first

155   hour was not used for analyses and served to ensure that the mice were accustomed to the

156   experimental procedures. In the following three hours, flow rate was set to one of the three

157   predetermined rates for one hour each.

158   Energy expenditure was determined by measuring the rate of O_2 consumption as a

159   proxy of MR using a portable O_2 analyser (FoxBoxC, Sable Systems International, USA). The

160   metabolic chamber was connected to the O_2 analyser (inbuilt pump and flow meter; pull mode;

161   order: metabolic chamber, pump, needle valve, flow meter, oxygen analyser) with airtight tubes

162   (Tygon R-3606, Saint-Gobain, Paris, France). Water vapour was removed from the air prior to

163   entering the analyser and the flow meter using silica gel. The O_2 analyser was calibrated

164   immediately before the experiment (single-point calibration as recommended by the

165   manufacturer). To account for any drift of the O_2 sensor, we used a gas switch (RM8

166   Multiplexer, Sable Systems International, US) to switch between reference air (baseline: 5 min)

167   and measured sample air for 55 min (sampling frequency every 60 sec). Energy expenditure of

168 mice was calculated in Watt using the data acquisition program Expedata (Sable Systems

169 International, USA) by using the Weir 'RQ-free' method proposed by Kaiyala et al. (2019),

170 following the equation MR (Watt) = 0.3 * FR * ΔO_2 * 1.162; where FR is flowrate in mL*min^{-1}

171 and ΔO_2 is delta O_2 expressed as a fractional concentration. Multiplying by 1.162 converts

172 the output from Kcal*hr^{-1} to Watt. For each of the six experimental conditions, the mean energy

173 expenditure was calculated for each individual from the lowest consecutive 20 % of the

174 readings within this cycle to exclude periods of activity.

175   During measurements temperature was recorded every 5 min with calibrated iButtons

176 (see above) inside the climate chamber, the climate cabinet, the metabolic chamber (glued to

177 the top of the chamber), and the tubes leading from the climate chamber into the metabolic

178 chamber (T_{flow}). The average body mass of the individuals did not differ between the

179 temperature treatments (t-test: $t_{13} = 1.04$, $P = 0.32$). Mean average body mass of the mice was

180 34.8 ± 10.3 g (N = 14).

181

182 *2.4 Data analysis*

183 Statistical analyses were performed with R (version 3.1-117, R Development Core Team,

184 2014). All values are reported as means $\pm$ SD. Data were tested for normality using Shapiro

185 tests. Differences in temperatures between the metabolic chambers and climate cabinet in each

186 treatment were tested with paired t-tests for dependent samples. To analyse potential

187 differences in whole-organism energy expenditure caused by different flow rates or T_{flow} we

188 performed a linear mixed-effects model, in which energy expenditure in Watts was used as the

189 response variable and interaction between flow rate and the T_{flow} was tested (package 'nlme';

190 Pinheiro et al. 2014) followed by a type 3 ANOVA; we also included animal identity as random

191 factor and controlled for body mass by using body mass as a covariate. Mass-specific metabolic

192 rates were only calculated for presentation in the text. Normal distribution and homogeneity of

193   variance of model residuals were tested using Shapiro-Wilk tests and Levene's tests

194   (leveneTest in library 'car', Fox and Weisberg 2011), respectively.

195

196   The study was carried out under permit 37/13 from the Amt für Verbraucherschutz, Hamburg.

197

198   **3. Results**

199   Despite the same mean temperature in the climate chamber between treatments (T_a: 20.7 °C $\pm$

200   0.5 °C; t-test: t_{13} = 0.10, P = 0.92; N = 14), the mean temperature in the metabolic chamber

201   was slightly, but significantly colder during the measurements with T_{flow} = 10 °C than during

202   the measurements with T_{flow} = 19 °C (on average 1.5 °C; mean: 21.2 $\pm$ 0.4 °C vs. 22.7 $\pm$ 0.6

203   °C; t-test: t_{11} = 6.73, P < 0.0001; N = 12). We did not find a statistical difference in energy

204   expenditure between different flow rates (χ^2= 0.506, df= 1, P= 0.479), or for the interaction

205   term (χ^2=1.024 = 2.49, df= 1, P = 0.312). Interestingly, the 1.5°C difference in the metabolic

206   chambers was not significantly reflected in energy expenditure (mean for all flow rates: 10°C:

207   0.57 $\pm$ 0.16 Watt/0.016 $\pm$ 0.005 Watt g^{-1} vs 19°C: 0.58 $\pm$ 0.19 Watt/0.016 $\pm$ 0.006 Watt g^{-1};

208   Fig. 2; linear mixed model, energy expenditure corrected for body mass; χ^2= 1.114, df= 1,P =

209   0.291). Furthermore, mean energy expenditure of all individuals at all treatments was 0.57 $\pm$

210   0.17 Watt.

211

212   **4. Discussion**

213   Our results support the validity of data obtained by open-flow respirometry even when the

214   temperature in the immediate environment of the animal differs from the ambient temperature

215   (and thus of the incoming air), e.g., when animals retreat into burrows or built nests. Although

216   the microclimate in the metabolic chamber was slightly altered when the constant airflow was

217   at a lower temperature this did not discernibly influence energy expenditure of the animals.

Furthermore, different flow rates did not significantly change estimates of energy expenditure, underlining the robustness of the results of this method to potentially varying parameters. The three flow rates used in our study [50 L*h^{-1} (834 mL*h^{-1}), 60 L*h^{-1} (1000 mL*h^{-1}) and 70 L*h^{-1} (1167 mL*h^{-1})] are within the range routinely used for small mammals in studies measuring oxygen uptake (e.g. 50L*h^{-1} for rodents, see Wilz and Heldmaier 2000; 50-60 L*h^{-1} for primates, see Schmid and Speakman 2000). If we calculate the wind speed the animals would have been exposed to in their metabolic chambers, we get wind speeds between 0.06 and 0.08 m*s^{-1} that should not disturb the insulation properties of the fur. This presumably allows animals to change their conductance in response to the slight drop in chamber temperature caused by the cooler air stream thus requiring no additional endogenous heat production. The laboratory mice that we used as a model for small mammals in our study are assumingly more thermally sensitive than wild species, never having been exposed to fluctuating temperatures in their lives (Gibbs & Gefen, 2009). For comparison, a study looking into the effect of wind speed on metabolic heat production of the small desert rodent, *Spermophilus tereticaudus,* has found that thermal conductance does not change in ground squirrels when using wind speeds between 0.25 and 1 m*s^{-1} (Wooden & Walsberg 2000). This suggests that our data are indeed transferable to other small mammal species. Thus, as long as flow rates are precisely monitored and recorded for inclusion in later analyses, the specific airflow is less critical. Generally, lower flow rates are preferable, as long as the CO_2 content remains below critical values (≤ 1 % CO_2 accumulation) and diffusion is not a problem, because differentials in gas concentration become more pronounced, whereas one may face the problem of dealing with gas concentrations that are too low to give a clear signal when using high flow rates (Lighton 2008).

In the laboratory it may be possible to regulate the temperature of the air drawn through the metabolic chamber by adjusting the room temperature accordingly. However, the temperature in animal facilities is often routinely kept constant at about 20 °C, although it has

been shown that this temperature below thermoneutrality (Speakman and Keijer 2013) influences the phenotype and physiological responses of mice (Maloney et al. 2014), and few laboratories use a heat exchanger or similar equipment to regulate incurrent air during respirometry accordingly. In general, we would not expect a temperature differential between T_c and T_{flow} of more than 10 °C in the laboratory, even if no heat exchanger is used and air is pulled from outside of a building. In the field, on the other hand, when natural nesting sites of animals are used as metabolic chambers (e. g., Dausmann et al. 2009; Pretzlaff et al. 2010), this differential is influenced by the climatic conditions of the habitat and the structure of the nest. For both parameters, manipulations of the temperature of the incoming air might not be desired or possible in a study aiming for natural conditions, and thus microclimatic differentials can be substantial. Underground refuges are generally comparatively well buffered against cold [e.g., for arctic ground squirrels *Spermophilus parryii* or marmots *Marmota marmota* (Barnes 1989; Arnold et al. 1991)] or heat [e. g., for fennecs *Vulpes zerda* (Maloiy et al. 1982)]. However, nests above ground will be more influenced by ambient conditions. Lovegrove et al. (1991) found that the large stick nests of black-tailed tree rats (*Thallomys paedulcus*) living in eastern and southern Africa buffer minimum daily ambient temperature and the temperature in the nest was on average 2.7 °C higher than the minimum air temperature and 6.3 °C lower than the maximum air temperature. Tree holes used by grey mouse lemurs (*Microcebus murinus*) buffered outside ambient temperature on average by 0.6 to 2.5 °C (Schmid 1998). We could not find data describing the preferred nest temperature of *M. musculus* in the wild, however, a study on nesting behaviour on different strains of laboratory mice showed a preferred nest temperature of between 26 °C and 29 °C at a ambient temperature of 20 °C, therefore maintaining a differential of between 6 °C and 9 °C (Gaskill et al. 2012). This result is comparable to the differential between 7 °C and 11 °C that we observed in this study and might reflect the limitations of nest building capacities in *M. musculus*. We measured the mice at their

268   usual housing temperature of 20 °C, which, as stated above, is below their TNZ and our values

269   therefore do not represent basal MR. However, as we aimed to address potential pitfalls of

270   respirometry in field studies, we chose this more realistic temperature range. Similar to our

271   experimental scenario nest temperatures of free-ranging animals will –at least in winter- often

272   be below the TNZ and thus large changes in temperature should affect energy expenditure as

273   animals have to compensate for the increased T_a-T_b differential. If we had kept our mice within

274   the TNZ and would have used a 10°C lower airflow temperature, it would have been unlikely

275   to see the real effect that flow temperature has on thermoregulation and MR, as within the TNZ

276   small changes of temperature should even less require changes in energy expenditure due to

277   the characteristic plateau of MR within this thermal range. Nevertheless, low temperature of

278   the airflow and the resulting slight drop in nest temperature could potentially compromise the

279   data if through this temperature shift the threshold of the lower critical temperature of the TNZ

280   is crossed, initiating active heat production. Although, a change of 1-2°C is unlikely to have a

281   large effect on energy expenditure, the effect is likely to be larger in a field setting as our setup

282   included a climate cabinet that would have counteracted larger temperature variations that

283   might accumulate over an extended period of time.

284   Our study emphasises the appropriateness and importance of the use of open-flow

285   respirometry when investigating energy budgets and physiological responses of animal species

286   to ambient conditions in the laboratory, as well as in the field. Nevertheless, subtle changes in

287   nest temperature caused by this method are detectable and may influence behaviour and

288   physiology of the animals. Thus, the constant airflow during open-flow respirometry and the

289   possible change in nest temperature should be kept in mind (and measured).

290

294

Conflict of interest

297

Funding

300

301 **Literature**

302 Arnold W., G. Heldmaier, S. Ortmann, H. Pohl, T. Ruf, and S. Steinlechner. 1991. Ambient

303   temperatures in hibernacula and their energetic consequences for alpine marmots

304   (*Marmota marmota*). J Therm Biol 16:223-226.

305 Barnes B.M. 1989. Freeze avoidance in a mammal: Body temperatures below 0°C in an

306   Arctic hibernator. Science 244:1593-1595.

307 Bartholomew G.A. and J.R.B. Lighton. 1986. Oxygen consumption during hover-feeding in

308   free-ranging Anna hummingbirds. J Exp Biol 123:191-199.

309 Berg W., O. Theisinger and K.H. Dausmann. 2017. Acclimatization patterns in tropical

310   reptiles: uncoupling temperature and energetics. Sci Nat 104:91

311 Cheviron Z.A., G.C. Bachman and J.F. Storz 2013. Contributions of phenotypic plasticity to

312   differences in thermogenic performance between highland and lowland deer mice. J

313   Exp Biol 216:1160-1166

314 Clark T. D., E. Sandblom and F Jutfelt 2013. Aerobic scope measurements of fishes in an era

315   of climate change: respirometry, relevance and recommendations. J Exp Biol 216:

316   2771-2782

317 Dausmann K.H., J. Glos, and G. Heldmaier. 2009. Energetics of tropical hibernation. J Comp

318   Physiol B 179:345-357.

319 Enstipp M.R., S. Ciccione, B. Gineste, M. Milbergue, K. Ballorain, Y. Ropert-Coudert, A.

320   Kato, V. Plot and J-Y Georges 2011. Energy expenditure of freely swimming adult

321   green turtles (*Chelonia mydas*) and its link with body acceleration. J Exp Biol 214:

322   4010-4020

323 Fox, J. and S. Weisberg, 2011. An companion to applied regression. 2 ed. Thousand Oaks,

324   CA: Sage.

325 Gaskill B.N., C.J. Gordon, E.A. Pajor, J.R. Lucas, J.K. Davis, and J.P. Garner. 2012. Heat or

326   Insulation: behavioral titration of mouse preference for warmth or access to a nest.

327   PlosOne 7:e32799.

328 Geiser F., J. Wen, G. Sukhchuluun, Q.S. Chi and D.H. Wang 2019. Precocious torpor in an

329   altricial mammal and the functional implications of heterothermy during

330   development. Front Physiol 10: 469, doi.org/10.3389/fphys.2019.00469.

331 Gibbs A.G. and E. Gefen 2009. Physiological adaptation in laboratory environments. In: T.

332   Garland, M.R. Rose (Eds.), Experimental Evolution, University of California Press,

333   Berkeley.

334 Gilbert C., D. McCafferty, Y. Le Maho, J.M. Martrette, S. Giroud, S. Blanc, and A. Ancel.

335   2010. One for all and all for one: the energetic benefits of huddling in endotherms.

336   Biol Rev 85:545-569.

337 Kaiyala K.J., B.E. Wisse and Y.R.B. Lighton. 2019. Validation of an equation for energy

338   expenditure that does not require the respiratory quotient. PLOS ONE 14(2):

339   e0211585. https://doi.org/10.1371/journal.pone.0211585.

340 Kleiber M. 1961. The Fire of Life. Wiley, New York.

341 Langer F., N. Havenstein, J. Fietz 2018. Flexibility is the key: metabolic and

342   thermoregulatory behaviour in a small endotherm. J Comp Physiol B 188:553-563.

343 Lighton J.R.B. 1996. Discontinuous gas exchange in insects. Ann Rev Entomol 41:309-324.

344 ——— 2008. Measuring Metabolic Rates: a manual for scientists Oxford University Press,

345 Oxford New York.

346 Lighton J.R.B. and F.D. Duncan. 2002. Energy cost of locomotion: Validation of laboratory

347   data by in situ respirometry. Ecology 83:3517-3522.

348 Lighton J.R.B. and Halsey, L.G. 2011. Flow-through respirometry applied to chamber

349   systems: Pros and cons, hints and tips. Comp Biochem Phys A 158: 265-275.

350   Lovegrove B.G., G. Heldmaier, and M. Knight. 1991. Seasonal and circadian energetic

351   patterns in an arboreal rodent, *Thallomys paedulcus*, and a burrow-dwelling rodent,

352   *Aethomys namaquensis*, from the Kalahari Desert. J Therm Biol 16:199-209.

353   Maloiy G.M.O., J.M.Z. Kamau, A. Shkolnik, M. Meir, and R. Arieli. 1982. Thermoregulation

354   and metabolism in a small desert carnivore: the fennec fox (*Fennecus zerda*)

355   (Mammalia). J Zool 198:279-291.

356   Maloney S.K., A. Fuller, D. Mitchell, C. Gordon, and J.M. Overton. 2014. Translating animal

357   model research: does it matter that our rodents are cold? Physiology 29:413-420

358   McNab B.K. and K.A. Weston. 2018. The energetics of torpor in a temperate passerine

359   endemic to New Zealand, the Rifleman (*Acanthisitta chloris*). Journal Comp Physiol

360   B 188:855-862

361   Nowack J., Delesalle M., Stawski C. and F. Geiser. 2016. Can hibernators sense and evade

362   fires? Olfactory acuity and locomotor performance during deep torpor. Sci Nat 103:

363   73

364   Payne N.L., E.P. Snelling, R. Fitzpatrick, J. Seymour, R. Courtney, A. Barnett, Y.Y.

365   Watanabe, D.W. Sims, L. Squire, and J.M. Semmens. 2015. A new method for

366   resolving uncertainty of energy requirements in large water breathers: the 'mega-

367   flume' seagoing swim-tunnel respirometer. Met Ecol Evol 6: 668-677.

368   Pinheiro J., D. Bates, S. DebRoy, D. Sarkar, and R Core Team. 2014. nlme: Linear and

369   Nonlinear Mixed Effects Models. R package version 3.1-117, URL: http://CRAN.R-

370   project.org/package=nlme.

371   Pretzlaff I., G. Kerth, and K.H. Dausmann. 2010. Communally breeding bats use

372   physiological and behavioural adjustments to optimise daily energy expenditure.

373   Naturwissenschaften 97:353-363.

374   R Development Core Team. 2014. R: a language and environment for statistical computing.

375   Vienna, Austria: R Foundation for Statistical Computing. http://www.R-project.org.

376   Reher, S., Ehlers, J., Rabarison, H., and Dausmann, K.H. (2018). Short and hyperthermic

377   torpor responses in the Malagasy bat *Macronycteris commersoni* reveal a broader

378   hypometabolic scope in heterotherms. Journal of Comparative Physiology B 188,

379   1015-1027.

380   Rödel H.G., K.H. Dausmann, A. Starkloff, M. Schubert, D. von Holst, and R. Hudson. 2012.

381   Diurnal nursing pattern of wild-type European rabbits under natural breeding

382   conditions. Mammal Biol 77:441-446.

383   Schmid J. 1998. Tree holes used for resting by gray mouse lemurs (*Microcebus murinus*) in

384   Madagascar: Insulation capacities and energetic consequences. Int J Primatol 19:797-

385   809.

386   Schmid J. and J.R. Speakman. 2000. Daily energy expenditure of the grey mouse lemur

387   (*Microcebus murinus*): a small primate that uses torpor. JCP B 170:633-641.

388   Speakman J.R. and J. Keijer. 2013. Not so hot: optimal housing temperatures for mice to

389   mimic the thermal environment of humans. Mol Metabol 2:5-9.

390   Tomlinson S., S.K. Maloney, P.C. Withers, C.C. Voigt, and A.P. Cruz-Neto. 2013. From

391   doubly labelled water to half-life; validating radio-isotopic rubidium turnover to

392   measure metabolism in small vertebrates. Met Ecol Evol 4:619-628.

393   Wilz M. and G. Heldmaier 2000. Comparison of hibernation, estivation and daily torpor in

394   the edible dormouse, *Glis glis*. J Comp Physiol B 170:511-521.

395   Wooden K.M. and G.E. Walsberg 2000. Effect of wind and solar radiation on metabolic heat

396   production in a small desert rodent, *Spermophilus tereticaudus*. J Exp Biol 203:879-

397   888.

Figure Legends

Figure 1 Experimental setup with airflow at either a) the same temperature as the metabolic chamber or b) colder than the metabolic chamber (~10 °C differential); Temperature of the climate chamber had been set at 20 °C, but was measured as about 19 °C. T_a: temperature in the climate chamber; T_c: temperature in the climate cabinet; T_{flow}: temperature of the airflow.

Figure 2 Energy expenditure of mice at three different flow rates with airflow at either the same temperature as the metabolic chamber [temperature of airflow (19 °C) ≈ temperature of metabolic chamber (20 °C); *white boxplots*] or colder than in the metabolic chamber [temperature of airflow (10 °C) < temperature of metabolic chamber (20 °C); *grey boxplots*]. N = 14 for each treatment. There were no statistical differences between any of the treatments.
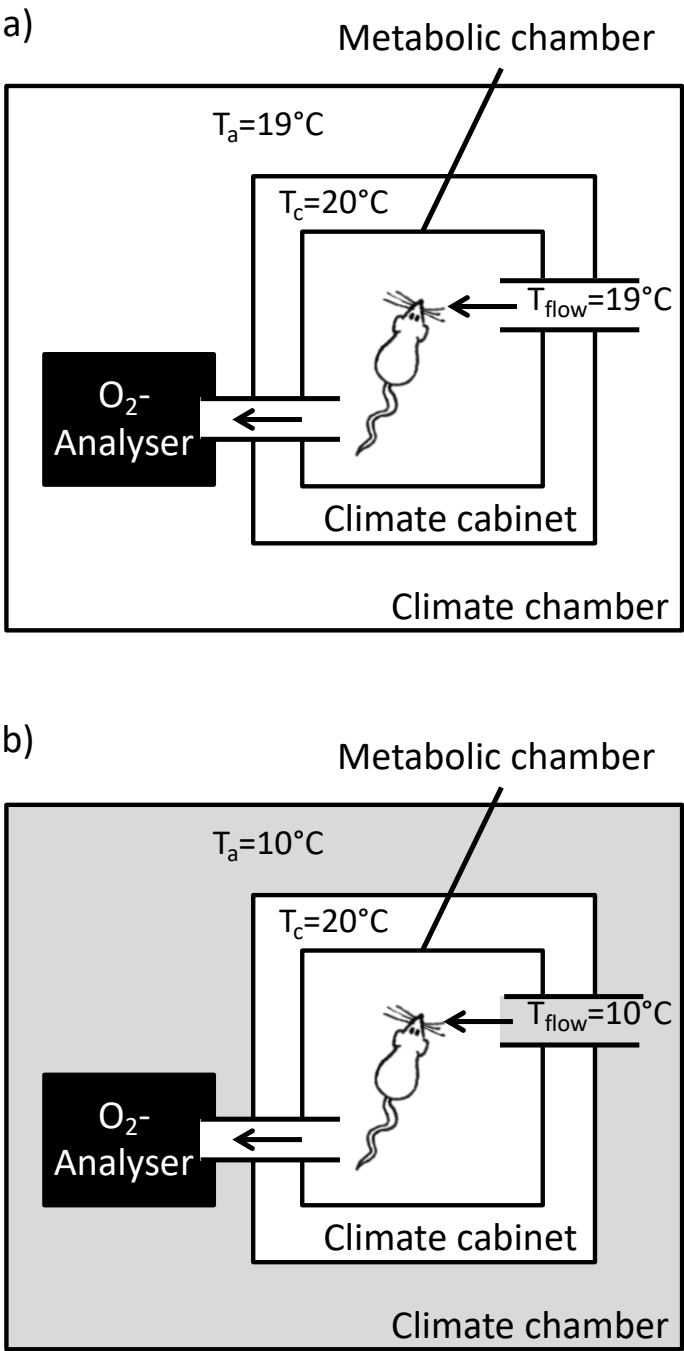
Figure 1

a)

Metabolic chamber

$T_a=19°C$

$T_c=20°C$

$T_{flow}=19°C$

O$_2$-Analyser

Climate cabinet

Climate chamber

b)

Metabolic chamber

$T_a=10°C$

$T_c=20°C$

$T_{flow}=10°C$

O$_2$-Analyser

Climate cabinet

Climate chamber

Figure 2