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Open-flow respirometry under field conditions: how does the airflow through the nest influence our results?

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Abstract

Open-flow respirometry is a common method to measure oxygen-uptake as a proxy of energy expenditure of organisms in real-time. Although most often used in the laboratory it has seen increasing application under field conditions. Air is drawn or pushed through a metabolic chamber or the nest with the animal, and the O_2 depletion and/or CO_2 accumulation in the air is analysed to calculate metabolic rate and energy expenditure. Under field conditions, animals are often measured within the microclimate of their nest and in contrast to laboratory work, the temperature of the air entering the nest cannot be controlled. Thus, the aim of our study was to determine the explanatory power of respirometry in a set-up mimicking field conditions. We measured O_2 consumption of 14 laboratory mice (*Mus musculus*) using three different flow 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 different temperatures of the inflowing air; either the same as the temperature inside the metabolic chamber (no temperature differential; 20 °C), or cooler (temperature differential of 10 °C). Our results show that the energy expenditure of the mice did not change significantly in relation to a cooler airflow, nor was it affected by different flow rates, despite a slight, but significant decrease of about 1.5 °C in chamber temperature with the cooler airflow. Our study emphasises the validity of the results obtained by open-flow respirometry when investigating energy budgets and physiological responses of animals to ambient conditions. Nevertheless, subtle changes in chamber temperature in response to changes in the temperature and flow rate of the air pulled or pushed through the system were detectable. Thus, constant airflow during open-flow respirometry and consequent changes in nest/chamber temperature should be measured.

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

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

a potential disturbance of the fur insulation of animals) and the temperature of air flow on the microclimate of the metabolic chamber or nest, and the energy expenditure of the animal measured in a laboratory set-up mimicking field conditions.

2. Material and Methods

2.1 Model species and housing conditions

The experiments were conducted with 14 young adult (~ 6 weeks old) mice (*Mus musculus;* 8 females, 6 males). Mice were chosen as a model for small mammalian species as they are easy to obtain and maintain. Throughout the study, the animals were housed individually at an ambient temperature of 22 °C under a L:D cycle of 12 h:12 h, provided with water and fed *ad libitum* using standard animal lab chow. The cages (260 x 260 x 140 mm) were equipped with wood shavings, nesting material and terracotta plant pots with a small entrance hole (diameter: 90 mm) placed upside down to serve as a nest.

2.2 Pre-experiment: Assessment of microclimate differentials

To estimate naturally occurring nest temperatures and temperature differentials, the nest temperatures of a subset of eight of the fourteen mice were determined by mounting temperature loggers (Hygrochron iButtons/DS1923, Dallas Semiconductor, USA; programmed to log every 10 min, accuracy ± 0.0625 °C) inside the plant pots but above the animals to avoid any body contact. All temperature loggers were calibrated against a mercury thermometer in a water bath in steps of 3 °C ranging from 1 to 40 °C prior to measurements. Animals were then placed inside their usual cages (which included the plant pots) with commercially available hamster wool in a climate chamber (WK 21‘, Firma Weiss Umwelttechnik GmbH, Germany) set at 10 °C (temperature climate chamber: T_a) and with the regular photoperiod (12 h:12 h) for 22 h. This temperature was chosen to thermally challenge

the mice, without jeopardizing their survival. One mouse did not use the plant pot as a nest and was excluded from the analyses, reducing the sample size to N=7. To estimate the temperature differential between T_a and the temperature an individual was experiencing in the nest, we used the 20 highest nest temperature measurements to ensure that only data with mice present in the nest were used in the analysis.

We found that the average nest temperature at a T_a of 10 °C varied between 16.9 °C and 20.6 °C for the seven mice and mean nest temperature was 18.6 ± 1.4 °C (N = 7). Thus, mice established a differential of almost 10 °C between nest temperature and T_a. The information about the naturally established temperature differential between ambient and nest temperature was used as the basis of our main study.

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

During the experiments, all fourteen individuals were transferred into individual airtight polythene boxes of 1.5 L volume (170 mm x 170 mm x 83 mm), with an air-inlet and outlet, to serve as metabolic chambers. The polythene containers were equipped with wood shavings, but no nesting material or nest structure (i.e. also no terracotta pot) to prevent nest constructions and allow unimpaired airflow through the box, as the nest temperature conditions were already mimicked by the relevant temperature (see above) in the temperature cabinet. A slice of apple (~ 15 g fresh mass) was also provided in the chamber.

All experiments were conducted separately, i.e. with one animal at a time, during the resting period of the mice (1000 h - 1400 h) to keep effects of activity to a minimum. Measurements were performed in a randomized order to counteract any potential circadian effects. Animals were weighed to an accuracy of 0.5 g (Cubis Precision Balance, Satorius, Göttingen, Germany) and placed in individual polythene boxes inside a climate cabinet (WTB, 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

mice was calculated in Watt using the data acquisition program Expedata (Sable Systems International, USA) by using the Weir 'RQ-free' method proposed by Kaiyala et al. (2019), following the equation MR (Watt) = 0.3 * FR * ΔO_2 * 1.162; where FR is flowrate in $mL*min^{-1}$ and ΔO_2 is delta O_2 expressed as a fractional concentration. Multiplying by 1.162 converts the output from $Kcal*hr^{-1}$ to Watt. For each of the six experimental conditions, the mean energy expenditure was calculated for each individual from the lowest consecutive 20 % of the readings within this cycle to exclude periods of activity.

During measurements temperature was recorded every 5 min with calibrated iButtons (see above) inside the climate chamber, the climate cabinet, the metabolic chamber (glued to the top of the chamber), and the tubes leading from the climate chamber into the metabolic chamber (T_{flow}). The average body mass of the individuals did not differ between the temperature treatments (t-test: $t_{13} = 1.04$, $P = 0.32$). Mean average body mass of the mice was 34.8 ± 10.3 g (N = 14).

2.4 Data analysis

Statistical analyses were performed with R (version 3.1-117, R Development Core Team, 2014). All values are reported as means ± SD. Data were tested for normality using Shapiro tests. Differences in temperatures between the metabolic chambers and climate cabinet in each treatment were tested with paired t-tests for dependent samples. To analyse potential differences in whole-organism energy expenditure caused by different flow rates or T_{flow} we performed a linear mixed-effects model, in which energy expenditure in Watts was used as the response variable and interaction between flow rate and the T_{flow} was tested (package 'nlme'; Pinheiro et al. 2014) followed by a type 3 ANOVA; we also included animal identity as random factor and controlled for body mass by using body mass as a covariate. Mass-specific metabolic rates were only calculated for presentation in the text. Normal distribution and homogeneity of

variance of model residuals were tested using Shapiro-Wilk tests and Levene's tests (leveneTest in library 'car', Fox and Weisberg 2011), respectively.

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

3. Results

Despite the same mean temperature in the climate chamber between treatments (T_a: 20.7 °C ± 0.5 °C; t-test: $t_{13} = 0.10$, P = 0.92; N = 14), the mean temperature in the metabolic chamber was slightly, but significantly colder during the measurements with T_{flow} = 10 °C than during the measurements with T_{flow} = 19 °C (on average 1.5 °C; mean: 21.2 ± 0.4 °C vs. 22.7 ± 0.6 °C; t-test: $t_{11} = 6.73$, P < 0.0001; N = 12). We did not find a statistical difference in energy expenditure between different flow rates (χ^2= 0.506, df= 1, P= 0.479), or for the interaction term (χ^2=1.024 = 2.49, df= 1, P = 0.312). Interestingly, the 1.5°C difference in the metabolic chambers was not significantly reflected in energy expenditure (mean for all flow rates: 10°C: 0.57 ± 0.16 Watt/0.016 ± 0.005 Watt g^{-1} vs 19°C: 0.58 ± 0.19 Watt/0.016 ± 0.006 Watt g^{-1}; Fig. 2; linear mixed model, energy expenditure corrected for body mass; χ^2= 1.114, df= 1,P = 0.291). Furthermore, mean energy expenditure of all individuals at all treatments was 0.57 ± 0.17 Watt.

4. Discussion

Our results support the validity of data obtained by open-flow respirometry even when the temperature in the immediate environment of the animal differs from the ambient temperature (and thus of the incoming air), e.g., when animals retreat into burrows or built nests. Although the microclimate in the metabolic chamber was slightly altered when the constant airflow was 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. 50$L*h^{-1}$ 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

usual housing temperature of 20 °C, which, as stated above, is below their TNZ and our values therefore do not represent basal MR. However, as we aimed to address potential pitfalls of respirometry in field studies, we chose this more realistic temperature range. Similar to our experimental scenario nest temperatures of free-ranging animals will –at least in winter- often be below the TNZ and thus large changes in temperature should affect energy expenditure as animals have to compensate for the increased T_a-T_b differential. If we had kept our mice within the TNZ and would have used a 10°C lower airflow temperature, it would have been unlikely to see the real effect that flow temperature has on thermoregulation and MR, as within the TNZ small changes of temperature should even less require changes in energy expenditure due to the characteristic plateau of MR within this thermal range. Nevertheless, low temperature of the airflow and the resulting slight drop in nest temperature could potentially compromise the data if through this temperature shift the threshold of the lower critical temperature of the TNZ is crossed, initiating active heat production. Although, a change of 1-2°C is unlikely to have a large effect on energy expenditure, the effect is likely to be larger in a field setting as our setup included a climate cabinet that would have counteracted larger temperature variations that might accumulate over an extended period of time.

Our study emphasises the appropriateness and importance of the use of open-flow respirometry when investigating energy budgets and physiological responses of animal species to ambient conditions in the laboratory, as well as in the field. Nevertheless, subtle changes in nest temperature caused by this method are detectable and may influence behaviour and physiology of the animals. Thus, the constant airflow during open-flow respirometry and the possible change in nest temperature should be kept in mind (and measured).

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Conflict of interest

The authors declare no conflict of interest.

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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

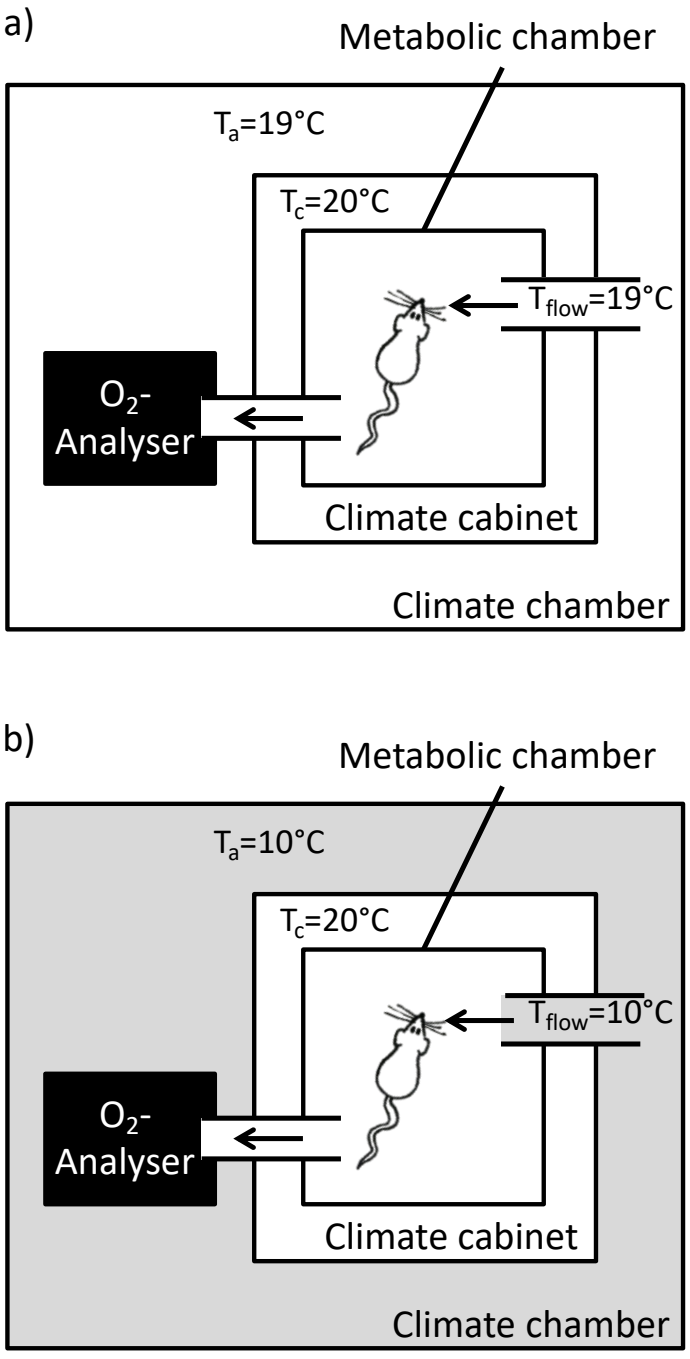
a)
Metabolic chamber
T_a=19°C
T_c=20°C
T_{flow}=19°C
O_2-Analyser
Climate cabinet
Climate chamber
b)
Metabolic chamber
T_a=10°C
T_c=20°C
T_{flow}=10°C
O_2-Analyser
Climate cabinet
Climate chamber

Figure 2