Exogenous hydrogen sulfide gas does not induce hypothermia in normoxic mice

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Hydrogen sulfide (H2S, 80 ppm) gas in an atmosphere of 17.5% oxygen reportedly induces suspended animation in mice; a state analogous to hibernation that entails hypothermia and hypometabolism. However, exogenous H2S in combination with 17.5% oxygen is able to induce hypoxia, which in itself is a trigger of hypometabolism/hypothermia. Using non-invasive thermographic imaging, we demonstrated that mice exposed to hypoxia (5% oxygen) reduce their body temperature to ambient temperature. In contrast, animals exposed to 80 ppm H2S under normoxic conditions did not exhibit a reduction in body temperature compared to normoxic controls. In conclusion, mice induce hypothermia in response to hypoxia but not H2S gas, which contradicts the reported findings and putative contentions.

Hibernation is a hypometabolic state characterized by a regulated decrease in core body temperature (Tb) (i.e., hypothermia) towards ambient temperature (Ta) and consequent reduction in oxygen (O2) consumption and carbon dioxide (CO2) production. It is engaged by several mammalian species to protect the organism from (environmental) stressors such as extreme cold, hypoxia and starvation and ultimately death.

The regulated decrease in Tb, which is termed anapryrexia, encompasses the downmodulation of the ‘internal thermostat’ outside of the thermoneutral zone. The thermoneutral zone constitutes a temperature range in which heat production (from basal metabolism) is in equilibrium with heat loss to the environment. The organism functions best when the Tb resides in the thermoneutral zone, but engages anapryrexia as a coping mechanism. How the anapryrexic signaling is biochemically and physiologically regulated and how the ‘internal thermostat’ is circumvented is largely elusive and hypothetical, but the ultimate outcome is unequivocally a state of hypometabolism. The natural purpose of the hypometabolism is to temporarily realign energy needs with reduced energy/O2 supply under conditions of stress in order to sustain life under circumstances that could otherwise have lethal consequences.

The state of cold hypometabolism is believed to be a result of systematic deviation from homeothermy, which in turn is caused by a reduction in or cessation of metabolism. The resulting hypothermia assists, or propagates, the hypometabolic state in accordance with Arrhenius’ law. This law states that the rate of chemical reactions (i.e., metabolism) decreases when the temperature decreases. Consequently, both the consumption of substrate (in this case O2) and the formation of product (in this case CO2, toxic metabolites such as lactate, and reactive O2 species) are reduced during hypothermia, as has been confirmed in natural hibernators during hibernation in terms of expired CO2. The alignment of metabolic demand with supply as well as the decreased formation of cytotoxic metabolites confer sustenance of life and cytoprotection in the stress-exposed organism.

In line with the above, mimicking these natural phenomena in non-hibernators such as humans by artificially inducing hypometabolism holds tremendous potential in medicine, aviation and space travel, and sports. An artificially induced hypometabolic state has been hypothesized to impart similar protective effects on otherwise stressed cells. Accordingly, numerous studies have focused on identifying agents that are capable of inducing hypometabolism in non-hibernating mammals (i.e., anapryrexic agents), which have yielded 5’-AMP, DADLE, 2-deoxyglucose, thyronamines, and exogenous hydrogen sulfide (H2S) as potential

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Exogenous H$_2$S has been proposed to induce hypometabolism that is associated with a state of suspended animation\textsuperscript{20,21}. Mice that were subjected to a gas mixture composed of 17.5% O$_2$, 80% nitrogen (N$_2$), and 80 ppm H$_2$S exhibited a 22°C reduction in $T_b$ (Fig. 1A) after 4 h of exposure, yielding a $T_b$ that was slightly above the $T_i$ of 13°C. At this point CO$_2$ production and O$_2$ consumption had decreased by approximately 90%, suggesting that the animals had reached a state of hypometabolism by anapyrexia. Moreover, this state was reversible inasmuch as all metabolic parameters reverted to baseline within 4 h after the exposure to H$_2$S was abrogated. During this recovery phase at 17.5% O$_2$ in both groups (6–10 h, right part of red vertical line). The $T_b$ was decreased during the exposure phase (dotted line). In (B) similar experiments were performed as in (A) but at fixed $T_d$ of 27°C (open diamonds, n = 4) or 35°C (open diamonds, n = 3). The 6-h H$_2$S exposure phase was followed by a 3-h recovery phase in air at a $T_d$ of 27°C (6–9 h, right part of red vertical line). Data modified from\textsuperscript{20,21}.

Figure 1. Previously reported temperature effects of H$_2$S. Temperature effects of inhaled H$_2$S gas (80 ppm) on the $T_b$ of mice as a function of exposure time as reported by Blackstone et al.\textsuperscript{20} (A) and Volpato et al.\textsuperscript{21} (B). In (A) mice were exposed to 80 ppm H$_2$S and 17.5% O$_2$ (n = 7) or 17.5% O$_2$ (n = 4) for 6 h, followed by a recovery phase at 17.5% O$_2$ in both groups (6–10 h, right part of red vertical line). The $T_b$ was decreased during the exposure phase (dotted line). In (B) similar experiments were performed as in (A) but at fixed $T_d$ of 27°C (closed diamonds, n = 3) or 35°C (open diamonds, n = 4). The 6-h H$_2$S exposure phase was followed by a 3-h recovery phase in air at a $T_d$ of 27°C (6–9 h, right part of red vertical line). Data modified from\textsuperscript{20,21}.

Results

To test the hypothesis that exogenous H$_2$S-induced hypothermia emanates from hypoxia and not H$_2$S, we performed experiments in 48 female C57BL/6 mice using a similar approach as was employed by Blackstone et al.\textsuperscript{20}. The experiments, which are outlined in Fig. 2 and the Materials & Methods section of this paper, encompassed the following groups: (A) 80 ppm H$_2$S in 21% O$_2$ and 79% N$_2$ (H$_2$S in 21% O$_2$ group; N = 12 mice); (B) 80 ppm H$_2$S in 17% O$_2$ and 83% N$_2$ (H$_2$S in 17% O$_2$ group; N = 6 mice); (C) 5% O$_2$ and 95% N$_2$ (5% O$_2$ group; N = 12 mice); (D) 17% O$_2$ and 83% N$_2$ (17% O$_2$ group; N = 6 mice); and (E) 21% O$_2$ and 79% N$_2$ (normoxia group;
N = 12 mice). The effects of exogenous H2S and a hypoxic atmosphere on Tb at a Ta of ~21 °C were measured non-invasively with a thermographic camera and the locomotor activity of the animals was quantitated with dedicated motion analysis software.

As shown in Fig. 3 and Supplemental Video S1, hypothermia and reduction in locomotor activity only occurred in mice subjected to hypoxic conditions. 5% O2-exposed animals immediately dropped their Tb to approximately 2 °C above the Ta (Fig. 3B, P < 0.0001) and reduced their locomotor activity to nearly nil compared to the H2S in FIO2 21% and normoxia groups (Fig. 3C, P < 0.0001) during the entire exposure period. The exogenous H2S in 21% FIO2 group did not differ from the normoxia group during 6 h of 80 ppm H2S gas exposure in neither superficial temperature nor locomotor activity. At 3 h of exposure, however, animals in the H2S in 17% FIO2 group started to drop their Tb to approximately 4 °C above Ta, in contrast to FIO2 17%-exposed control animals (Fig. 3B, P < 0.0001).

Alleviation of the hypoxic conditions during the restoration phase resulted in complete reversal of the superficial temperature to baseline levels within 1 h in the FIO2 5% group, which is in agreement with previous reports 20,21 (Fig. 1). During the 3 h of restoration at normoxic atmosphere, the H2S in FIO2 17%-exposed animals remained hypothermic and only restored Tb to the level of the FIO2 17% and 21% control groups at 9 h (Fig. 3B, P < 0.01). Mice in the H2S groups exhibited some discomfort during H2S exposure, as evidenced by the cringed posture, which occasionally concurred with vigorous locomotion (Supplemental Video S1).

Peripheral vasodilation is one of the cooling mechanisms that are autonomically regulated in response to a mismatch between the Tb and the internal thermostat (i.e., Tb > thermoneutral zone) 3,35,36. Peripheral vasodilation is integral to anapyrexia3, which enables cooling. The cooling process is in turn facilitated by the blockade of thermogenic effectors and the enabling of peripheral vasodilation36–38. Therefore, the extent of peripheral vasodilation was determined by measuring the change in tail temperature at baseline and at approximately 4 min after initiation of H2S- or hypoxia exposure.

The tail of 5% O2-exposed animals warmed up right after the start of exposure (+2.1 ± 0.5 °C, N = 3, P < 0.05 versus the H2S group, unpaired student’s t-test), while the tail of H2S in 21% O2-exposed animals (−1.3 ± 1.1 °C, N = 3) and normoxia-exposed animals (+0.1 °C, N = 1) did not exhibit changes in temperature (P > 0.05, unpaired student’s t-test) (Fig. 4). These results provide compelling evidence for the induction of peripheral vasodilation by hypoxia but not exogenous H2S, and hence for hypoxia-mediated anapyrexic signaling. The absence of a vasodilatory response in the exogenous H2S group is in agreement with the surface temperature data, which encompassed an absence of hypothermia (Fig. 3).

Figure 2. Schematic illustration of the experimental setup and design. The animals were allocated to one of the following experimental groups: (A) 80 ppm H2S in 21% O2 and 79% N2 (H2S in 21% O2 group, N = 12); (B) 80 ppm H2S in 17% O2 and 83% N2 (H2S in 17% O2 group, N = 6); (C) 5% O2 and 95% N2 (5% O2 group, N = 12); (D) 17% O2 and 83% N2 (17% O2 group, N = 6); and (E) 21% O2 and 79% N2 (normoxia group, N = 12). The experiments were performed at a mean ± SD Ta of 21.2 ± 0.6 °C, measured with a thermistor. During the whole experiment the mice were solitarily housed in a custom-built airtight cage and recorded with a thermographic camera (CAM) for 10 min every hour (red markers) for skin temperature- and locomotor activity analysis. After 1 h of baseline 21:79% O2:N2 exposure, each mouse was exposed to a gas mixture (A–E) for 6 h that was passed through the airtight cage, after which 6 of the animals in each group were allowed to recover at 21:79% O2:N2 for 3 h. The other 6 animals of group (A, C and E) were sacrificed for another study.
Discussion

Based on the experimental evidence, namely $T_b$, tail temperature, and locomotion, it can be concluded that inhalation of H$_2$S gas at 80 ppm in a native atmosphere of 21% O$_2$ and 79% N$_2$ does not induce hypothermia in mice, which contradicts what has been reported previously$^{20,21}$. Hypoxia, on the other hand, is a very potent inducer of hypothermia that, given the peripheral vasodilation observed in the tail vasculature, may comprise part of an anapyrexic response$^{2,3}$. The subclinical thermal effects of mild hypoxia, however, are potentiated by combined 80 ppm H$_2$S gas exposure.

One consistent finding in mouse studies on the pharmacological induction of hypothermia is that the animal's $T_b$ or surface temperature approximates the $T_a$ and subsequently enters a plateau phase that is sustained in the
and Volpato of exogenous H$_2$S to the lungs may further compromise pulmonary blood flow during hypoxic conditions, which was observed in our experiments. Regardless of what actually caused the hypothemic signaling in the experiments by Blackstone et al. and Volpato et al., the $T_a$ was in all instances downmodulated to a depth at which the $T_b$ was more or less in equilibrium with the $T_a$, irrespective of the magnitude of the $T_a$ (i.e., 13 °C, 27 °C, or 35 °C). The same pattern was observed in our experiments ($T_a = 21$ °C), suggesting that the hypothermia may have been mediated via a common mechanism. Moreover, this decline-plateau pattern suggests that the cooling process is passive once the thermogenic effectors have been shut off. The cooling is halted upon reaching a thermodynamic equilibrium where $T_b = T_a$, i.e., a point at which the organism is not equipped to cool further. Unlike under normophysiological circumstances, where $T_b$ is tightly regulated via engagement of cooling effectors or thermogenic effectors, the hypothermic state seems to sustain itself through passive heat transfer only.

The main differences between the results of Blackstone et al. and Volpato et al., and our results are the rate of cooling and subsequently the time required to reach the plateau phase ($T_b = T_a$). The cooling rate was approximately 1.3 °C/h and 4.0 °C/h in the experiments of Volpato et al. and Blackstone et al., respectively, whereas in our experiments the cooling rate was approximately 5.3 °C/h. The convergence of $T_b$ with $T_a$ required ~6 h in the study of Blackstone et al., ~4 h in the study of Volpato et al., and 2 h in our study (Fig. 3). The same animal species with similar animal weights were employed in all studies. Hence, it is unlikely that these discrepancies arose from differences related to physical laws such as Galilei’s square-cube law, the implication of which is that animals in line with our results obtained in the 17% $F_iO_2$ groups, the exacerbation likely occurred in the experiments by Blackstone et al. and Volpato et al. for four possible reasons. First, as explained in the Introduction section, H$_2$S may aggravate the circulatory hypoxia caused by subatmospheric $F_iO_2$ levels and culminate in a hypoxic state that is considerable enough to trigger anapyrexia in mice, unless such mild hypoxic conditions are exacerbated by exogenous H$_2$S.

In light of the finding that exogenous H$_2$S is not an inducer of hypothermia, the question that remains to be answered is “why did Blackstone et al. and Volpato et al. observe hypothermia in H$_2$S-exposed mice?” Volpato et al. was able to reproduce the hypothemic effects of 80 ppm H$_2$S of Blackstone et al. Consequently, we do not question the methodology and validity of their results. In our opinion, the answer lies in the hypoxic conditions that were induced by the combination of subatmospheric $F_iO_2$ and the various mild forms of exogenous H$_2$S-induced hypoxia. The 3.5% lower $F_O_2$ versus native atmospheric $F_O_2$ (17.5% versus 21%), respectively is, in itself, not sufficient to trigger anapyrexia in mice, unless such mild hypoxic conditions are exacerbated by exogenous H$_2$S.

In line with our results obtained in the 17% $F_O_2$ groups, the exacerbation likely occurred in the experiments by Blackstone et al. and Volpato et al. for four possible reasons. First, as explained in the Introduction section, H$_2$S can induce histotoxic hypoxia by inhibiting cytochrome c oxidase and corollary ATP production, resulting in reduced metabolic supply (energy). Consequently, the organism is forced to adapt its metabolic demand to survive by means of e.g., hypothermia (Arrhenius’ law). Secondly, H$_2$S can limit the binding of O$_2$ to hemoglobin’s O$_2$ binding sites, thereby causing O$_2$ affinity hypoxia. Thirdly, H$_2$S reduces cardiac output through its derogulatory and negative chronotropic effects on cardiac rhythm, which leads to circulatory hypoxia. Fourthly, H$_2$S is pulmonotoxic and may impair pulmonary O$_2$/CO$_2$ exchange and the extent of O$_2$ saturation, which in turn may aggravate the circulatory hypoxia caused by the cardiovascular effects. In addition, based on ex vivo experiments, H$_2$S seems to play an essential role in hypoxic pulmonary vasoconstriction. Therefore, administration of exogenous H$_2$S to the lungs may further compromise pulmonary blood flow during hypoxic conditions, which can augment hypoxemic hypoxia. Accordingly, all these forms of H$_2$S-mediated hypoxia may add to the mild hypoxia caused by subatmospheric $F_O_2$ levels and culminate in a hypoxic state that is considerable enough to trigger anapyrexia. As addressed in Dirkes et al., circulatory hypoxia is sensed through carotid bodies located in the carotid artery that, under non-hypometabolism-inducing hypoxic conditions, relay arterial O$_2$ tension ($P_{O_2}$)-related information to the brain. The brain subsequently (hyper)activates certain physiological functions to remediate the hypoxia, which include panting and tachycardia. How this is blocked during the induction of anapyrexia is currently unclear.
Endogenous H$_2$S as well as intracerebrally administered exogenous H$_2$S analogues inhibit the ventilatory and thermal response to hypoxia in the hypothalamus and brain stem. Contrastingly, microinjection of Na$_2$S (H$_2$S precursor) in the anteroventral preoptic hypothalamus of rats potentiates hypothemic signaling by hypoxia, but does not alter $T_b$ under normoxic conditions$^{55}$. Microinjection of the endogenous H$_2$S production inhibitor amino-oxycetate in the sympathetic excitatory rostral ventrolateral medulla of rats attenuates hypoxia-induced hypothermia$^{56}$. As H$_2$S passes the blood-brain barrier freely, central effects of inhaled H$_2$S could have contributed to hypoxia-induced anapnoeia via the hypothalamus or brain stem$^{22}$ albeit an unequivocal mechanistic explanation remains warranted in light of the contrasting results.

In the experiments of Blackstone et al. and Volpato et al., $T_b$ was determined by telemetry devices that record the core temperature (i.e., intra-abdominal temperature). In our experiments, the superficial temperature was determined. We believe that this approach is valid for the purpose of this study inasmuch as we were interested in temperature trends as a function of exposure time and gas composition, and not the real $T_b$ per se. Since all groups were thermographically analyzed in the same manner, the resulting data yield credence to our conclusions. Moreover, the use of thermographic imaging has some benefits over intra-abdominal temperature determination, such as the determination of thermoregulatory vasoactivity by tail temperature measurement (Fig. 4).

Although this paper focused on the hypometabolic properties of H$_2$S gas, several animal studies on the effects of liquid H$_2$S analogues NaHS and Na$_2$S have been published. After inhalation, H$_2$S gas diffuses freely across the alveolar membrane and enters the blood as predominantly HS$^-$ and H$_2$S$^{57}$. Appropriately, intravenously administration of solubilized H$_2$S precursors/analogues is believed to follow the same pharmacodynamics as administration through inhalation, only without the detrimental effects on local pulmonary physiology and toxicity. The hypothermic effects of NaHS and Na$_2$S in small as well as in large animals have been reviewed before$^{57}$. Continuous administration of NaHS is assumed to induce hypothermia in anesthetized rats; although these studies lack essential control groups$^{58,59}$. The evidence considering the hypothermic and hypometabolic effects of NaHS in large animals has been conflicting; in a pig study a small hypothermic effect was observed following 8 continuous hours of NaHS administration$^{29}$, whereas in several other studies in pigs$^{65,66}$ and sheep$^{67}$ such hypothermic effects were not reproducible. The differences between the effects of H$_2$S in small and large animals have been contemplated by Dirkes et al. and are explained by the inability of large animals to lose heat sufficiently due to the low body surface:mass ratio$^{68}$.

In this paper, the tail temperature was used as a measure of central activation of peripheral cooling mechanisms (i.e., peripheral vasodilatation), as has been before in the determination of thermoregulatory peripheral vasoactivity in pyrexic mice$^{68}$. However, as reviewed by Liu et al., H$_2$S has biphasic effects on the vascular tone: at low concentrations H$_2$S induces vasoconstriction and at higher doses vasodilation is induced, as evidenced in mouse and rat aortic tissue$^{69–71}$. Consequently, the absence of thermoregulatory vasodilation and a consequent increase in the tail temperature of 3 animals (Fig. 4) could also be a direct vasoconstrictive effect of low-dose H$_2$S. Nevertheless, H$_2$S-induced vasoconstriction is unlikely to be responsible for the absence of H$_2$S-induced hypothermia in our experiments. A ‘masked’ thermoregulatory vasodilative response would be accompanied by deactivation of brown adipose tissue (BAT) and shivering thermogenesis (i.e., major source of heat in mice at a $T_b$ of 21 °C)$^{72}$. Subsequently, the cessation of thermogenesis would be reflected in the $T_b$/superficial temperature of H$_2$S-exposed animals, which was not observed (Fig. 3).

In conclusion, exogenous H$_2$S is not a hypometabolism-inducing agent. The hypometabolism induced in mice that were subjected to exogenous H$_2$S was caused by hypoxia. At subatmospheric F$O_2$ levels, exogenous H$_2$S exacerbates the hypoxic conditions to such a degree that anapnoeia and hypothermia are triggered. Accordingly, exogenous H$_2$S is a hypometabolizable adjuvant rather than a hypometabolism-inducing agent.

Materials and Methods

Animals. Forty-eight female C57BL/6 mice (Charles River, L’Arbresle, France; 10–12 weeks of age) were acclimated for 2 weeks under standardized laboratory conditions with a 12 h light/dark cycle, a constant ambient temperature ($T_a$) of approximately 21 °C, and ad libitum access to standard chow and drinking water. The experimental protocol was evaluated and approved by the animal ethics and welfare committee of the Academic Medical Center, University of Amsterdam under protocol number BEX 102753. Animals were treated in compliance with institutional guidelines and the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (NIH publication No. 86–23, revised 2011).

Experimental setup and gas mixtures. The hydrogen sulfide (H$_2$S) and 17% oxygen (O$_2$) gas mixtures were obtained from Westfalen (Münster, Germany) and consisted of (1) 80 ppm H$_2$S, 21% O$_2$, and 79% N$_2$; (2) 80 ppm H$_2$S, 17% O$_2$, and 83% N$_2$; or (3) 17% O$_2$, and 83% N$_2$. The 5% O$_2$ gas mixture was obtained from Linde Gas (The Linde Group, Munich, Germany) and consisted of 5% O$_2$ and 95% N$_2$. Normo-atmospheric air (21% O$_2$, and 79% N$_2$) was used as control.

An experimental setup was custom-built to allow controlled gas exposure while unobtrusively assessing body temperature ($T_b$) with a thermographic camera (TheraCAM SC2000, FLIR Systems, Wilsonville, OR) in non-anesthetized mice. The setup consisted of gas-tight polypropylene chambers (Fig. 5A, length × depth × height of 109 mm × 109 mm × 61 mm) that were sealed at the imaging end with a thin, infrared light-permeable polyethylene sheet to permit thermal imaging from outside (Fig. 5B). Metal wires were secured longitudinally so that the animals could not reach the polyethylene sheet (Fig. 5C). Gas inflow and outflow tubes were connected to each box at the posterior end for modulation of experimental conditions (Fig. 5E). The gas permeability of the chambers was tested by air pressure decline experiments. Also, a thermistor (Fluke 51 II, Fluke Corporation, Everett, WA) was secured in the posterior wall (Fig. 5D) to facilitate the measurement of the temperature in the chamber. The thermistor was used as a calibrator for the thermographic camera images, as the thermographic images display the temperature of the copper bolt retaining the thermistor.
To ascertain sufficient inflow of gas in all experiments and prevent CO$_2$ accumulation, the flow rates were controlled on the basis of CO$_2$ outflow concentrations (<600 ppm, CO$_2$ Meter, Ormond Beach, FL). The system was also connected to an O$_2$ and H$_2$S meter (model OdaLog 7000, App-Tek International, Brendale, Australia), which was calibrated by a certified company prior to the experiments (Carltech, Maarheeze, the Netherlands). The O$_2$ and H$_2$S meter was post hoc tested for measurement accuracy. The experiments were performed at a mean ± SD $T_a$ of 21.2 ± 0.6 °C.

**Experimental procedure.** To test the hypothesis that H$_2$S-induced hypothermia emanates from hypoxia and not H$_2$S, all 48 animals were randomly divided among 5 experimental groups. Group A was exposed to 80 ppm H$_2$S in 21% O$_2$ and 79% N$_2$ (H$_2$S in 21% O$_2$ group, N = 12), group B was exposed to 80 ppm H$_2$S in 17% O$_2$ and 83% N$_2$ (H$_2$S in 17% O$_2$ group, N = 6), group C was exposed to 5% O$_2$ and 95% N$_2$ (5% O$_2$ group, N = 12), group D was exposed to 17% O$_2$ and 83% N$_2$ (17% O$_2$ group, N = 6), and group E was exposed to 21% O$_2$ and 79% N$_2$ (normoxia group, N = 12).

Mice were placed in the chambers individually. After 1 h of exposure to normoxia (21% O$_2$ and 79% N$_2$), the mice were exposed to one of the gas mixtures (A – E) for 6 h, after which 6 of the animals per group were allowed to recover at normoxic conditions for 3 h before being terminated. The other 6 animals of group A, C and E were terminated immediately after the 6 h of exposure for another study.

No anesthetics were used before or during the experimental procedure.

**Thermal imaging and data processing.** Animals were filmed every hour for 10 min with a thermographic camera (ThermaCAM SC2000, FLIR Systems, Wilsonville, OR)(Fig. 2). Thermographic camera images (3 images per second) were processed and analyzed in ThermaCAM Researcher 2001 (FLIR Systems). The mean maximum superficial temperature was calculated per time point per group.

The tail temperatures of animals in group A (N = 3), C (N = 3), and E (N = 1) were obtained from the thermographic camera images at 0 h, just before the start of exposure, and approximately 4 min after the start of exposure. We noticed the intergroup differences in tail temperature during the experiments, as a result of which the tail temperature was measured in only 7 animals. The mean difference in tail temperature between both time points was calculated and compared for group A and C.

Locomotor activity was assessed per time point using the same thermal images as were used for the calculation of superficial temperature. An analytics program was written in LabVIEW (LabVIEW, National Instruments, Austin, TX). The thermographic camera images were converted to grayscale images and loaded into LabVIEW. Locomotor activity was calculated per animal per time point (−1 up to 9 h) on the basis of fluctuations in pixel intensity. A pixel was considered to reflect ‘motion’ when the grayscale intensity difference between direct temporally consecutive pixels exceeded 7 on a scale of 0 to 255. The intensity difference of at least 7 was based on the

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**Figure 5.** Components of the experimental setup. (A) The bare polypropylene chamber, consisting of the main chamber and the lid (back part of the chamber). (B) The bottom of the main chamber was removed and replaced with a polyethylene sheet. (C) Metal wires were inserted in front of the polyethylene sheet at a distance at which the mice could not pass through. (D) A bolt was inserted into the back panel to measure the actual temperature in the chamber. The bolt was connected to a thermistor. (E) Configuration of the tubing that was connected to the different gas-containing cylinders and used to modulate the chamber atmosphere during the experiments.
disappearance of background scatter present as intensity differences between 1 and 6. Values were expressed as the mean ± SEM amount of pixels with 'motion' per group per time point.

**Statistical analysis.** Statistical analyses were performed using MatLab 2013a (MathWorks, Natick, MA). Homogeneity of variance in each group was tested using the Bartlett's test. Based on equality of variances, either a one-way ANOVA or a Kruskal-Wallis test was performed, followed by a Tukey's range test or Dunn's test, respectively, to compare ordinal variables related to maximum superficial temperature and locomotor activity between groups. Tail temperature values were compared using an unpaired student's t-test. P-values less than 0.05 were considered significant. All values were presented as mean ± SEM, unless otherwise mentioned.

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Author Contributions
S.D.H. prepared and performed the experiments, analyzed the data, and co-wrote the manuscript. M.C.D. is responsible for the conceptual design of the experiments and supervised the experiments. M.H.N.V. analyzed the thermographic data and RB designed the motion analysis software. T.M.G. supervised the project. M.H. supervised the project and wrote the manuscript. All authors critically read the manuscript and approved the final version.

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