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A novel whole-body thermal stress test for monitoring cardiovascular responses in guinea pigs

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ABSTRACT

Cardiovascular disease is a leading cause of morbidity and mortality worldwide. Stress tests are frequently employed to expose early signs of cardiovascular dysfunction or disease and can be employed, for example, in the context of preterm birth. We aimed to establish a safe and effective thermal stress test to examine cardiovascular function. Guinea pigs were anaesthetized using a 0.8% isoflurane, 70% N_2O mix. ECG, non-invasive blood pressure, laser Doppler flowmetry, respiratory rate, and an array of skin and rectal thermistors were applied. A physiologically relevant heating and a cooling thermal stress test was developed. Upper and lower thermal limits for core body temperature were set at 41.5 ^{O}C and 34 ^{O}C, for the safe recovery of animals. This protocol therefore presents a viable thermal stress test for use in guinea pig models of health and disease that facilitates exploration of whole-system cardiovascular function.

1. Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality, worldwide (Okwuosa et al., 2016; Yusuf et al., 2015). While 'traditional' risk factors, such as smoking or poor lifestyles, explain much of this morbidity and mortality, novel non-modifiable risk factors are still being identified. Perinatal challenges, encompassed by the so-called Developmental Origins of Health and Disease hypothesis, are one such example (Hanson and Gluckman, 2008). Those born preterm (<37 weeks' gestation) or growth restricted (fetal weight below 10th percentile for gestational age) have been shown to exhibit an elevated risk profile for developing CVD (Chan et al., 2010; Crump, 2020; Longo et al., 2013; Singhal, 2006). The mechanisms contributing to this risk profile are still being identified (Crump, 2021).

As with 'traditional' CVD populations, animal models provide insight into the specific mechanisms contributing to this CVD risk in those born preterm. Functional assessments, or stress tests, provide further insight in these models by expanding mechanistic findings to the systems level. Exercise stress testing has proven effective in some models of CVD (Feng et al., 2019); however, this is not suitable for all animal models. Expanding on the utility of our previously established guinea pig model of preterm birth (Berry et al., 2015; Morrison et al., 2018), we have developed a thermal stress test that facilitates examination of central cardiac- and peripheral micro-vascular response to stress.

Exercise stress is an established cardiovascular stressor in animal models (Feng et al., 2019). However, to-date few studies have examined the cardiovascular consequences of thermal stress, with investigations using thermal stress in animals typically focussing on thermal tolerance, heat acclimation, and heat stroke (Adolph, 1947; Fewell et al., 1997; Gordon, 1986; Hinckel and Schroder-Rosenstock, 1982; Laughter and Blatteis, 1985; Leon et al., 2005; McKechnie and Wolf, 2019). However, cardiovascular function is a critical component in thermoregulatory heat loss, with CVD and dysfunction impairing thermoeffector function (Balmain et al., 2018; Ikaheimo, 2018). Given that microvascular dysfunction often precedes clinical manifestation of cardiovascular compromise, the ability to examine the microvasculature is a strength of thermal stress testing. Importantly, microvascular dysfunction is also considered a crucial pathway in the development and progression of cardiometabolic disease and is associated with increased cardiovascular mortality (Houben et al., 2017). Furthermore, exposure to both heat and cold stress uncovers different cardiovascular warning signs, allowing for more nuanced understanding of CVD risk (Cui and Sinoway, 2014; Greaney et al., 2017; Hess et al., 2009; Minson et al., 1998; Wilson et al., 2014). This is clinically manifest in the different mechanisms contributing to excess cardiovascular deaths associated with heat waves and cold snaps (Hess et al., 2009; Huynen et al., 2001; Smith et al., 2014).

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Irrespective of the thermoregulatory characteristics between species, the role of the cardiovascular system is evident. As such, the aim of the current study was two-fold: 1) to design a thermal wrap capable of eliciting tightly controlled changes in core body (T_C) and skin temperature (T_{sk}) in a standard laboratory species; and 2) using the thermal wrap to design reproducible hyper- and hypo-thermic challenges capable of inducing cardiovascular stress via a thermoregulatory homeostatic response suitable for use in both healthy and vulnerable animals.

2. Methods

2.1. Animals

Outbred Dunkin-Hartley guinea pigs were sourced from the Biomedical Research Unit, University of Otago Wellington. All procedures were prospectively approved by the University of Otago Wellington Animal Ethics Committee. All studies were carried out in accordance with the Health Research Council of New Zealand code of practice for the care and use of animals for scientific purposes, and are reported according to the ARRIVE guidelines (Kilkenny et al., 2012).

Term-born animals (gestational age: 67–70 d) were recruited from stock animals. Preterm-born animals were generated via our standard induction of labour protocol and neonatal care (Berry et al., 2015). In brief, preterm animals received around-the-clock care for the first 7 days (to term equivalent age), with intervals of care (feeding, facilitating urination/faecal production) increasing with improvements in welfare. Upon graduating from intensive care, preterm pups and dams were housed alongside term pups in a 12:12 light and temperature-controlled environment, with ad libitum access to standard guinea pig chow (Specialty Feeds, Glenforest, Australia) and vitamin C-enriched water for 21 days before being weaned into sex-specific housing. At time of testing, preterm animals were free of overt dysfunction (e.g., normal B.A.R. (Bright, Alert, Responsive) score and weight, and normal resting heart rate and blood pressure).

Development of the thermal challenge comprised three heating iterations and two cooling. Graphical representation of the succeeding protocol iterations is presented in Supplemental Figs. 1 and 2. Animals were familiarized to all waking baseline procedures for three days prior to challenge and fasted for a minimum of 4 h prior to initiation of all study iterations.

2.2. Setup

2.2.1. Anaesthesia

Animals were anaesthetized for the duration of thermal stress testing, using our optimized anaesthetic protocol, described previously (Sixtus et al., 2021). In brief, animals were induced under 4.0% isoflurane in 50% O_2 at 2 L min^{-1} and titrated to a maintenance dose of 0.8–1.0% isoflurane +70% N_2O across 30 min (Datex Ohmeda Aestiva 5, GE Healthcare, Chicago, IL, USA). Spontaneous breathing was maintained in the prone posture for the duration of testing. Anaesthesia was withdrawn under medical air (21% O_2) upon conclusion of thermal challenge recovery phase, and peripheral SpO_2 monitored until spontaneous arousal. Anaesthetic depth was assessed using a combination of minimum alveolar concentration (MAC = 1.5%), physiological parameters (heart rate (HR), respiration (RR)), and monitored signs (body tone, palpebral reflex). Anaesthetic plane was maintained below surgical thresholds such that animals were responsive to pain, therefore pedal withdraw was not assessed.

2.2.2. Apparatus

Water-perfused suits have been used since the 1960s to bridge the gap in methodologies between water-immersion and exposure to extreme environmental conditions – whether natural or artificial (Brengelmann et al., 1977; Crandall and Wilson, 2015; Rowell et al., 1969). Although water-immersion clamps skin temperature to that of the water, the hydrostatic pressure confounds investigation of microvascular perfusion. Water-perfused suits enable a tightly controlled thermal stressor to be applied in an inexpensive, rapid and uncompensable manner (Crandall and Wilson, 2015) while allowing comprehensive physiological monitoring of the cardiovascular response (Rowell et al., 1969, 1970). Although not previously described, the water-perfused system is easily adaptable to a wide variety of species by simply modifying the size and fit of the 'suit'. The wrap in which animals were secured consisted of a 30 cm × 30 cm, 2 mm neoprene wrap, used for its insulative properties, upon which was sewed a tight weave of 3 mm tubing spaced ~1 cm apart (Fig. 1). Tubing was connected to a precision-controlled circulating water bath (R-1, Anova Industries, Texas, USA), pumped at 5 L min^{-1}. The wrap was secured with Velcro fasteners, adjusted to ensure a secure connection between skin and tubing.

2.2.3. Equipment

A comprehensive array of cardiovascular, thermoregulatory, and respiratory measures was used to examine cardiovascular function (Fig. 1). Cardiovascular measures included three-lead ECG (needle probe, ADInstruments, Dunedin, New Zealand) positioned at right (negative) and left (ground) clavicle/neck, and on the left lateral thorax (lateral border of costal margin); non-invasive blood pressure (NIBP; rat tail cuff, NIBP, ADInstruments) was placed on the right forelimb; and laser Doppler flowmetry (LDF; Probe 457, PeriFlux 5001, Perimed, Jarfalla, Sweden) at a distal (ear) and proximal (interscapular) skin site. Temperature was recorded rectally (RET-1, ADInstruments), 4 cm past the anus (T_{re} *depth = Total Body Length (mm) x 0.2*, derived from Czaja and Butera (1986)), and at four skin sites (right ear, interscapulum,

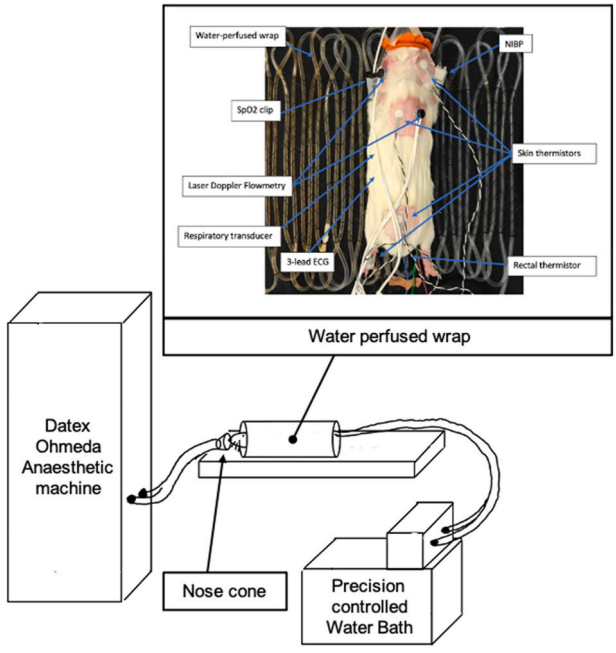

Fig. 1. Methodological setup for comprehensive cardiovascular monitoring during thermal challenges.
ECG was positioned according to Shiotani et al. (2007), configuration 6. The NIBP cuff was placed over the right forelimb and pulse pressure sensor on the paw. LDF was placed at a distal (ear) and proximal (interscapular) skin site. A pulse transducer was placed under the abdomen at the level of the diaphragm. SpO_2 was measured at the left forepaw. Skin thermistors were positioned over ear, interscapular, rump, and foot skin. Finally, a rectal thermistor was positioned 4 cm past the anus.
N.b. LDF: laser Doppler flowmetry; NIBP: Non-invasive blood pressure; SpO_2: Oxygen saturation.

rump, left hind foot) using insulated thermistors (skin thermistor, ADInstruments). Respiratory rate was measured via a transducer (Finger pulse transducer, ADInstruments) positioned externally at the level of the diaphragm, and peripheral oxygen saturation (SpO_2; ADInstruments) from the left forelimb. Hairy skin sites were depilated (Veet hair removal cream, RB Healthcare UK, England) prior to positioning probes. Blood gas variables (CG4+, iStat, Abbot Point of Care, Illinois, USA), were measured pre- and post-anaesthesia from arterialized capillary samples obtained from ear vessels (Zavorsky et al., 2007). Serial blood gas measures throughout the challenge were not possible due to the nature of the wrap and interference with probes.

2.3. Stabilizing T_C for thermal challenges

Thermoregulatory defence thresholds are roughly inversely proportional to inhaled isoflurane concentration (Støen and Sessler, 1990; Xiong et al., 1996). Additionally, response to both the heating and cooling thermal challenges is inextricably tied to T_C at baseline, and this is complicated by anaesthesia. As such, the stabilization phase aimed to maintain core body temperature (T_C; assessed as rectal temperature, T_{re}) and mean skin temperature (T_{sk}; *Mean* $T_{sk}(^OC)$ = *(0.3 x (ear + back)) + (0.2 x (rump + foot))*, modified for guinea pigs from Ramanathan (1963)) close to conscious levels upon beginning challenge baseline. As per the anaesthetic protocol (Sixtus et al., 2021), anaesthetic induction was begun in an acrylic chamber, then maintained at ~3–4% isoflurane on a nose cone throughout equipment set up, while lying on the water-perfused wrap. With equipment applied, animals were secured in the wrap and anaesthetic titration begun. Time from induction to challenge baseline was ~50 min (~8 min induction, ~10 min setup, 30 min titration). T_C stabilization was ensured during induction and

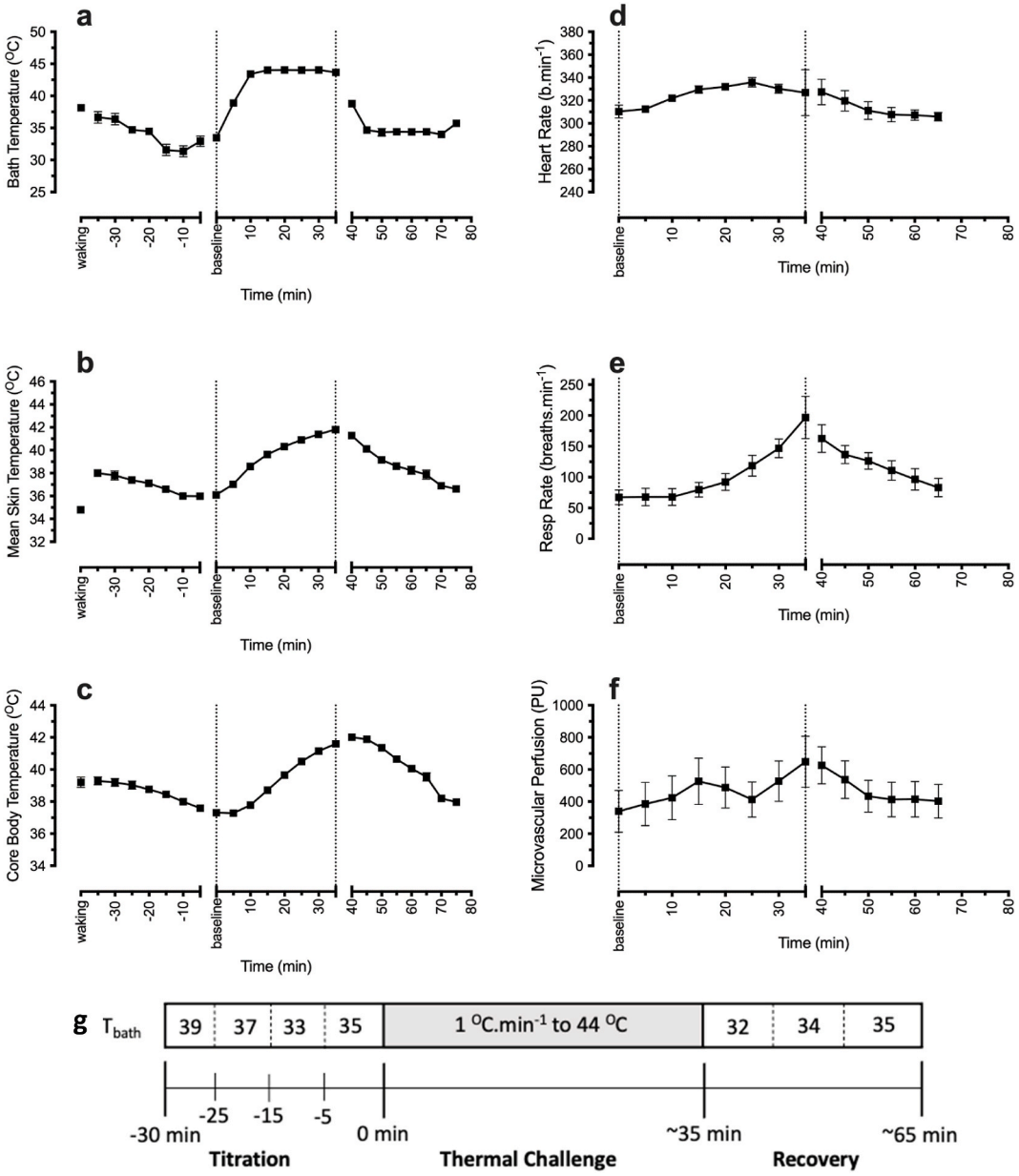

Fig. 2. Design and key parameters of the final heating challenge (H3).
Thermal parameters through induction, challenge and recovery phases are presented in panels a/b/c. 'a' represents bath temperature, 'b' represents mean skin temperature, and 'c' represents core body (rectal) temperature. Panels d/e/f present cardiorespiratory parameters during the thermal challenge and recovery phases. 'd' represents heart rate change, 'e' represents respiratory rate, and 'f' represents microvascular perfusion at the distal skin site. The protocol is depicted on panel 'g'. Data are presented as mean ± SEM. Error bars on thermal parameters are present, but extremely tight.

titration in the final protocol design using a temperature- and humidity-controlled chamber (Incubator 8000 SC, Dräger, Lübeck, Germany) set at 39 ^{O}C, to eliminate a thermal gradient. This temperature was reduced to 33 ^{O}C during titration. Circulating wrap temperature via T_{bath} began at 39 ^{O}C and was reduced during titration to 37 ^{O}C at −20 min, and 35 ^{O}C and −10 min (Figs. 2 and 3).

2.4. Heating thermal challenge

We sought to elicit a clearly defined thermal stimulus (Fig. 2g). Beginning from a baseline T_{sk} of 36 ± 0.25 ^{O}C, three female and three male guinea pigs underwent a 1 ^{O}C. min^{-1} ramp of T_{bath}, from 35 ^{O}C to 44 ^{O}C, whereupon T_{bath} was maintained until T_{re} attained the upper limit (41.5 ^{O}C). Upon achieving a T_{re} of 41.5 ^{O}C, animals began recovery to cool and stabilize T_{re} (T_{bath}, recovery start: 32 ^{O}C, 20 min: 34 ^{O}C, 25 min: 35 ^{O}C). This protocol sought to balance the fatal thermal loading from the first iteration (H1; Supplemental Fig. 1a, 3a/b/c and 4a/b/c), and the inadequate loading from the second (H2; Supplemental Fig. 1b, 3d/e/f and 4d/e/f).

To ensure the safety of this proposed challenge in vulnerable animals, two female and two male preterm guinea pigs underwent the challenge. Extended welfare monitoring (B.A.R. score [Bright, Alert, Responsive]) was performed for three days following each challenge to ensure that preterm animals recovered successfully.

2.5. Cooling thermal challenge

The cooling challenge protocol is presented in Fig. 4g. A lower T_{re} thermal threshold was set at 34 ^{O}C to limit complications of excess hypothermia observed in pilot work (C1; Supplemental Fig. 2, 5a/b/c, and 6a/b/c). Three female and three male guinea pigs were included. The protocol consisted of a 1 ^{O}C. min^{-1} T_{bath} ramp from 35 ^{O}C to 15 ^{O}C, with the challenge ceased at the lower T_{re} threshold. Recovery was then begun with T_{bath} set to 36 ^{O}C and adjusted to 38 ^{O}C after 20 min. As with the heating challenge, a subgroup of two female and two male preterm guinea pigs underwent the cooling challenge as described.

2.6. Data analysis

Physiological measures were sampled continuously throughout anaesthesia. ECG, NIBP, respiration, T_{re} and T_{sk} were sampled using PowerLab (ADInstruments) at a rate of 1 k.s^{-1}, with an analogue notch filter applied, and recorded in LabChart (ADInstruments). HR and RR were derived using peak-to-peak analysis. LDF, collected alongside

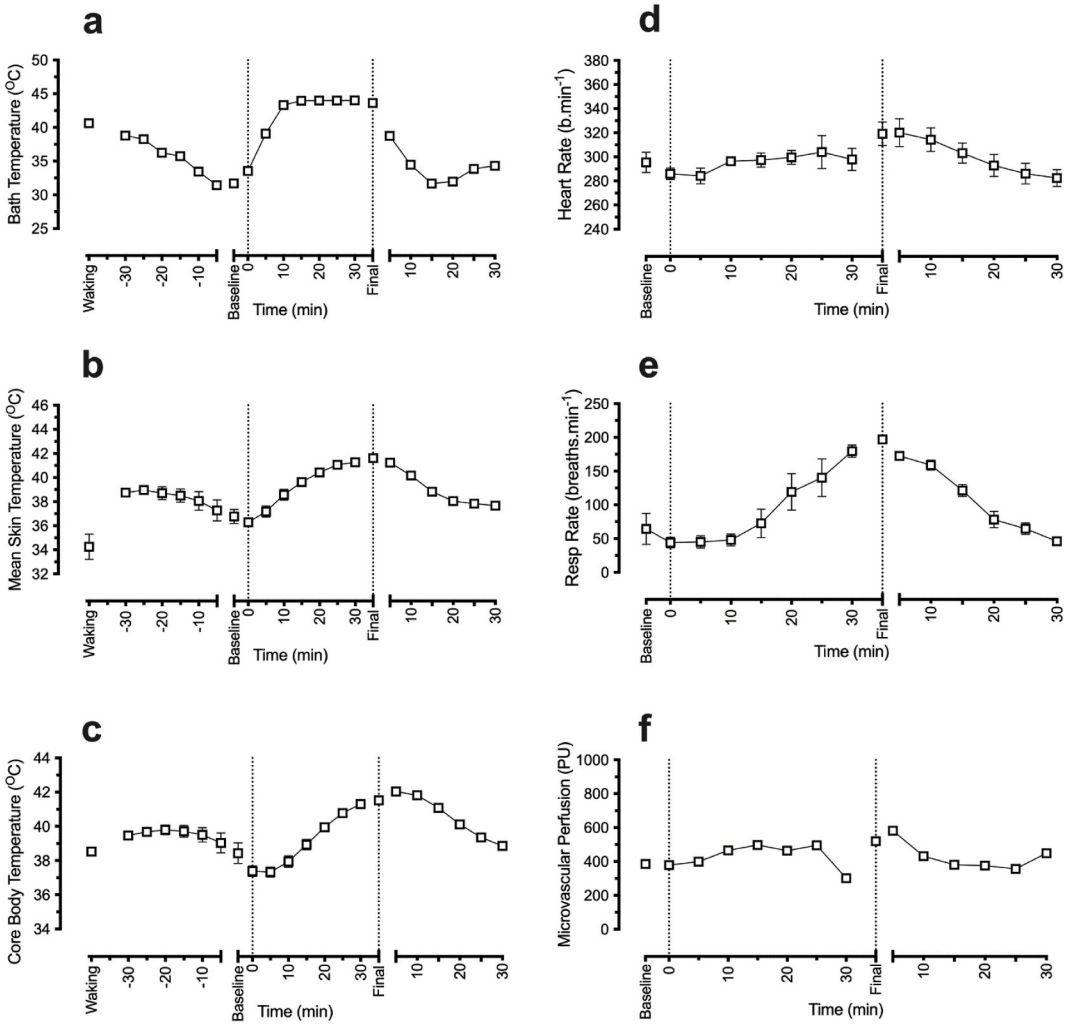

Fig. 3. Preterm thermal and cardiovascular response to the heating challenge (H3).
Thermal parameters through induction, challenge and recovery phases are presented in panels a/b/c. 'a' represents bath temperature, 'b' represents mean skin temperature, and 'c' represents core body (rectal) temperature. Panels d/e/f present cardiorespiratory parameters during the thermal challenge and recovery phases. 'd' represents heart rate change, 'e' represents respiratory rate, and 'f' represents microvascular perfusion at the distal skin site. Data are presented as mean ± SEM. Error bars on thermal parameters are present, but extremely tight.

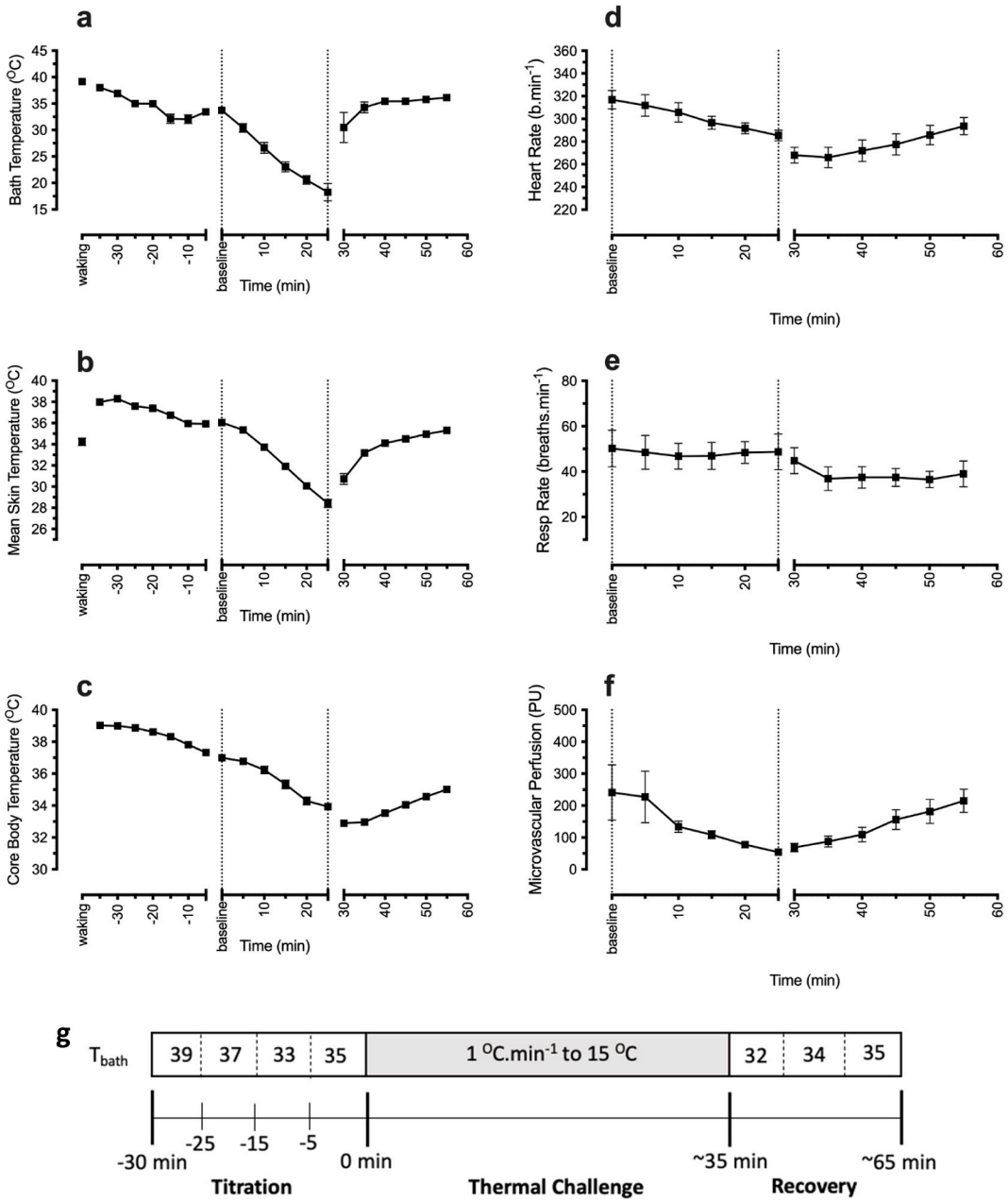

Fig. 4. Design and key parameters of the final cooling challenge (C2).
Thermal parameters through induction, challenge and recovery phases are presented in panels a/b/c. 'a' represents bath temperature, 'b' represents mean skin temperature, and 'c' represents core body (rectal) temperature. Panels d/e/f present cardiorespiratory parameters during the thermal challenge and recovery phases. 'd' represents heart rate change, 'e' represents respiratory rate, and 'f' represents microvascular perfusion at the distal skin site. The protocol is depicted on panel 'g'. Data are presented as mean ± SEM. Error bars on thermal parameters are present, but extremely tight.

central cardiovascular assessments, was measured at 32 Hz with a time constant of 0.03 s, assessed in arbitrary perfusion units (PU; equipment calibration checked prior to each recording and calibrated when values ± 0.5 PU outside of calibration norm, or at least monthly), and analysed using custom software (PSW2; Perimed). Measures were assessed in 1 min blocks every 5 min throughout titration, challenge, and recovery across all iterations. Due to changes in challenge format, data analysis differed in the challenge phase of each iteration. Selected blocks were representative, artefact-free data. Data are expressed as mean ± S.E.M. Study findings are reported primarily as descriptive statistics; where relevant, Students T-tests and linear regressions have been used to determine significance of change from challenge start.

3. Results

3.1. Animal characteristics across thermal challenges

Physical characteristics are presented in Table 1. Animals ranged in age from 32 d to 46 d (39 ± 1 d), and weighed 337.0 ± 9.2 g.

3.2. Development and efficacy of thermal stabilization using a water perfused wrap in rodents

The water-perfused thermal wrap proved sufficient for both supporting T_{re} during titration, and in manipulating T_{re} throughout challenges (Figs. 2c and 3c). Post induction heat loss was eliminated using

Table 1
Animal characteristics across heating and cooling challenge iterations.

Protocol	N	Sex	Age (d)	Weight (g)	Comment
Heating	6	M/F[a]	37 ± 1	355.4 ± 50.2	Term animals
	4	M/F**	44 ± 1	323.3 ± 13.2	Preterm animals
Cooling	6	M/F[a]	36 ± 3	349.5 ± 63.5	Term animals
	4	M/F**	44 ± 1	313.5 ± 5.4	Preterm animals

N.b. 'excessive stress' relates to uncompensable thermal loading induced by the heating and cooling challenges. An n of 1 was used in each challenge in *Study I* as the challenge resulted in a fatal outcome. *Study II*, 'inadequate loading' relates to inadequate thermal loading. *Study II* was not replicated beyond n = 1 due to clear limitations in study design.

[a] N = 3 male, 3 female; **N = 2 male, 2 female preterm guinea pigs. Presented as mean ± SEM.

the incubator (39.4 ± 0.7 ^{O}C), though T_{re} decreased to 37.3 ± 0.2 ^{O}C at 0 min/baseline. Preterm animals reacted well to T_{re} maintenance, increasing 0.9 ^{O}C from waking to 39.5 ± 0.3 ^{O}C. At 0 min/baseline preterm T_{re} was 37.4 ± 0.3 ^{O}C.

3.3. Heating challenge

T_{bath} increased at 0.7 ^{O}C. min^{-1} (set rate: 1 ^{O}C. min^{-1}), achieving 44.0 ^{O}C in 15 min and was maintained for 15–20 min. Thermal and cardiorespiratory results are presented in Fig. 2. T_{re} rose at 0.15 ^{O}C. min^{-1} after 5 min of challenge. HR rose 1.1 b min^{-1} (r^2 = 0.98, 310 ± 6 b min^{-1} to 336 ± 4 b min^{-1}) across the first 25 min of challenge at 7 b min^{-1}. ^{O}C^{-1} (r^2 = 0.90), declining after T_{re} exceeded 40.5 ± 0.2 ^{O}C, to complete challenge at 326 ± 20 b min^{-1}. Unlike humans, the relationship between T_{re} and HR of many laboratory species exposed to heat stress, including guinea pigs, does not appear linear (Adolph, 1947). Distal microvascular perfusion increased 91% across the challenge, rising at a rate of 7 PU. min^{-1} (r^2 = 0.83; P = 0.15), while proximal perfusion increased 29% across the challenge at a rate of 1 PU. min^{-1} (r^2 = 0.9; P = 0.60). RR rose 192% across the challenge completing at 197 ± 34 breaths. min^{-1} (P < 0.0001). During recovery phase, T_{re}, HR, and RR returned toward baseline levels within the 30 min (T_{re}: 38.0 ± 0.0 ^{O}C; HR: 327 ± 20 b min^{-1}; RR: 83 ± 15 breaths. min^{-1}).

Blood gas concentrations were markedly altered by the heating challenge (Table 2). pH reduced from 7.464 ± 0.028 at baseline to nadir at 7.384 ± 0.041 at 3 h post challenge. Base excess, corresponding to significant reductions in both P_{CO2} (baseline: 36.3 ± 0.8 mmHg; 10 min post: 29.0 ± 0.7 mmHg; P = 0.01) and HCO_3 (baseline: 26.1 ± 1.1; 10 min post: 20.2 ± 1.0; P = 0.05), reduced immediately post challenge to −4.0 ± 1.5 mmol.L^{-1}. All variables recovered by 24 h post challenge (pH: 7.479 ± 0.023; Base Excess: 3.0 ± 1.2 mmol.L^{-1}; P_{CO2}: 35.9 ± 1.7 mmHg; HCO_3: 26.6 ± 1.0 mmol.L^{-1}; blood glucose: 9.6 ± 0.5 mmol.L^{-1}; Lactate: 2.5 ± 0.1 mmol.L^{-1}).

The sub-group of preterm animals (Fig. 3) displayed significant changes in T_{sk} and T_C with to the heating (R^2 = 0.85, P < 0.0001; R^2 = 0.74, P < 0.0001) and cooling (R^2 = 0.91, P < 0.0001; R2 = 0.82, P < 0.0001) challenges, respectively. Heat stress resulted in a 30.6 ± 11.2% rise in distal perfusion, accompanied by a HR elevation of 11.5 ± 2.6% above baseline (challenge end: 319 ± 10 b. min-1). RR increased 239% above baseline to finish challenge at 197 ± 4 breaths. min^{-1}. With recovery, both HR and RR reduced linearly with time, finishing at 282 ± 7 b min^{-1} and 46 ± 5 breaths. min^{-1} (1.2 ± 2.3% below and 17 ± 26% above baseline, respectively). All preterm animals recovered successfully, rousing from anaesthesia within 5 min of offset and normalising behaviour within 1hr (B.A.R.).

3.4. Cooling challenge

The cooling challenge followed the effective implementation of the heating challenge. Thermal and cardiovascular results are summarized in Fig. 4. Animals began the thermal challenge with T_{re} of 37.6 ± 0.2 ^{O}C (conscious: 38.6 ± 0.5 ^{O}C). During the challenge, T_{bath} reduced −0.6 ^{O}C. min^{-1} to nadir at 25 min (15.9 ^{O}C). T_{re} declined significantly in a biphasic manner (P < 0.0001), reducing −0.08 ^{O}C. min^{-1} until 10 min, then −0.16 ^{O}C. min^{-1} until challenge end (25 min: 33.9 ^{O}C). HR decreased significantly alongside T_{re} at 9 b min^{-1}. ^{O}C^{-1} (r^2 = 0.80; P = 0.0003), beginning challenge at 317 ± 8 b min^{-1} and completing at 285 ± 5 b min^{-1}. Distal microvascular perfusion declined 78% across the challenge, reaching its minimum at challenge end (baseline: 241 ± 87 PU; challenge end: 54 ± 4 PU). Perfusion then recovered back to −11% below baseline levels after 30 min of recovery (215.5 ± 36 PU). RR remained stable across the challenge, beginning at ~48 ± 3 breaths. min^{-1}; P = 0.85). In contrast to C1, all animals recovered following the challenge.

In response to cooling, the sub-group of preterm animals (Fig. 5) exhibited a significant 58.6 ± 10.9% reduction in distal perfusion, reducing from 254 ± 50 PU to 94 ± 13 PU (R^2 = 0.49, P < 0.0001). Proximal perfusion, however, remained stable throughout both challenge and recovery, remaining at 4.1 ± 7.7% above baseline at challenge end and 21.3 ± 10.5% above baseline following recover. HR also remained stable throughout the challenge remaining 2.6 ± 7.1% above baseline at challenge end (baseline: 264 ± 5 b min^{-1}; challenge end: 251 ± 3 b min^{-1}). RR increased, rising 55.7 ± 29.0% above baseline levels to finish challenge at 43 ± 6 breaths. min^{-1}. Following the recovery period, preterm animals remained below baseline across all parameters (HR: −7.6 ± 2.6%; RR: −12.6 ± 15.8%; microvascular perfusion: −24.0 ± 12.5%). All preterm animals recovered successfully.

Blood gas concentrations are presented in Table 2 pH was reduced immediately following challenge (baseline: 7.476 ± 0.027; 10 min post: 7.418 ± 0.011; 3 h post: 7.39-±0.036). Correspondingly, base excess was significantly reduced immediately post challenge (baseline: 2.5 ± 1.0 mmol.L^{-1}; 10 min post: −1.8 ± 1.3 mmol.L^{-1}; P = 0.048), alongside HCO_3 (baseline: 26.0 ± 1.1 mmol.L^{-1}; 10 min post: 22.9 ± 1.3 mmol.L^{-1}; 3 h post: 23.7 ± 1.1 mmol.L^{-1}). Acid-base balance was restored by

Table 2
Blood gas variables (mean ± SEM) following final heating and cooling challenge iterations.

		pH	Base Excess	P_{CO2}	HCO_3	Blood Glucose	Lactate
Heating	Pre	7.464 ± 0.028	2.5 ± 1.5	36.3 ± 0.8	26.1 ± 1.1	8.6 ± 0.4	2.8 ± 0.7
	Post 30 min	7.448 ± 0.029	−4.0 ± 1.5	29.0 ± 0.7**	20.2 ± 1.0*	9.9 ± 1.3	3.9 ± 1.6
	Post 3hr	7.384 ± 0.041	−3.0 ± 2.3	36.9 ± 2.3	22.2 ± 1.9	9.2 ± 0.4	5.3 ± 1.7
	Post 24hr	7.479 ± 0.023	3.0 ± 1.2	35.9 ± 1.7	26.6 ± 1.0	9.6 ± 0.5	2.5 ± 0.1
Cooling	Pre	7.476 ± 0.027	2.5 ± 1.0	35.5 ± 2.7	26.0 ± 1.1	9.7 ± 1.3	3.7 ± 0.5
	Post 30 min	7.418 ± 0.011	−1.8 ± 1.3*	35.4 ± 1.8	22.9 ± 1.3	10.5 ± 1.0	2.9 ± 0.3
	Post 3hr	7.390 ± 0.036	−1.3 ± 1.5	39.1 ± 2.2	23.7 ± 1.1	9.3 ± 0.2	3.0 ± 0.5
	Post 24hr	7.467 ± 0.018	4.0 ± 1.0	38.3 ± 0.7	27.7 ± 0.7	9.5 ± 0.7	2.8 ± 0.1

Units are expressed as per Abbott Point of Care CG4+ cartridge: Base excess: mmol.L^{-1}; P_{CO2}: mmHg; HCO_3: mmol.L^{-1}; blood glucose: mmol.L^{-1}; Lactate: mmol.L^{-1}.
N.b. n = 4 for both heating and cooling challenge iterations. Significance indicates change from baseline, *P = 0.05–0.02. **P = 0.01–0.002.

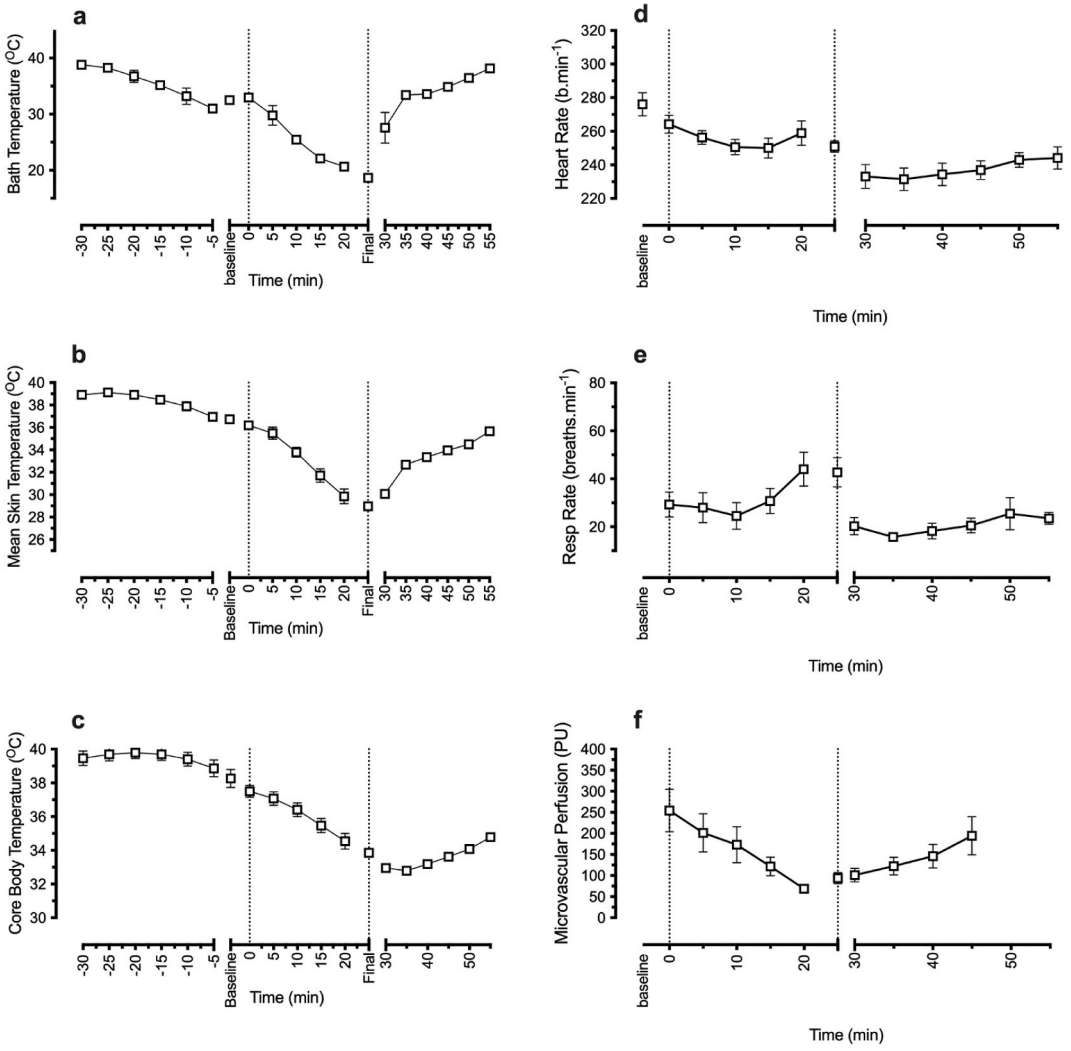

Fig. 5. Preterm thermal and cardiorespiratory response to the final cooling challenge (C2).
Thermal parameters through induction, challenge and recovery phases are presented in panels a/b/c. 'a' represents bath temperature, 'b' represents mean skin temperature, and 'c' represents core body (rectal) temperature. Panels d/e/f present cardiorespiratory parameters during the thermal challenge and recovery phases. 'd' represents heart rate change, 'e' represents respiratory rate, and 'f' represents microvascular perfusion at the distal skin site. Data are presented as mean ± SEM. Error bars on thermal parameters are present, but extremely tight.

24 h post challenge (pH: 7.467 ± 0.018; Base Excess: 4.0 ± 1.0 mmol. L^{-1}; P_{CO2}: 38.3 ± 0.7 mmHg; HCO_3: 27.7 ± 0.7 mmol.L^{-1}; blood glucose: 9.5 ± 0.7 mmol.L^{-1}; Lactate: 2.8 ± 0.1 mmol.L^{-1}).

4. Discussion

We have successfully used a water perfused wrap, modified for the guinea pig, to demonstrate: 1) a non-invasive method for stabilizing T_{re} during anaesthetic induction using a thermoneutral environment (incubator ≈ 39 ^{O}C ≈ T_C) in conjunction with high wrap settings (39 ^{O}C ≈ T_C); 2) we have set upper and lower T_{re} limits for the safe recovery of a small animal model from thermal stress (41.5 ^{O}C and 34 ^{O}C, respectively); and 3) established a physiologically meaningful challenge capable of reproducibly eliciting significant cardiovascular stress in the guinea pig, a well characterized model for studying human health and disease.

4.1. Stabilization of core body temperature

Anaesthetic agents blunt both thermoregulatory and cardiovascular control in a graded manner to the minimum alveolar concentration of that agent (Constantinides et al., 2011; Seagard et al., 1983; Yang et al., 2014). While anaesthetic agents blunt thermoregulatory thresholds (vasoconstriction (Goto et al., 1999; Xiong et al., 1996); shivering (Imamura et al., 2003)), these effects can be minimized by reducing the dose or mixing the agent with an adjuvant to offset its depressive effects (e.g., adding N_2O to isoflurane (Ozaki et al., 1995; Sixtus et al., 2021; Vahle-Hinz et al., 2007),).

We previously determined that 70% N_2O adjuvant significantly improves cardiorespiratory stability in guinea pigs under isoflurane anaesthesia (Sixtus et al., 2021). This returns thermoregulatory thresholds back toward conscious levels and restores the cardiorespiratory response to physiological challenges. However, induction and titration of anaesthetic agents requires much greater concentrations, and the slow titration method currently employed (to prevent complications associated with anaesthetic gas mixtures and problematic respiratory responses known to guinea pigs (Sixtus et al., 2021)) produced a prolonged state of near abolished thermoeffector thresholds ranges (H1 and C1 baseline T_{re} ≈ 34 ^{O}C; Supplemental Figs. 3 and 5). This necessitated the introduction of a controlled thermoneutral environment (via neonatal incubator set at 39 ^{O}C, roughly equivalent to waking T_{re}) and graded reduction of T_{bath}. Doing so greatly improved thermal stability

prior to maintenance anaesthetic doses. This allowed all animals to begin the challenges with T_{re} close to that of an awake, conscious state.

4.2. Thermoeffector thresholds

All homeothermic mammals regulate and maintain their internal T_C within tight physiological limits using a combination of autonomic and behavioural thermal defences (Gagge and Gonzalez, 2011; Hensel et al., 1974; Lv and Liu, 2007; Morrison, 2016; Romanovsky, 2007; Schlader et al., 2018; Werner, 2010). Between species, the regulated T_C varies little, raising or lowering based on metabolic rate. The guinea pigs' T_C is maintained at around 39 ^{O}C (Fewell et al., 1997; Gordon, 1986). This internal temperature, irrespective of mammalian species, lies very close to the upper limit of survivability but comparatively far from the lower thermal limit (Crandall and Gonzalez-Alonso, 2010; McKechnie and Wolf, 2019). The primary response to deviations in T_C among mammals is cardiovascular (e.g., vaso-dilation or -constriction). While divergent thermoeffectors are recruited between mammalian species, the reliance on sensible heat loss, and associated cardiovascular strain remains functionally comparable (Adolph, 1947).

With heat exposure and insipient hyperthermia, the body transitions from compensable heat stress to uncompensable heat stress as the thermal load exceeds the capacity of thermoeffectors to rectify (Leon and Bouchama, 2015). Compensable heat stress typically becomes uncompensable due to impediments in the heat loss process (e.g., excess clothing, or a water perfused wrap maintained at 44 ^{O}C). As T_C proceeds into uncompensable heat stress (>40 ^{O}C) a cascade of events occurs, including tissue thermal injury, coagulopathy, and uncontrolled systemic inflammation stimulated by progressively leaky membrane barriers (e.g., inflammatory response to endotoxemia) (Leon and Bouchama, 2015). Lethal T_C in experimental animals have been reported to range from ~44.5 ^{O}C in monkeys (Gathiram et al., 1987), 43.5 ^{O}C in cats (Adolph, 1947), 40.5–45.4 in rats (Adolph, 1947; DuBose et al., 1983; Lord et al., 1985), and 43.9 ^{O}C in guinea pigs (Adolph, 1947; Romanovsky and Blatteis, 1996). Despite ceasing the challenge at T_{re} = 42 ^{O}C, T_{re} in H1 continued to rise to peak at 44.7 ^{O}C, even with aggressive cooling. In most cases once initiated, recovery from the cascade of events associated with this level of heat stroke is poor, with multi-organ damage continuing across days to weeks (Leon and Bouchama, 2015). As such, following H1, an upper T_{re} limit of 41.5 ^{O}C was set to ensure further challenges represent a physiologically relevant but recoverable thermal loading. As T_{re} exhibits a temporal delay from 'actual' T_C during dynamic challenges (Taylor et al., 2014), this limit would prevent the latent rise of T_{re} beyond survivable limits (e.g., H3 T_{re} typically rose ~0.5 ^{O}C to 42 ^{O}C following cessation of challenge).

Whereas homeotherms lie close to the upper limit of thermal survivability, the lower limit is less well defined. While hypothermia is, physiologically, comparatively safe, risks obviously remain. In humans, hypothermia begins at 35 ^{O}C (stage 1), with this progressing to stage 2 at 32 ^{O}C, stage 3 at 28 ^{O}C, and stage 4 below 24 ^{O}C (Brown et al., 2012). As guinea pigs have a higher resting T_C (~2 ^{O}C), it is plausible that the hypothermic threshold is similarly shifted. Shivering thermogenesis ceases in stage 2 hypothermia, with invasive rewarming recommended from stage 3 (e.g., extracorporeal membrane oxygenation, cardiopulmonary bypass) (Brown et al., 2012). As such, the lower thermal limit was set at 34 ^{O}C once the limits of hypothermic loading were established. Reversibility of hypothermia with complete recovery of neurological capacity has been demonstrated in isolated cases from as low as 13.7 ^{O}C in humans (Gilbert et al., 2000). Although, in humans cardiac arrest is common below T_C of 24 ^{O}C and organ failure occurs frequently during, or following, re-warming, even at less severe extremes of hypothermia (Brown et al., 2012). As is the case in humans, the excessive hypothermic load in C1 was not survivable (Supplemental Figs. 5 and 6). Modification of the protocol to ensure physiologically meaningful, but not excessive, thermal loading ensured that C2 (Figs. 4 and 5) was a reliable test of multi-organ response to a controlled hypothermic insult.

4.3. Thermal stress

In terms of absolute change in T_C, the final iteration of each challenge proved sufficient to drive uncompensable heat gain (H3, Figs. 2 and 3) and heat loss (C2, Figs. 4 and 5), respectively. Indeed, guinea pigs exposed to both heating and cooling, attained T_{re}'s known to be at the limit of physiological recovery in their respective conditions: heat stroke (>41 ^{O}C) and hypothermia (<35 ^{O}C). Across the iterations of the heating challenge, H1 produced findings similar to that of Adolph (1947) and Romanovsky and Blatteis (1996), whereby sustained thermal loading exceeded the thermal capacity of the guinea pig resulting in lethargy, low body tone, respiratory complications (repeated gasping/agonal breathing), T_C instability and ultimately, an unrecoverable thermal load. Conversely, H2 produced such mild thermal stimulus (T_{re} ~0.2 ^{O}C. min^{-1}) as to be entirely compensable and inadequate for the purposes of examining multi-system responses to a thermal challenge (Crandall and Wilson, 2015). This was rectified in H3, whereby the rapid and sustained increase to 44 ^{O}C did not permit compensation. Additionally, momentum of the rising T_{re} produced a latent rise of 0.5 ^{O}C to ~42 ^{O}C following the challenge. We have shown this remains a recoverable T_C, as also observed in other rodents (Quinn et al., 2015). Interestingly, in humans the association between T_C and HR is directly proportional (e.g., Rowell et al. (1969) in man, HR increased 122% with the same method of whole body heating). But in guinea pigs, the relationship between HR and T_C change was less tightly linked (increasing only 13%, ~40 b min^{-1} above baseline levels in H3). This again appears to corroborate previous findings in guinea pigs, where HR increased only moderately with increased T_{re}, but RR rose promptly under heat stress (Adolph, 1947). Increased respiratory drive, in excess of the change in T_C, is indicative of panting, regardless of whether it is polypneic or hyperventilatory (i.e., physiological or maladaptive) (Romanovsky and Blatteis, 1994, 1996). Examination of hemodynamic factors, including those attributable to heat stress, including systemic inflammation, tissue damage and endotoxins, would further elucidate the thermovascular response (See review for suggested markers of cardiovascular damage (Leon and Bouchama, 2015)).

While less immediately life threatening, cold exposure can induce significant strain on the cardiovascular system through dramatic reductions in cutaneous blood flow, increased central blood pressure, and a flood of circulating catecholamines (Stocks et al., 2004). This response is complicated by increased activity of metabolically hungry tissues for heat production (e.g., shivering, or non-shivering thermogenesis). In the current study, from the onset of cooling vasoconstrictor tone increased dramatically in both C1 and C2. This shift in blood volume from the periphery to the core was accompanied by a reciprocal reduction in HR. While not formally quantified, shivering was observed during cooling. As shivering and non-shivering thermogenesis are among the few upregulated systems during progressive T_C cooling, they are likely the primary drivers of sustained rather than reducing RR (irrespective of anaesthesia (Imamura et al., 2003; Ozaki et al., 1995);). The effects of shivering could be better quantified in the future using electromyography (Zhang et al., 1995). Furthermore, continuous recordings of blood pressure, hemodynamic profiles and circulating factors would elucidate the systemic stress of the cold response. Regardless, this challenge serves as a solid platform for more in-depth physiological examinations of the acute cold response.

4.4. Limitations

Despite efforts to mitigate its impact (Sixtus et al., 2021), the effect of anaesthesia cannot be discounted in any of the measured variables. However, as with all physiological assessments in animal models, researchers must weigh up the cost of sedative effects with the risk of confounding stressors alongside the responsibilities of meeting animal welfare needs. We have previously reported our optimized anaesthetic regime for examining physiological function with minimal

anaesthetic-induced cardiorespiratory depression (Sixtus et al., 2021). Nonetheless, the degree of T_C modulation may have been influenced by the anaesthetic-induced depression of thermoregulatory thresholds. The degree to which these thresholds are affected is ultimately modulated by the anaesthetic dose, and this was minimized by our current anaesthetic regime, with our aim to induce a cardiovascular stress which superseded these anaesthetic-induced physiological limits.

However, by utilizing anaesthesia, a key method of heat loss which may have confounded study results was avoided - that of salivary spreading (McKechnie and Wolf, 2019). This is a strength of the method of testing, both in terms of comparative physiology and in terms of examining the cardiovascular response. Water-perfused suits eliminate the efficacy of evaporative heat loss via sweating in humans by clamping T_{sk} to T_{bath} in a similar manner to how whole-body water immersion eliminates sweating efficacy, as sweat relies on a thermal gradient, convection, and radiation to wick moisture away from the skin. However, when enveloped in a water-perfused suit, each of these modes of heat exchange is severely limited. In humans, therefore, water-perfused wraps negate the evaporative cooling effects of sweat glands, producing a challenge focused on cardiovascular function separate from the most powerful autonomic effector (Brengelmann et al., 1977; Detry et al., 1972; Rowell et al., 1969). In the current study, the wrap is similarly efficacious, as the wrap largely overcomes the buffer provided from fur, by securing the wrap in close contact with the skin (and eliminating 'loft' and air entrapment in the fur). As the animal is anaesthetized, evaporative heat loss from salivary spreading is also removed, meaning the anaesthetized guinea pig is limited to the same cardiovascular mechanisms as humans, albeit with widened interthreshold zones (Foëx, 1988; Goto et al., 1999).

In terms of thermoregulatory measures, the wrap did not perfectly clamp skin- and circulating bath-temperatures. This produced a significant lag in the T_{sk} response following changes in T_{bath}, which may have lessened the magnitude of the challenge. However, the challenge design ensured a predictable and significant T_{sk} was elicited such that T_{re} was manipulated. T_{sk} may have also been influenced by the close apposition of thermistors to the tubing, this confounder was minimized using insulated thermistors with multiple tape layers but cannot be excluded. Additionally, while no effect of brown adipose tissue activity was observed in T_{sk} or perfusion, future studies should consider brown adipose tissue deposition in relation to skin thermistor sites as this may unduly impact interpretation of thermal data. Finally, T_{re} as a measure of dynamic T_C change is admittedly poor (Taylor et al., 2014). Of all 'gold standard' measures, T_{re} exhibits the greatest phase delay (other gold standard measures including nasoesophageal, gastrointestinal via radiotelemetry, or pulmonary artery (Taylor et al., 2014)). Unfortunately, there are few valid non-invasive methods for obtaining a corollary of T_C in animal models, with T_{re} proving the best, most cost-effective, and versatile measure of T_C. It should be noted that in humans, despite a greater range of available non-invasive measures of T_C (Fogt et al., 2017; Taylor et al., 2014), T_{re} remains frequently used during dynamic stress tests, largely for the same reasons as with animal models. Critically, any delay in T_{re} would only underestimate the magnitude of the challenge currently used.

5. Conclusions

We have developed a novel physiologically relevant assessment of cardiovascular function allowing for multiparameter assessment in response to a physiological stressor: environmental heat and cold. By challenging both ends of the thermovascular response, we are able to examine a wide range of systemic responses across the cardiovascular system, especially the vasculature. In developing this thermal challenge, we have overcome significant hurdles, including stabilizing the T_C effects of anaesthetic induction agents, and producing a safe, reproducible. Finally, this methodology permits dynamic examination specifically of the vasculature under physiological stress, which is not feasible under exercise modalities. This will allow for much better characterization of the integrated vascular stress response alongside the central cardiovascular response in small animal models of human disease and disease risk.

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Competing interest

The authors have declared that no competing interests exist.

CRediT authorship statement

Conception and design of study: RP Sixtus, CL Gray, MJ Berry, RM Dyson; Acquisition of Data: RP Sixtus, RM Dyson; Analysis and/or interpretation of data: RP Sixtus, RM Dyson; Drafting the manuscript: RP Sixtus, RM Dyson; Revising the manuscript critically for important intellectual content: RP Sixtus, RM Dyson, CL Gray, MJ Berry; Approval of the version of the manuscript to be published: RP Sixtus, CL Gray, MJ Berry, RM Dyson.

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Appendix A. Supplementary data

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