

1 **Systemic application of the TRPV4 antagonist GSK2193874 induces tail vasodilation in a**
2 **mouse model of thermoregulation**

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15 **Abstract**

16 In humans the skin is a primary thermoregulatory organ, with vasodilation leading to rapid
17 body cooling, whereas in Rodentia the tail performs an analogous function. Many
18 thermodetection mechanisms are likely to be involved including transient receptor potential
19 vanilloid-type 4 (TRPV4), a widely distributed ion channel with both mechanical and
20 thermosensitive properties. Previous studies have shown that TRPV4 can act as a
21 vasodilator by local action in blood vessels, and in this study, we investigated whether
22 TRPV4 activity effects *mus musculus* tail vascular tone and thermoregulation. We
23 measured tail blood flow by pressure plethysmography in lightly sedated *mus musculus*
24 (CD1 strain) at a range of ambient temperatures, with and without intraperitoneal
25 administration of the blood brain barrier crossing TRPV4 antagonist GSK2193874. We also
26 measured heart rate and blood pressure with and without GSK2193874. As expected for a
27 thermoregulatory organ, we found that tail blood flow increased with temperature.
28 However, unexpectedly we found that the TRPV4 antagonist GSK2193874 increased tail
29 blood flow at all temperatures, and we observed changes in heart rate variability. Since
30 TRPV4 activation stimulates the relaxation of peripheral resistance arteries (vasodilation)
31 that would increase tail blood flow, these data suggest that increases in tail blood flow
32 resulting from the TRPV4 antagonist may arise from a site other than the blood vessels
33 themselves, perhaps in central cardiovascular control centres such as the hypothalamus.

34 **Introduction**

35 Thermoregulation is one of the defining homeostatic processes common to mammals; core
36 body and brain temperatures are well maintained despite challenges such as changing
37 ambient temperature and exercise to the degree that brain temperature rarely changes
38 outside of a 3 °C range [1-3]. Mammals detect temperatures at both central and peripheral
39 sites and responses to changing temperatures can result both from local responses and
40 central, hypothalamus-co-ordinated autonomic responses [4-6]. Typical thermogenic
41 effector mechanisms include liver thermogenesis and skeletal muscle shivering whereas
42 cooling mechanisms including behavioural changes and redistribution of blood from core to
43 peripheral vessels [4, 5, 7]. Rodents use basal metabolic rate and non-shivering
44 thermogenesis as their principle mechanisms for heat production, mainly because of their
45 small size [8]. In terms of heat loss, transfer of excess heat to the environment is facilitated
46 by so-called heat transfer zones, which are usually found at the body extremities, for
47 example, in humans, typically, acute heat loss is mediated by redistributing blood to
48 cutaneous vascular beds [5]. The location of critical heat transfer zones are somewhat
49 species specific, so for example, the ear for elephants and rabbits [9, 10], head vasculature
50 in large dinosaurs [11, 12] and the feet [13] and tail for rodents [14-16]. The tail of rodents
51 is ideal as a heat transfer zone due to its glabrous nature [16]. It thought that
52 vasoconstriction rather than counter-current heat exchange provides the major barrier to
53 core-to-tail heat flow [17].

54 In this work, we have investigated the role of *mus muscularis* TRPV4 in this homeostatic
55 system using a potent and selective TRPV4 inhibitor, GSK2193874. TRPV4 is one of several
56 temperature sensitive ion channels and expressed in both the hypothalamus and the
57 vasculature, in both smooth muscle and endothelial cells. Recently, there has been
58 considerable interest in the immune, neuromodulatory, cardiovascular and
59 thermoregulatory potential of small molecule TRPV4 modulatory drugs, such as
60 GSK2193874 and HC-067047 [18-25].

61 TRPV4 is a relatively non-selective Ca²⁺ channel (PCa/PNa 6-10) that was first characterised
62 as mechanosensory [26, 27], however, it is also activated by temperatures >30 °C, and so, at
63 physiological temperatures, it would be expected to be constitutively active under basal
64 conditions [28-30]. Activation of TRPV4 leads to vasodilation [31-34] and logically, therefore,

65 transgenic elimination of TRPV4 (TRPV4^{-/-} knock out) would be expected to increase blood
66 pressure, but it does not [18, 31].

67 The precise contribution of TRPV4 to thermosensing and thermoregulation *in vivo* remain
68 unclear. No changes in escape latency from heat stimuli were observed in the hotplate
69 challenge [35, 36]. However, post subcutaneous injection of capsaicin or carrageenan,
70 TRPV4^{-/-} mice showed longer escape latencies from the hot surface compared to wild-type
71 [36]. In another study, it was shown that TRPV4 is required for normal thermal
72 responsiveness *in vivo*; on a thermal gradient, TRPV4^{-/-} mice selected warmer floor
73 temperatures. In addition, TRPV4^{-/-} mice also exhibited prolonged withdrawal latencies
74 during acute tail heating [37].

75 In terms of pharmacological manipulations, activation of TRPV4 with topological RN1747
76 decreased core temperature of *rattus norvegicus* and increased tail vasodilatation [38]. The
77 effects of a TRPV4 inhibitor (HC067047), in the same study were mixed with increases of
78 core body temperature with ambients of 26 and 30[°]C, but not 22 and 32[°]C.

79 In this study we had aimed to investigate whether the small molecule TRPV4 inhibitor,
80 GSK2193874, would decrease tail vasodilation response to elevated ambient temperatures.
81 As a surrogate for tail vasodilation we used tail blood flow measured by volume
82 plethysmography [39]. We also investigated frequency domain heart rate variability (HRV).
83 HRV is a sensitive tool that assesses the time difference between consecutive heart beats to
84 evaluate autonomic nervous system modulation [40, 41]. Accumulating data suggests that
85 ultra-short-range HRV can be successfully derived from as low as 30s of human ECG [42, 43]
86 and pulse rate variability (estimation of variation in heart rate from *photoplethysmography*)
87 has recently been successfully measured from the rat tail [44]. Potentially, measurement of
88 HRV from tail-cuffs would be a useful 3Rs advancement, since surgery is not required.
89 Therefore, we sought to, for the first time, (a) establish, empirically, the length of heart rate
90 (HR) record necessary for HRV in mice and (b) perform HRV from mouse tail volume
91 plethysmography using the CODA apparatus. HRV reflects homeostasis in thermoregulation
92 and blood pressure control and has been shown to be modulated by thermal stimuli in
93 humans [45].

94 Surprisingly, we found the TRPV4 inhibitor increased tail blood flow when measured above
95 mouse thermoneutrality, and we saw temperature dependent changes in ultra-short-range

96 HRV raising the possibility that TRPV4 ion channels expressed outside of the vasculature, for
97 example in the central nervous system, may also be involved with rodent thermoregulation.
98

99 **Methods**

100 *Animals*

101 Fourteen female adult CD1-mice (Charles River, UK) were used. All experimental procedures
102 were ethically approved by the University's Animal Welfare Committee and performed
103 under a UK Home Office Scientific Procedures licence (70/8746).

104 *Volume pressure plethysmography (VPR) recording*

105 We used the CODA tail volume plethysmography (VPR) system (Kent Scientific, Torrington,
106 CT, USA) on control CD1-mice and mice that had received the selective TRPV4 antagonist
107 GSK2193874. Full details of warming methodology and VPR methods are included in the
108 supplementary materials. Note, all temperatures reported are ambient temperatures read
109 from the thermocouple.

110 *Statistical Analyses*

111 Blood pressure (MAP), heart rate (HR) and blood flow statistical comparisons were made
112 with the nlme package in R, which incorporates a repeated measures design. For HRV
113 statistical comparisons, we used MANOVA in Minitab (PA, USA). $p \leq 0.05$ was taken as
114 significant.

115 *Drugs*

116 Midazolam was supplied by our animal service unit, but GSK2193874 (300 $\mu\text{g}/\text{kg}$, *i.p.*) and
117 DMSO were obtained from Sigma-Aldrich. GSK2193874 was dissolved in DMSO at 20 mg/ml
118 stock then diluted 1:100 before *i.p.* injection (0.2 mg/ml), following [23, 34]. "Control"
119 includes 1% DMSO and volume of injection was dependent upon animal weight.

120

121 **Results**

122 We measured MAP, HR and blood flow (Flow) in 14 animals with and without GSK2193874
123 over the ambient temperature range of 31°C to 36°C. These are plotted in two-factor
124 (treatment, temperature) format and analysed with a repeated-measures, mixed effects
125 design. There was a statistically significant effect of temperature on all parameters
126 measured, MAP (**Figure 1A**: Temperature $F=5.34$, $p\leq 0.05$; Drug $F=0.38$ $p>0.05$, Drug x
127 Temperature $F=0.17$, $p>0.05$), HR (**Figure 1B**: Temperature $F=7.37$, $p\leq 0.05$; drug $F=0.68$
128 $p>0.05$, Drug x Temperature $F=0.23$, $p>0.05$) and tail blood flow (**Figure 1C**: Temperature
129 $F=13.21$, $p\leq 0.005$; drug $F=5.57$, $p\leq 0.05$, Drug x Temperature $F=14.00$, $p\leq 0.0005$). In the
130 cases of HR and MAP there was no significant effect of treatment with the TRPV4 antagonist
131 (GSK2193874). However, with tail blood flow there was both a significant increase with
132 GSK2193874 treatment and a very highly significant interaction between temperature and
133 GSK2193874 treatment.

134 Since we were able to derive beat-by-beat heart rate records for several seconds (for
135 example Supplementary Figure 1), we investigated whether HRV could be captured over
136 such short periods. To test whether this was feasible, we simulated mouse heart rate
137 interval records of decreasing length using a modified version of McSharry *et al.*, 2003 [46]
138 and then measured HRV spectral powers by the Lomb-Scargle method [47, 48] over 3000
139 simulations. **Figure 2A** shows that just a few seconds of ECG are sufficient to obtain a
140 picture of the heart rate variability in a mouse, in so far as, increasing the simulation
141 duration beyond this does not greatly affect the HRV spectra. We therefore measured HRV
142 power in the 0.1 to 1.9Hz bands in our samples of control and GSK2193874 records (**Figure**
143 **2B, C**) and compared these statistically with a MANOVA model, over a range of
144 temperatures. There was no over-all statistical difference with temperature, however there
145 was a statistically different set of spectra between control and GSK2193874 treated spectra.
146 Furthermore, with univariate analyses, there was a significant difference between
147 treatment and control at each individual frequency except the 0.5Hz banding.

148

149 **Discussion**

150 In this work we investigate the role of TRPV4 in rat tail blood flow with a systemic inhibitor
151 of TRPV4, GSK2193874. Surprisingly, we find that tail blood flow is increased by
152 GSK2193874. Inspection of Figure 1C., suggests that there is little effect of temperature, in
153 control conditions, above (\geq) 32°C, however the largest numerical increase in flow occurred
154 above 35°C ambient. Possibly suggesting that this quite high ambient temperature was
155 necessary to see the changes in TRPV4 activity. There was a detectable effect also on HRV,
156 but no significant change in blood pressure or HR themselves.

157 *Blood flow, HR and MAP effects*

158 GSK2193874 is a small lipid soluble inhibitor of TRPV4 [19] that crosses the blood-brain
159 barrier well (brain:plasma ratio = 0.6, personal communication with Dr David Behm of GSK)
160 and so there are several locations at which TRPV4 could potentially influence the control of
161 blood flow in response to elevated temperatures. A non-exhaustive list of possible sites of
162 action could include the vasculature or cardiovascular control neurones.

163 TRPV4 is expressed in both vascular smooth muscle and the endothelial cell lining [49].
164 Activation of these channels leads to clear vasodilatation. Whilst the mechanism of this
165 vasodilatation is complex, involving both endothelial and smooth muscle cells, potential
166 release of EDRF/EDHF and, ultimately, small local increases of Ca^{2+} activate potassium
167 channels which hyperpolarize the muscle cells and allow relaxation/vasodilatation [31-33]. A
168 TRPV4 inhibitor would therefore be expected to cause vasodilation (or have no effect if
169 there was no constitutive TRPV4 activity) and so it seems unlikely the increase in tail blood
170 flow we report in this study results from direct action on the vasculature. Furthermore, if
171 the effect of GSK2193874 were primarily on blood vessels to cause dilation, we would have
172 expected to see an over-all drop in MAP, and possibly then a reflex increase in HR since the
173 baroreceptor loop features in established mechanisms of cardiovascular control as well as,
174 specifically, thermoregulation [50, 51]. We saw no change in blood pressure or heart rate,
175 although multivariate analysis detected a small change in short-range HRV analysis. The
176 potential for us to have missed such a baroreceptor mediated effect due Type II errors is
177 discussed in the *limitations* section below.

178 A second location of TRPV4 channels that may be of relevance is the central nervous
179 system, for example the hypothalamus [52]. It is known that other transient receptor

180 potential channels influence the cardiovascular system via changes in sympathetic activity
181 [53, 54]. Our own work shows that TRPV4 channels are located on pre-autonomic neurones
182 of the hypothalamic paraventricular nucleus (PVN) and can influence cardiovascular control
183 in response to osmotic challenge [55, 56] and this effect was abolished with a TRPV4
184 inhibitor [55]. At the neuronal level, we have shown that the action current frequency of
185 parvocellular PVN neurones is dramatically reduced when TRPV4 channels are inhibited
186 [56]. To date, there have been no studies that have explored thermoregulatory roles for
187 TRPV4 in central cardiovascular control neurones.

188

189 *HRV effects*

190 HRV analysis is an increasingly common method for cardiovascular assessment. In humans,
191 for example, decreased HRV (i.e., a very steady pulse) is an independent predictor of cardiac
192 mortality [57]. In animals too it is proving increasingly useful in a range of contexts
193 including phenotyping transgenic animals [58], investigating cardiovascular effects of drugs
194 [59] and predicting arrhythmias [60]. Whilst there are many papers analysing HRV in mice
195 using radiotelemetry [61] we investigated here whether it was possible to do this with VPR. It
196 has previously been shown that relatively long *photoplethysmography* recordings could be
197 used for HRV, with high accuracy, but the present study is the first to systematically analyse
198 how long a recording needs to be. The derivation of this short-range HRV from non-invasive
199 apparatus may prove a useful advance in 3Rs. Since the average mouse heart rate is some 8
200 times that of a human, an 8s segment would be equivalent to the standard 1 minute of
201 recording necessary to detect higher frequency components of human ECG [41]. Here,
202 simulation shows that periodograms from very short segments of ECG are similar to that of
203 conventional 1-minute records (Figure 2A), and these data themselves and this approach
204 may be of field interest. In terms of the response to temperature, we did not see an overall
205 effect on HRV, probably because temperature typically effects the low frequency powers,
206 beyond the scope of ultra-short-range recording [45], however, GSK2193874 did
207 significantly alter overall frequency power.

208

209 *Limitations*

210 We measured only ambient temperature and not core temperature. We felt that the loss of
211 this important information was necessary to avoid the disturbance of rectal thermocouple

212 of mice in the non-invasive recording equipment. Also, we used sedation that could
213 influence the whole animal responses and only *female mice*, unlike many males only studies
214 [23, 34]. Furthermore, to keep the study manageable, we opted for a one antagonist dose
215 study rather than a full in vivo dose-response curve, which would have been useful. It is
216 difficult to predict accurately the local concentration that an ion channel will “see” when a
217 drug given systemically will reach, but if we assume that GSK2193874 has a typical volume
218 of distribution of between 1 and 10L/kg, our 300µg/kg dose would translate to
219 approximately 40 to 400nM, in the order of the maximal dose for GSK2193874 on TRPV4
220 channels [23]. Although GSK2193874 is highly selective for TRPV4 compared to the other
221 200+ proteins it has been assayed against [23], repeating our studies with TRPV4-/- lines
222 [31] would be the only way to confirm with certainty that the true target was indeed TRPV4.
223 We encountered technical challenges too, e.g., recording VPR data below 30°C (ambient)
224 was unreliable, so we report a relatively limited temperature range rather than strictly hot
225 vs cold. These limitations could be addressed by a telemetric study, but large motivation for
226 our current approach was to utilise a non-invasive blood pressure design, for 3Rs ethical
227 reasons. Furthermore, as in many physiological studies, statistical power was an issue. Our
228 initial design (see supplementary information) included a power analysis for heart rate and
229 blood pressure; which made a number of assumptions but passed 80% power with around 8
230 or 9 animals. We then used 14, however, we were not able to get all conditions for all
231 animals and so the final statistical power could be below 80%. We have hypothesised that
232 an increase of blood flow, by TRPV4 antagonist in the absence of significant changes in
233 MAP/HR would be compatible with a central mechanism of vasodilation. However, if we
234 simply missed changes due to a type II error, the vasodilation could result from baroreflex-
235 mediated mechanisms. This could be addressed by either increasing animal numbers or by
236 repeating similar experiments with surgical or pharmacological block of the baroreceptor
237 reflex [62].

238

239 In conclusion, this whole animal study shows that a TRPV4 antagonist has a significant effect
240 on tail blood flow, in the context of thermoregulation, but its site of action, and the
241 mechanism of such modulation remain to be determined. We also demonstrate non-
242 invasive measurement of frequency domain HRV analysis from very short-range data that
243 may prove useful in future 3Rs friendly research.

244

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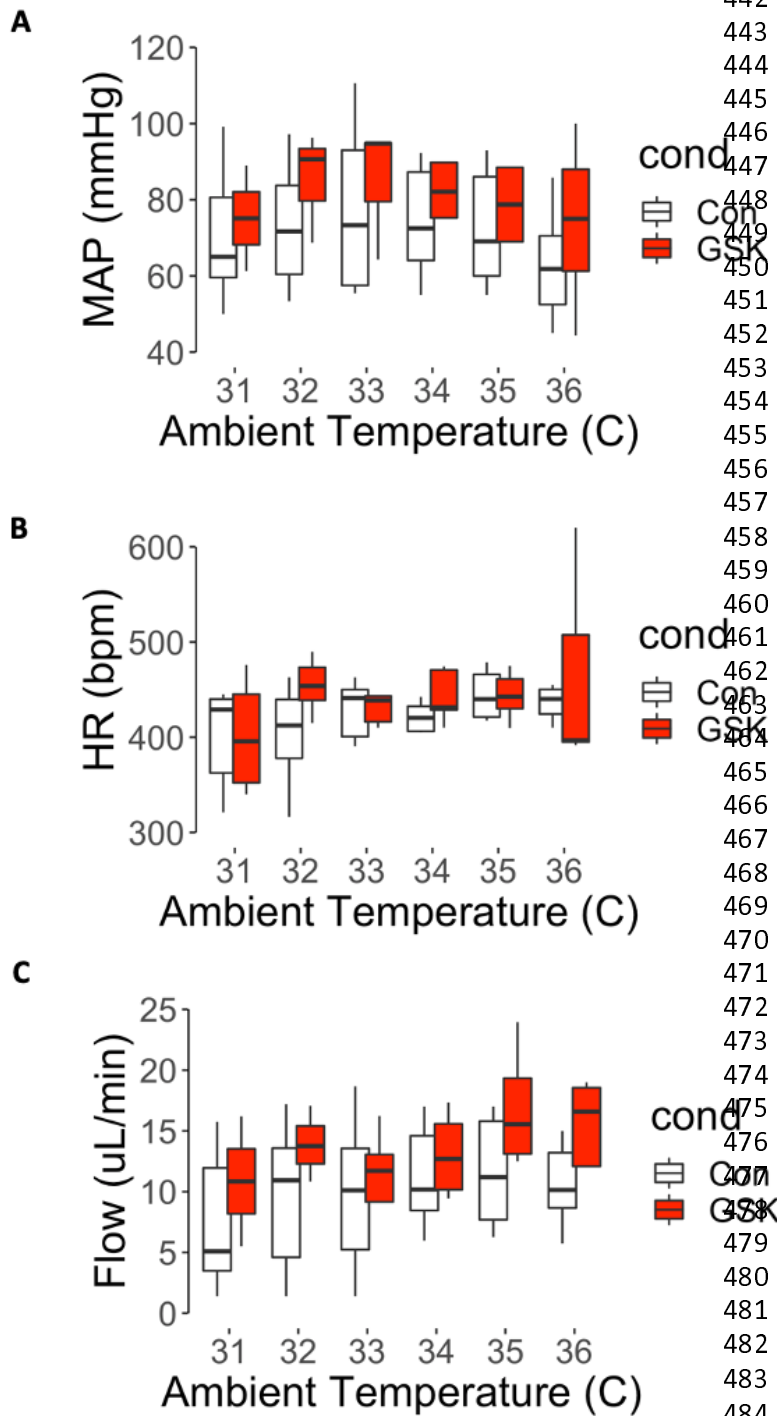
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- 440

441 **Figures**



442 **Figure 1. Effects of**
443 **Pharmacological inhibition**
444 **of TRPV4 on blood**
445 **pressure, heart rate and**
446 **blood flow.**

447 (A) Mean arterial pressure
448 at a range of ambient
449 temperatures in control and
450 GSK2193874, there was a
451 significant association with
452 temperature, but not drug.
453 There was also no
454 significant association
455 between temperature and
456 drug (Mixed effects model:
457 See main text for details).

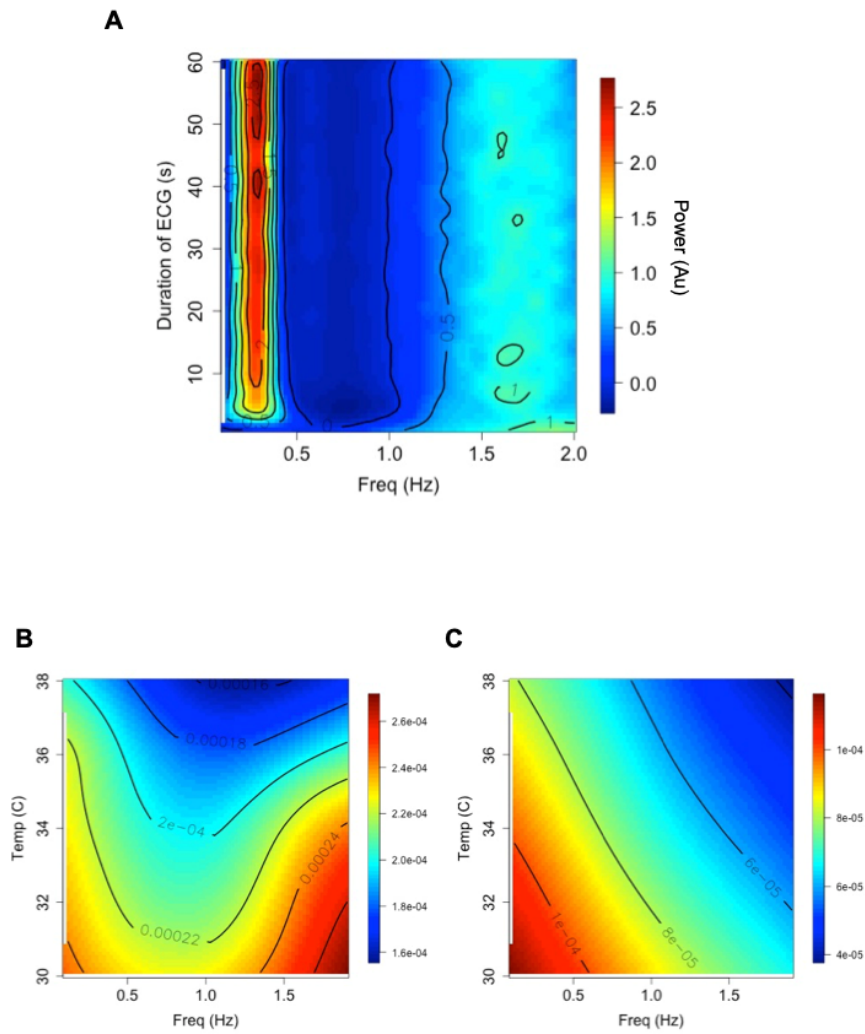
458 (B) Mean heart rate across
459 a range of ambient
460 temperature in control and
461 GSK2193874, over-all there
462 was a significant change of
463 heart rate with
464 temperature, but no
465 significant difference with
466 drug and no significant
467 association between drug
468 and temperature (Mixed
469 effects model: See main
470 text for details).

471 (C) Tail blood flow across a range
472 of ambient temperature in
473 control and GSK2193874.
474 Overall, there was a
475 statistically significant
476 increase of blood flow with
477 temperature and with
478 GSK2193874 and a
479 significant interaction
480 (Mixed effects model: See
481 main text for details).
482 Overall, $n = 14$ animals or
483 for each temperature; 31°C
484 $n = (9,4)$ 32°C $n = (10,4)$,

485 33°C $n = (11,6)$, 34°C $n = (10,7)$, 35°C $n = (11,7)$ and 36°C $n = (12,5)$. To investigate specific
486 temperature points that were different to the 31°C value, we treated temperature as a factor and
487 ran the estimated-marginal means method with the R-package Emmeans. This consists of 66
488 pairwise comparisons and we used Benjamini-Hochberg multiple comparison correction. In control,
489 no individual flow is significantly greater than that at 31°C however, in GSK2193874, blood flow at
490 both 35°C and 36°C was significantly greater than at 31°C ($p < 0.05$).

491

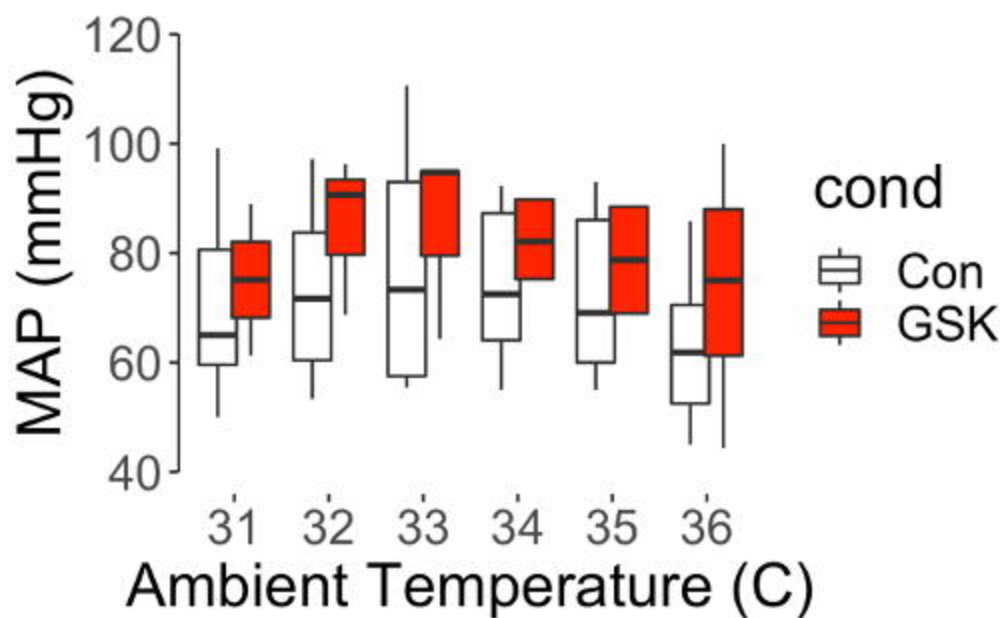
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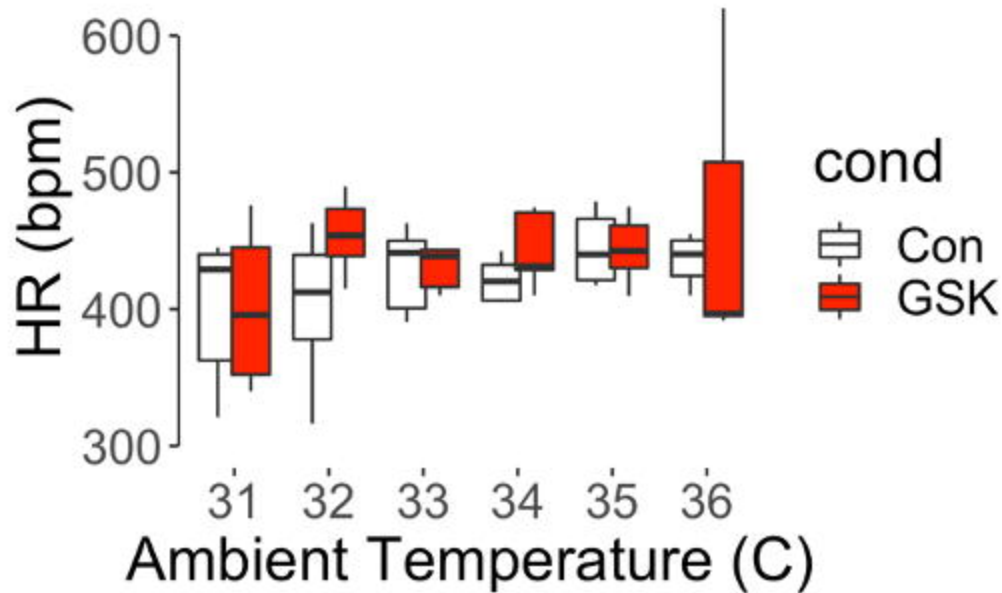
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494 **Figure 2. Short range heart rate variability analysis. (A)** Stacked Lomb periodograms for
495 3000 simulated ECG inter-beat interval datasets. Y-axis is the duration of the simulated ECG
496 record, X-axis is the frequency component of each Lomb-Scargle. The periodograms have
497 been normalized and scaled therefore the power (colour bar on the right) is in arbitrary
498 units. Below are periodogram surfaces recorded at different temperatures under control
499 **(B)**, or after injection GSK2193874 **(C)**. Power given in the scale-bars. MANOVA (Minitab)
500 analyses show these two distributions are significantly different, Wilks lambda $p < 0.05$.
501 Overall, $n = 14$ animals.

A



B



C

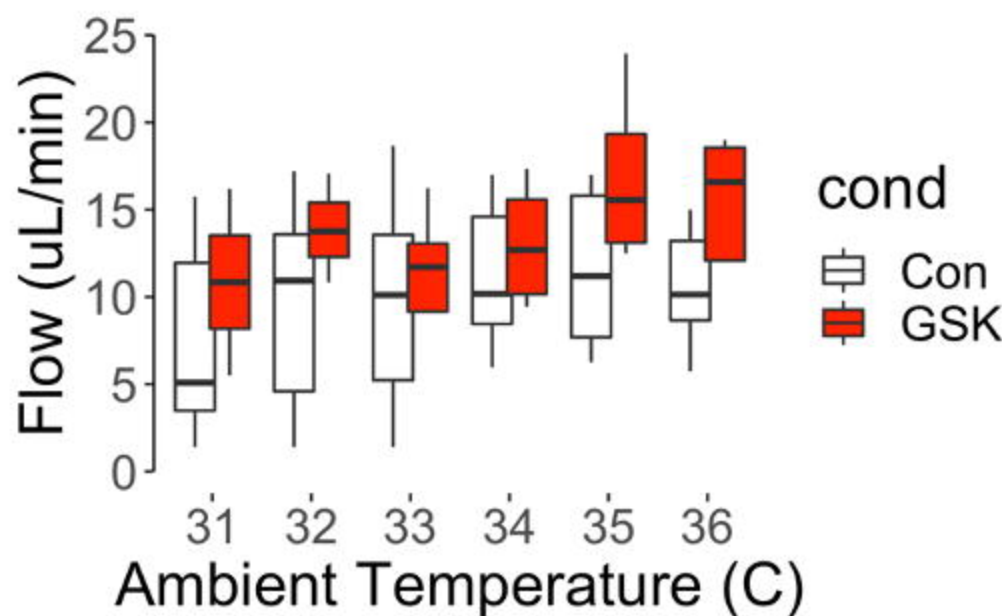
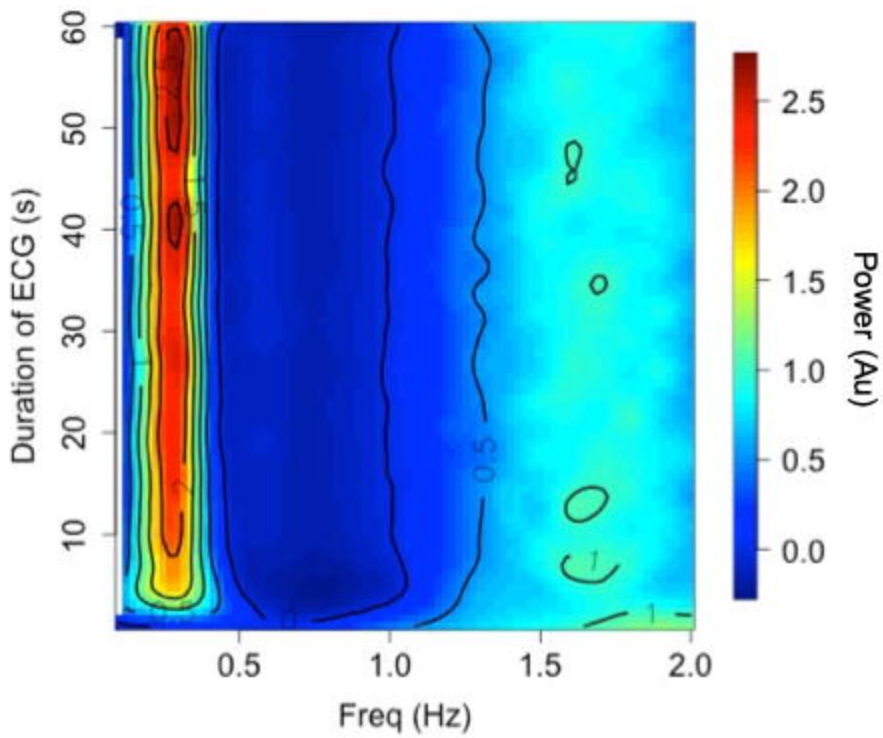
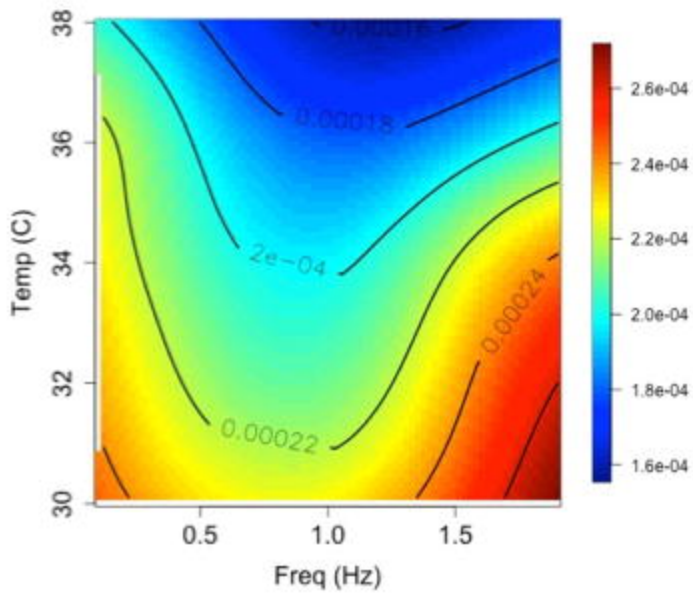


Figure 1

A



B



C

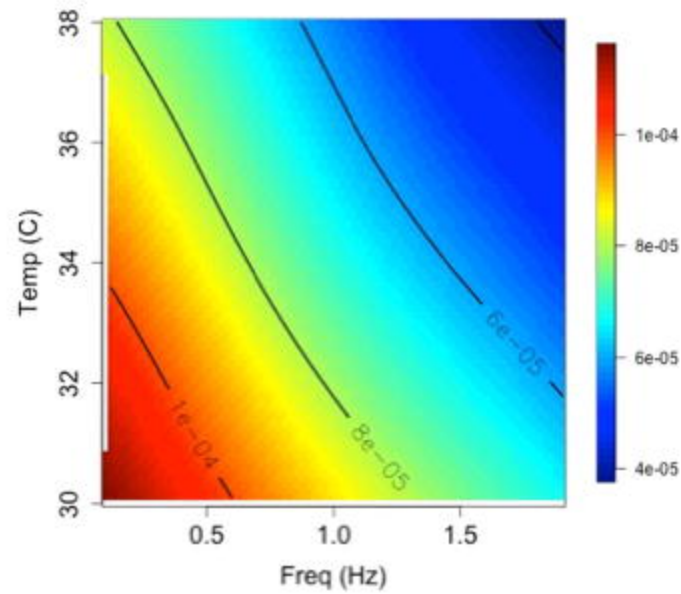


Figure 2