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

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Abstract

In humans the skin is a primary thermoregulatory organ, with vasodilation leading to rapid body cooling, whereas in rodents the tail performs an analogous function. TRPV4 is a widely distributed ion channel with both mechanical and thermosensitive properties. Previous studies have shown that TRPV4 can act as vasodilator by local action in blood vessels, and in this study we investigated whether there was a constitutive role for TRPV4 in mouse tail vascular tone and thermoregulation.

We measured tail blood flow by pressure plethysmography in lightly sedated mice at a range of ambient temperatures, with and without intraperitoneal administration of the blood brain barrier crossing TRPV4 antagonist GSK2193874. We also measured heart rate and blood pressure with and without GSK2193874.

As predicted, we found that tail blood flow increased with temperature. However, unexpectedly we found that the TRPV4 antagonist GSK2193874 increased tail blood flow at all temperatures.

Since arterial TRPV4 activation is known to cause vasodilation that would increase tail blood flow, these data suggest that increases in tail blood flow resulting from the TRPV4 antagonist must arise from a site other than the blood vessels themselves, perhaps in central cardiovascular control centres such as the paraventricular nucleus of the hypothalamus.

Introduction

Thermoregulation is one of the key homeostatic processes common to mammals. Strict control of temperature is critical to a number of biological processes such as enzyme function and neuronal activity. The body core and brain temperatures are well maintained despite challenges such as changing ambient temperature and exercise to the degree that brain temperature rarely changes outside of a 3 ˚C range (Fuller, Carter, & Mitchell, 1998; Fuller et al., 1999; Walters, Ryan, Tate, & Mason, 2000). Typically, animals detect temperatures at both central and peripheral sites and responses to changing temperatures can result both from local responses and central, hypothalamus-co-ordinated autonomic responses (Hemingway & Price, 1968; Liedtke, 2017; Tan & Knight, 2018). In turn, a temperature regulating effector system acts to return the core temperature (Tc) towards the set point (Hammel, 1968), which changes with time of day (Alawi et al., 2015; Lee, Iida, Mizuno, Suzuki, & Caterina, 2005; C. A. Sand, A. Starr, M. Nandi, & A. D. Grant, 2015; Tan & Knight, 2018). Typical thermogenic effector mechanisms include liver thermogenesis, skeletal muscle shivering whereas cooling mechanisms include counter-current mechanisms in some species, behavioural changes and redistribution of blood from core to peripheral vessels (Hammel, 1968; Hemingway & Price, 1968; Tan & Knight, 2018).

In humans, typically, acute heat loss is mediated by redistributing blood to cutaneous vascular beds (Hemingway & Price, 1968), but in rodents, this role is largely fulfilled by the tail (Grant, 1963). The tail is ideally suited to this role due to its glabrous nature, but unlike the similarly glabrous ear it has the necessary arterio-venous anastomoses (Grant, 1963). Vasoconstriction and dilatation of the tail arteries are in turn controlled by increases and decreases (respectively) of tail sympathetic nerve activity (SNA) (Shafton, Kitchener, McKinley, & McAllen, 2014). There is a strong correlation between each of abdominal temperature and tail SNA, skin temperature and tail SNA and brain temperature and tail SNA, but the latter has the steepest correlation slope (Shafton et al., 2014)). In this work, we have investigated the role of TRPV4 in this homeostatic system using a potent and selective TRPV4 inhibitor, GSK2193874. TRPV4 is one of several temperature sensitive ion channels and expressed in both the hypothalamus and in the vasculature in both smooth muscle and endothelial cells. Recently, there has been considerable interest in the immune, neuromodulatory, cardiovascular and thermoregulatory potential of small molecule TRPV4

modulatory drugs, such as GSK2193874 and HC-067047 (Cheung et al., 2017; Dias et al., 2019; Li et al., 2019; C. A. Sand et al., 2015; Shannon et al., 2020; Thorneloe et al., 2012; Thorneloe, Cheung, Holt, & Willette, 2017; Zhu & Pei, 2018). TRPV4 is a relatively non-selective Ca^{2+} channel (PCa/PNa 6-10) that was first characterised as mechanosensory (Voets et al., 2002; Watanabe et al., 2002). However, it is also activated by temperatures above approximately 30 ˚C, and so would be expected to be constitutively active, under basal conditions (Chung, Lee, & Caterina, 2003). Nevertheless, activation of TRPV4 leads to vasodilation (Campbell & Fleming, 2010; Earley et al., 2009; Filosa, Yao, & Rath, 2013). This is the opposite to what one might expect for activation of a Ca^{2+}/non-specific cation channel in the vasculature, since they may be expected to induce depolarisation which in turn induces contraction of vascular smooth muscle (Nelson & Quayle, 1995). The anomaly can easily be explained as a relatively conserved intracellular coupling between TRPV4 and Ca^{2+} activated potassium channels, seen in a wide range of cells such as chondrocytes, neurones, endothelial and smooth muscle cells (Earley et al., 2009; Feetham, Nunn, Lewis, Dart, & Barrett-Jolley, 2015; Lewis et al., 2011; Nilius & Droogmans, 2001; Sonkusare et al., 2012). A small influx of Ca^{2+} is insufficient to cause depolarisation, but sufficient to activate nearby Ca^{2+}-activated potassium channels (Fakler & Adelman, 2008), which lead to hyperpolarisation and a reduction in over-all Ca^{2+} entry. Additionally, TRPV4 action on endothelial cells has been proposed to induce both direct coupling to vascular smooth muscle cells (and dilatation) and release of endothelially derived relaxant factors such as EDRF/EDHF (Campbell & Fleming, 2010; Earley et al., 2009). There is a consensus amongst several studies that TRPV4 activation is vasodilatory. Therefore, the logical extension of this body of evidence would be that transgenic elimination of TRPV4; TRPV4 -/- knock out would increase blood pressure, but it does not (Earley et al., 2009). Although counter-intuitive data could be explained as resulting from potential compensatory changes of expression in related ion channels, paradoxically, general linear model analyses of the publicly available data from Sand *et al.,* 2015 (C. A. Sand et al., 2015) shows that over a 24hr period mean arterial blood pressure is lower in TRPV4 -/- mice (Claire A. Sand, Anna Starr, Manasi Nandi, & Andrew D. Grant, 2015). Furthermore, in terms of temperature regulation, TRPV4 -/- does not have altered core temperature. On the other hand, activation of TRPV4 with topological RN1747 decreases core temperature of rats and increased tail vasodilatation (Vizin et al., 2015). The effects of a TRPV4 inhibitor (HC067047), in the same

study were mixed with increases of core body temperature with ambients of 26 and 30°C, but not 22 and 32°C.

In this study we therefore had a straightforward aim; to investigate whether the small molecule TRPV4 inhibitor, GSK2193874, would decrease tail vasodilation at response to elevated ambient temperatures. As a surrogate for tail vasodilation we used tail blood flow measured by volume plethysmography (Feng et al., 2008). Surprisingly, we found the TRPV4 inhibitor increased tail blood flow when measured above mouse thermoneutrality, raising the possibility that the TRPV4 ion channels expressed outside of the vasculature, for example in the central nervous system, may also be involved with rodent thermoregulation.

Methods

Animals

Adult CD1 mice (Charles River, UK) were housed at 22-24°C in a 12 h light/dark cycle-controlled facility at the University of Liverpool animal facilities with *ad libitum* access to food and water. All experimental procedures were performed under a UK Home Office licence (Home Office licence number 70/8746, approved 09/11/2015) and complied with the UK Animals (Scientific Procedures) Act 1986 and were ethically approved by the University's Animal Welfare Committee (AWERB).

Volume pressure recording (VPR) plethysmography

For recording cardiovascular parameters via tail volume plethysmography (VPR), which included blood pressure and tail blood volume (CODA VPR system; Kent Scientific, Torrington, CT, USA), mice were lightly sedated with midazolam (5mg/kg, *i.p*) and kept in thermoneutral conditions (approximately 30°C) during an acclimatisation period. Ambient temperature was increased slowly using heating pads under their housing and insulting the lids. Temperature was monitored by thermocouple during the experiment whilst the CODA device was continuously recording. Readings below 15 ml tail volume displacement were disregarded for tail flow and blood pressure recordings, since accurate readings could not be acquired in this range.

Statistical Analyses

All data on graphs are shown as mean ± SEM. Statistical comparisons were made with general linear model (GLM) or MANOVA in Minitab (Minitab Inc, PA, USA) or using a two-tailed Student's paired t-test as appropriate. A value of $p < 0.05$ was taken as significant.

Drugs

Midazolam was issued by our animal service unit, but GSK2193874 (300 µg/kg, *i.p*) and DMSO were obtained from Sigma-Aldrich.

Results

To investigate the role of TRPV4 in the cardiovascular responses to temperature change, we employed volume pressure recording plethysmography. Using the CODA VPR system, occlusion pressure was recorded in control CD1 mice as well as mice that had received the selective TRPV4 antagonist GSK2193874. A representative example of the occlusion cuff (inflated to 250 mmHg) deflating over 15 seconds in control and GSK2193874 treated CD1 mice is shown in Figure 1A. As pressure in the tail cuff decreases over this time, there comes a point at which blood pressure exceeds the tail cuff pressure and blood enters the tail. This is then detected in a second pressure sensor, the VPR sensor itself that records tail blood volume (change). From such data several features can be captured by the CODA software including blood pressure, tail blood flow, tail volume and heart rate. Figure 1B shows an expanded view of the data in A, and figure C shows how with sufficient amplification and filtering individual pulse can be extracted, and peaks detected to allow heart rate measurement.

Figure 2 shows tail blood flow measurements. Tail flow, measured by the VPR is a surrogate for vasodilation, since in the context of the tail, the greater the dilation the greater the blood flow into the tail. Figure 2A shows the mean tail blood flow at 36˚C just above thermoneutrality (34˚C) for a mouse (Ganeshan & Chawla, 2017). Pharmacological inhibition of TRPV4 with the blood brain barrier permeable drug GSK2193874 (300 µg/kg) significantly increase mean tail blood flow at 36˚C (n=4, *p<0.05) and the sensitivity of this parameter to temperature (n=4, **p<0.01). Note that these parameters often could not be captured at lower temperatures, where tail blood flow becomes too low to acquire reliable readings.

In terms of mean arterial blood pressure (Figure 3A), there were no significant differences between treatment and control over all or at any one temperature. For heart rate there was a small but statistically lower heart rate with GSK2193874 (300 µg/kg) over-all and also at each of 31, 32, 33 and 34˚C (Figure 3B).

Since we were able to derive beat-by-beat heart rate records for several seconds (for example Figure 1B), we investigated whether heart rate variability (HRV) could be captured over such a short period. To test whether this was feasible, we simulated mouse heart rate interval records of decreasing length using a modified version of McSharry et al 2003 (McSharry,

Clifford, Tarassenko, & Smith, 2003) and then measured HRV spectral powers by the Lomb-Scargle method (Lomb, 1976; Scargle, 1982) for these over 3000 simulations. Figure 4A shows that just a few seconds of ECG are necessary to get a reasonably accurate picture of the heart rate variability in a mouse. We therefore measured this in our samples of control and GSK2193874 records (Figure 4B, C) and analyzed these statistically with a general linear model, over a range of temperatures. There was again a small, but statistically different set of spectra between control and GSK2193874 assessed by MANOVA ($p<0.05$).

Discussion

In this work we investigate the role of TRPV4 in rat tail blood flow with a systemic inhibitor of TRPV4, GSK2193874. Surprisingly, we find that tail blood flow is increased by GSK2193874 at temperatures above thermoneutrality. There was a very small, but statistically detectable effect also on heart rate, but no significant changes in blood pressure.

Using a potent drug inhibitor rather than constitutive transgenic knock down of TRPV4 has the advantage that, realistically, in the time frame of an experiment there will be no compensatory expression of functionally related proteins. The trade-off is selectivity. Whilst GSK2193874 is considered highly selective for TRPV4 compared to the other 200+ proteins it has been assayed against (Thorneloe et al., 2012), there could still be unknown off target effects. GSK2193874 is a small lipid soluble inhibitor of TRPV4 (Cheung et al., 2017) that crosses the blood-brain barrier well (brain:plasma ratio = 0.6, personal communication with Dr David Behm of GSK) and so there are several locations at which TRPV4 could potentially influence the control of blood flow in response to elevated temperatures. A non-exhuastive list of possible sites of action could include the vasculature, sensory neurones or cardiovascular control neurones; we will now discuss each of these in turn. The best studied of these potential sites of action is perhaps the vasculature itself. TRPV4 is expressed in both vascular smooth muscle and the endothelial cell lining (White et al., 2016). Activation of these channels leads to clear vasodilatation. Whilst the mechanism of this vasodilatation is complex, involving both endothelial cells and smooth muscle cells, potential release of local transmitters and tight junctions, ultimately, small local increases of Ca^{2+} activate potassium channels which hyperpolarize the muscle cells and allow relaxation/vasodilatation (Campbell & Fleming, 2010; Earley et al., 2009; Filosa et al., 2013). If there were to be no constitutive activity of TRPV4, an inhibitor would have little effect on tail blood flow via this site of action, if however, there is constitutive activity, an inhibitor would be expected to promote channel closure, vasoconstriction and decreased tail blood flow. It therefore seems unlikely the increase in tail blood flow we report in this study results from direct action on the vasculature. Furthermore, if the effect of GSK2193874 were simply on blood vessels to cause dilation, we would have expected to see an over-all drop in blood pressure, and possibly then a reflex increase in heart rate. We saw no change in blood pressure, but there was a small change in heart rate, reflected in both the mean heart rate itself and the short-range HRV analysis.

A second common site of action of TRPV4 channels is sensory neurones (Brierley et al., 2008; Kim et al., 2016) and have been implicated in temperature nocifensive responses, but only in situations of heat hyperalgesia (Todaka, Taniguchi, Satoh, Mizuno, & Suzuki, 2004). Escape latencies (the time it takes before animals avoid a noxious stimuli) at temperatures of 35-50˚C are unaltered in TRPV4 -/- knock-out mice, but these were delayed following induction of heat hyperalgesia. Together, these data offer no evidence that the TRPV4 inhibitor vasodilating effect we see is due to modulation of sensory neurones.

A third location of TRPV4 channels that may be of relevance is the central nervous system, for example the hypothalamus (Feetham, O'Brien, & Barrett-Jolley, 2018). It is known that other transient receptor potential channels do influence the cardiovascular system via changes in sympathetic activity (Alawi et al., 2015; Aubdool et al., 2014). Our own work also shows that TRPV4 influence cardiovascular control in response to osmotic challenge (Feetham et al., 2015), and are located on pre-autonomic neurones of the hypothalamic paraventricular nucleus (PVN). Changes in heart rate and thermoregulatory response independent of blood pressure would likely require involvement of central control neurones, but the effect we observed here was rather small despite being statistically significant. Also this is the first recorded use of HRV analysis from such short segments of heart rate recording. We justify this in two ways; (i) since the average mouse heart rate is some 8 times that of a human a, 8s segment would be equivalent to the 1 minute of recording necessary to detect higher frequency components of human ECG (Malik, 1996). (ii) There is no clear law that states how long a segment should be to faithfully recover a given frequency component, since this is the *opposite* of the Nyquist theorem which sets the minimum sample frequency. The landmark cardiac Electrophysiology Task-Force report stated that “recording should last for at least 10 times the wavelength of the lower frequency” (Malik, 1996), but without evidence. Here, simulation shows that periodograms from very short segments of ECG are similar to that of conventional 1-minute records (Figure 4A), and these data themselves and this approach may be of field interest.

Therefore, in future work it will be important to investigate the behavior of hypothalamic TRPV4 channels in response to changes in temperature.

In conclusion, this non-invasive whole animal study shows that the TRPV4 antagonist GSK2193874 has a significant effect on tail blood flow, in the context of thermoregulation, but its site of action, and the mechanism of such modulation remains to be determined.

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Figure Legends

Figure 1. Non-invasive volume plethysmography recording (VPR) of heart rate, blood pressure and tail volume from CD1 mice.
(A) Representative examples of complete (single) VPR. The system comprises two tail cuffs per animal an occlusion cuff and a VPR-sensor cuff. the occlusion cuff is places at the base of the tail and the VPR sensor cuff over the middle of the tail. Initially the occasion cuff is inflated to approximately 250mmHg. This prevents blood entering the tail and the pressure in the VPR sensor steadily decreases. Once the occlusion cuff drops sufficiently blood returns to the tail and causes activation of the VPR sensor. this is factory calibrated to allow calculation of blood flow/volume. The black trace shows a control experiment following injection of vehicle whereas the red trace shows this in the presence of GSK2193874. The two peaks on the right-hand side are where the tail volume increased. Following the VPR pressure-peak, the pulse pressure can be seen in the VPR-pressure traces. An enlarged view is shown in (B). (C) This section of recording can then be filtered and amplified to allow each pulse pressure peak to be identified (red circles) and further analyzed. This is done automatically by the CODA software, but this figure was created via a MATLAB script that allows more detailed heart rate analysis.

Figure 2. Pharmacological inhibition of TRPV4 increases tail blood flow in CD1 mice
Tail blood flow was measured in the absence and presence of GSK2193874. (A) Tail blood flow was significantly elevated with GSK2193874 alone (at 36˚C) and (B) change of blood flow with temperature (n=4, mean ± SEM, *p<0.05). (C) Representative example mean data showing tail blood volume in control (black) and GSK2193874 treated (red) of CD1 mice as temperature is increased from 34-37 ˚C (n=4, mean ± SEM, **p<0.01).

Figure 3. Effects of Pharmacological inhibition of TRPV4 on heart rate and blood pressure.
(A) Mean arterial pressure at a range of ambient temperatures in control and GSK2193874, there was no significant difference between these two samples (general linear model, GLM, Minitab). (B) Mean heart rate across a range of ambient temperature in control and GSK2193874, over-all there was no change of heart rate with temperature. Overall, there was a small but statistically significant increase of heart rate with temperature with GSK2193874 (GLM p<0.05).

Figure 4. Short range heart rate variability analysis. (A) Stacked Lomb periodograms for 3000 simulated ECG interbeat interval datasets. Y-axis is the duration of the simulated ECG record, X-axis is the frequency component of each Lomb-Scargle. The periodograms have been normalised and scaled therefore the power (colour bar on the right) is in arbitrary units. Below are periodogram surfaces recorded at different temperatures under control **(B)**, or after injection GSK2193874 **(C)**. Power given in the scale-bars. MANOVA (Minitab) analyses show these two distributions are significantly different, Wilks lambda p<0.05.

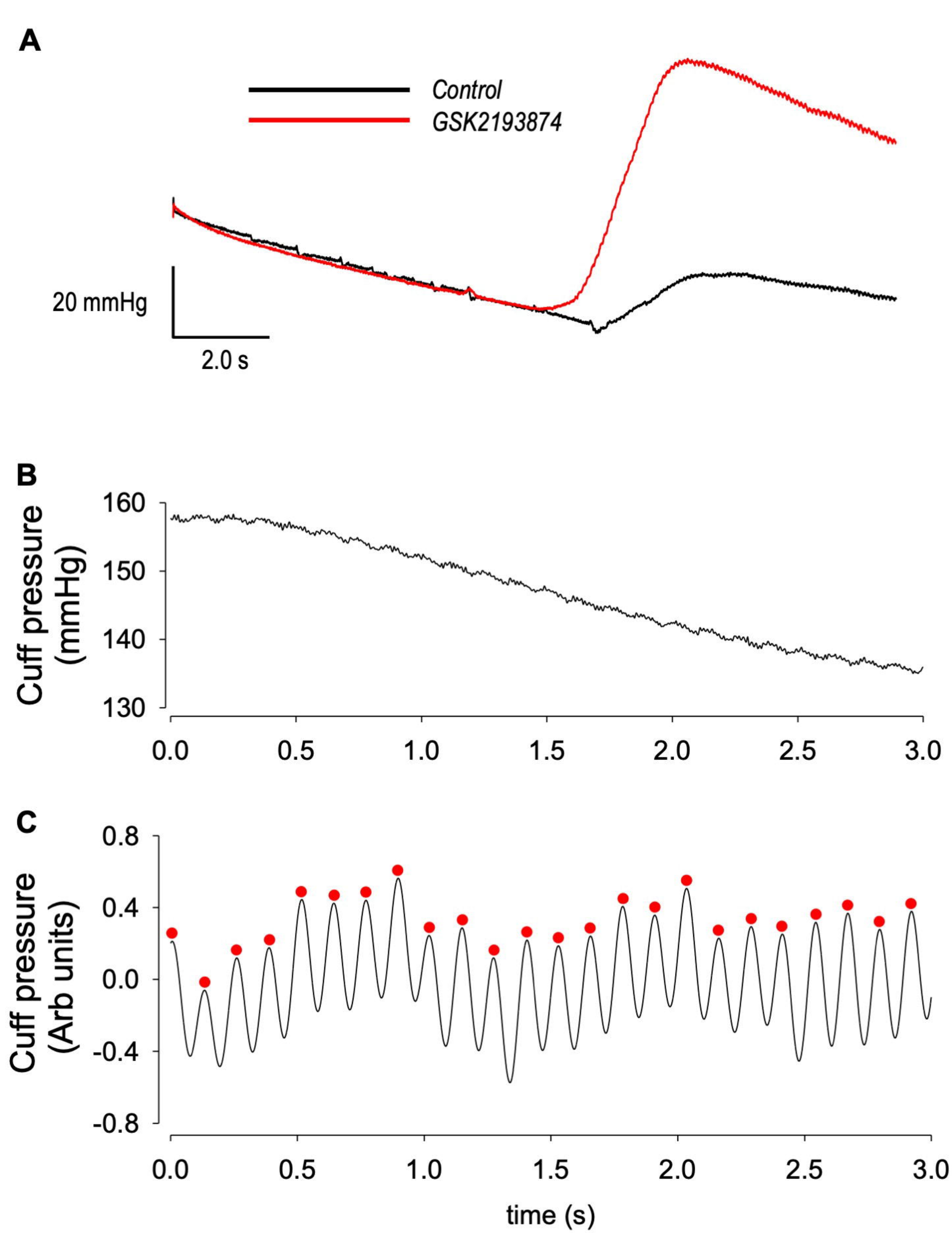

A
Control
GSK2193874
20 mmHg
2.0 s
B
Cuff pressure (mmHg)
160
150
140
130
0.0
0.5
1.0
1.5
2.0
2.5
3.0
C
Cuff pressure (Arb units)
0.8
0.4
0.0
-0.4
-0.8
0.0
0.5
1.0
1.5
2.0
2.5
3.0
time (s)

Figure 1

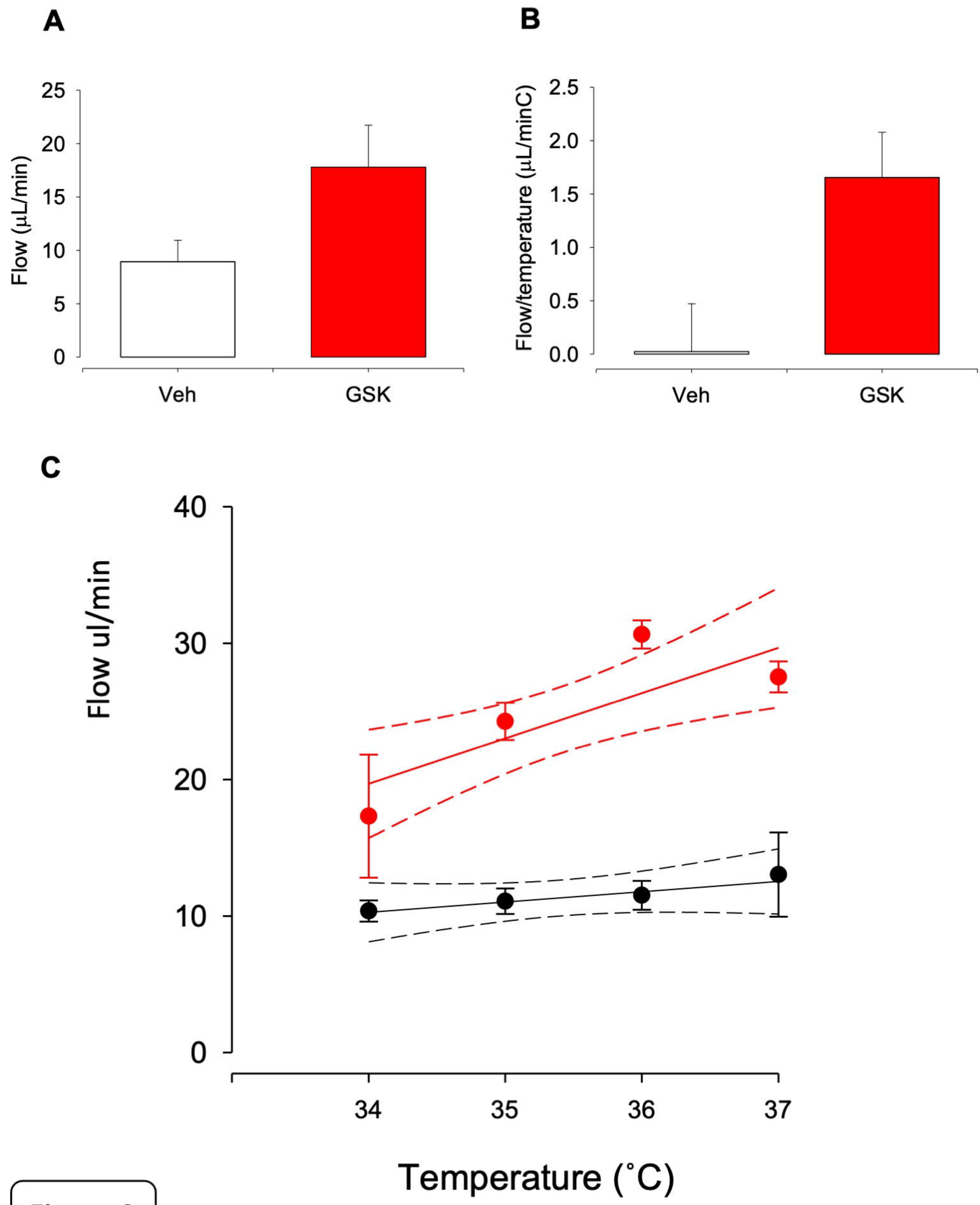
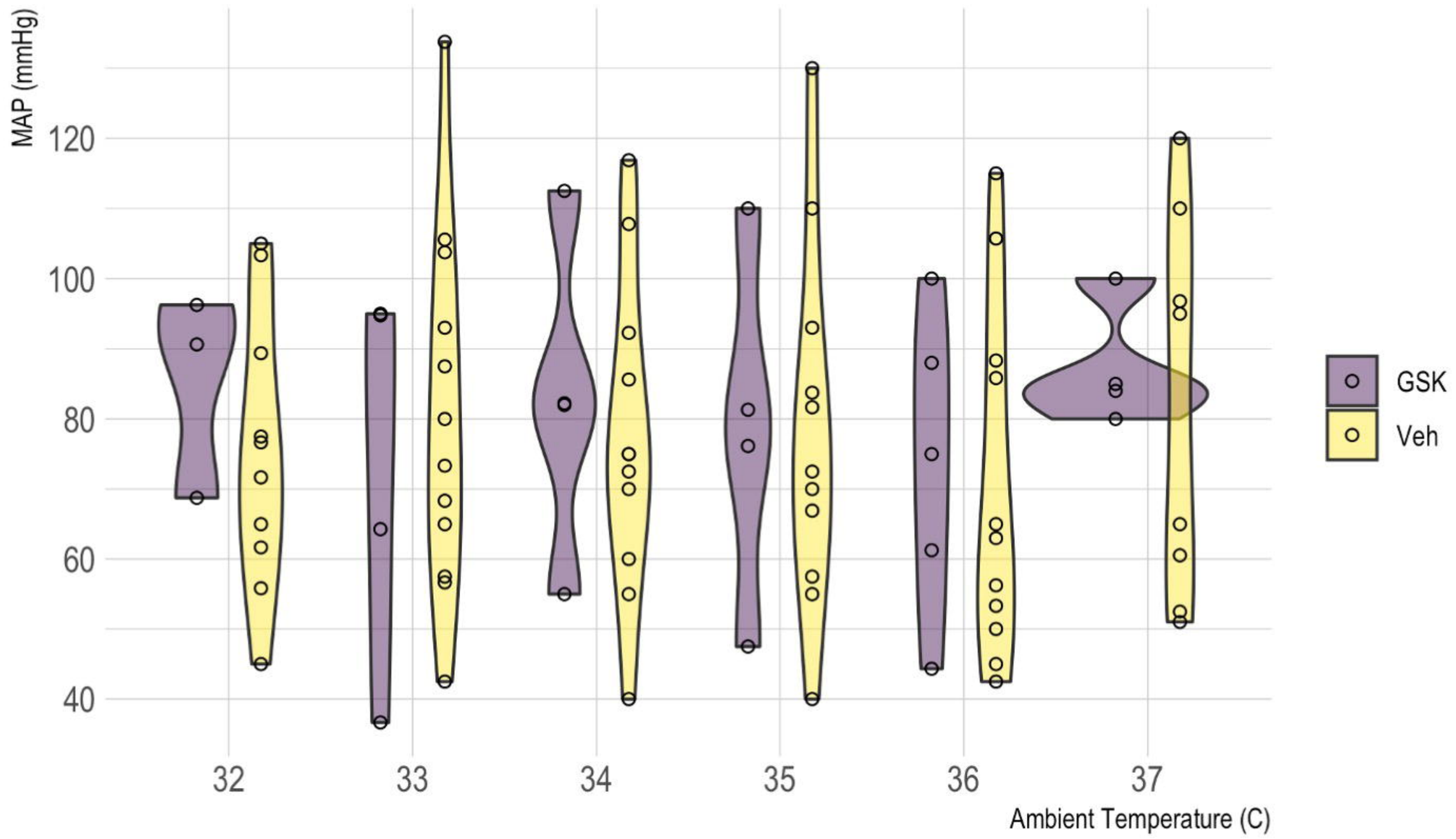

A
Flow (µL/min)
25
20
15
10
5
0
Veh
GSK
B
Flow/temperature (µL/minC)
2.5
2.0
1.5
1.0
0.5
0.0
Veh
GSK
C
Flow ul/min
40
30
20
10
0
34
35
36
37
Temperature (˚C)

Figure 2

A


MAP (mmHg)
120
100
80
60
40
32
33
34
35
36
37
Ambient Temperature (C)
GSK
Veh

B

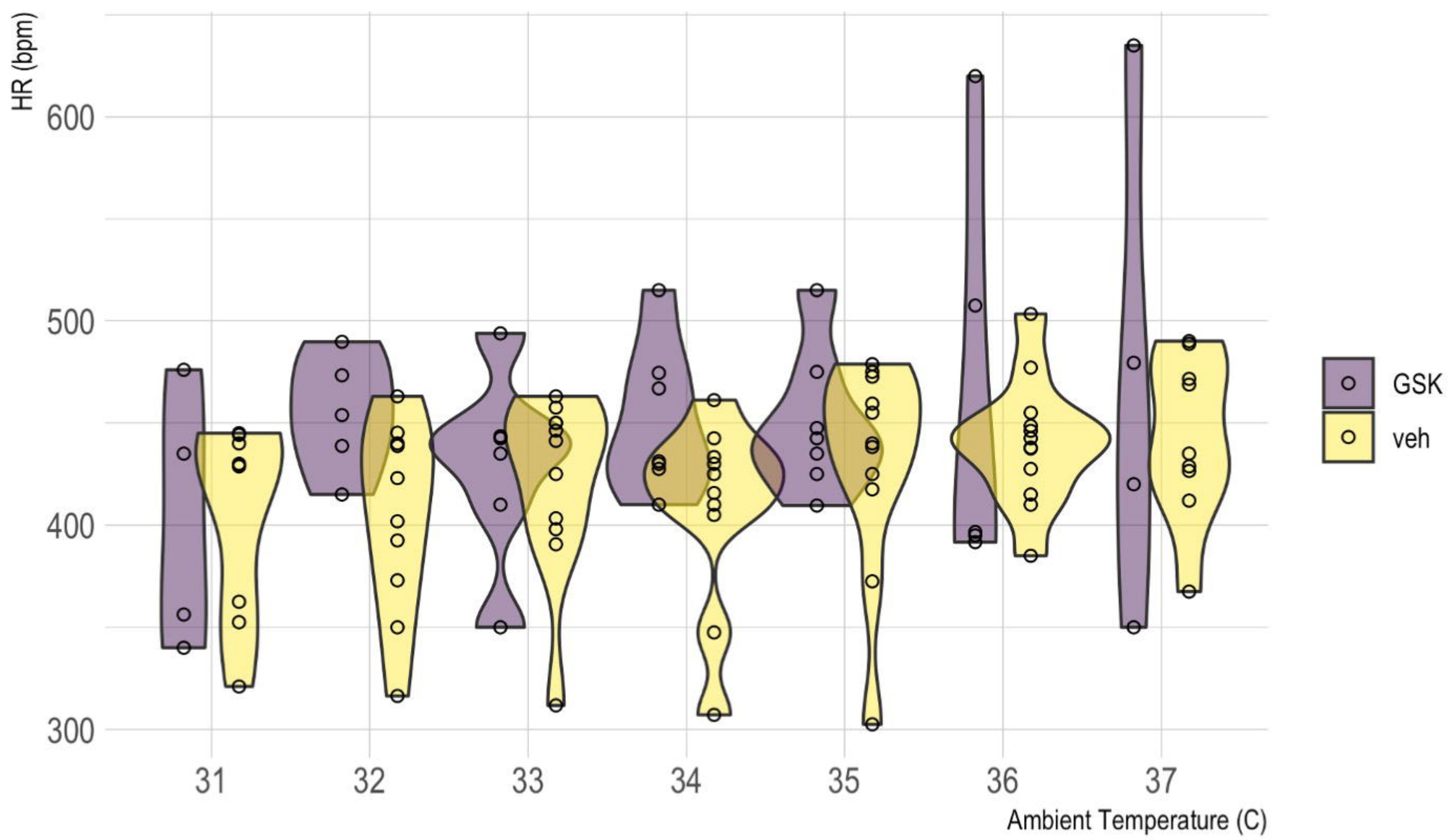

HR (bpm)
600
500
400
300
31
32
33
34
35
36
37
Ambient Temperature (C)
GSK
veh

Figure 3

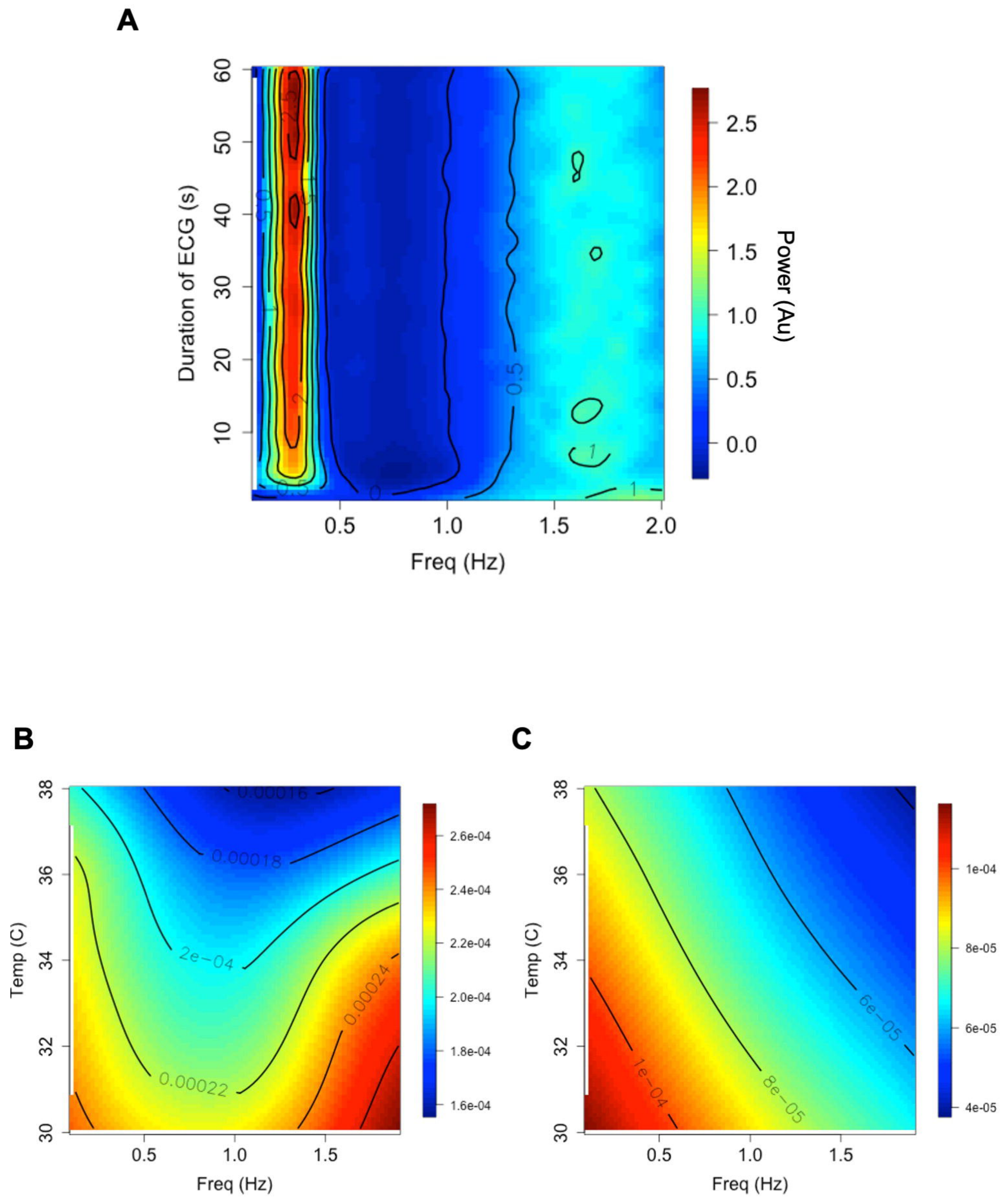

A
Duration of ECG (s)
Freq (Hz)
Power (Au)
B
Temp (C)
Freq (Hz)
C
Temp (C)
Freq (Hz)

Figure 4