1 **A thymus tumour impairing hibernation**

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Abstract

Background

Hibernation is a physiological and behavioural adaptation that permits survival during periods of reduced food availability and extreme environmental temperatures. This is achieved through cycles of metabolic depression and reduced body temperature (torpor) and rewarming (arousal). Rewarming from torpor is achieved through the activation of brown fat (BAT) associated with a rapid increase in ventilation frequency. Here, we studied the rate of rewarming in the European hamster by measuring both BAT temperature, core body temperature and ventilation frequency.

Results

Temperature was monitored in in parallel in the BAT (IPTT tags) and peritoneal cavity (iButtons) during hibernation torpor-arousal cycling. We found that increases in brown fat temperature preceeded core body temperature rises by about 47 min, and this was accompanied by a significant increase in ventilation frequency. The rate of rewarming was slowed by the presence of a spontaneous thymus tumour in one of our animals. Core body temperature re-warming was reduced by 6.2°C*h^{-1} and BAT rewarming by 12°C*h^{-1}. Ventilation frequency was increased by 77% during re-warming in the thymus tumour animal compared to a healthy animal. Inspection of the position and size of the tumour indicated that the lungs and heart were obstructed.

Conclusions

We validated a minimally invasive method to monitor BAT temperature during hibernation. Using this method we showed compromised re-warming from hibernation in an animal with a thymus tumour, the likely cause of which is obstruction of the lungs and heart leading to inefficient ventilation and circulation.

33 **Background**

34 Hibernation is a physiological and behavioural adaptation that permits survival during periods of
35 reduced food availability and extreme environmental temperatures. This is achieved through cycles of
36 metabolic depression characterised by reduced body temperature (torpor) and rewarming (arousal).
37 Entrance into torpor precisely is controlled by decreases in heart rate, ventilation frequency and
38 oxygen consumption [1, 2]. Arousal occurs in a coordinated manner with increased ventilation
39 frequency and oxygen consumption subsequently followed by heart rate, blood pressure and core
40 body temperature rise [1]. Rewarming from torpor is achieved through the activation of subcutaneous
41 brown fat reserves on top of the scapulae (classical BAT) and within the intra-scapular region (intra-
42 scapular BAT) [3]. The primary function of brown fat is heat generation through non shivering
43 thermogenesis [4], which has a high energy (oxygen) demand [5, 6]. During arousal from torpor
44 temperature increases in brown fat correlate with increased oxygen consumption (respiration rate) in
45 the brown bat (*Eptesicus fuscus*) and the golden-mantled ground squirrel (*Callospermophilus lateralis*),
46 and precede rectal or muscle temperature increases [7–10].

47 Activation of brown fat is thought to originate in the thermo-sensing regions in the
48 hypothalamus coupled to a sympathetic nervous pathway, which activates beta adrenergic receptors
49 and in turn the brown fat mitochondria [11]. The thermogenic capacity of brown fat comes from the
50 use of proton leak (uncoupling) in mitochrondria as opposed to the coupled oxidative phosphorylation
51 pathway which produces ATP [12]. The uncoupled pathway depends on the BAT-specific expression of
52 the uncoupling protein, UCP1 transporter in the mitochondrial membrane. Initiation of this process
53 requires good oxygen/energy supply to the BAT, and a marked increase in ventilation is an early event
54 in the rewarming process [13]. The heat generated from BAT rewarms the anterior of the animal first,
55 and then increases in heart rate and circulation are required to warm the rest of the animal.
56 Subsequently, shivering thermogenesis is initiated to help the animal to reach normal body
57 temperature (euthermy) quickly [5, 6, 14]. In Syrian hamsters (*Mesocricetus auratus*) the initiation of

58 shivering thermogenesis can only occur once the body temperature is above 16℃ [5] and in the
59 marmot (*Marmota marmota*) shivering is only observed when the subcutaneous BAT temperature
60 reaches 16℃ [4]. These data highlight the importance of non-shivering thermogenesis by BAT in the
61 initial stages of the re-warming process.

62 Here, we validated a minimally invasive method to monitor both BAT temperature (T_{BAT}) and
63 core body temperature (Tb) in a well-established hibernation model, the European hamster (*Cricetus*
64 *cricetus*) [15–17]. We demonstrate that increases in T_{BAT} precede increases in Tb and that the
65 ventilation frequency correlates with the rate of T_{BAT} re-warming. Furthermore, in one animal bearing
66 a thymus tumour, impaired ventilation led to a marked slowing of the re-warming process.

67 **Results**

68 *Re-warming in brown fat compared to the core body*

69 We placed Implantable Programmable Temperature Transponder (IPTT) tags subcutaneously to
70 measure the temperature of classical BAT (T_{BAT}). In the same animals we surgically implanted iButtons
71 into the intraperitoneal cavity to monitor core body temperature (Tb). To initiate the preparation for
72 hibernation we transferred animals from long photoperiod (14L:10D, 14 hours of light per 24 hours)
73 and 22℃ (LP22), to short photoperiod (10L:14D, 10 hours of light per 24 hours) and 22℃ (SP22). After
74 8 weeks, we reduced the room temperature to 10℃ (SP10, Figure 1A). All animals showed a
75 hibernation phenotype within 4 weeks of transfer to SP10, which was preceded by "test drops" in Tb
76 of approximately 10 to 15℃ below euthermy before initiating the multi-day torpor-arousal cycling
77 characteristic of the hibernation season (Figure 1B). Once hibernating, the European hamster drops its
78 Tb to near ambient room temperature for multiple days (approximately 25℃ below euthermy) and
79 arouses at intervals returning to euthermy (Figure 1B).

80 We confirmed the initiation of the hibernation season and torpor-arousal cycling for at least 2
81 weeks before we started monitoring T_{BAT} during the periodic arousals. These data were time matched
82 with Tb from the iButtons. Plotting the data from 5 arousing individuals we observe that both T_{BAT} and
83 Tb show similar curve trajectories until euthermy is reached (Figure 2A, non-linear asymmetric
84 sigmoidal model, $T_{BAT:}$ r^2= 0.9778; Tb: r^2= 0.9878). We found the maximum re-warming rate (RWR_{MAX})
85 was also similar for BAT (RWR_{MAX} = 20.9℃*h^{-1} ; 95% confidence interval (CI): 20.3℃ to 21.5℃) and
86 core body (RWR_{MAX} = 21.0℃*h^{-1} ; CI: 15.4℃ – 26.5℃), however Tb RWR_{MAX} showed more individual
87 variance. We show that brown fat re-warming precedes subsequent core body temperature re-
88 warming by 47 minutes (CI: 44-49 minutes) (Figure 2A). The mean ventilation frequency (VF) for the 5
89 individuals was calculated for each quartile of re-warming showing a clear increase in VF during re-
90 warming (Figure 2A). Using visual assessment, the onset of shivering in muscles adjacent to the BAT

91 during arousal was noted, an average BAT temperature of 15℃ was recorded at the onset of shivering
92 (Figure 2B).

93 We plotted data from one representative individual and calculated the RWR_{max} in brown fat as
94 19.3℃*h^{-1}, a similar rate was observed in the core body temperature (18.1℃* h^{-1}, Figure 2C). We also
95 monitored the ventilation frequency during arousal which increased from 13 breaths per minute (bpm)
96 to a maximum of 86 bpm (Figure 2C). Ventilation frequency increases correlated with T_{BAT} (pearson r=
97 0.899, p-value= 0.002).

98 *Brown fat re-warming is compromised by the presence of a spontaneous thymus tumour*

99 We identified one individual in our study with a spontaneous thymus tumour (Figure 3A). When
100 comparing the amount of time spent torpid or aroused the tumour bearing animal spent 63.5%
101 aroused and 14.5% torpid, compared to an average of 25.9% aroused and 54.6% torpid for the healthy
102 animals (Figure 3B). The RWR_{max} of brown fat was reduced by 12℃*h^{-1} relative to a healthy animal
103 (Figure 3C). The RWR_{max} of the core body was less compromised showing a reduction of 6.2℃*h^{-1}.
104 Ventilation frequency increases still correlated with T_{BAT} (pearson r= 0.873, p-value= 0.002) but the
105 tumour animal showed a 77 % higher maximum ventilation frequency compared to a healthy animal
106 (Figure 2B compared to Figure 3C). Brown fat re-warming precedes core body temperature re-warming
107 by 53.9 minutes in the tumour animal, this is outside the confidence intervals for the healthy animals,
108 suggesting that the efficiency of BAT re-warming of the core is compromised.

109 **Discussion**

110 Our study has validated a minimally invasive method to measure BAT temperature during hibernation.
111 We show that there is a significant correlation between T_{BAT} and Tb, with T_{BAT} increases preceding that
112 of Tb. Compared to intraperitoneal implantation of iButtons the IPTT tags offer a minimally invasive
113 method to monitor temperature, furthermore there is a significant advantage to monitoring BAT
114 temperature in hibernation studies instead of core body temperature because the earliest arousal
115 events can be detected almost immediately.

116 The increases in T_{BAT} and T_b are both correlated with increased ventilation frequency.
117 Hyperventilation has been previously observed in the thirteen-lined ground squirrel but BAT
118 temperature was not studied [13]. Our T_b RWR_{max} are similar to previous observations in rodents and
119 European hamsters [18] but no data on BAT temperature is available.

120 Re-warming efficiency of the BAT in the tumour bearing animal was only 38% of that in the
121 healthy animal, whereas the rewarming efficiency of the core body was 67% of that in the healthy
122 animal. We also observed a greatly increased ventilation frequency compared to a healthy animal. We
123 suggest that the size and position of the thymus tumour obstructed the lungs, reducing ventilation
124 volume, thereby compromising the animal's ability to deliver the necessary oxygen to support the
125 activity of BAT. This probably resulted in increased ventilation frequency in an attempt to meet tissue
126 demands. It has been observed that the early arousal events show a reliance on increased ventilation
127 frequency and that increases in heart rate occur later as peripheral circulation increases [13]. The
128 generally lower re-warming efficiency in both BAT and core body might be interpreted as evidence for
129 compromised cardiac function leading to reduced circulation efficiency. In support of a decreased rate
130 of re-warming of the BAT and core body due to decreased circulation we see re-warming rates in the
131 thymus tumour animal comparable to that of the much larger Marmot (5000g compared to 500g) [18].

132 The discrepancy between BAT re-warming efficiency and core body re-warming is intriguing,
133 and possibly relates to complicated relationship and order of events in arousal which start with

134 increased ventilation to increase oxygen availability, followed by vasodilation, and increased heart
135 rate, to re-perfuse the circulation and re-warm the whole animal. Low oxygen availability increases
136 vasodilation [19] therefore in the tumour bearing animal lower oxygen availability may have increased
137 vasodilation leading to a compensated re-warming efficiency of the core body. Vasodilation would
138 also benefit the BAT by supplying more oxygen in the blood. In support of this the tumour bearing
139 animal in the initial stages of BAT re-warming from 10 to 15℃ appears to show a gentler slope
140 compared to 20 to 30℃ (Figure 3C), indicating a lower efficiency in the early arousal stage which would
141 not benefit from vasodilation, this distinction is not seen in the healthy animals. Another explanation
142 for the discrepancy between BAT and core body re-warming efficiency may be the additional
143 contribution of shivering thermogenesis to core body temperature increases.

144 The thymus is a lymphoid organ involved in T-cell maturation , it is located in front of the heart
145 and found in all vertebrates (reviewed in: [20]). In general, thymus tumours are rare but are reported
146 in domestic, laboratory and wild contexts (including wild European hamsters)[21]. Characterisation of
147 European hamster thymus tumours has noted they closely match human thymic epithelial tumours
148 [22]. Human thymus tumour cases often present with tumours that obstruct the heart and lungs,
149 similar to our observations. Also in humans, an association of thymus tumour with
150 hypothermia/defective re-warming has been reported twice [23, 24]. Impressively in one of the cases
151 the patient experienced a body temperature between 32.8℃ to 35℃ (Ambient room temperature:
152 22℃) [23]. However, the cause of hypothermia in these cases is unknown.

153 **Conclusions**

154 In conclusion we have used temperature logging in BAT and intraperitoneal cavity to study progression
155 of arousal in hibernating hamsters. We show compromised re-warming from hibernation in an animal
156 with a thymus tumour, the likely cause of which is obstruction of the lungs and heart leading to
157 inefficient ventilation and circulation.

158 **Methods**

159 *Animals and ethics statement*

160 European hamsters were bred from stock animals at the Chronobiotron, an animal facility dedicated
161 to the study of biological rhythms. Animals were housed in an environment controlled room in
162 separate cages according to legislations provided in European Commission directive 2010/63/EU.
163 They were provided with *ad libitum* access to food (Safe®) and water throughout the study
164 period. The animals were kept under a long photoperiod (14L:10D) and at 22℃ (LP22) until the start
165 of the experiment. To initiate the preparation for hibernation (pre-hibernal period) the animals were
166 transferred to a short photoperiod (10L:14D) at 22℃ (SP22). After 8 weeks, the temperature was
167 lowered to 10℃ (SP10), all animals exhibited torpor-arousal cycling, and were kept in this condition
168 to the end of the experiment (10 weeks, Figure 1A). The experimental procedure was validated by a
169 local ethical committee and further validated by the Ministry of Higher Education, Research and
170 Innovation (APAFIS#21424-2019070219421923 v3).

171 *Calibration of IPTT tags*

172 The accuracy of each IPTT tag (BMDS IPTT-300®) was verified from manufacturer to be ±1℃ of actual
173 temperature in the range between 21 and 30℃. During torpor-arousal the tags would measure
174 between 10-38℃ therefore to ensure accuracy between the iButtons and IPTT tags we created an
175 individual calibration curve for each IPTT tag allowing for *post hoc* correction at a 0.1℃ resolution. A
176 similar method was used by Wacker et al [25]. Each tag was calibrated using a water bath containing
177 two iButtons (thermochron DS1922L, Maxim integrated) set to 16-bit resolution (0.0625℃) and
178 sampling rate of 2 seconds, the IPTT tag was scanned every 2-3 seconds. The water bath was then
179 placed on a heating plate with a magnetic stirrer and heated, the temperature range recorded was 5℃
180 to 39℃. The resulting data was plotted in Graphpad Prism v8.0 and a regression analysis was done to
181 allow for calibration of the IPTT tag recordings.

182 *iButton and IPTT tag surgery*

183 Surgeries were performed on each animal to implant iButtons (thermochron DS1922L, Maxim
184 integrated) and IPTT tags. Each animal was anesthetized by 3 % isoflurane, surgery was performed
185 under 3% isoflurane and 95 % oxygen. The IPTT-tag was implanted subcutaneously into the classical
186 brown adipose tissue (BAT) depot, using a standard IPTT-tag injector (supplied by the manufacturer).
187 Post-mortem verification ensured the tag was in contact with the BAT. The iButton was implanted in
188 the abdominal cavity with the use of laparotomy. Local anaesthetic agents (lidocaine and bupivacaine)
189 was injected intraperitoneally (2.5 mg/kg) before the incision was made. Analgesic agent (Metacam®,
190 injectable, 2mg/kg) was dorsally injected subcutaneously. In addition, analgesic agent was dissolved in
191 drinking water and provided for 3 days post-surgery (Metacam® drinkable 1.5 mg/ml, 1 mg/kg). The
192 animals were extensively monitored after surgery and allowed to recover for two weeks.

193 *Behaviour and temperature monitoring*

194 To keep track of individual torpor-arousal patterns, behavioural recordings were made twice per day;
195 1 to 2 hours after lights on and 9-10 hours after lights on. We defined torpid as a ventilation frequency
196 (VF) <10 per minute, curled up position in hibernacula, immobile, unresponsive and an IPTT reading
197 <13.5℃. Signs of arousal were; increased IPTT tag temperature and increased ventilation frequency.
198 We identified animals beginning to arouse and monitored IPTT tag temperatures every two minutes,
199 ventilation frequency was counted in regular intervals for one minute. IButton core temperature
200 recordings were collected post-mortem and time matched with the calibrated IPTT measurements.
201 Graphpad Prism v8.0 was used for statistical analysis and data plotting.

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263

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271 **Author contributions**

272 **FM -** Experimental design, analysed the data, collected samples, prepared figures, and revised
273 manuscript**.** **VJM –** Experimental design, analysed the data, collected samples, prepared manuscript**.**
274 **BB –** performed surgeries, technical expertise, revised the manuscript. **DGH -** conceived and designed
275 the experiments, supervised, and revised the manuscript. **VS -** conceived and designed the
276 experiments, supervised, provided funding and revised the manuscript. **SHW**– conceived and designed
277 the experiments, supervised, provided funding and prepared the manuscript.

278 **Figure legends**

279 **Figure 1: European hamster displays hibernation after exposure to short photoperiod and low**
280 **ambient temperature.** A.) Study design to induce the hibernation phenotype. Animals were
281 transferred from long photoperiod (14 hours of light per day, 14:10) and 22℃ to short photoperiod
282 (10 hours of light per day, 10:14) for 8 weeks. The ambient temperature (Ta) was lowered to 10°C, a
283 temperature of 9.6 ± 1°C was observed. B.) Core body temperature (Tb) measurement from a European
284 hamster displaying torpor-arousal cycling. The grey dotted line indicates the average ambient
285 temperature (Ta) over the experiment.

286 **Figure 2: Brown adipose tissue re-warming precedes core body temperature increases.** A) Core body
287 temperature (Tb – iButton, diamond symbol) and BAT temperature recordings (T_{BAT} - IPTT tag, circle
288 symbol) from 5 individuals during an arousal from torpor (each individual represented by a different
289 colour). The mean ventilation frequency (VF) at each quartile of arousal, as defined by a non-linear
290 curve fit for all individuals, is indicated by black arrows. Mean maximum re-warming rate (RWR_{MAX}) for
291 core body temperature (Tb) = 21.0C (confidence intervals: 15.4 – 26.5) & brown adipose tissue (T_{BAT}) =
292 20.9C (CI: 20.3 – 21.5). B) BAT temperature recording corresponding to onset of BAT adjacent skeletal
293 muscle shivering. Observations and recordings were done in 8 separate arousal events in 5 different
294 animals. C) A representative individual showing core body temperature (Tb), BAT temperature (T_{BAT})
295 and ventilation frequency (VF) changes during arousal from torpor. Grey dotted line shows room
296 temperature (Ta). Inset graph shows the linear portion of the arousal that was used to calculate
297 maximum re-warming rate (RWR_{max}) in ℃ per hour.

298 **Figure 3: Rewarming from torpor is compromised in an animal with a thymus tumour.** A.) Post-
299 mortem image of exposed thoracic cavity of a European hamster with a thymus tumour (indicated by
300 the white arrow). For reference, white * is placed on lungs and ^ on heart. B.) Heat-map of core body
301 temperature recorded by iButtons over the course of the experiment. Each line is an individual animal,
302 the first animal has the tumour. The percentage time spent above 35.5℃ (Euthermic – aroused) and

below 12℃ (Torpid) is indicated, the remainder is time spent between 35 to 12 ℃ (entering or arousing from torpor) for each individual. Grey indicates no data was recorded in this period. C.) Core body temperature (Tb), BAT temperature (T_{BAT}) and ventilation frequency (VF) changes during arousal from torpor in an animal with a thymus tumour. Grey dotted line shows room temperature (Ta). Inset graph shows the linear portion of the arousal that was used to calculate maximum re-warming rate (RWR_{max}) in ℃ per hour.

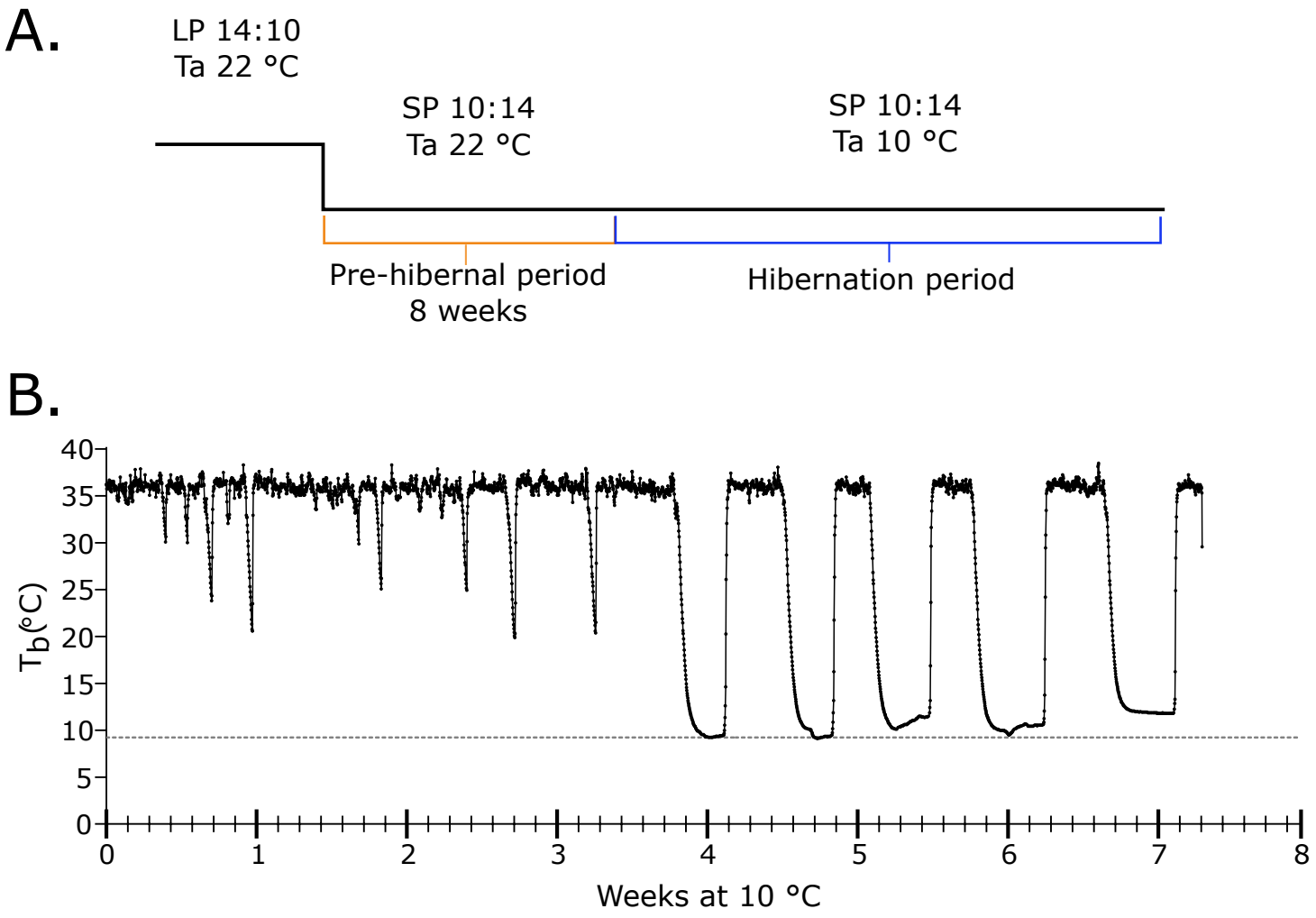

A.
LP 14:10
Ta 22 °C
SP 10:14
Ta 22 °C
SP 10:14
Ta 10 °C
Pre-hibernal period
8 weeks
Hibernation period
B.
T_b(°C)
40
35
30
25
20
15
10
5
0
0
1
2
3
4
5
6
7
8
Weeks at 10 °C

Fig. 1

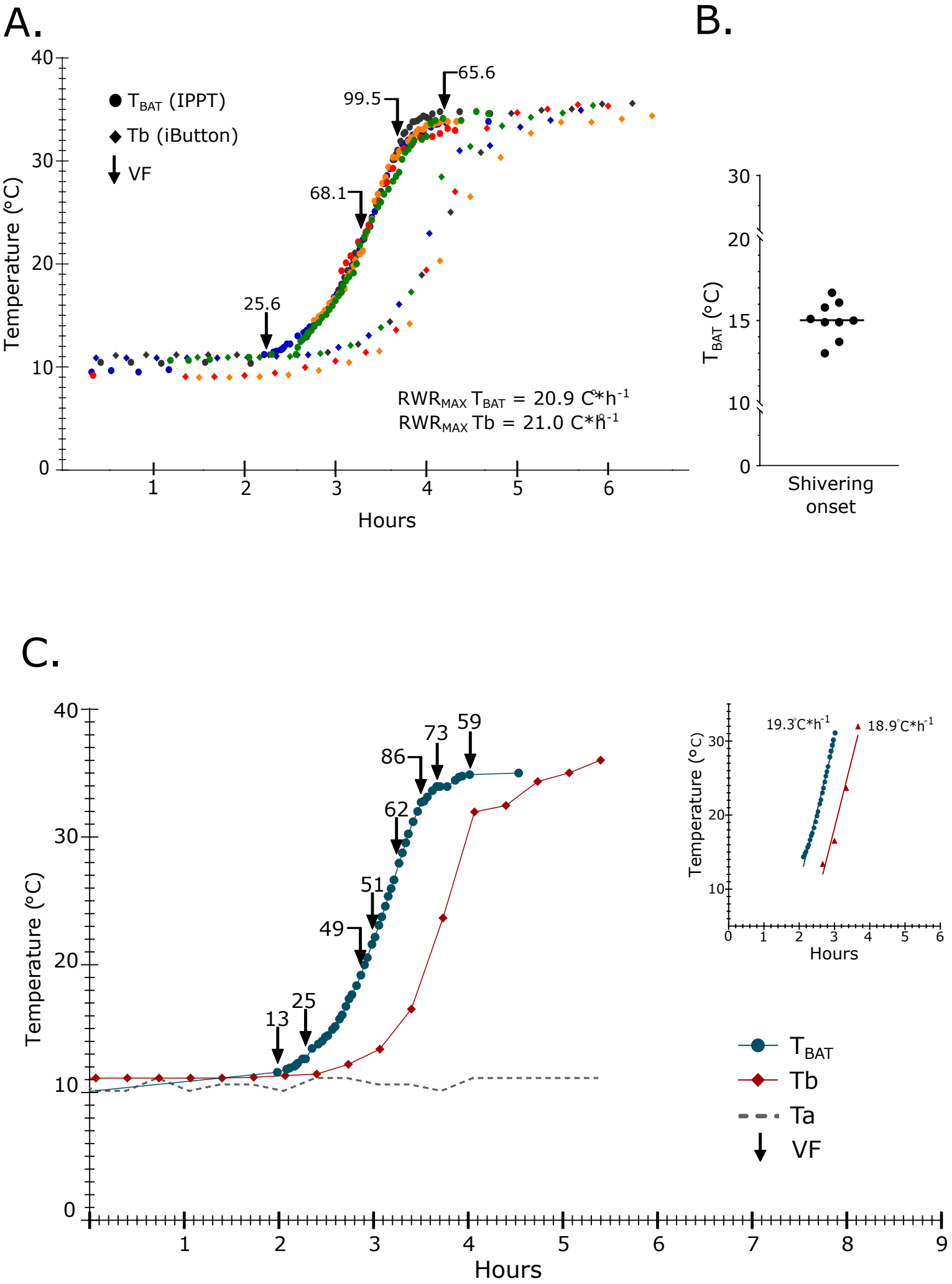
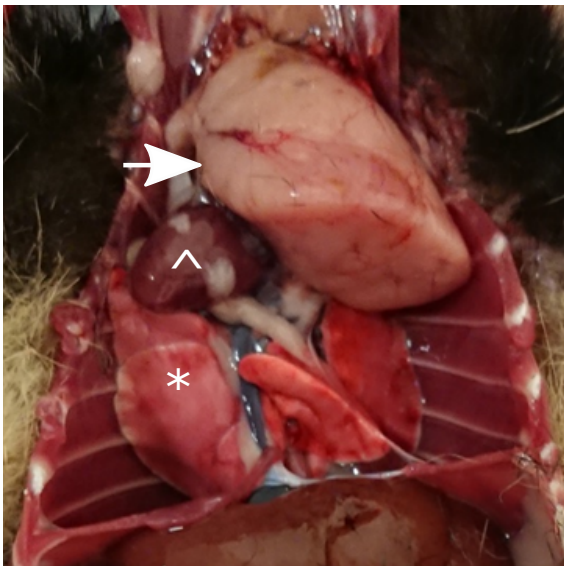
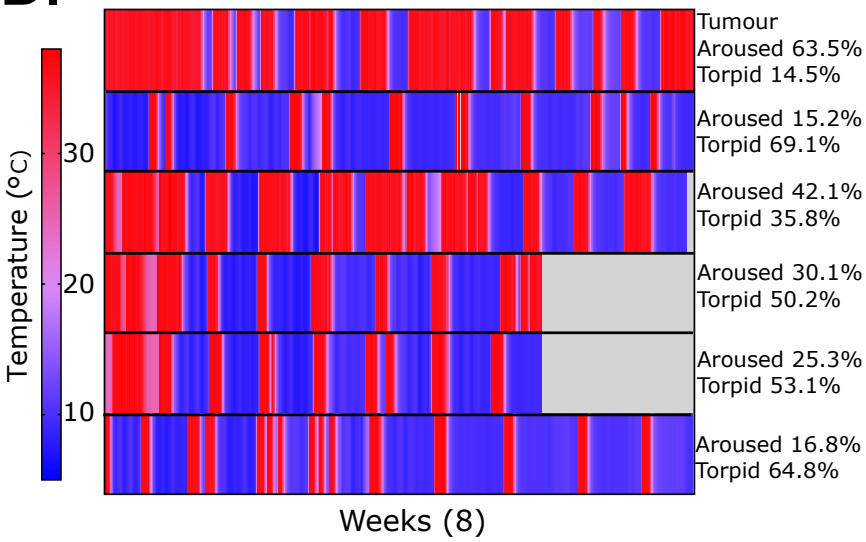

A.
B.
C.
T_{BAT} (IPPT)
Tb (iButton)
VF
Temperature (°C)
Hours
25.6
68.1
99.5
65.6
RWR_{MAX} T_{BAT} = 20.9 C°*h^{-1}
RWR_{MAX} Tb = 21.0 C°*h^{-1}
T_{BAT} (°C)
Shivering onset
13
25
49
51
62
86
73
59
19.3°C*h^{-1}
18.9°C*h^{-1}
T_{BAT}
Tb
Ta
VF

Fig. 2

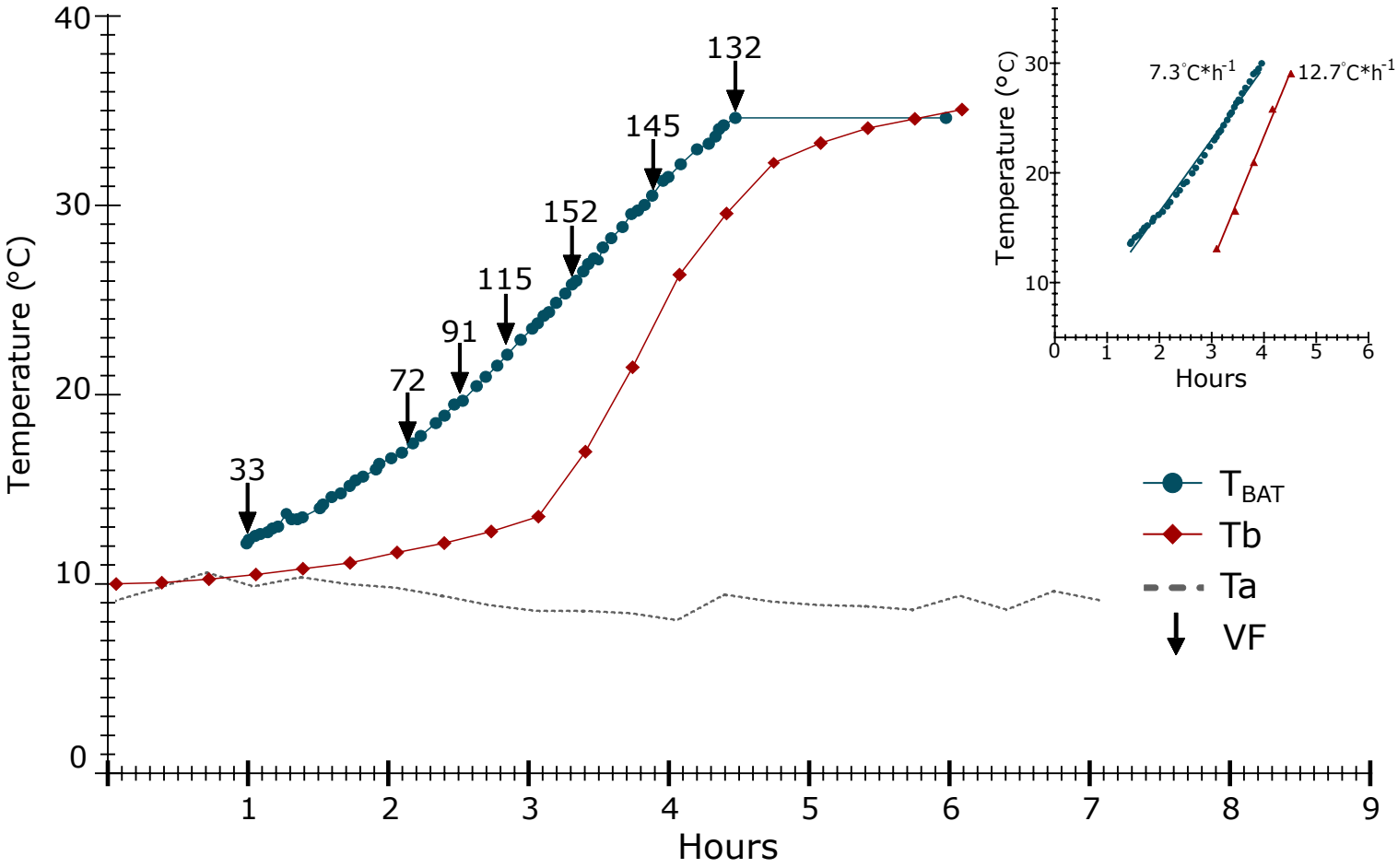
A.



B.


Temperature (°C)
30
20
10
Weeks (8)
Tumour
Aroused 63.5%
Torpid 14.5%
Aroused 15.2%
Torpid 69.1%
Aroused 42.1%
Torpid 35.8%
Aroused 30.1%
Torpid 50.2%
Aroused 25.3%
Torpid 53.1%
Aroused 16.8%
Torpid 64.8%

C.


Temperature (°C)
40
30
20
10
0
Hours
1
2
3
4
5
6
7
8
9
33
72
91
115
152
145
132
T_{BAT}
Tb
Ta
VF
7.3°C*h-1
12.7°C*h-1