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1 **Muscle nonshivering thermogenesis in a feral mammal**

2 Running head: Evidence of muscle NST in wild boar

3 Julia Nowack[1], Sebastian G. Vetter[1], Gabrielle Stalder[1], Johanna Painer[1,2], Maria Kral[2], Steve Smith[2],
4 Minh Hien Le[1], Perica Jurcevic[1], Claudia Bieber[1], Walter Arnold[1], Thomas Ruf[1]

5 [1]Department of Integrative Biology and Evolution, Research Institute of Wildlife Ecology, University
6 of Veterinary Medicine, Vienna, Austria
7
8 [2]Department of Integrative Biology and Evolution, Konrad Lorenz Institute for Ethology, University of
9 Veterinary Medicine, Vienna, Austria
10
11 Corresponding author: Julia Nowack; contact: Julia.nowack@vetmeduni.ac.at

12 ***Abstract***

13 While small mammals and neonates are able to maintain an optimal body temperature (T_b)
14 independent of ambient conditions by producing heat via nonshivering thermogenesis (NST) in the
15 brown adipose tissue (BAT), larger mammals and other mammals lacking BAT were long believed to
16 rely primarily on shivering and behavioural adaptations. However, recently, a second mechanism of
17 NST was found in skeletal muscle that could play an important role in thermoregulation of such
18 species. Muscle NST is independent of muscle contractions and produces heat based on the activity
19 of an ATPase pump in the sarcoplasmic reticulum (SERCA1a) and controlled by the protein sarcolipin.
20 To evaluate whether muscle NST could indeed play an important role in thermoregulation in species
21 lacking BAT, we investigated the thermogenic capacities of new-born wild boar piglets. During cold
22 exposure over the first 5 days of life, total heat production was improved while shivering intensity
23 decreased, indicating an increasing contribution of NST. Sampling skeletal muscle tissue for analyses
24 of SERCA activity as well as gene expression of SERCA1a and sarcolipin, we found an age-related
25 increase in all three variables as well as in T_b. Hence, the improved thermogenesis during the
26 development of wild boars is not due to shivering but explained by the observed increase in SERCA
27 activity. Our results suggest that muscle NST may be the primary mechanism of heat production
28 during cold stress in large mammals lacking BAT, strengthening the hypothesis that muscle NST has
29 likely played an important role in the evolution of endothermy.

30

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31 ***Keywords***

32 Calcium slippage, Cold exposure, Heat production, SERCA, Shivering, Sarcolipin

33 ***Introduction***

34 The regulation of a high and stable body temperature (T_b) independent of climatic conditions is one
35 of the most important mechanisms that arose during the evolution of mammals and birds. After
36 decades of intensive research, it is now well-understood how mammals possessing brown adipose
37 tissue (BAT) - a specialised thermogenic organ - are able to maintain an optimal T_b even in cold
38 environments by using nonshivering thermogenesis (NST) [reviewed in 1]. However, only ~20% of
39 endothermic birds and mammals actually possess BAT [2]. NST in BAT requires thermogenic
40 functional uncoupling protein 1 (UCP1) that alters proton conductance in the inner mitochondrial
41 membrane, leading to heat generation instead of ATP production [3, 4]. Functional UCP1 has not
42 been found, however, in marsupials or monotremes [5] and typically substantial amounts of BAT are
43 present only in neonates of large-bodied mammals [6, 7, but see: 8]. Furthermore, a recent study has
44 shown that UCP1-inactivating mutations have occurred in various mostly large-bodied placental
45 mammals [9]. Large mammals, such as pigs, are likely able to cope well with cold exposure even
46 without possessing BAT. Their juveniles, on the other hand, have high surface area-to-volume ratios
47 and need a high capacity for heat production to maintain a constantly high T_b [10]. Inside the
48 mother´s womb juveniles are protected against thermal variations and they are exposed to a cold
49 environment for the first time after birth. In fact, it is known that neonates of many large-bodied
50 species in which BAT depots are negligible in adults, possess large amounts of BAT [6, 7].

51 It was long believed that species without thermogenic functional UCP1 rely solely on
52 shivering, a process that on its own is insufficient for the maintenance of a stable T_b during cold
53 exposure in UCP1 knockout mice [11]. However, a second mechanism of NST in muscle, which had
54 been studied in vitro for a long time [12-15], has recently been shown to play an important role in
55 thermoregulation in mice lacking functional BAT [11]. The mechanism is so far only confirmed as an
56 additional form of NST in rodents in which the wildtype possesses BAT [11, 16-20], but assumed to

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57 occur in all mammals [2, 21]. Furthermore, muscle NST is discussed as a heat production mechanism
58 that played an important role for the evolution of a high T_b, i.e. endothermy, in mammals [2, 21], as
59 endothermy in this group evolved before UCP1- mediated NST [22].
60 In short, muscle NST is based on the activity of the Ca^{2+}-ATPase pump in the sarcoplasmic
61 reticulum (SERCA). SERCA1a, the major isoform occurring in skeletal muscle [23], is involved in
62 muscle contraction via the transport of Ca^{2+}-ions from the cell lumen into the sarcoplasmic reticulum
63 [24, 25]. But ATP hydrolysis by SERCA1a can be uncoupled from actual transmembrane transport of
64 Ca^{2+} by the regulatory protein sarcolipin (SLN) causing the release of the Ca^{2+}-ions bound to SERCA
65 back to the cytoplasmic side of the membrane (so called "slippage") rather than into the
66 sarcoplasmic reticulum [reviewed in 26]. This results in increased ATP hydrolysis and heat production
67 in muscle through SERCA1a activity without actual Ca^{2+}-transport and without muscle contraction
68 [13, 26-29]. Studies on laboratory mice have shown that muscle NST can compensate for the loss of
69 UCP1, but muscle NST in wildtype mice is largely masked by heat production in BAT [30, 31]. This
70 raises the question whether the thermogenic capacity of muscle NST alone can enable mammals to
71 maintain a stable T_b under cold conditions.
72 We investigated whether muscle NST is an important and effective mechanism to generate
73 heat in wild-type mammals lacking BAT, using new-born wild boars (*Sus scrofa*), naturally lacking BAT
74 [32] and the UCP1-dependent NST [33, 34] as model species. Importantly, juvenile wild boars are
75 born in early spring, when ambient temperatures (T_a) can still be around or below 0°C and
76 thermoregulatory demands are high. We firstly hypothesized that, if NST plays a significant role for
77 thermogenesis in piglets, heat production should increase during cold exposure, whereas shivering
78 should remain constant or may even decrease. We secondly hypothesized that, if heat production is
79 based on muscle NST, expression levels of SERCA1a and sarcolipin as well as SERCA activity should
80 be elevated during cold exposure.

81 ***Material and Methods***

82 *Experimental setup*

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83 Piglets were born in March 2017 by five sows kept and bred in outdoor enclosures at the Research
84 Institute of Wildlife Ecology (48.22° N, 16.28° E) of the University of Veterinary Medicine Vienna in
85 Austria. Each sow was provided with a roofed shelter outfitted with straw in which they gave birth.
86 Shelters were equipped with IP-cameras and activity of the sows was monitored to know the exact
87 time of birthing. All animals were exposed to natural T_a but piglets typically huddled with each other
88 or with adults. Mean daily T_a during the 5 days of our study ranged from 5.9 °C to 14.7 °C and nightly
89 minimum T_as were between 1.5 °C and 9.4 °C.

90 We temporarily removed 19 of 29 new-born wild boars from their mothers within the first 24
91 hours after birth (age 8.5-24 h) and again four days later. Rectal T_b was taken within 5-10 minutes
92 after removal from the mother by inserting a thermometer with rectal gel approximately 2 cm into
93 the rectum. Piglets were weighed to an accuracy of 1 g (Sartorius, Göttingen, Germany) and
94 equipped with a custom-made acceleration logger (see below) that was firmly attached to their
95 abdomen with a cohesive bandage (Henry Schein, New York, USA) before being placed individually
96 into metabolic chambers (40 x 25x 22 cm, volume: 20 l). The metabolic chambers were located inside
97 a walk-in climate chamber that was set to + 10 °C (mean 10.3 ± 0.7 °C). Individuals were exposed to
98 cold for approximately 60-90 min and heat production and shivering intensity during cold exposure
99 were determined (see below). After measurements rectal T_b was determined again and piglets were
100 transferred to a surgery room where muscle biopsies were taken (see below).

101 *Metabolic measurements*

102 All experiments were conducted between 0800h and 1630h. Metabolic measurements lasted for 60-
103 90 min (depending on the activity level of the animal). Animals were allowed to acclimate to cold
104 conditions for 10 min, before the measurement was started. Energy expenditure was determined by
105 measuring the rate of O_2 consumption (VO_2) as a proxy of metabolic rate using an O_2 and CO_2
106 analyser (Servopro 4100, Servomex, Crowborough, UK). The metabolic chamber was connected to
107 the analyser (pull mode; order: metabolic chamber, pump, needle valve, flow meter, O_2 analyser, CO_2
108 analyser) with airtight tubes. Water vapour was removed from the air prior to analysis using silica

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109 gel. A gas switch allowed measurement of air from six metabolic cages and one reference air channel
110 for one minute each. The analyser was calibrated once a week using a high precision gas-
111 proportioning pump (H. Wösthoff, Bochum, Germany, type 55A27/7a). Air was continuously drawn
112 through the cages with pumps at a flow rate of 250 on day 1 and - 330 l h^{-1} on day 5. Flow rate
113 through each metabolic chamber was measured using calibrated thermal mass-flow meters (FMA
114 3100, Omega Engineering, Stamford, CT, USA). VO_2 was calculated by a self-written R program [35]
115 using equation 10.6 by Lighton [36] and converted to heat production (watts) assuming 20.1 J ml^{-1} O_2
116 consumption [37]. VO_2 were computed from the mean of the three lowest consecutive values per
117 measurement. To standardize measurements we only used the first 30 min of the measurement for
118 calculations.

119 *Shivering*

120 We successfully measured shivering on day 1 and day 5 of 12 cold-exposed piglets by attaching a
121 custom-made acceleration logger (three axis acceleration sensor ADXL345; 3 x 1 x 45 mm; Li-polymer
122 rechargeable Battery LP-402025-1S-3; weight with battery of 6.4 g). The sensor of our acceleration
123 logger measured acceleration in three orthogonal axes (x, y, and z). Sampling rate of the acceleration
124 sensor was set to 1600 Hz, acceleration range was ± 16 *g* and had a resolution of 4 m*g* per axis. The
125 battery had a capacity of 155 mAh limiting the runtime to approx. 6 hours, which was sufficient for
126 our needs. We analyzed the acceleration sensor data entirely in the frequency domain. For this
127 purpose, we calculated the resulting acceleration from the x, y and z-components:

128
$$a(t) = \sqrt{a_x(t)^2 + a_y(t)^2 + a_z(t)^2}\,.$$

129 Then we subtracted the mean acceleration stemming from gravitation and applied a Fast Fourier
130 Transformation (FFT) in R (V3.4.2) [38] (*fft* in package 'signal' [39]) to consecutive segments of 6
131 second intervals in the time domain (see supplementary figure S1). From every FFT output we
132 extracted the following parameters:

133 1. Maximum Amplitude (m/s^2)

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134 2. Frequency at Maximum Amplitude (in Hz)

135 Exposure to 10°C without the opportunity to huddle with conspecifics caused clearly visible shivering
136 with short intermitted phases of suspension of shivering. To calculate shivering intensity (the
137 maximum amplitude) we used the same time frame as for metabolic rate calculations, i.e. a total of
138 30 min, starting after 10 min of acclimatization. The frequency of muscle activation during shivering
139 thermogenesis is correlated with body mass in mammals [40] and is reported to be: $\log f = 1.85 - 0.18$
140 $\log m$, where f is the mean shivering frequency in Hz and m is the mean body mass in g [40]. Based on
141 this equation the shivering frequency of day 1 to day 5 old piglets with a mean body mass of 1700 g ±
142 306 g is predicted to be 18.55 Hz. We therefore restricted our analyses to frequencies between 10 Hz
143 and 30 Hz and excluded frequencies above or below this range from the analysis. Measured mean
144 shivering frequency at maximum amplitude for piglets was with 17.97 ± 1.66 Hz (N= 24) in the
145 predicted range and did not differ between days (χ^2= 0.540, df= 1, p= 0.462).

146 *Biopsies*

147 80-100 mg muscle tissue was taken with a biopsy needle from the thigh region of the piglets
148 (*Musculus semimembranosus*) under standard surgical conditions. The procedure was conducted
149 under general anaesthesia in combination with local anaesthesia. The anaesthesia was induced by
150 placing a mask over the piglets mouth and nose, using an Isofluran gas (Isofluran, Isoba, MSD animal
151 health, Vienna, Austria) inhalation machine and medical oxygen. Pain relief was achieved by injecting
152 local anaesthetics (Lidocain, Xylocain 2 % with Epinephrin 1:100,000. Mibe GmbH, Brehna, Germany)
153 10 min prior biopsy in the proximal region of the *M. semimembranosus* and the skin. Anti-
154 inflammatory and pain relief treatment was furthermore gained by injecting Meloxicam 10 min prior
155 biopsy intramuscularly (Meloxicam 0.4 mg/kg, Metacam 2% inj., Boehringer Ingelheim Vetmedica,
156 Ingelheim, Germany). During the entire procedure, vital parameters (respiration rate, peripheral
157 haemoglobin oxygen saturation as measured by pulse oximetry (SpO2), heart rate, T_b) and
158 anaesthetic depth were monitored. The skin and muscle fascia incisions were closed separately in
159 two layers with absorbable sutures (Surgicryl USP 2/0 PGA, SMI AG, Hünningen, Belgium). Piglets

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160 were marked with an ear tag for further individual recognition. After the biopsy the juveniles were
161 monitored and kept in a warm environment for recovery from anaesthesia until they were moved to
162 their enclosure. Piglets were fed with 1 mL of glucose solution orally (50 % glucose infusion, B.Braun
163 AG, Melsungen, Germany) before being returned to their mothers. Piglets were never kept away
164 from their mother for more than 3-4 hours. The muscle tissue sample was split for biochemical as
165 well as genetic analyses. We were able to obtain samples on both days of 17 piglets. The two animals
166 for which we only obtained one sample were excluded from further analyses regarding SERCA1a and
167 SLN. For two further animals tissue material was too small to be used for biochemical analyses, which
168 reduced the sample size for SERCA activity to N= 15.

169 *Biochemical analysis*

170 Approximately 50 mg of muscle tissue of 15 piglets were snap frozen within 10 min and later stored
171 at -80°C until used to prepare muscle homogenates according to the procedures described in Giroud
172 et al. [41]. Homogenates were used to measure SERCA activities by a standard coupled enzyme
173 assay, in which the rate of SERCA ATP hydrolysis was calculated from spectrophotometric recording
174 (method previously described by Simonides et al. [42]). While this assay is measuring overall ATPase
175 activity of all isoforms of SERCA present in the sample, SERCA activity is assumed to primarily reflect
176 SERCA1a activity, because SERCA1a is the major isoform found in fast twitching muscles, such as *M.*
177 *semimembranosus* [23]. SERCA activity was divided by total protein concentration (determined with
178 Bradford method [43]) in order to normalize the ATPase activity values for variations in total protein
179 concentrations among samples. More details on isolation of muscle homogenates and SERCA activity
180 measurements can be found in the supplementary methods.

181 *Genetical analyses*

182 Further, 30 mg muscle tissue of 17 piglets were directly stored in RNAlater®, kept in the fridge for 24
183 hours and stored at -80°C until RNA was extracted using the RNeasy Fibrous Tissue Mini Kit (Qiagen,
184 Hilden, Germany). Gene expression levels for SLN and SERCA1a were analysed from cDNA via Droplet

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185 Digital PCR (ddPCR™). RNA was reverse-transcribed with MultiScribe™ Reverse Transcriptase (High
186 Capacity cDNA Reverse Transcription Kits, ThermoFisher Scientific) using random hexamer primers.
187 Primer sequences for the target gene, SLN, were available from Vangheluwe et al. [44]. No primer
188 sequence was available for SERCA1a so we designed suitable primers from the reference sequence
189 NM_001204393.1 with the assistance of the NCBI primer design tool [45]. Primers for candidate
190 reference genes *Hypoxanthine Phosphoribosyltransferase 1 (HPRT1)* and *Glucuronidase Beta (GUSB)*
191 were available from [46]. All primer sequences as well as additional methodological details can be
192 found in the supplementary methods and table S1. Data acquisition was accomplished by the
193 QX200™ Droplet Reader (Bio-Rad), and analysed using the Bio-Rad Droplet Digital™ PCR QuantaSoft
194 software. Expression levels are given as the relative ratio of the concentration (copies/µl) of the
195 assay target gene over the concentration of the reference gene. Importantly, SLN protein expression
196 has been shown to correlate well with SLN mRNA expression [31].

197 *Data analyses*

198 Data are presented as mean ± 1 S.E.; N denotes the number of individuals. Data were analysed in R
199 (V3.4.2)[38]. We first tested whether SERCA1a gene expression, SLN gene expression, SERCA activity
200 and T_b increased with age (using group "day 1" and "day 5") by employing a linear-mixed effect
201 model followed by type II sum-of-squares ANOVA (lme in library 'nlme' [47]; Anova in library 'car'
202 [48]). All models were corrected for non-independence by including the individual's mother as well
203 as the individual´s ID as nested random effects. To investigate whether SERCA activity was explained
204 by SLN and SERCA1a gene expression, we additionally computed linear-mixed effect models
205 including random effects as described above (pseudo r^2 was calculated using sem.model.fits in library
206 'piecewiseSEM' [49]). For the cold exposure experiments we tested whether there was an age
207 depended change in heat production, shivering intensity and shivering frequency as described above.
208 In tests for changes of total heat production, we included body mass as a covariate. Mass-specific
209 rates of heat production were computed for graphical presentation, but not used for statistical tests.
210 To test T_b regulation over the time of cold exposure we computed a linear mixed effect model, again

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211 with ID and mother as random effects, T_b at the end of exposure as the response variable, and day (1
212 or 5), initial T_b, and duration of cold exposure (60-90 min) as fixed effects. We used Shapiro-Wilk
213 tests to access the normality of model residuals. If needed, data were Box-Cox transformed.

214 *Results*

215 *Cold exposure experiment*

216 Heat production during cold exposure did not significantly differ between day 1 and day 5 when
217 adjusted for the increase in body mass (χ^2= 1.92, df= 1, p= 0.165; N= 19; Fig. 3a), although shivering
218 intensity decreased between both measurements by 50% (χ^2= 9.00, df= 1, p= 0.003; N= 12; Fig. 3b).
219 T_b after cold exposure was dependent on T_b at start of the experiment (Tab. 2; χ^2= 8.01, df= 1,
220 p=0.0046), but did not significantly differ between the days (χ^2= 3.33, df= 1, p= 0.0679) and was not
221 dependent on the duration of cold exposure (χ^2= 1.86, df= 1, p= 0.173).

222

223 *SERCA1a, SLN and SERCA activity*

224 SERCA activity, as well as SERCA1a and SLN mRNA expressions increased significantly from day 1 to
225 day 5 of the juveniles´ life (Tab. 1; SERCA1: χ^2= 15.29, df= 1, p<0.0001; SLN: χ^2= 9.63, df= 1, p= 0.002;
226 SERCA activity: χ^2= 16.81, df= 1, p<0.0001) and SERCA1a and SLN expression levels were positively
227 correlated (χ^2= 32.2, df= 1, p<0.0001, marginal pseudo r^2: 0.51; Fig.1). Importantly, the age-related
228 increase of SERCA activity was linked to the increase in SLN gene expression, when tested in a single-
229 predictor model (χ^2= 13.68, df= 1, p<0.001) as well as in multiple regression together with SERCA1
230 (χ^2= 6.57, df= 1, p= 0.01; Fig.2a); SERCA1a gene expression was only significantly influencing SERCA
231 activity when used in a single-predictor model (χ^2= 7.23, df= 1, p= 0.007), but not when tested
232 together with SLN (χ^2= 0.002, df= 1, p= 0.967; Fig. 2b). T_b of piglets increased significantly from day 1
233 to day 5 (χ^2= 41.01, df= 1, p<0.001; Tab.2) and this increase was linked to SERCA activity (χ^2= 5.22, df=
234 1, p= 0.02).

235

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236 ***Discussion***

237 Our study revealed that shivering intensity decreases from day 1 to day 5 in juveniles exposed to
238 10°C, while heat production during cold is increasing proportional to body mass and the level at
239 which T_b is maintained increases. This result is clear evidence for an increasing contribution of NST to
240 thermogenesis during cold exposure in piglets over the first days of life. The finding that
241 simultaneously, SERCA activity and the expression of SERCA1a and SLN were recruited points to
242 muscle NST and increased SERCA activity as the principal source of heat production, as we can rule
243 out UCP1- mediated NST in this species.

244 Our statistical analysis suggests that the increase in SERCA activity between day 1 and day 5
245 was mainly due to an SLN controlled up-regulation of ATP hydrolysis by SERCA1a instead of an
246 increase in SERCA1a molecules. This is in accordance with previous data on mice in which
247 upregulated SLN expression led to an increasing contribution of SERCA-based Ca^{2+}-slippage to heat
248 production [11, 17].

249 In mice and rats, in which neonates are born blind and naked ('altricial' neonates), the
250 transcription and translation of SLN is highest after birth and gradually decreases with development
251 when kept at normal housing conditions (~23°C) [16, 31], while the amount of BAT is successively
252 recruited [1]. However, when kept under cold conditions juvenile mice keep SLN up-regulated for
253 improved thermoregulatory capacity [31], suggesting that both mechanisms of NST are necessary for
254 an effective maintenance of a high T_b during cold exposure in new born rodents. In adult mice,
255 however, both mechanisms of NST, UCP1-mediated as well as muscle NST, can compensate for the
256 loss of one system [50]. In contrast, our data indicate that muscle NST, in combination with some
257 shivering, is already sufficient to maintain a stable T_b for short-term cold exposure in juvenile wild
258 boar, which are markedly larger than juvenile mice and are already born with fur and much better
259 thermogenic abilities ('precocial' juveniles). Interestingly, in other precocial species, such as sheep
260 and goats that possess functional UCP1, BAT is recruited already before birth [1]. Furthermore,

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261 reconstituted function of UCP1 can further improve thermoregulatory function of cold exposed 6-
262 months old Bama pigs, a cold-sensitive pig breed.

263 Our finding that muscle NST is involved in thermoregulation of juvenile wild boars and allows
264 a near stable T_b even during short-term cold exposure supports the hypothesis that muscle NST may
265 be the primary mechanism of heat production during cold-exposure in large mammals lacking BAT.
266 While the evolution of BAT has often been related to the ability of small placental mammals to
267 colonize colder habitats [4, 51, 52], a recent study has shown that UCP1-inactivating mutations have
268 occurred in at least eight of the 18 placental mammalian orders, mainly in larger-bodied species [53],
269 such as pigs. It therefore appears that the combination of shivering and muscle NST is sufficient for
270 heat production in large mammals. Pigs, for example, likely lost UCP1 function and the ability to use
271 BAT for thermoregulation because of absent or only weak selection for this mechanism in a warm
272 climate [54]; all species except the wild boar live only in tropical or subtropical habitats. In addition
273 to heat production via muscle NST, wild boar apparently evolved compensatory mechanisms to cope
274 with adverse thermal conditions in northern habitats, such as larger adult body size [55], building
275 insulating nests for offspring, and synchronizing reproduction within social groups or enabling piglets
276 to huddle in large groups of combined litters [54, 56]. Behavioural thermoregulation is less
277 energetically costly than NST [57] and a study on winter mortality of juvenile wild boar has shown
278 that the negative effects of cold winters can be compensated by high availability of food resources
279 [55].

280 In addition to our finding that SLN-mediated NST in skeletal muscle is involved in piglet
281 thermoregulation, recent studies on domestic pig breeds suggest that SERCA2b (another isoform of
282 SERCA) and UCP3 might also influence pig thermoregulation [58, 59]. However, so far the importance
283 of both mechanisms is unclear [e.g. 2] and the evolution of a compensatory mechanism after the pigs
284 colonized cold habitats is likely [58], while muscle NST is discussed as a potentially evolutionary old
285 heat production mechanism [e.g. 2, 21]. Whether and to what extend domestic pig breeds also
286 possess muscle NST remains speculative. While piglets of wild boar are accustomed to deal with

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287 temperatures around or below zero degrees, domestic pigs are kept under warm conditions (20-
288 35°C). Therefore, it cannot be ruled out that the extreme susceptibility of pigs to cold is partly due to
289 inadvertent selection against high thermogenic capacity during domestication. Previous studies on
290 thermoregulation of juvenile domestic pigs have also found that shivering intensity decreased during
291 the first days after birth while heat production and blood flow to muscles simultaneously increased
292 [60-62]. While this was originally attributed to an increase in shivering efficiency [60, 61], it seems
293 questionable whether an increased thermogenesis by increased efficiency of shivering is physically
294 possible. Our data now suggest that the improved thermogenesis found in domestic pigs, similarly to
295 wild boar, was not due to an increase in shivering efficiency, but explained by an increase in muscle
296 NST.

297 Taken together, our data show for the first time that muscle-based NST via SERCA1a plays a
298 role in the thermoregulation of wild type mammals lacking BAT and that muscle NST can replace
299 UCP1-mediated NST. The function of UCP1 as a thermogenic protein has occurred after the
300 divergence between placental and marsupial mammals [22], suggesting that the evolution of
301 endothermy in ancestral mammals was independent of heat production in BAT. Although the earth
302 was likely warmer, ancestral mammals still would have experienced daily and yearly fluctuations in
303 T_a, likely similar to temperatures found in tropical areas today, which can get rather cold during the
304 night. Therefore, while heat produced as a by-product of metabolic processes as well as basking [63]
305 would have allowed the establishment of a stable T_b during a big part of the day, muscle NST was
306 likely important during the colder night hours.

307 *Ethics statement*

308 The study was approved by the institutional ethics and animal welfare committee and the national
309 authority according to §§ 26ff. of Animal Experiments Act, Tierversuchsgesetz 2012 – TVG 2012
310 (BMWFW-68.205/0171-WF/V/3b/2016).

311 *Data accessibility*

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312 The data will be made available at figshare upon acceptance of the publication.

313 *Acknowledgements*

314 We thank Peter Steiger and Michaela Salaba for their help with animal maintenance, Joy Einwaller,
315 Jessica S. Cornils and Arne Müller for help during the experiments, Omid Hekmat and Michael
316 Hämmerle for biochemical analyses and Martin Olesch, Thomas Paumann and Radovan Kovacki for
317 infrastructural support. The study was supported by funding from the Humboldt foundation to JN
318 and by the University of Veterinary Medicine, the City of Vienna, and the Government of Lower
319 Austria.

320 *Author Contributions*

321 JN and TR designed the experiments, JN conducted the experiments, analysed the data and wrote
322 the manuscript, SV was involved in the performance of the experiments and helped with statistical
323 analyses, GS and JP performed the biopsies, ML and OH conducted the biochemical analyses, MK and
324 SS conducted the genetic analyses, JP designed the accelerometers and computed the shivering
325 intensities, CB designed the enclosures and organised logistics, CB and WA were involved in the
326 discussion of the experimental plan. All authors commented on the manuscript.

327 *Declaration of Interests*

328 The authors declare no competing interests.

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329 *References*

330 1. Cannon, B. and J. Nedergaard, *Brown adipose tissue: function and physiological*
331 *significance.* Physiological Reviews, 2004. **84**(1): p. 277-359.
332 2. Nowack, J., et al., *Muscle non-shivering thermogenesis and its role in the evolution*
333 *of endothermy.* Frontiers in Physiology, 2017. **8**(889).
334 3. Hansen, E. and J. Knudsen, *Parallel measurements of heat production and*
335 *thermogenin content in brown fat cells during cold acclimation of rats.* Bioscience
336 Reports, 1986. **6**(1): p. 31-38.
337 4. Heaton, G., et al., *Brown-adipose-tissue mitochondria: photoaffinity labelling of the*
338 *regulatory site of energy dissipation.* European Journal of Biochemistry, 1978. **82**:
339 p. 515-521.
340 5. Hayward, J.S. and P.A. Lisson, *Evolution of brown fat: its absence in marsupials and*
341 *monotremes.* Canadian Journal of Zoology, 1992. **70**: p. 171-179.
342 6. Vatnick, I., et al., *Regression of brown adipose tissue mitochondrial function and*
343 *structure in neonatal goats.* American Journal of Physiology-Endocrinology and
344 Metabolism, 1987. **252**(3): p. E391-E395.
345 7. Alexander, G., J. Bennett, and R. Gemmell, *Brown adipose tissue in the new‐born*
346 *calf (Bos taurus).* The Journal of Physiology, 1975. **244**(1): p. 223-234.
347 8. Gilsanz, V., H.H. Hu, and S. Kajimura, *Relevance of brown adipose tissue in infancy*
348 *and adolescence.* Pediatric research, 2013. **73**(1): p. 3-9.
349 9. Gaudry, M.J., et al., *Inactivation of thermogenic UCP1 as a historical contingency in*
350 *multiple placental mammal clades.* Science Advances, 2017. **3**(7): p. e1602878.
351 10. Barnett, S.A. and A.C. Neil, *The growth of infant mice at two temperatures.* Journal
352 of Reproduction and Fertility, 1972. **29**(2): p. 191-201.
353 11. Bal, N.C., et al., *Sarcolipin is a newly identified regulator of muscle-based*
354 *thermogenesis in mammals.* Nature Medicine, 2012. **18**(10): p. 1575-1579.
355 12. de Meis, L., *Ca^{2+}-ATPases (SERCA): energy transduction and heat production in*
356 *transport ATPases.* The Journal of Membrane Biology, 2002. **188**(1): p. 1-9.
357 13. de Meis, L., *Uncoupled ATPase activity and heat production by the sarcoplasmic*
358 *reticulum Ca^{2+}-ATPase. Regulation by ADP.* Journal of Biological Chemistry, 2001.
359 **276**(27): p. 25078-25087.
360 14. de Meis, L., *Role of the sarcoplasmic reticulum Ca^{2+}-ATPase on heat production and*
361 *thermogenesis.* Bioscience Reports, 2001. **21**(2): p. 113-137.
362 15. de Meis, L., A. Arruda, and D. Carvalho, *Role of sarco/endoplasmic reticulum Ca^{2+}-*
363 *ATPase in thermogenesis.* Bioscience Reports, 2005. **25**(3-4): p. 181-190.
364 16. Babu, G.J., et al., *Differential expression of sarcolipin protein during muscle*
365 *development and cardiac pathophysiology.* Journal of Molecular and Cellular
366 Cardiology, 2007. **43**(2): p. 215-222.
367 17. Bal, N.C., et al., *Increased reliance on muscle-based thermogenesis upon acute*
368 *minimization of brown adipose tissue function.* Journal of Biological Chemistry,
369 2016. **291**(33): p. 17247-17257.
370 18. Pant, M., N.C. Bal, and M. Periasamy, *Sarcolipin: a key thermogenic and metabolic*
371 *regulator in skeletal muscle.* Trends in Endocrinology & Metabolism, 2016.
372 **27**(12): p. 881-892.
373 19. Anderson, K.J., *Multi-omic analysis of hibernator skeletal muscle and regulation of*
374 *calcium handling*. 2016, University of Minnesota.
375 20. Anderson, K.J., et al., *Proteogenomic analysis of a hibernating mammal indicates*
376 *contribution of skeletal muscle physiology to the hibernation phenotype.* Journal of
377 Proteome Research, 2016. **15**(4): p. 1253-1261.

bioRxiv preprint doi: https://doi.org/10.1101/377051; this version posted July 25, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

378 21. Rowland, L.A., N.C. Bal, and M. Periasamy, *The role of skeletal-muscle-based*
379 *thermogenic mechanisms in vertebrate endothermy.* Biological Reviews, 2014.
380 **10.1111/brv.12157**.
381 22. Saito, S., C.T. Saito, and R. Shingai, *Adaptive evolution of the uncoupling protein 1*
382 *gene contributed to the acquisition of novel nonshivering thermogenesis in*
383 *ancestral eutherian mammals.* Gene, 2008. **408**(1–2): p. 37-44.
384 23. Periasamy, M. and A. Kalyanasundaram, *SERCA pump isoforms: their role in*
385 *calcium transport and disease.* Muscle & Nerve, 2007. **35**(4): p. 430-442.
386 24. Hasselbach, W. and M. Makinose, *Die Calciumpumpe der Erschlaffungsgrana des*
387 *Muskels und ihre Abhängigkeit von der ATPSpaltung.* Biochemische Zeitschrift,
388 1961. **333**: p. 518-528.
389 25. Periasamy, M. and S. Huke, *SERCA pump level is a critical determinant of* Ca^{2+}
390 *homeostasis and cardiac contractility.* Journal of Molecular and Cellular
391 Cardiology, 2001. **33**(6): p. 1053-1063.
392 26. Mall, S., et al., *The presence of sarcolipin results in increased heat production by*
393 Ca^{2+}*-ATPase.* Journal of Biological Chemistry, 2006. **281**(48): p. 36597-36602.
394 27. Asahi, M., et al., *Sarcolipin regulates sarco(endo)plasmic reticulum* Ca^{2+}*-ATPase*
395 *(SERCA) by binding to transmembrane helices alone or in association with*
396 *phospholamban.* Proceedings of the National Academy of Sciences, 2003. **100**(9):
397 p. 5040-5045.
398 28. Maurya, S.K., et al., *Sarcolipin is a key determinant of the basal metabolic rate, and*
399 *its overexpression enhances energy expenditure and resistance against diet-induced*
400 *obesity.* Journal of Biological Chemistry, 2015. **290**(17): p. 10840-10849.
401 29. MacLennan, D.H., M. Asahi, and A.R. Tupling, *The regulation of SERCA-type pumps*
402 *by phospholamban and sarcolipin.* Annals of the New York Academy of Sciences,
403 2003. **986**(1): p. 472-480.
404 30. Bal, Naresh C., et al., *Mild cold induced thermogenesis: are BAT and skeletal muscle*
405 *synergistic partners?* Bioscience Reports, 2017. **37**(5): p. BSR20171087.
406 31. Pant, M., N.C. Bal, and M. Periasamy, *Cold adaptation overrides developmental*
407 *regulation of sarcolipin expression in mice skeletal muscle: SOS for muscle-based*
408 *thermogenesis?* Journal of Experimental Biology, 2015. **218**(15): p. 2321-2325.
409 32. Jastroch, M. and L. Andersson, *When pigs fly, UCP1 makes heat.* Molecular
410 Metabolism, 2015. **4**(5): p. 359-362.
411 33. Berg, F., U. Gustafson, and L. Andersson, *The uncoupling protein 1 gene (UCP1) is*
412 *disrupted in the pig lineage: a genetic explanation for poor thermoregulation in*
413 *piglets.* PLoS Genet, 2006. **2**(8): p. e129.
414 34. Hou, L., et al., *Pig has no uncoupling protein 1.* Biochemical and Biophysical
415 Research Communications, 2017. **487**(4): p. 795-800.
416 35. R Core Team, *R: A language and environment for statistical computing*. 2014, R
417 Foundation for Statistical Computing: Vienna, Austria.
418 36. Lighton, J.R.B., *Measuring metabolic rates. A manual for scientists*. 2008, New
419 York: Oxford University Press.
420 37. Schmidt-Nielsen, K., *Animal physiology: adaptation and environment.* 1983,
421 Cambridge: Cambridge University Press.
422 38. R Development Core Team, *R: a language and environment for statistical*
423 *computing*. 2014, R Foundation for Statistical Computing: Vienna, Austria.
424 39. Developers, S. *Signal: signal processing*. 2014; Available from: http://r-forge.r-
425 project.org/projects/signal/.
426 40. Spaan, G. and F.W. Klussmann, *Die Frequenz des Kältezitterns bei Tierarten*
427 *verschiedener Größe.* Pflügers Archiv, 1970. **320**(4): p. 318-333.

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428 41. Giroud, S., et al., *Membrane phospholipid fatty acid composition regulates cardiac*
429 *SERCA activity in a hibernator, the Syrian hamster (Mesocricetus auratus).* PloS
430 ONE, 2013. **8**(5): p. e63111.
431 42. Simonides, W.S. and C. van Hardeveld, *An assay for sarcoplasmic reticulum Ca^{2+}-*
432 *ATPase activity in muscle homogenates.* Analytical Biochemistry, 1990. **191**(2): p.
433 321-331.
434 43. Bradford, M.M., *A rapid and sensitive method for the quantitation of microgram*
435 *quantities of protein utilizing the principle of protein-dye binding.* Analytical
436 Biochemistry, 1976. **72**(1): p. 248-254.
437 44. Vangheluwe, P., et al., *Sarcolipin and phospholamban mRNA and protein*
438 *expression in cardiac and skeletal muscle of different species.* Biochemical Journal,
439 2005. **389**(Pt 1): p. 151-159.
440 45. Ye, J., et al., *Primer-BLAST: a tool to design target-specific primers for polymerase*
441 *chain reaction.* BMC Bioinformatics, 2012. **13**: p. 134-134.
442 46. Charron, S., et al., *Identification of region-specific myocardial gene expression*
443 *patterns in a chronic swine model of repaired tetralogy of fallot.* PLOS ONE, 2015.
444 **10**(8): p. e0134146.
445 47. Pinheiro, J., et al., *Linear and nonlinear mixed effects models*. 2014.
446 48. Fox, J. and S. Weisberg, *An companion to applied regression*. 2 ed. 2011, Thousand
447 Oaks, CA: Sage.
448 49. Lefcheck, J.S., *piecewiseSEM: Piecewise structural equation modelling in r for*
449 *ecology, evolution, and systematics.* Methods in Ecology and Evolution, 2016. **7**(5):
450 p. 573-579.
451 50. Rowland, L.A., et al., *Uncoupling protein 1 and sarcolipin are required to maintain*
452 *optimal thermogenesis, and loss of both systems compromises survival of mice*
453 *under cold stress.* Journal of Biological Chemistry, 2015. **290**(19): p. 12282-
454 12289.
455 51. Chaffee, R.R.J., et al., *Studies on thermogenesis in brown adipose tissue in*
456 *temperature-acclimated Macaca mulatta.* Comparative Biochemistry and
457 Physiology Part A: Physiology, 1975. **50**(2): p. 303-306.
458 52. Foster, D. and M. Frydman, *Nonshivering thermogenesis in the rat. II.*
459 *Measurements of blood flow with microspheres point to brown adipose tissue as the*
460 *dominant site of the calorigenesis induced by noradrenaline.* Canadian Journal of
461 Physiology and Pharmacology, 1978. **56**: p. 110-122.
462 53. Gaudry, M., et al., *Inactivation of thermogenic UCP1 as a historical contingency in*
463 *multiple placental mammal clades.* bioRxiv, 2016.
464 54. Berg, F., U. Gustafson, and L. Andersson, *The uncoupling protein 1 gene (UCP1) is*
465 *disrupted in the pig lineage: a genetic explanation for poor thermoregulation in*
466 *piglets.* PLoS Genetics, 2006. **2**(8): p. e129.
467 55. Vetter, S.G., et al., *What is a mild winter? Regional differences in within-species*
468 *responses to climate change.* PloS ONE, 2015. **10**(7): p. e0132178.
469 56. Graves, H.B., *Behavior and ecology of wild and feral swine (Sus scrofa).* Journal of
470 Animal Science, 1984. **58**(2): p. 482-492.
471 57. Terrien, J., M. Perret, and F. Aujard, *Behavioral thermoregulation in mammals: a*
472 *review.* Frontiers in Bioscience, 2011. **16**: p. 1428-1444.
473 58. Lin, J., et al., *Cold adaptation in pigs depends on UCP3 in beige adipocytes.* Journal
474 of Molecular Cell Biology, 2017(1-12).
475 59. Ikeda, K., et al., *UCP1-independent signaling involving SERCA2b-mediated calcium*
476 *cycling regulates beige fat thermogenesis and systemic glucose homeostasis.* Nature
477 Medicine, 2017. **23**: p. 1454.

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478 60. Berthon, D., et al., *Metabolic changes associated with sustained 48-hr shivering*
479 *thermogenesis in the newborn pig.* Comparative Biochemistry and Physiology Part
480 B: Biochemistry and Molecular Biology, 1996. **114**(4): p. 327-335.
481 61. Berthon, D., P. Herpin, and J. Le Dividich, *Shivering thermogenesis in the neonatal*
482 *pig.* Journal of Thermal Biology, 1994. **19**(6): p. 413-418.
483 62. Herpin, P., M. Damon, and J. Le Dividich, *Development of thermoregulation and*
484 *neonatal survival in pigs.* Livestock Production Science, 2002. **78**(1): p. 25-45.
485 63. Geiser , F., et al., *Phoenix from the ashes: fire, torpor, and the evolution of*
486 *mammalian endothermy.* Frontiers in Physiology, 2017.
487

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Table 1: Comparison of physiological SERCA activity and the amount of mRNA expression of SLN and SERCA1 at day 1 and day 5 after birth. Sample sizes of tested wild boar piglets are given in brackets. Different letters indicate significant differences. Statistical test results are reported in the text.
* Gene expression is reported in copies per µl target gene/copies per µl reference gene (HPRT1).
+ SERCA activity is reported as ATP hydrolyses per minute and mg total protein.

	Body mass (g)	**SERCA activity**+	**SERCA1a mRNA expression***	**SLN mRNA expression***
Day 1	1411.9 ± 16.5 (N=19)	0.36 ± 0.07 (N=15)[a]	109.6 ± 19.7 (N=17)[a]	40.9 ± 7.8 (N=17)[a]
Day 5	1987.6 ± 29.0 (N=19)	0.96 ± 0.12 (N=15)[b]	320.1 ± 47.2 (N=17)[b]	93.7 ± 14.5 (N=17)[b]

Table 2: Body temperature (T_b) regulation during cold exposure at 15°C (mean ±SD). Statistical test results are reported in the text.

	Day 1	**Day 5**
T_b (°C) before cold exposure	38.6 ± 0.09 (N=19)	39.2 ± 0.07 (N=19)
T_b (°C) after cold exposure	38.6 ± 0.09 (N=19)	39.0 ± 0.07 (N=18)

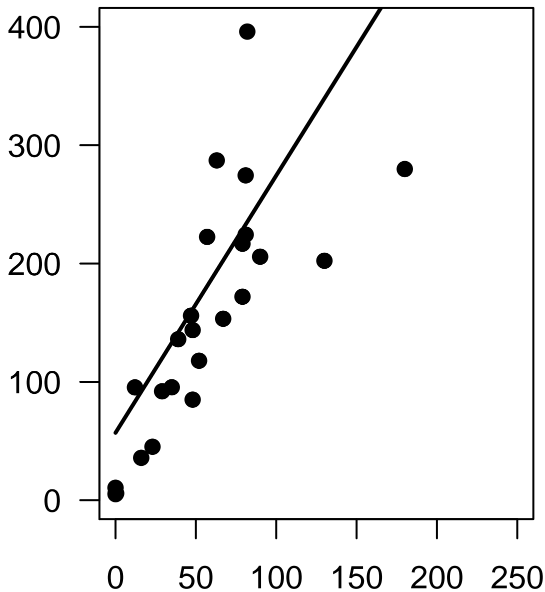
Figure legends

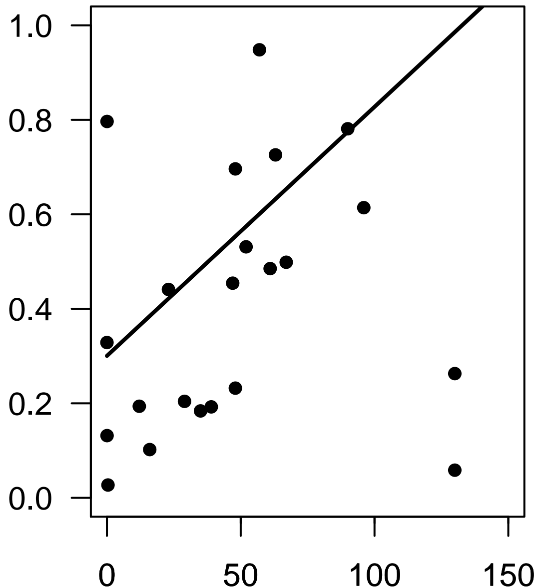
Figure 1: Correlation of SERCA1a and SLN gene expression. The expression of both genes was significantly correlated (χ^2=32.2, df=1, p<0.0001, marginal pseudo r^2: 0.51). Gene expression is reported in copies per µl target gene/copies per µl reference gene (HPRT1).

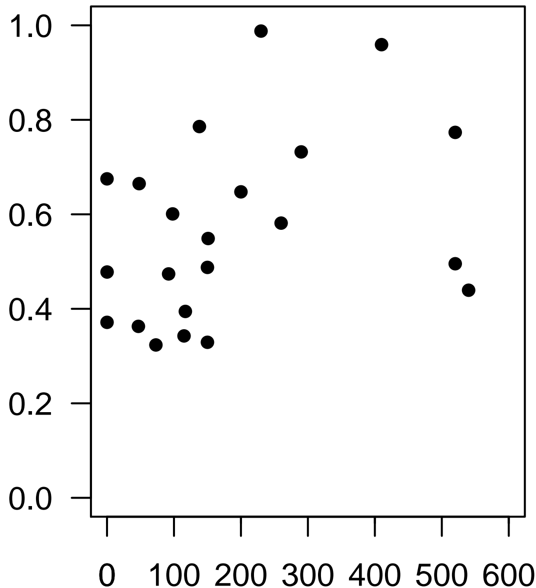
Figure 2ab: Partial regression plots of the effect of SLN and SERCA1a gene expression on SERCA activity. The increase of SERCA activity was linked to SLN gene expression (χ^2=6.57, df=1, p=0.01), while SERCA1a gene expression had no significant effect on SERCA activity when tested together with SLN (χ2=0.002, df=1, p=0.967). Gene expression is reported in copies per µl target gene/copies per µl reference gene (HPRT1), SERCA activity as ATP hydrolyses per minute and mg total protein.

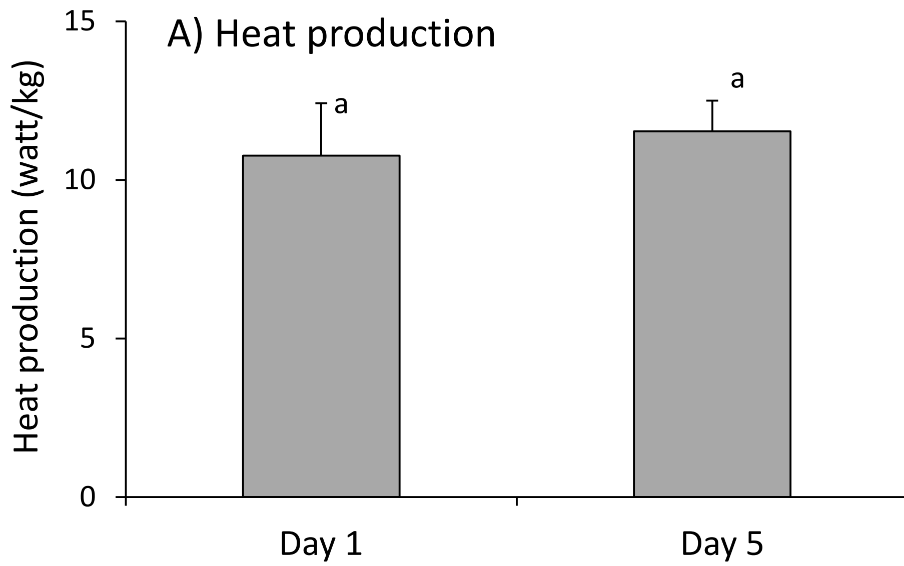
Figure 3ab: Change in heat production and shivering between day 1 and day 2. Different letters indicate significant differences. Heat production, adjusted for the increase in body mass, did not significantly differ between days (N=19; χ^2=1.92, df=1, p= 0.165), whereas shivering significantly decreased (N=12; χ^2=9.00, df=1, p=0.003).

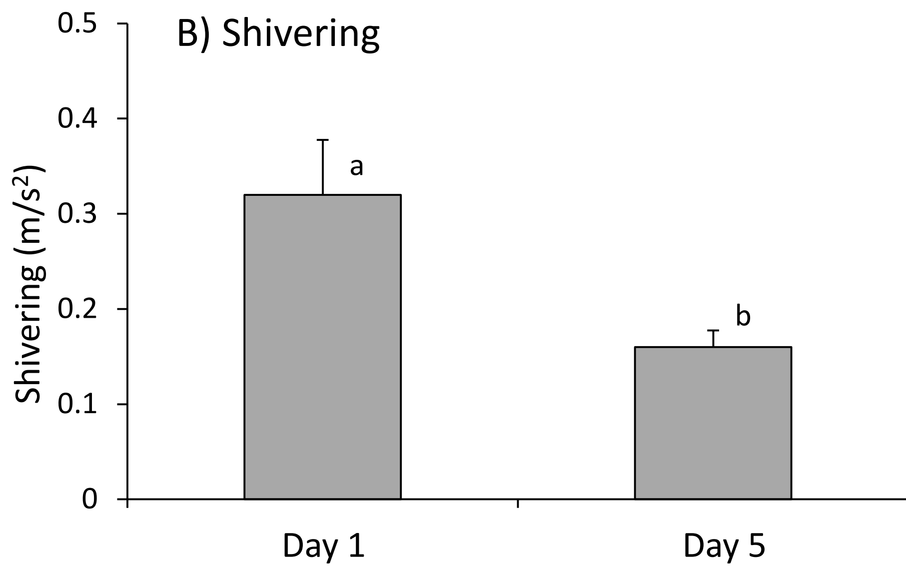
488


400
300
200
100
0
SERCA1 gene expression
0
50
100
150
200
250
SLN gene expression


SERCA activity (?mol ATP mg^{-1} min^{-1})
1.0
0.8
0.6
0.4
0.2
0.0
0
50
100
150
SLN gene expression


SERCA activity (?mol ATP mg−1 min−1)
1.0
0.8
0.6
0.4
0.2
0.0
0
100
200
300
400
500
600
SERCA1 gene expression


A) Heat production
Heat production (watt/kg)
15
10
5
0
a
a
Day 1
Day 5


B) Shivering
Shivering (m/s2)
0.5
0.4
0.3
0.2
0.1
0
a
b
Day 1
Day 5