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# Muscle metaboreflex activation during hypercapnia modifies nonlinear heart rhythm dynamics, increasing the complexity of the sinus node autonomic regulation in humans

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1 **Muscle metaboreflex activation during hypercapnia**  
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5  
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21 led the research. M.I. gave advice on the study design, performed the experiments,  
22 analyzed the results, and discussed and critiqued the manuscript. K.W. gave  
23 advice on the experimental setup and performed the experiments. N.F. led the  
24 experimental setup, performed the experiments, analyzed the results and  
25 discussed the manuscript. T.N. gave advice on the study conception and design as  
26 well as the data processing and discussed and challenged the manuscript.

27 **Running head:** HRV during interaction of the muscle metaboreflex and  
28 hypercapnia

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34 **ABSTRACT [250 words]**

35

36 Muscle metaboreflex activation during hypercapnia leads to enhanced pressive effects that  
37 are poorly understood while autonomic responses including baroreflex function are not  
38 documented. Thus, we assessed heart rate variability (HRV) that is partly due to  
39 autonomic influences on sinus node with linear tools (spectral analysis of instantaneous  
40 heart period), baroreflex set point and sensitivity with the heart period–arterial pressure  
41 transfer function and sequences methods, and system coupling through the complexity of  
42 RR interval dynamics with nonlinear tools (Poincaré plots and approximate entropy  
43 (ApEn)). We studied ten healthy young men at rest and then during muscle metaboreflex  
44 activation (MMA, postexercise muscle ischemia) and hypercapnia (HCA, PetCO<sub>2</sub>=+10  
45 mmHg from baseline) separately and combined (MMA+HCA). The strongest pressive  
46 responses were observed during MMA+HCA, while baroreflex sensitivity was similarly  
47 lowered in the three experimental conditions. HRV was significantly different in  
48 MMA+HCA compared to MMA and HCA separately, with the lowest total power spectrum  
49 ( $p<0.05$ ), including very low frequency ( $p<0.05$ ), low-frequency ( $p<0.05$ ), and high  
50 frequency (tendency) power spectra decreases, and the lowest Poincaré plot short term  
51 variability index (SD1): SD1=36.2 ms (MMA+HCA) vs. SD1=43.1 ms (MMA,  $p<0.05$ ) and  
52 SD1=46.1 ms (HCA,  $p<0.05$ ). Moreover, RR interval dynamics complexity was  
53 significantly increased only in the MMA+HCA condition (ApEn increased from 1.04±0.04,  
54 1.07±0.02, and 1.05±0.03 to 1.10±0.03, 1.13±0.04, and 1.17±0.03 in MMA, HCA, and  
55 MMA+HCA conditions, respectively;  $p<0.01$ ). These results suggest that in healthy young  
56 men, muscle metaboreflex activation during hypercapnia leads to interactions that reduce  
57 parasympathetic influence on the sinus node activity but complexify its dynamics.

58

59 **KEYWORDS:** muscle metaboreflex, hypercapnia, heart rate variability, heart rhythm  
60 dynamics, nonlinear

## 61 INTRODUCTION

62 Patients with chronic obstructive pulmonary disease, obstructive sleep apnea syndrome,  
63 and heart failure are known not only to develop muscle metaboreflex activation and  
64 hypercapnia but also to have increased arrhythmic morbidity and mortality rates  
65 [GAGNON P 2012<sup>1</sup>, OLSON TP 2014<sup>2</sup>, BRUCE RM 2016<sup>3</sup>, GIANNONI A 2016<sup>4</sup>, UENO-  
66 PARDI LM 2017<sup>5</sup>]. Nonetheless, the effects of muscle metaboreflex activation during  
67 hypercapnia on the heart are poorly documented.

68 Inspired by the pioneering works of Ponikowski et al. [PONIKOWSKI P 2001<sup>6</sup>] and Lykidis  
69 et al. [LYKIDIS CD 2009<sup>7</sup> and 2010<sup>8</sup>], we previously documented the cardiovascular  
70 response to forearm muscle metaboreflex activation during hypercapnia in healthy  
71 subjects [DELLIAUX S 2015<sup>9</sup>]. We reported partially additive pressor effects due to the  
72 attenuation of chronotropic and vasoconstrictive responses, the underlying mechanisms of  
73 which were not obvious.

74 Muscle metaboreflex activation in humans by postexercise muscle ischemia (PEMI)  
75 simultaneously increases parasympathetic and sympathetic influences on sinus activity  
76 [NISHIYASU T 1994<sup>10</sup>, PIEPOLI M 1995<sup>11</sup>, IELLAMO F 1999<sup>12</sup>, FISHER JP 2010<sup>13</sup>].  
77 However, this increase in the cardiac parasympathetic influence on sinus activity might  
78 reflect, at least in part, the buffering effects of the arterial baroreflex stimulated by the  
79 increased blood pressure [O'LEARY 1993<sup>14</sup>, NISHIYASU 1994<sup>10</sup>, IELLAMO F 1999<sup>12</sup>, KIM  
80 JK 2005<sup>15,16</sup>, WATANABE K 2010<sup>17</sup>, INCOGNITO AV 2017<sup>18</sup>]. In addition, slight  
81 hypercapnia in dogs strongly increased both vagal and sympathetic discharges to the heart  
82 [KOLLAI M 1979<sup>19</sup>]. More recent pharmacological studies have confirmed the increasing  
83 effects of hypercapnia on cardiovagal activity [IKENOUE T 1981<sup>20</sup>, SICA AL 2002<sup>21</sup>], and  
84 similar results have been found in humans [SASANO N 2002<sup>22</sup>, BROWN SJ 2007<sup>23</sup>].

85 While muscle metaboreflex and hypercapnia generate signals that converge on common  
86 areas in the brain [SPYER KM 1994<sup>24</sup>] and stimulate cardiovascular function through the  
87 autonomic nervous system, the effects of muscle metaboreflex activation during  
88 hypercapnia on sinus node activity, its autonomic regulation and, therefore, heart rate  
89 dynamics in humans still need to be documented and characterized. To fill this gap, we  
90 tested the hypothesis that these combined cardiovascular stimuli increase  
91 parasympathetic modulation of sinus node activity.

92 To assess the neural component of sinus node activity control, we performed RR interval  
93 (RRI) and systolic blood pressure (SBP) time series analyses including heart rate

94 variability, mainly through spectral analysis. Because RRI time series are the result of a  
95 dynamically interacting complex system including muscle metaboreflex, hypercapnia, and  
96 arterial baroreflex inputs, the behavior of these time series might be nonlinear. To capture  
97 the nonlinear dynamics of heart rhythm, we also performed Poincaré plot analysis and  
98 approximate entropy (ApEn) estimation. ApEn is a family of statistics that quantify  
99 entropy and particularly the concept of changing complexity of the system [PINCUS SM  
100 1991<sup>25,26</sup>, 1994<sup>27,28</sup>]. ApEn has already been used and validated in the field of cardiac  
101 autonomic neuroscience [PINCUS SM 1992<sup>29</sup>, FLEISHER LA 1993<sup>30</sup>, AUBERT AE 2001<sup>31</sup>,  
102 BOGAERT C 2001<sup>32</sup>, BECKERS F 2001<sup>33</sup>].

103

## 104 **Materials and methods**

### 105 *Subjects*

106 Ten healthy young males were tested in this study. Their mean ( $\pm$  SEM) age, height  
107 and weight were  $24\pm 1$  years,  $1.73\pm 0.01$  m and  $64\pm 1$  kg, respectively. All subjects had no  
108 history of smoking or taking any medication. Subjects were asked not to exercise for two  
109 days before their participation and not to drink alcohol, coffee or tea 12 hours before the  
110 experiment started. The study was approved by the Ethical Committee of the University  
111 of Tsukuba and was conducted in accordance with the Declaration of Helsinki. Each  
112 subject provided written consent. All experiments were conducted in the presence of a  
113 medical doctor.

### 114 *Experimental procedures*

115 Experimental procedures have been previously described [DELLIAUX S 2015<sup>9</sup>], and data  
116 were reanalyzed in an effort to better understand the mechanisms whereby the interaction  
117 between the metaboreflex and hypercapnia resulted in a hypo-additive heart rate  
118 response. Interventions consisted of measuring maximal voluntary contraction (MVC),  
119 stimulating the muscle metaboreflex, and performing a hyperventilation challenge.  
120 Subjects were asked to grip a hand dynamometer (digital dynamometer; TKK 5101; Takei,  
121 Niigata, Japan) and to perform three maximal voluntary contractions (MVCs) using their  
122 dominant arm. The highest of the three measurements was used in the experiment. Local  
123 circulatory occlusion was used to trap metabolites produced by contracting muscles,  
124 thereby evoking the muscle metaboreflex. Local circulatory occlusion with a suprasystolic  
125 pressure of 240 mmHg was achieved with a rapidly inflatable cuff placed on the upper

126 part of the dominant arm. The cuff pressure reached 240 mmHg in less than 1 s. The  
127 voluntary hyperventilation challenge consisted of calibrated hyperventilation. Breathing  
128 frequency (BF) was maintained at 20 breaths per min regulated by an electronic  
129 metronome, and a 1,500 ml tidal volume (Vt) was targeted (with visual feedback of the  
130 instantaneous Vt). N<sub>2</sub> and CO<sub>2</sub> supplies were adjusted to ensure normoxia and  
131 normocapnia or hypercapnia.

132 In a single experimental day, subjects performed three protocols in random order. Each  
133 protocol entailed a sequence of three periods: control, reflex activation and recovery. The  
134 control period was designed to provide 5 min of resting baseline data with normoxia,  
135 normocapnia and spontaneous breathing. Reflex activation was characterized by a 6-min  
136 experimental stimulus: 1) muscle metaboreflex activation (MMA), which consisted of a 1-  
137 min isometric handgrip exercise at 50% MVC (set from previous experiments and  
138 preliminary data) followed by a 5-min rest, all during normocapnia; 2) normoxic  
139 hypercapnia (HCA), which consisted of a 6-min rest with +10 mmHg end-tidal CO<sub>2</sub> partial  
140 pressure (PetCO<sub>2</sub>) from baseline; and 3) muscle metaboreflex activation during  
141 hypercapnia (MMA+HCA). In all three protocols, dominant upper-arm ischemia induced  
142 by local circulatory occlusion and voluntary hyperventilation were maintained throughout  
143 the 6 min of Reflex Activation. Recovery consisted of a 5-min rest period (no exercise, no  
144 ischemia) under free air breathing conditions. The protocols were separated by rest periods  
145 of at least 30 min.

#### 146 *Measurements*

147 Exclusive mouth breathing was ensured by having the subjects wear a nose clip. A  
148 mouthpiece was connected to a pneumotachograph and a T-shaped non-rebreathing  
149 Rudolph valve. N<sub>2</sub> and CO<sub>2</sub> supplies were controlled by manual flow meters connecting the  
150 inspiratory path to N<sub>2</sub> and CO<sub>2</sub> tanks. The expiratory path was opened to the room air.  
151 Inspiratory and expiratory gases were sampled at the mouthpiece level, and composition  
152 was analyzed by a mass spectrometer (Arco 2000; Arco System, Chiba, Japan). Analog  
153 signals for the respiratory variables, electrocardiogram (3-lead; Nihon Kohden, Tokyo,  
154 Japan), and arterial pressure waveform (finger photoplethysmography; Finometer;  
155 Finapres Medical System, Amsterdam, The Netherlands) were digitized at a sampling  
156 frequency of 1000 Hz (PowerLab 16/30 & LabChart Pro; ADInstruments Japan Inc.,  
157 Nagoya, Japan) and fed into a personal computer.

158 We used Doppler ultrasound to evaluate stroke volume (SV). We measured  
159 ascending aortic blood velocity with a hand-held transducer probe (operating frequency: 2  
160 MHz) connected to a Doppler ultrasound system (Echodopler; HDI 3500; Atl/Philips  
161 Ultrasound, Bothwell, WA, USA). SV was calculated as the product of the aortic blood  
162 velocity (cm/s) and aortic cross-sectional area (cm<sup>2</sup>), while aortic diameter was measured  
163 in a separate resting session.

#### 164 *Data analysis*

165 *Cardiorespiratory variables.* We assessed the hemodynamic profile by measuring  
166 MAP (using digital photoplethysmography), heart rate (HR, using ECG), and SV (using  
167 echo Doppler), then calculated cardiac output as  $CO = SV \times HR$ , from which we calculated  
168 total peripheral resistance as  $TPR = MAP / CO$ . We assessed the ventilatory profile  
169 through measured Vt, BF and Ve, and PetCO<sub>2</sub> and end-tidal O<sub>2</sub> partial pressure (PetO<sub>2</sub>)  
170 obtained from respiratory flows (using pneumotachography) and respiratory gas analysis  
171 (using mass spectrometry), respectively. The ratio of inspiratory time to expiratory time  
172 (Ti/Te) has been used as an index of respiratory drive.

173 *Baroreflex operating point and sensitivity.* Baroreflex control of HR was determined  
174 from the relation and its spontaneous fluctuations (spontaneous arterial baroreflex)  
175 between HR and systolic pressure. The operating point was determined as the HR and  
176 SBP average values over 5 min for each period of the protocol, i.e., control, reflex activation  
177 (during the 5-min local circulatory occlusion), and recovery. Systolic pressure was used  
178 because HR correlates closely with systolic pressure but not with diastolic pressure  
179 [RUDAS L 1999<sup>34</sup>]. To ensure the reliability of spontaneous arterial baroreflex sensitivity  
180 (BRS) assessment, we applied two different methods, the sequences and the transfer  
181 function methods [BERTINIERI G 1985<sup>35</sup>, PARATI G 1988<sup>36</sup>, PINNA GD 2001<sup>37</sup>].  
182 Concerning the sequences method, we first analyzed RRI and SBP beat-to-beat time series  
183 and identified sequences, i.e., all joint RRI and SBP increases or decreases involving at  
184 least 3 consecutive beats. The threshold change was set to 4 ms for RRI and 1 mmHg for  
185 SBP. For each sequence, linear regression between RRI and SBP was performed, and the  
186 regression coefficient was considered only if the correlation coefficient was  $\geq 0.85$ . Mean  
187 value of the absolute values of the regression coefficients, i.e., the mean slope of rectified  
188 regression lines, was taken as a measure of the BRS. Concerning the transfer function  
189 method, we estimated BRS as the mean of RRI-SBP transfer function gains that had a  
190 squared coherence  $\geq 0.85$  in the frequency range of 0.05-0.15 Hz. The transfer function  
191 between RRI and SBP was calculated as

192 
$$H(f) = S_{\text{RRI,SBP}}(f)/S_{\text{RRI,RRI}}(f)$$

193 where  $S_{\text{RRI,SBP}}(f)$  is the cross-spectrum of RRI and SBP signals and  $S_{\text{RRI,RRI}}(f)$  is the auto-  
194 spectrum of the RRI signal. Transfer function gain (magnitude) was obtained from the real  
195 part of the complex function  $H(f)$ , and the squared coherence function, which expresses  
196 the linear relationship between the input and output powers of the studied signals over  
197 the frequencies of interest, was calculated as

198 
$$\text{Coh}(f) = |S_{\text{RRI,SBP}}(f)|^2/[S_{\text{RRI,RRI}}(f) \cdot S_{\text{SBP,SBP}}(f)]$$

199 where  $S_{\text{RRI,SBP}}(f)$  is the cross-spectrum of RRI and SBP signals, and  $S_{\text{RRI,RRI}}(f)$  and  
200  $S_{\text{SBP,SBP}}(f)$  are the auto-spectra of the RRI and SBP signals, respectively.

201 *Autonomic influences on heart period.* Heart rate variability (HRV) due to  
202 sympathovagal and more generally to autonomic effects on the sinoatrial node activity was  
203 assessed by spectral analysis according to guidelines [TASK FORCE 1996<sup>38</sup>]. We first  
204 visually checked all signals to remove and correct artifacts or too many frequent ectopic  
205 beats when necessary. Beat-to-beat RRI discrete time series were generated and then  
206 presented as a function of time. Cubic spline interpolation and resampling at 4 Hz  
207 converted the non-equidistantly sampled RR interval time series to 1200 points at equal  
208 time intervals. Then, we applied Welch's periodogram method for spectral density  
209 estimation. The RRI time series was divided into five equal ~66% overlapping segments of  
210 512 points each. Linear detrending, Hanning windowing, and fast-Fourier transform were  
211 applied to each segment. The five generated spectra were averaged to provide spectrum  
212 estimates of the initial beat-to-beat RRI discrete time series. The spectral resolution was  
213 ~0.008 Hz. Total power (TP; 0–0.4 Hz), very low-frequency (VLF; <0.04 Hz) power, low-  
214 frequency (LF; 0.03–0.15 Hz) power and high-frequency (HF; 0.15–0.35 Hz) power were  
215 calculated by integrating spectral estimates over the frequency ranges of interest. LF and  
216 HF power were also expressed in normalized units (LFnu and HFnu, n.u.) and were  
217 calculated according to  $\text{LF} / (\text{TP} - \text{VLF}) \times 100$  and  $\text{HF} / (\text{TP} - \text{VLF}) \times 100$ . HF in  $\text{ms}^2$  and  
218 n.u. Finally, the ratio of LF [ $\text{ms}^2$ ] to HF [ $\text{ms}^2$ ] was calculated.

219 *Poincaré plot.* In some physiological and pathological states, concomitant  
220 sympathetic and parasympathetic activation can occur, leading to more complex  
221 autonomic modulation of sinus node activity. Acetylcholine and norepinephrine have a  
222 complex interaction at the level of the sinus node, resulting in a typical condition favoring  
223 the occurrence of complex HR dynamics (LEVY MN 1971<sup>39</sup>, LEVY MN 1995<sup>40</sup>). The  
224 Poincaré plot, which is a diagram in which each RR interval of a tachogram is plotted as

225 a function of the previous RR interval (first recurrence map), seems to be a useful tool in  
226 such conditions [TULPPO MP 1998<sup>41</sup>, BRENNAN M 2001<sup>42</sup>, KOICHUBEKOV B 2017<sup>43</sup>].  
227 An ellipse was fitted to the plot; the standard deviation (SD) of the plot data on the minor  
228 axis (SD1) described the instantaneous beat-to-beat variability, while the SD on the major  
229 axis (SD2) described the continuous long-term variability of the data. The ratio SD1/SD2  
230 was also computed and characterizes the shape of Poincaré plots said to highlight  
231 nonlinear properties.

232 *Regularity.* We assessed the complexity of the nonlinear dynamical system that is  
233 the cardiovascular system by calculating the amount of regularity with approximate  
234 entropy (ApEn) [PINCUS M 1991<sup>25,26</sup>, PINCUS M 1994<sup>27,28</sup>]. Entropy refers to system  
235 randomness, regularity and predictability and allows systems to be quantified by the rate  
236 of information loss or generation. ApEn more specifically measures the likelihood that  
237 runs of patterns that are close will remain close for subsequent incremental comparisons.  
238 In the present study, ApEn was calculated according to Pincus' formula [PINCUS M  
239 1991<sup>25</sup>] with fixed input variables  $m = 2$  and  $r = 15\%$  ( $m$  being the length of the compared  
240 runs and  $r$  being a filter) [PINCUS SM 1994<sup>27,28</sup>, GOLDBERGER AL 1994<sup>44</sup>]. Higher  
241 values of ApEn indicate a more complex structure in the time series. In this sense, ApEn  
242 rises with complexity and with interactions when applied to complex adaptive systems  
243 characterized by nonlinearly interacting components. ApEn was calculated as

$$244 \quad \text{ApEn}(m, r, N) = \Phi^m(r) - \Phi^{m+1}(r)$$

245 where  $\Phi^m(r) = (N - m + 1)^{-1} \cdot \sum_{i=1}^{N-m+1} \ln C_i^m(r)$ , with  $N$  being the number of measured  
246 RRIs constituting the time series,  $m$  being a positive integer representing the length of  
247 the compared runs of data (i.e., the embedding dimension, where  $m=2$ ),  $r$  is a positive real  
248 number specifying the filtering level (here,  $r=0.2$ . SD of RRI constituting the time series),  
249 and  $C_i^m(r)$  is the relative number of vectors of length  $m$  for which  $d(u_i, u_j) \leq r \forall i \in$   
250  $(1, 2, \dots, N - m + 1)$  and  $\forall j$ .

### 251 *Statistics*

252 Before any analysis we checked relevance of sample size for our purposes. To achieve a  
253 power of 80% (type II error) and a level of significance of 5% (two-sided type I error), for  
254 detecting an effect size of 1 (strong effect) on the approximate entropy (ApEn) between  
255 pairs as defined by Cohen's  $d$  formula, the study should have required a sample size of 11  
256 (number of pairs). A posteriori calculation of Cohen's  $d$  showed that effect size effect was

257 very strong (equal to 4) in our experiment and that sample size = 10 was sufficient. Data  
258 are presented as the means  $\pm$  SEMs. Statistical analyses were performed using 2-way  
259 repeated-measures ANOVA. The two factors for ANOVA were the period (control and  
260 reflex activation) and the experimental stimulus during reflex activation (HCA, MMA and  
261 MMA+HCA). When main effects were observed, post hoc procedures (Tukey's test after  
262 Bonferroni's correction) compared pairs of means. When appropriate, paired two-tailed t  
263 tests were also used. Values of  $p < 0.05$  were considered significant. Because of the poor  
264 quality of the echo Doppler signal during some periods of the voluntary hyperventilation  
265 challenge in 1 subject, some of his hemodynamic data were excluded.

266

## 267 **RESULTS**

### 268 *Coactivation and cardiorespiratory macro-variables*

269 The data during control and then during reflex activation periods in MMA, HCA and  
270 MMA+HCA conditions are summarized in table 1. Accordingly, there was no difference in  
271 any end-tidal, respiratory or hemodynamic variable at baseline across the three protocols,  
272 and the three experimental conditions led to HR, DBP, MBP, SBP, and CO increases  
273 ( $p < 0.001$ ). When compared to HCA, MMA pressive response led to higher DBP, MBP, and  
274 SBP but to similar chronotropic effects with comparable HR. In contrast, MMA+HCA led  
275 to the strongest cardiovascular response, including the highest HR, DBP, MBP, SBP, and  
276 CO increases, and interestingly led to the only SV significant increase from the control  
277 ( $p = 0.017$ ).

### 278 *Coactivation and baroreflex function*

279 Assessment of spontaneous arterial baroreflex function through determination of its  
280 operating point and sensitivity during control and then during MMA, HCA, and  
281 MMA+HCA is summarized in figure 1. In this figure, only the baroreflex sensitivities  
282 obtained from transfer function analysis are reported (oblique straight lines), and those  
283 from sequence analysis are strongly stackable to them and not significantly different  
284 ( $p = 0.937$ ). As shown, operating points as baroreflex sensitivities were similar across the  
285 three protocols at baseline. In contrast, reflex activation periods modified operating points  
286 accordingly to the experimental condition, i.e., on the one hand MMA and HCA and on the  
287 other hand MMA+HCA. MMA+HCA led to the strongest operating point change ( $p < 0.001$ ),  
288 including the highest SBP increase ( $p < 0.001$ ) and the deepest RRI decrease ( $p < 0.001$ ). In

289 addition, spontaneous arterial baroreflex sensitivity was lowered compared to the control  
290 ( $p < 0.001$ ) but similarly by MMA, HCA and MMA+HCA ( $p = 0.894$ ), from  $19.71 \pm 2.99$ ,  
291  $18.76 \pm 3.02$ , and  $18.09 \pm 3.18$  to approximately  $13.33 \pm 1.97$ ,  $12.94 \pm 1.82$ , and  $13.17 \pm 1.63$   
292  $\text{ms} \cdot \text{mmHg}^{-1}$ , respectively, i.e., approximately -30%.

### 293 *Coactivation and HR dynamics*

294 *HRV and autonomic influences.* RRIs were significantly and markedly lowered  
295 during MMA, HCA and MMA+HCA compared to the control ( $p < 0.001$ ), from  $984 \pm 30\text{ms}$ ,  
296  $1000 \pm 30\text{ms}$  and  $968 \pm 29\text{ms}$  to  $870 \pm 20\text{ms}$ ,  $833 \pm 15\text{ms}$ , and  $789 \pm 15\text{ms}$ , respectively, as shown  
297 in figure 1. The evolution of the power spectra of the RRI time series according to the three  
298 protocols is summarized in table 2. On one hand, overall variability, as expressed by TP,  
299 which is equal to total variance, was significantly lowered only during the MMA+HCA  
300 condition ( $p = 0.0262$ ), reflecting mainly VLF and LF power decreases ( $p = 0.042$  and  
301  $p = 0.0188$ , respectively), HF power tending to decrease only ( $p = 0.068$ ). On the other hand,  
302 LFn<sub>u</sub> and HFn<sub>u</sub> were significantly lowered and increased, respectively, during  
303 MMA+HCA ( $p = 0.0189$ ) only. Therefore, the LF/HF ratio was significantly lowered only  
304 during the MMA+HCA condition ( $p = 0.0495$ ).

305 *Nonlinear HR behavior.* An illustrative example of a Poincaré plot from a  
306 representative subject is shown in figure 2, and statistics are reported in table 2. SD1,  
307 SD2, and SD1/SD2 were similar at baseline across the three protocols. Concerning SD1  
308 and SD2, the MMA+HCA condition led to significant decreases ( $p = 0.0495$  and  $p = 0.02316$ ,  
309 respectively), while MMA and HCA tend to increase SD1 and had no significant effects on  
310 SD2. In contrast, SD1/SD2 was significantly ( $p < 0.001$ ) and similarly higher during MMA,  
311 HCA, and MMA+HCA.

312 *Complexity.* The evolution of ApEn during the protocol is summarized in Figure 3.  
313 Reflex activation periods increased ApEn ( $p = 0.000263$ ), but while MMA and HCA alone  
314 only tended to increase ApEn, the MMA+HCA condition was the only one to significantly  
315 increase ApEn ( $0.00633$ ), and the sum of the ApEn increases induced by MMA on one side  
316 and HCA on the other side was lower ( $p = 0.000572$ ) than the ApEn increase induced by the  
317 MMA+HCA condition.

318

319 **DISCUSSION**

320 To better understand how muscle metaboreflex activation during hypercapnia leads to  
321 hypo-additive pressive and chronotropic effects [DELLIAUX S 2015<sup>9</sup>], we assessed the  
322 autonomic modulation of cardiovascular function from RRI and SBP time series analysis,  
323 including HRV analysis, spontaneous arterial baroreflex operating point and sensibility  
324 determination, and system coupling evaluation through RRI dynamics complexity in  
325 healthy young men. MMA+HCA led to the strongest pressive and chronotropic changes,  
326 while baroreflex sensitivity was similarly lowered in the three experimental conditions.  
327 The major findings of this study are the significant HR dynamics changes observed in the  
328 only MMA+HCA condition, including concomitant spectral powers (depending on  
329 parasympathetic system activity mainly) decrease and ApEn (complexity) increase. These  
330 findings suggest that in healthy young men, when the metaboreflex is activated under  
331 hypercapnic conditions, stimuli interactions lead to enhanced autonomic regulation of the  
332 cardiovascular function with a decreased parasympathetic influence on sinus node activity  
333 and a resetting of the spontaneous arterial baroreflex.

#### 334 *Complexity of HR dynamics*

335 Our results strongly suggest otherwise demonstrate that muscle metaboreflex activation  
336 during hypercapnia leads to interacting cardiovascular responses. The increase in ApEn  
337 in the MMA+HCA condition indicates an interacting dynamical system. ApEn assesses  
338 and classifies complex systems, including both determinist chaotic and stochastic  
339 processes, according to their complexity [PINCUS SM<sup>25,26,27,28</sup>]. Large values of ApEn  
340 indicate the complex structure of a time series and the complex behavior of the system  
341 generating the time series. The more interacting the components of a system, the higher  
342 the complexity of the system [BARANGER M<sup>45</sup>]. The higher the complexity of the system  
343 is, the higher the ApEn [PINCUS SM<sup>25,26,27,28</sup>]. The present data show that complexity in  
344 HR dynamics increased during the MMA+HCA condition and that the sum of ApEn  
345 increases induced by MMA and HCA taken alone was lower ( $p < 0.001$ ) than the ApEn  
346 increase of MMA+HCA. These results support a synergistic interaction and therefore  
347 evoke rather a centrally-located interaction. This hypothesis seems to be corroborated by  
348 a recent study [SHAFER BM 2021<sup>46</sup>] performed on 13 young healthy males that reported  
349 a preserved muscle sympathetic nerve activity response to metaboreflex activation after  
350 acute hypercapnic hypoxia. Increased ApEn measures of RRI time series are rarely  
351 available in the literature. Nevertheless, it has been shown that under varying  
352 physiological conditions and in response to pharmacological denervation of arterial  
353 baroreceptors, the entropy of RRI time series could reflect parasympathetic modulation of

354 HR in conscious dogs [PALAZZOLO JA 1998<sup>47</sup>] and in humans [PENTTILA J 2003<sup>48</sup>].  
355 What's more, high values of ApEn have been shown to decrease from childhood (highest  
356 values) to senescence (lowest values) [PIKKUJAMSA SM 1999<sup>49</sup>], and exercise has been  
357 associated with increased ApEn [TULPPO MP 2001<sup>50</sup>]. Accordingly, we can consider that  
358 during metaboreflex activation and hypercapnia, the sinus node undergoes the influence  
359 of various direct and indirect reflexes (metaboreflex, chemoreflex, baroreflex etc.) that  
360 "complexify" the behavior of heart rate on a beat-by-beat basis. Depending on the  
361 physiological scenario, parasympathetic and sympathetic components of the autonomic  
362 nervous system can work simultaneously and have cross-influence that, *in fine*, can be  
363 inhibitory or excitatory. This could be the case in our experiments and could explain why  
364 the complexity of the RR time series increases that cannot be precisely assessed via the  
365 linear indexes of HRV.

### 366 *Autonomic influences*

367 Indexes from RRI time series spectral analysis are known to be markers of the autonomic  
368 nervous system influences on the sinus node rhythmic activity. We found that TP and  
369 VLF, LF, and HF spectral powers decrease during the MMA+HCA condition. Besides, we  
370 found that LFnu and HFnu decrease and increase, respectively, during the MMA+HCA  
371 condition. According to first HRV guidelines [TASK FORCE 1996<sup>38</sup>], one could interpret  
372 these results as a sympathovagal balance change in aid of parasympathetic influence. On  
373 the contrary, at least three elements should alert us to a most judicious interpretation.  
374 First, a more recent and excellent review [REYES DEL PASO G 2013<sup>51</sup>] reminds us that  
375 scientific literature evidence pleads in favor of a mainly parasympathetic-determined  
376 HRV when assessed by spectral analysis indexes. Second, mathematical constructs of  
377 HRV normalized metrics can lead to confusing changes depending on whether LF and HF  
378 each other relatively vary as  $LFnu = \frac{LF}{TP - VLF} \approx \frac{LF}{LF+HF}$  and  $HFnu = \frac{HF}{TP - VLF} \approx \frac{HF}{LF+HF}$ . Third,  
379 Poincaré plot short term variability index SD1 was lowered during MMA+HCA condition.  
380 SD1, that is the standard deviation of the plot data on the minor axis is mathematically  
381 equal to root mean square of successive differences of RRI duration described the  
382 instantaneous beat-to-beat variability that is neurally supported by the only vagal nerve  
383 [TULPPO MP 1996<sup>52</sup>]. This interpretation is more coherent with MMA+HCA effects on  
384 cardiovascular macro-variables as MMA+HCA led to significantly higher HR and MBP  
385 compared to MMA and HCA taken alone.

386 During PEMI, cardiac sympathetic [PIEPOLI M 1995<sup>11</sup>, IELLAMO F 1999<sup>12</sup>] and  
387 parasympathetic [O'LEARY DS 1993<sup>14</sup>, NISHIYASU T 1994<sup>10</sup>] influences have been  
388 shown to be simultaneously increased and to result from muscle metaboreflex and arterial  
389 baroreflex activations [FISHER JP 2010<sup>13</sup>, WATANABE K 2010<sup>17</sup>, DIPLA K 2013<sup>53</sup>] and  
390 their interactions [ICHINOSE M 2002<sup>54</sup>, 2004<sup>55</sup>, 2005<sup>56</sup>]. Usually, this leads to enhanced  
391 blood pressure, but PEMI-induced parasympathetic outflow to the sinus node overpowers  
392 the metaboreflex-induced cardiac sympathetic activation, limiting HR increase. Similarly,  
393 hypercapnia is known to be associated with increased HR and increased vagal and  
394 sympathetic modulation of sinus node activity [SASANO N 2002<sup>22</sup>], independent of any  
395 main direct effect on the baroreflex [COOPER VL 2005<sup>57</sup>, SIMMONS GH 2007<sup>58</sup>]. In our  
396 study, we observed significantly lowered LF and SD2 in the only MMA+HCA condition,  
397 while BRS sensitivity was similar in the three experimental conditions. This suggests  
398 globally inhibitory effects on combined parasympathetic/sympathetic/baroreflex  
399 modulation [SHAFFER F 2017<sup>59</sup>] of sinus node activity. Because muscle metaboreflex and  
400 hypercapnia alone did not significantly decrease LF and SD2 powers, one can suppose that  
401 responses to MMA+HCA were triggered by their interactions. In our experiment, the  
402 complex system that led to RRI time series included at least three potentially interacting  
403 components: muscle metaboreflex, hypercapnia with direct humoral cardiovascular effects  
404 (hypercapnia *per se*) and indirect neurally supported cardiovascular effects (hypercapnia-  
405 evoked chemoreflex), and arterial baroreflex.

#### 406 *Baroreflex function*

407 Even if the sum of the outputs of two systems taken separately is different from the output  
408 of two systems taken together, it cannot be assumed that they interact directly. In  
409 particular, our previous work could not exclude a limiting effect of the arterial baroreflex,  
410 such that the mechanisms of the observed hemodynamic responses were still unclear.  
411 Here, baroreflex resetting during reflex activation was different according to the  
412 experimental condition. In contrast, hemodynamic variable changes during MMA+HCA,  
413 which we previously attributed to reflex interactions [DELLIAUX S 2015<sup>9</sup>], seem not to be  
414 influenced by baroreflex function, as we found that BRS was similar in the three  
415 experimental conditions during the reflex activation periods, possibly as a result of  
416 resetting. Moreover, as the three experimental conditions led to the same magnitude of  
417 BRS decrease, it would be reasonable to think that a lower limit of baroreflex sensitivity  
418 was reached (a floor value, below which any measurements are out of range). The BRS  
419 values we observed are not unusual and as it has been previously reported that muscle

420 metaboreflex activation enhances BRS [SAMORA M<sup>60</sup>] and that hyperventilation lowers  
421 BRS (VAN DE BORNE P 2000<sup>61</sup>), it is more likely that a physiological condition common  
422 to the three experimental conditions could have driven BRS lowering. An obvious  
423 explanation could be the same respiratory pattern we set in the three experimental  
424 conditions.

#### 425 *Limitations and strengths of the study*

426 A potential limitation of this study is that we did not directly assess sympathetic and  
427 parasympathetic tones. We used indirect indexes (spectral powers) from spectral analysis  
428 of RRI time series that are known to capture only linear properties of HR dynamics.  
429 Nevertheless, in stationary conditions, LF and HF, expressed in ms<sup>2</sup> as well as in  
430 normalized units, are largely thought to reflect sympathetic and parasympathetic  
431 modulatory effects on sinus node activity [TASK FORCE 1996<sup>38</sup>]. But as afore mentioned,  
432 a more recent review study [REYES DEL PASO G 2013<sup>51</sup>] compiling effects of  
433 manipulations affecting sympathetic and vagal activity on HRV, predictions of group  
434 differences in cardiac autonomic regulation from HRV, relationships between HRV and  
435 other cardiac parameters, and the theoretical and mathematical bases of the concept of  
436 autonomic balance evaluates the suitability of low LF heart rate variability as an index of  
437 sympathetic cardiac control and the LF/HF ratio as an index of autonomic balance.  
438 Authors concluded that available data challenge the interpretation of the LF and LF/HF  
439 ratio as indices of sympathetic cardiac control and autonomic balance, respectively, and  
440 suggest that the HRV power spectrum, including its LF component, is mainly determined  
441 by the parasympathetic system. Thus, LF and HF are said to be of limited interest in some  
442 physiological conditions, especially during interacting processes that, by definition, lead  
443 to nonlinear behavior [CASTIGLIONI P 2011<sup>62</sup>, SILVA LE 2017<sup>63</sup>]. In relation to the  
444 complexity of the sinus node activity modulation system, predominantly nonlinear  
445 behavior must be assumed. Thus, the detailed description and classification of dynamic  
446 changes using time and frequency measures is often not sufficient. SD1 is known to be  
447 correlated with short-term HR variability [COPIE X 1996<sup>64</sup>] and HF [CONTRERAS P  
448 2007<sup>65</sup>, GUZIK P 2007<sup>66</sup>]. SD1 is also proposed as a marker of vagal activity [TOICHI M  
449 1997<sup>67</sup>]. But Poincaré plot analysis and SD1 have been described as limited in their ability  
450 to capture nonlinear behavior, as the underlying metric is linear [BRENNAN M 2001<sup>42</sup>,  
451 CICCONE AB 2017<sup>68</sup>], even if lag return maps are usually used to assess phase space in  
452 nonlinear phenomena. In contrast, ApEn is a tool that can assess nonlinear properties  
453 [PINCUS SM 1991<sup>25,26</sup>, 1994<sup>27,28</sup>]. In general, high entropy is a characteristic of healthy

454 complex systems, in which regulatory mechanisms are working on their best performance  
455 and, therefore, are capable of adapting to adverse conditions. Entropy has been described  
456 to be correlated with vagal activity through pharmacological and physiological tests  
457 [PENTILLÄ J 2003<sup>48</sup>, BOLEA J 2014<sup>69</sup>, SILVA LE 2017<sup>70</sup>] and at short scales would reflect  
458 vagal modulation of HR [BARI V 2014<sup>71</sup>, SILVA LE 2016<sup>70</sup>, 2017<sup>63</sup>]. But observed ApEn  
459 should not be considered as a mere expression of vagal activation since exercise, which is  
460 known to notoriously lead to vagal withdrawal and sympathetic activation, is associated  
461 with ApEn increase [TULPPO MP 2001<sup>50</sup>]. Evidence is still lacking to understand  
462 precisely what are the changes in the sympathetic and parasympathetic modulations of  
463 heart during MMA+HCA conditions.

464 Previous studies highlighted that HRV is dependent on the mean HR [PLATISA MM  
465 2006<sup>72,73</sup>, STAUSS HM 2014<sup>74</sup>, MONFREDI O 2014<sup>75</sup>, SACHA J 2014<sup>76</sup>, GASIOR JS  
466 2015<sup>77</sup>]. It is reasonable to think that the lower the heart period is, the more restricted its  
467 range of variation will be. Nevertheless, autonomic influences on HRV cannot be  
468 disregarded to the extent of considering HRV a surrogate of HR [DIAS DA SILVA VJ  
469 2015<sup>78</sup>, YANIV Y 2014<sup>79</sup>]. Here, we showed that even if MMA+HCA led to the highest HR  
470 (shortest heart period), MMA+HCA also led to the increase in ApEn. Now, ApEn is  
471 insensitive to HR variance. The ApEn method extracts the intrinsic dynamic properties of  
472 the RR time series, and the algorithm disregards total variability through the tolerance  
473 factor. This factor ( $r$ ) was taken as a percentage of the time series standard deviation,  
474 introducing a normalization procedure. Therefore, the results presented here can be  
475 assumed to be related to cardiac autonomic modulation and not to the effect of different  
476 mean HRs among subjects and conditions.

477 A second limitation of the study could be the necessary precaution or even caution to take  
478 before generalizing these results. Three points needs to be addressed to consolidate  
479 conclusions: even if our sample size was sufficient for a data re-analysis, it has not been  
480 calculated on all variables of interest of the study that has not been designed accordingly;  
481 female gender was not represented in our volunteer subjects, and all subjects were healthy  
482 young men having daily physical activity. Further studies are needed to confirm and  
483 generalize our first results.

#### 484 *Perspective and Significance*

485 In conclusion, this study showed that, in healthy young men, the muscle metaboreflex  
486 during hypercapnia modifies HRV dynamics. The interaction of physiological functions led

487 to highly nonlinear HRV behavior that needed to be assessed by appropriate tools. In this  
488 study, the variable of ApEn coupled with HRV spectral indexes and Poincaré plot SD1  
489 inde highlighted that the muscle metaboreflex during hypercapnia modifies autonomic  
490 influences on the heart, particularly decreasing vagal modulation of the sinus node  
491 activity and complexifying its dynamics. In addition, our results strongly encourage the  
492 use of such nonlinear tools to study interactions between physiological functions.

493 **LEGENDS**

494 **FIGURE 1. Spontaneous arterial baroreflex set point and sensitivity during control and reflex**  
495 **activation periods**

496 Set points (5-min average RRI (ms) as a function of 5-min average SBP (mmHg)) during control periods  
497 (circles) and reflex activation periods (triangles) are plotted according to the experimental condition,  
498 i.e., MMA (white), HCA (light gray), and MMA+HCA (dark grey). Set-points are shown as the means  
499 (markers)  $\pm$  SEMs (error bars). The BRS values of the control and reflex activation periods are  
500 represented by continuous and dashed lines, respectively. \*\*\*, significant difference vs. control at  
501  $p < 0.001$ ; ### significant difference vs. MMA at  $p < 0.001$ ; £££, ££, significant difference vs. HCA at  
502  $p < 0.001$  and  $p < 0.01$ , respectively. There was no difference in BRS across the three reflex activation  
503 periods: NS, nonsignificant.

504

505 **FIGURE 2. Representative subject data: tachograms, power spectra, and Poincaré plots under the**  
506 **MMA, HCA, and MMA+HCA experimental conditions**

507 Tachograms, power spectra, and Poincaré plots of a representative subject are reported in the upper,  
508 middle, and lower panels, respectively, with the MMA, HCA, and MMA+HCA experimental conditions  
509 presented in the left, middle, and right panels, respectively. Tachograms represent the RR interval (RRI,  
510 ms) as a function of time (min) of the running protocol and the corresponding phases that consisted  
511 of rest (5 min); muscle contraction, if any (1 min, MMA, and MMA+HCA); and venous occlusion (5 min)  
512 leading to PEMI when contraction occurred. Power spectra express the power density ( $\text{ms}^2$ ) of the RRI  
513 as a function of frequency (Hz) according to protocol periods, i.e., rest (dashed gray line) and reflex  
514 activation (continuous black line). Poincaré plots (scatter plots) represent the  $n^{\text{th}}$  RRI (ms) as a function  
515 of the  $n-1^{\text{th}}$  RRI (previous beat, ms) of the RRI time series corresponding to rest (gray markers and line)

516 and reflex activation (dark markers and line) periods. Lines are shown to illustrate the complexity  
517 trajectory of RRI pairs.

518

519 **FIGURE 3. Approximate entropy of the RR interval (RRI) during control and reflex activation periods**

520 Approximate entropy (ApEn, unitless) values are box plotted (median, 25-75<sup>th</sup> percentiles, and 1-99<sup>th</sup>  
521 percentiles) as a function of phase experiments (control and reflex activation) according to  
522 experimental condition: MMA (white), HCA (light gray), and MMA+HCA (dark gray). \*\*\* p<0.001 for  
523 reflex activation vs. control when MMA, HCA, and MMA+HCA data are aggregated (phase effect with  
524 ANOVA); \*\* p<0.01 for reflex activation vs. control in the MMA+HCA experimental condition (condition  
525 effect with ANOVA); # p<0.05 for MMA+HCA vs. MMA; <sup>†</sup> 0.05<p<0.1 for MMA+HCA vs. HCA. ApEn is  
526 significantly enhanced in the MMA+HCA condition.

527

528 **TABLE 1. Cardiorespiratory macro-variables during control (Baseline) and reflex activation (Reflex)**  
529 **periods**

530 Values are means ± SEMs. Control, resting period with free breathing and no stimulus; reflex activation,  
531 period defined by the experimental conditions (voluntary hyperventilation and local circulatory  
532 occlusion in the dominant arm plus hypercapnia or muscle metaboreflex activation or hypercapnia plus  
533 muscle metaboreflex activation). PetCO<sub>2</sub>, end-tidal partial pressure of CO<sub>2</sub>; PetO<sub>2</sub>, end-tidal partial  
534 pressure of O<sub>2</sub>; Ve, minute-ventilation calculated from the raw data and then rounded to the nearest  
535 significant digit; BF, breathing frequency; Vt, tidal volume; Ti/Te, inspiratory time/expiratory time; HR,  
536 heart rate; SBP, systolic blood pressure; MBP, mean blood pressure; DBP, diastolic blood pressure; CO,  
537 cardiac output; SV, stroke volume. \*, \*\*, \*\*\*, significant difference vs. control at p<0.05, p<0.01, and  
538 p<0.001, respectively; #, ##, ### significant difference vs. MMA at p<0.05, p<0.01, and p<0.001,  
539 respectively; <sup>£</sup>, <sup>££</sup>, <sup>£££</sup> significant difference vs. HCA at p<0.05, p<0.01, and p<0.001, respectively.

540

541 **TABLE 2. HRV during control (Baseline) and reflex activation (Reflex) periods**

542 Values are means (rounded to the nearest significant digit)  $\pm$  SEMs of spectral analysis and Poincaré  
543 plot indexes calculated from the 5-min RRI time series. Control, resting period with free breathing and  
544 no stimulus; reflex activation, period defined by the experimental conditions (voluntary  
545 hyperventilation and local circulatory occlusion in the dominant arm plus hypercapnia or muscle  
546 metaboreflex activation or hypercapnia plus muscle metaboreflex activation). HCA, hypercapnia;  
547 MMA, muscle metaboreflex activation; MMA+HCA, muscle metaboreflex activation during  
548 hypercapnia; TP, total power; VLF, very low-frequency power; LF, low-frequency power; HF, high-  
549 frequency power; LFnu, low-frequency power in normalized units; HFnu, high-frequency power in  
550 normalized units; LF/HF, low-frequency power to high-frequency power ratio; SD1, standard deviation  
551 of points along the minor axis (perpendicular to the line of identity) of a fitted ellipse; SD2, standard  
552 deviation of points along the major axis (along the line of identity) of a fitted ellipse; SD1/SD2, SD1 to  
553 SD2 ratio. <sup>†</sup>, \*, \*\*, \*\*\*, significant difference vs. control at 0.05<p<0.1, p<0.05, p<0.01, and p<0.001,  
554 respectively; #, ##, significant difference vs. MMA at p<0.05 and p<0.01, respectively; <sup>£</sup> significant  
555 difference vs. HCA at p<0.05.

556

557 **TABLE 1. Cardiorespiratory macro-variables during control (Baseline) and reflex activation (Reflex)**  
 558 **periods**

	MMA		HCA		MMA+HCA	
	Baseline	Reflex	Baseline	Reflex	Baseline	Reflex
<b>PetCO<sub>2</sub>, mmHg</b>	46.1±0.7	46.7±0.8	45.9±0.6	56.8±0.5 <sup>***,###</sup>	46.0±0.7	56.8±0.7 <sup>***,###</sup>
<b>PetO<sub>2</sub>, mmHg</b>	100±1	102±1	100±1	102±1	100±1	101±1
<b>Ve, L.min<sup>-1</sup></b>	8.5±0.4	29.5±0.5 <sup>***</sup>	8.1±0.4	31.4±0.5 <sup>***,##</sup>	8.2±0.3	31.9±0.4 <sup>***,##</sup>
<b>BF, min<sup>-1</sup></b>	13±1	20±0 <sup>***</sup>	12±1	20±0 <sup>***</sup>	12±1	20±0 <sup>***</sup>
<b>Vt, L</b>	0.7±0.0	1.5±0.0 <sup>***</sup>	0.7±0.0	1.6±0.0 <sup>***,#</sup>	0.7±0.1	1.6±0.0 <sup>***,#</sup>
<b>Ti/Te, no units</b>	68±4	97±3 <sup>***</sup>	62±3	90±2 <sup>***,##</sup>	71±5	85±3 <sup>***,##</sup>
<b>HR, min<sup>-1</sup></b>	61±3	69±3 <sup>**</sup>	60±3	72±4 <sup>***</sup>	62±3	76±4 <sup>***,###,££</sup>
<b>SBP, mmHg</b>	119±3	146±3 <sup>***</sup>	117±4	137±4 <sup>***,###</sup>	123±6	163±6 <sup>***,###,£££</sup>
<b>MBP, mmHg</b>	79±2	101±2 <sup>***</sup>	80±3	91±3 <sup>**,#</sup>	82±3	110±3 <sup>***,##,£££</sup>
<b>DBP, mmHg</b>	60±2	78±2 <sup>**</sup>	61±3	69±3 <sup>*,#</sup>	62±3	82±3 <sup>***,#,£</sup>
<b>CO, L.min<sup>-1</sup></b>	5.21±0.42	6.48±0.24 <sup>**</sup>	5.02±0.24	6.44±0.47 <sup>**</sup>	5.12±0.27	7.44±0.45 <sup>***,##,££</sup>
<b>SV, mL</b>	86±3	94±3	84±3	89±3	84±4	98±4 <sup>*,#,£</sup>

559

560

561 **FIGURE 1. Spontaneous arterial baroreflex set point and sensitivity during control and reflex**  
562 **activation periods**

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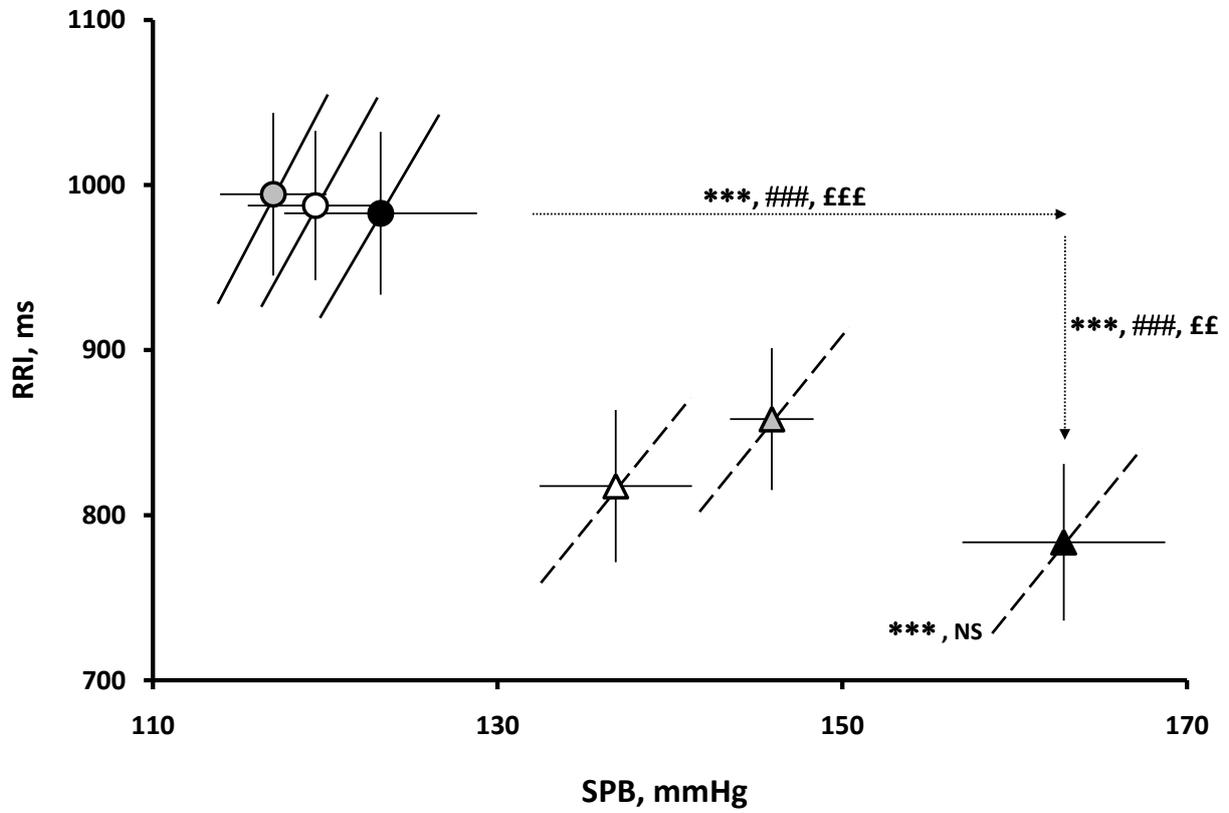


TABLE 2. HRV during control (Baseline) and reflex activation (Reflex) periods

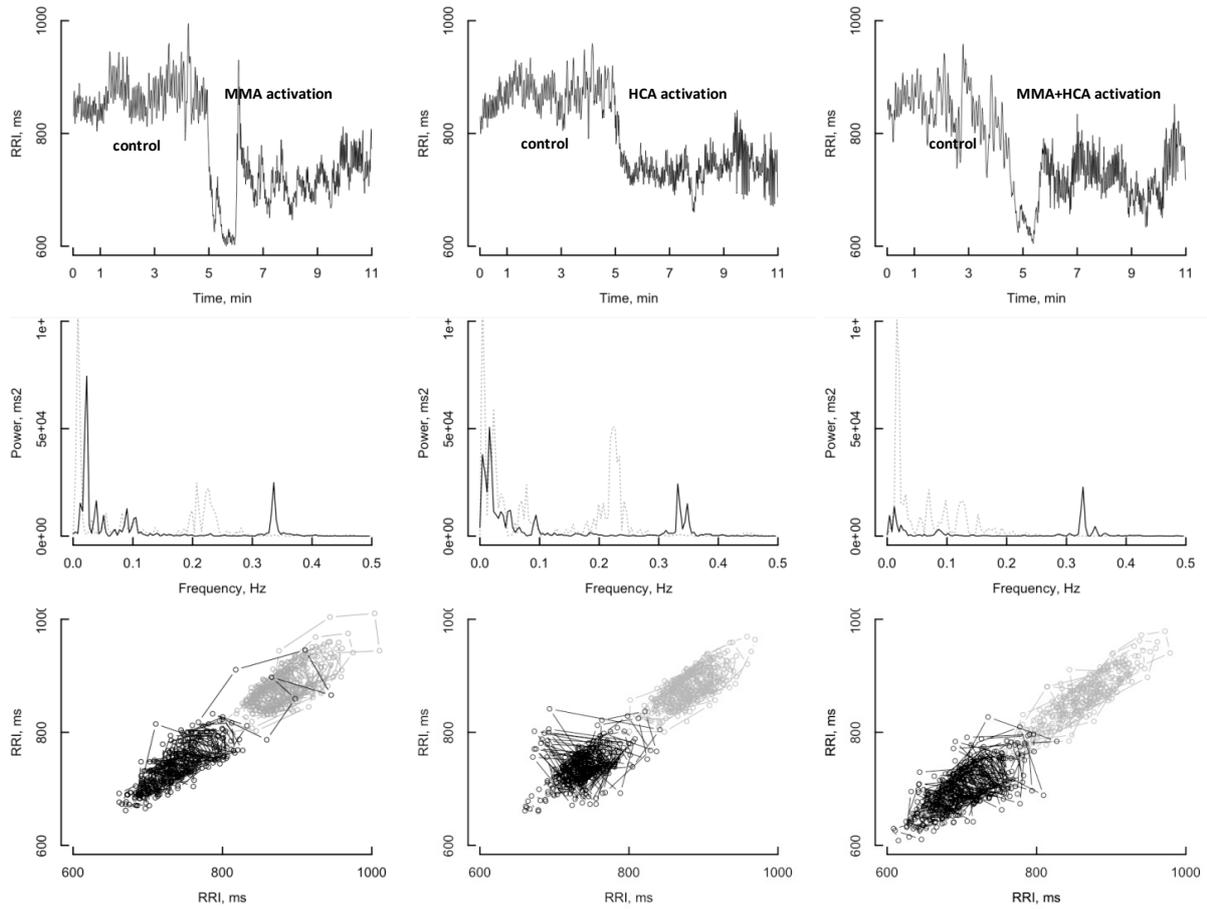
	MMA		HCA		MMA+HCA	
	Baseline	Reflex	Baseline	Reflex	Baseline	Reflex
<i>Spectral analysis</i>						
TP, ms <sup>2</sup>	4530±1277	2185±539	4256±1152	2311±803	4441±948	1485±297*
VLF, ms <sup>2</sup>	898±379	378±63	1205±396	507±100	1137±343	326±70*
LF, ms <sup>2</sup>	1891±1010	452±108	1361±619	316±74	1386±398	267±87* <sup>#</sup>
HF, ms <sup>2</sup>	1741±591	1355±501	1689±504	1489±671	1918±691	892±217
LFnu, n.u.	42.6±7.4	33.3±6	39.1±8.2	27.7±4.8	46.4±9.5	25.0±5.3* <sup>#,£</sup>
HFnu, n.u.	57.4±7.4	66.7±6	60.9±8.2	72.2±4.8	53.6±9.5	75.0±5.3* <sup>#,£</sup>
LF/HF	1.09±0.5	0.33±0.1 <sup>†</sup>	0.80±0.4	0.21±0.1	0.72±0.4	0.29±0.0*
<i>Poincaré plot</i>						
SD1, ms	38.3±7.1	43.1±7.0	35.5±7.6	46.1±9.4	39.8±8.2	36.2±6*
SD2, ms	70.9±9.9	52.6±5.0	66.8±12.3	53.0±6.4	74.6±11.5	47.6±3.9*
SD1/SD2	0.54±0.06	0.8±0.008***	0.53±0.04	0.87±0.09**	0.53±0.07	0.76±0.08**

569 **FIGURE 2. Representative subject data: tachograms, power spectra, and Poincaré plots under the**  
570 **MMA, HCA, and MMA+HCA experimental conditions**

571

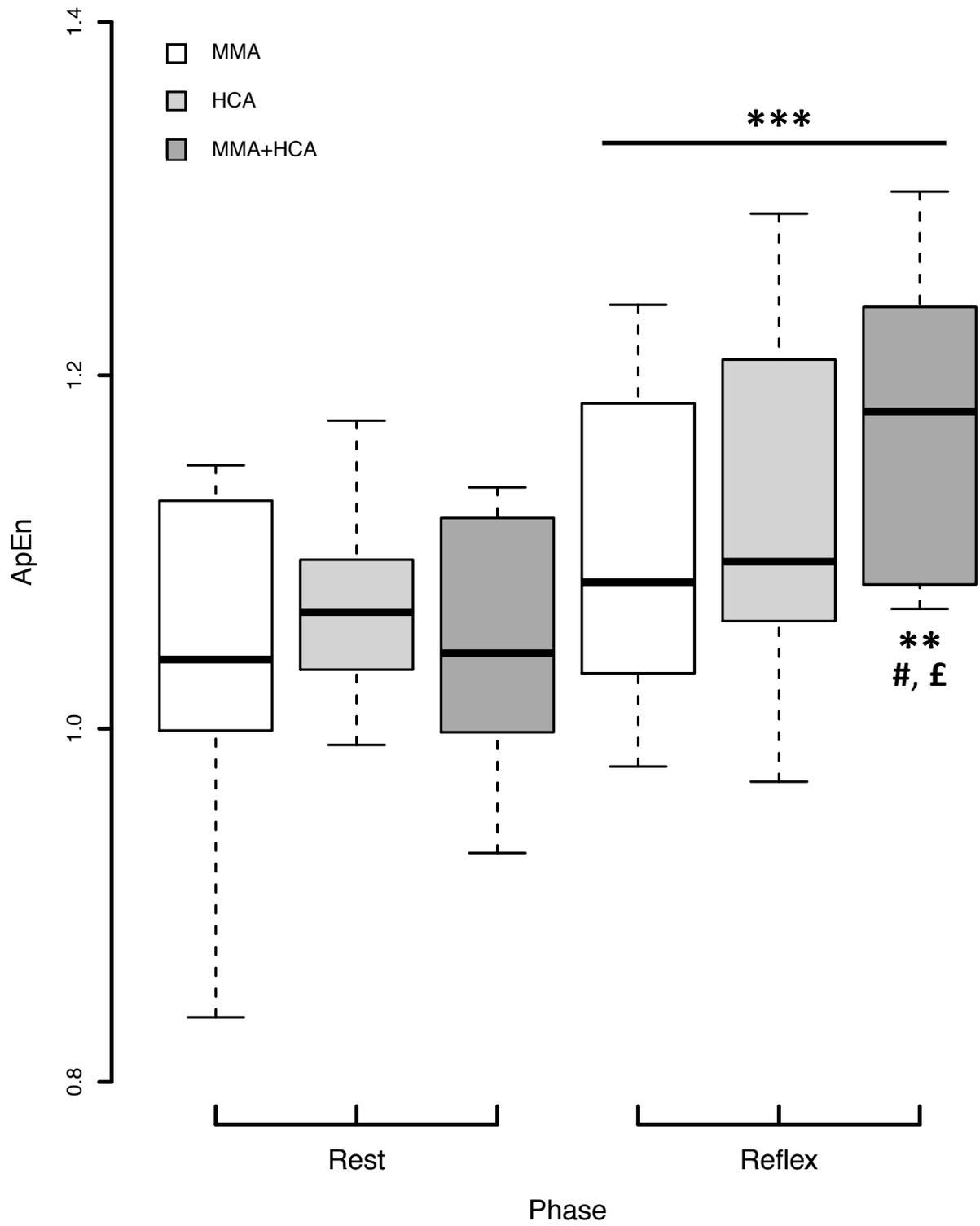
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573



574 **FIGURE 3. Approximate entropy of the RR interval (RRI) during control and reflex activation periods**

575



576

577

578

579 **DECLARATIONS**

580 *Ethical Approval*

581 The study was approved by the Ethical Committee of the University of Tsukuba and was  
582 conducted in accordance with the Declaration of Helsinki. Each subject provided written  
583 consent.

584 *Competing interests*

585 Authors declare to have no competing interests.

586 *Authors' contributions*

587 S.D. conceptualized and designed the study, performed the experiments, processed the  
588 data, analyzed the results, wrote the manuscript, and led the research. M.I. gave advice  
589 on the study design, performed the experiments, analyzed the results, and discussed and  
590 critiqued the manuscript. K.W. gave advice on the experimental setup and performed the  
591 experiments. N.F. led the experimental setup, performed the experiments, analyzed the  
592 results and discussed the manuscript. T.N. gave advice on the study conception and design  
593 as well as the data processing and discussed and challenged the manuscript.

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599 d'Avenir programme" AMX-21-IET-017.

600 *Availability of data and materials*

601 Data are available on reasonable request to the corresponding author.

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