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TITOLO DELLA TESI DI DOTTORATO

CARDIOVASCULAR RESPONSES AND ADAPTATIONS TO BREATH-HOLDING IN HUMANS

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Thanks to those who are close to me and believe in me.
The right word at the right time may worth a life.

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GENERAL INTRODUCTION

Physiological aspects of diving

Apnoea means cessation of breathing, which concerns only the mechanical action of alternate lung expansion and restriction, as alveolar gas exchange does not cease. This will result in a gradual reduction of the partial pressure of oxygen in alveolar air (P_AO_2) and in arterial blood (P_aO_2), with the simultaneous and unavoidable increase in the partial pressure of carbon dioxide in the alveoli (P_ACO_2) and in the arterial blood (P_aCO_2).

When the drop of P_AO_2 and P_aO_2 exceeds certain limits, oxygen delivery becomes insufficient to support metabolism adequately. Thus, metabolism is unable to provide the amount of energy that is necessary to sustain the cells' activities. When such circumstances are attained, there is loss of consciousness (syncope) and, in the absence of immediate medical intervention, progressively more serious brain damage, and even death.

The attainment of such circumstances, however, is generally prevented by the fact that, when there is a remarkable increase in P_aCO_2, chemoreceptor activation causes involuntary diaphragm contractions with subsequent urge to breathe. As soon as this becomes irresistible, the apnoea is interrupted. Moreover, it is postponed by some typical cardiovascular responses, which include bradycardia, extreme peripheral vasoconstriction and increase in arterial blood pressure, the consequence of which is a reduction of oxygen consumption, with subsequent decrease of the rate at which P_AO_2 and P_aO_2 fall. This contributes to protect nervous tissue from the effects of extreme hypoxia. The diagram below (figure 1) illustrates the anatomical and functional structures involved in the responses to the suspension of the breath.

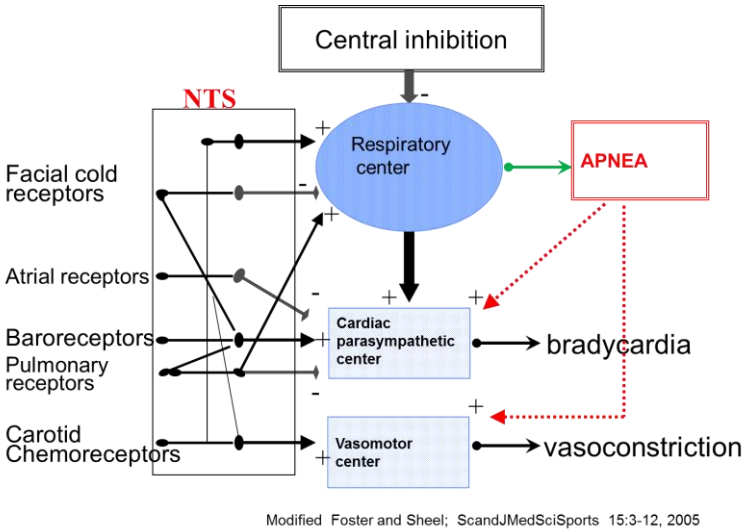

Figure 1. A schematic representation of the pathways assumed to be involved in cardiovascular control during apnoea. Modified after Foster and Sheel, 2005.

If the apnoea is performed during water immersion, most of the reduction of heart rate (HR) occurs within the first 20-30 meters of descent and the levels attained are maintained for the remainder of the dive. In this case, the initial bradycardia is linked to the so called "reflection plunger" or "diving reflex". This reflex is evoked by simple suspension of breathing and is exasperated by the association of cold stimulation of face receptors, for example by immersion of the face in water (Andersson et al., 2000). The afferent branch of the diving reflex is located in the trigeminal nerve, while the efferent branch includes a vagal component, which determines the bradycardia, and an adrenergic vasoconstriction of peripheral vessels.

Some authors (Javorka et al., 2002) have instead shown that during apnoeas without face immersion ("dry") there is an initial stage of tachycardia, lasting at most 10 s, followed by a progressive decrease of HR. The bradycardia is less if the apnoea follows the breathing of an oxygen-enriched gas mixture, indicating the importance of hypoxia. The progressive further reduction of the HR that follows the initial phase leads to a HR nadir similar to that observed for prolonged apnoea at depth, and is the result of a complex interaction between the cardiac inhibition starting from the aortic baroreceptors and cardiac acceleration starting from receptors that are located in the atrial walls (Lindholm and Lundgren, 2009).

The bradycardia is not associated with a compensatory increase in stroke volume (SV), with consequent decrease in cardiac output (CO). It is rather accompanied by a vasoconstriction of the visceral and peripheral vascular beds, and entails a reduction of oxygen consumption by the myocardium. Values as high as 280-300 mmHg for systolic pressure and 200 mmHg for diastolic

blood pressure were detected in elite divers (Ferrigno et el., 1997). It also noteworthy that peripheral vasoconstriction entails a redistribution of blood flow toward the vital organs. In particular there is an increase in carotid blood flow during apnoea (Pan et al., 1997) and a reduction in cutaneous blood flow (Andersson et al., 2002). All these mechanisms are aimed at saving oxygen stores to the advantage of the most sensitive tissues to hypoxic damage, i.e., heart and brain (Andersson and Schagatay 1998; Andersson 2002).

The reduction of CO is larger, the greater is the lung volume at which the apnoea is performed. The high intrathoracic pressure generated at elevated lung volumes results in a reduction of venous return (Andersson et al., 2002).

During extreme apnoea in elite divers, the bradycardia may be so intense that in some cases there may be a junctional rhythm and supraventricular or ventricular extra systoles may appear (Ferrigno et al., 1991). There is no prevalence of any specific arrhythmia, and these arrhythmias may occur even for apnoea in shallow water or in dry apnoeas. These extra systoles are mechanically effective, and so they are followed by heart contraction, thus contributing to the maintenance of an adequate blood pressure (BP); if all the extra systolic beats were to be effective, CO would be high enough to ensure the cerebral perfusion even when HR is as low as 20 b / min.

The BP increase during apnoea follows an increase in total peripheral resistance, in turn due to increased vascular sympathetic activity leading to massive peripheral vasoconstriction (Leuenberger et al., 2001). Vasoconstriction is also triggered by the suspension of breathing and amplified by stimulation of facial cold receptors. With prolonged apnoea, the stimuli of chemical nature (chemoreflexes) become predominant (Foster and Sheel, 2005).

If the apnoea is associated with water immersion, blood shifts from the periphery to the thorax ("blood shift"). Central blood volume increases, thus preventing the crushing of heart and lungs (Ferretti 2001).

Classically, a maximal apnoea was subdivided into two distinct phases, separated by the attainment of the physiological "breaking point" of apnoea, defined as the alveolar gas composition that is able to induce the first involuntary contractions of the inspiratory muscles (Lin et al., 1974; Parkes, 2006). The first phase of the apnoea is called "easy going phase", the second "struggle phase".

Some authors have demonstrated a parallel increase of blood lactate concentration at the end of an apnoea, suggesting the occurrence of anaerobic metabolism (Andersson et al., 2004). Others, however, did not detect any increase in blood lactate at the end of a dry apnoea (Ferretti, 2001), whereas such an increase is a common finding after deep breath-hold dives. The blood lactate accumulation in a condition in which the intensity of the performed exercise is as low as walking at optimal speed is considered a consequence of extreme vasoconstriction in peripheral districts, namely skin and muscles, such that blood flow and oxygen delivery to these districts are reduced to zero.

The spleen is a dynamic store of erythrocytes, of which it is able to deliver a large volume during exercise (Stewart et al., 2002), during immersion (Espersen et al., 2002) or in response to severe hypoxia (REF). The splenic contraction is mediated by adrenergic α2 receptor stimulation and leads to an increase in hematocrit and in hemoglobin concentration, thus improving oxygen transport capacity. The splenic contraction was in fact observed even during resting apnoea (Bakovic et al., 2003; Palada et al., 2007).

Unlike the diving reflex, which occurs immediately in the first seconds of suspension of the breath, the splenic contraction requires more than an apnoea to be activated at full. This is probably because the splenic contraction depends on the liberation of humoral factors, in particular noradrenaline (Schagatay E. et al., 2001). Some studies have shown the occurrence of a rapid reduction of the volume (about 20%) of the spleen and of the splenic content of erythrocytes during apnoea, without substantial alteration of the blood flow in the artery and the splenic vein. The return to baseline values is rather slow (it is still incomplete 60 minutes after the end of a maximal apnoea), and the splenic contraction could contribute to the prolongation of repeated apnoeas. In aquatic mammals, the splenic contraction occurs at depth and has the effect of liberating erythrocytes previously seized at ambient pressure.

Cardiovascular responses to prolonged apnoea

The kinetics of the cardiovascular response to prolonged apnoea at rest was recently studied on a beat-by-beat basis, both during dry apnoeas (Perini et al ., 2008), and during apnoea in immersion (Perini et al., 2010). The analysis of responses to dry apnoea at rest - performed in the supine position, which is typically used by divers for training and which from the point of view of cardiovascular function is similar to water immersion – has allowed to clearly characterise the

kinetics of adjustment periods precisely. Perini et al (2008) divided a maximal apnoea into three phases, called respectively Phase I, Phase II and Phase III. After the start of the apnoea there is a rapid transition phase, lasting no more than 30 seconds (phase I), followed by a period of about 120-140 seconds of cardiovascular balance (phase II) and by a final phase (phase III) during which there is a continuous modification of parameters. This last phase is, however, present only in individuals who are able to prolong the suspension of breath beyond two and a half minutes. An example of such a recording, with identification of the three phases of apnoea, is reported in Figure 2.

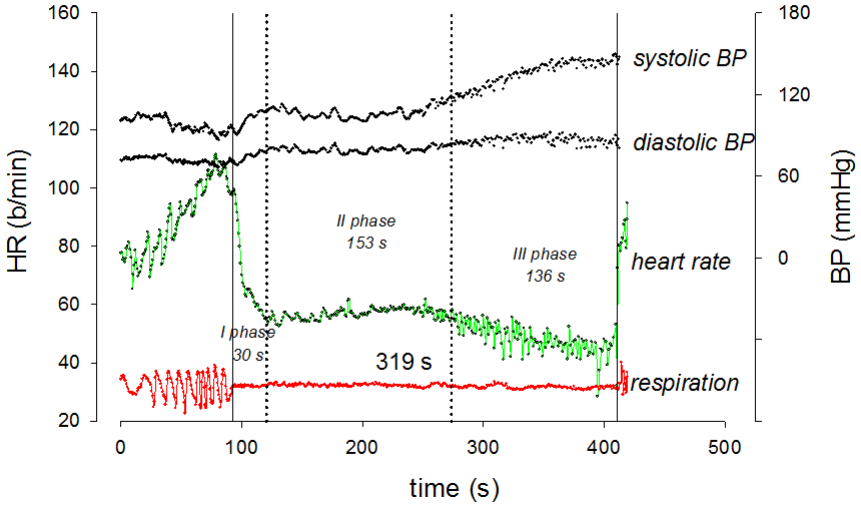

Figure 2. Beat-to-beat values of heart rate (HR) and systolic and diastolic blood pressure (SBP, DBP) during prolonged dry apnoea. Apnoea was divided in phases according to changes in cardiovascular parameters: phase I, transition phase; phase II, "steady state" phase; phase III, increases in SBP an DBP with slight changes in HR.

Results very similar were also obtained during the static apnoeas in immersion in water at 27 ° C, where the presence of the three phases was confirmed (Perini et al, 2010), with similar duration as in dry apnoeas.

In phase I, already in the first few seconds of apnoea, both dry and wet, there is a marked decrease in systolic blood pressure (SYS) and, although less severe, in diastolic blood pressure (DIA), that some authors attributed to the sharp reduction in venous return as a consequence of breathing suspension (Andersson and Schagatay, 1998; Palada et al., 2007). After the attainment of a minimum, BP increases rapidly. After 25-30 seconds SBP returns to its initial values, while DIA reaches values even higher than those before apnoea. It was also observed that, at the end of phase I, the mean arterial pressure (MAP) was higher than before the apnoea, suggesting vasoconstriction. Some authors have proposed that apnoea activates the sympathetic system that acts mainly on muscle vessels (Zbrozyna and Westwood, 1992; Foster e Sheel, 2005) and, during immersion in

water at temperature lower than the neutral, possibly also on cutaneous vessels (Pendergast and Lundgren, 2009), with a greater increase in resistance. In phase I there is also an immediate, or in some cases delayed by a few seconds, decrease in HR, so that at the end of phase I HR reaches values lower than those before apnoea (Andersson et al. , 2002; Hansel et al., 2009). A fast response of such entity is attributable to the inhibitory action of the vagus, also triggered by the suspension of breathing (Lindholm et al., 1999; Foster and Sheel, 2005). Noteworthy is the fact that in diving apnoeas the decrease of HR in the first 25-30 seconds is greater than in the dry apnoeas, presumably for the additive effect of the "diving reflex" (Foster and Sheel, 2005; Jay et al., 2007). The increase in BP does not precede the reduction in HR, suggesting that the role of the baroreceptor reflex, which normally regulates the interaction between HR and BP, is negligible, if not absent, in phase I. This is in agreement with the conclusion of Journeay et al., (2003) according to whom the cardiac and vascular controls are dissociated during apnoea.

The next phase (phase II), as said, is characterized by the presence of almost constant values of all cardiovascular variables, around the levels attained at the end of phase I. The presence of a steady state cardio during prolonged apnoea was not identified in previous studies, which were not carried out on a beat-by-beat basis. Something corresponding to phase II was even not observed during deep apnoeas, whether in sea (Ferrigno et al., 1991) or in a hyperbaric chamber (Ferrigno et al., 1997). Those studies in fact showed a continuous decrease in HR up to extremely low values. However, multiple other factors, such as water temperature and the depth of immersion, could influence the cardiovascular responses.

After about two minutes of stable values in phase II, phase III starts, indicating a state in which the balance is broken. In dry apnoeas there is a progressive, linear reduction of HR, associated with a progressive, almost linear increase in BP. This corresponds somehow to the very marked increase of BP observed during deep apnoeas (Ferrigno et al, 1997). At the end of phase III, the HR may reach extremely low values even in dry apnoeas, whereas SYS may attain values above 200 mmHg with DIA above 100 mmHg.

During dry apnoeas, S_aO_2 does not change up to about 2/3 of the phase II; the subsequent decrease leads to S_aO_2 at the end of phase II around 0.95. During phase III, the decline of S_aO_2 becomes sharper, so that values below 0.85mare often attained at the end of phase III.

The experimental data of the cardiovascular responses obtained on a beat-by-beat basis in dry apnoea lead to the hypothesis that the end of phase II, i.e. the breaking of the steady state for cardiovascular variables, may coincide with the physiological breaking point of apnoea (Lin et al, 1974). This is defined as the time when the first diaphragmatic contractions appear (Lin et al., 1974; Parkes, 2006; Palada et al., 2008), as a consequence of the activation of chemoreceptors by hypoxia and hypercapnia (Hong et al., 1971, Lin et al., 1974; Ferretti et al., 1991). The activation of chemoreflexes entail subsequent circulatory readjustments, with a progressive and continuous increase in BP accompanied by a gradual continuous decrease in HR (phase III). In support of this hypothesis is the observation that the rhythmic oscillations of HR and BP around their average value are progressively amplified during phase III (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996).

AIM OF THE THESIS

The aim of this thesis was to test the general hypothesis, detailed in the Introduction, that the end of phase II, i.e. the breaking of the steady state for cardiovascular variables, may coincide with the physiological breaking point of apnoea (Lin et al, 1974). This hypothesis was investigated by means of two, interrelated studies. Each of these studies analysed an experimental consequence of the general hypothesis, the one opposite with respect to the other.

The first experimental consequence to be tested was that the duration of phase II and phase III would be shorter when apnoeas are carried out during light exercise than at rest because of the increase in metabolic rate in the former case. Of course, we expected to find the same values for cardiovascular variables at end of phase II at exercise as at rest, with similar characteristics during phase III. The testing of this hypothesis is the object of the first article, which is currently under revision at the European Journal of Applied Physiology.

The second experimental consequence to be tested was that the duration of phase II and phase III would be longer when apnoeas are carried out when pure oxygen is breathed before apnoea instead of air, because of the increase in oxygen stores in the former case. Of course, we expected to find the same values for cardiovascular variables at end of phase II at exercise as at rest, with similar characteristics during phase III. The testing of this hypothesis is the object of the second article, which is currently under preparation.

METHODS

The methods of the two studies were the same. They are therefore presented together. Differences in protocols between the two studies are highlighted, when necessary.

Subjects

Experiment 1: Six male competitive divers volunteered for this study. Their age was 37.2 + 7.3 years, and they were 73.1 + 9.5 kg heavy and 176 + 4 cm tall. All divers were non-smokers and had no previous history of cardiovascular, pulmonary or neurological diseases. None of them was taking medications at the time of the study. All gave their informed consent after having received a detailed description of the methods and experimental procedures of the study. Local ethical approval was obtained.

Experiment 2: Seven apparently healthy male subjects volunteered for this study. The main inclusion criterion was the ability to carry out a dry apnoea at rest lasting at least 3 minutes. Two of them were not habitual free divers**,** the remaining five were agonist divers**.** Their age was 38.1 ± 14.1 years, they were 178.3 ± 7.3 cm tall and weighed 74.7 ± 8.3 kg. All subjects were non-smokers and had a negative history for cardiovascular, pulmonary or neurological disease**.** None of them was taking drugs at the time of the study, nor were habitual consumers of alcohol. All gave their informed consent after having received a detailed description of the methods and experimental procedures of the study. Local ethical approval was obtained.

Experimental procedure

Experiment 1. All tests were carried out in a thermostated room at 25–26°C, in the late afternoon. Upon arrival in the laboratory, after instrumentation, the subject took the sitting posture. Five minutes were allowed to achieve steady state conditions; then, 10 min of measurements were obtained with the subject lying at rest and spontaneously breathing (control). Then he was asked to perform two successive prolonged apnoeas. The second apnoea was requested to be maximal. Subjects undertook their pre-dive breathing routine before breath-holding, generally consisting of two-to-three deep respiratory acts. All apnoeas were initiated with a lung volume close to the subject's total lung capacity, so that the last breathing movement before breath-holding was a deep

inspiration and the first breathing movement at the end of apnoeas was an expiration, which the subjects were asked to perform as deep as possible. Then he moved on the cycle ergometer, where, after 3 min of quiet resting, he started pedalling at a power of 30 W. After attainment of the exercise steady state, 1 min of recordings during regular breathing was allowed. Then two maximal apnoeas were performed, with a recovery interval of 2 min between them. Again, two-to-three deep respiratory acts preceded the apnoea start, and the last breathing movement before breath-holding was a deep inspiration. After the end of the second apnoea, the subject kept exercising for 3 min. Both at rest and at exercise, the longest apnoea was retained for further analysis.

Experiment 2. All tests were carried out in a room at 25–26°C, in the late afternoon. Upon arrival in the laboratory, after instrumentation, the subject took the supine posture. Five minutes were allowed to achieve steady state conditions; then, 10 min of measurements were obtained with the subject sitting at rest and spontaneously breathing (control). Then he was asked to perform two successive prolonged apnoeas. The second apnoea was requested to be maximal. Subjects undertook their pre-dive breathing routine before breath-holding, generally consisting of two-to-three deep respiratory acts. All apnoeas were initiated with a lung volume close to the subject's total lung capacity, so that the last breathing movement before breath-holding was a deep inspiration and the first breathing movement at the end of apnoeas was an expiration, which the subjects were asked to perform as deep as possible. The same protocol was then completed in the sitting posture.

After completion of the tests in air, the same subjects repeated the protocol, on the following day, while breathing pure oxygen before and after performance of apnoeas. Inspired oxygen was administered from precision high pressure gas cylinders via an 80-l Douglas bag buffer. The gas flow from the cylinders was continuously adjusted to the subject's ventilation. Since the gas analyser could not measure inspired oxygen fraction, because it was outside the operational range of the gas analysers, the steady state oxygen consumption before the performance of apnoeas was not measured during this protocol, for which the metabolic rate was considered equivalent to that observed during the corresponding experiments in air.

Methodology

Arterial blood pressure profiles (PortaPres, TNO-TPD, Amsterdam, The Netherlands) were continuously recorded throughout the experiments. Arterial blood O_2 saturation (SaO_2) was also continuously monitored by infrared spectroscopy (BioPac System Inc., Goleta, CA, USA) at an earlobe. The signals were sampled at 100 Hz by using a 16-bit A/D converter (MP100 VS, BioPac

System Inc., Goleta, CA, USA) and stored on a personal computer for subsequent analysis. Metabolic rate and air flow at the mouth were monitored by means of a metabolic cart (Quark b_2, Cosmed, Italy) whenever the subject was breathing.

Data treatment

Arterial pressure profile and respiratory traces were analysed off line. Beat-to-beat values of HR, systolic, diastolic and mean blood pressures (SBP, DBP and MSP, respectively) were computed. The duration of each apnoea for each subject was calculated as the time over which the flow meter recorded no respiratory air flows. The pulse pressure profile was analysed by means of the Modelflow model (Wesseling et al, 1993 implemented in the Beatscope™ software (TNO-TPD, The Netherlands). Stroke volume (SV) and cardiac output (Q') were calculated. The ratio between MSP and Q' was calculated to estimate total peripheral resistance (TPR). Metabolic rate and ventilation were calculated at rest and exercise steady state as the mean of the breath-by-breath values over 1 min of regular breathing. Alveolar partial pressures of oxygen (PAO_2) and carbon dioxide ($PACO_2$) in experiment were taken as the end tidal value observed in the final part of the first deep expiration at the end of apnoea, which is known to consist of alveolar air.

The beat-by-beat data were analysed off-line to identify the different phases of apnoeas (Perini et al., 2008). An automated procedure implemented under Matlab (version 7.6.0.324, MathWorks, Natick, MA, USA) was used to this aim. The procedure was based on linear regression analysis, allowing detection of changes in slope between successive phases.

Statistical analysis

Data are presented as mean and standard deviation (SD). One-way ANOVA for repeated measures was used to evaluate the effect of time of apnoea on various variables at rest and exercise. Tukey test was used as post-hoc test to isolate the differences when necessary. Paired t-test was used to compare the duration of breath-holding at rest versus exercise. Differences that were below the $P<0.05$ were considered significant. Linear regression was used dynamic baroreflex sensitivity (Adami et al., 2013). Parameters of linear regression equations were calculated by the least-square method. The Stata 10.0 statistical software (StataCorp, College Station, TX, USA) was used.

STUDY 1

A BEAT-BY-BEAT ANALYSIS OF CARDIOVASCULAR RESPONSES TO DRY RESTING AND EXERCISE APNOEAS IN ELITE DIVERS

Paper submitted to the European Journal of Applied Physiology

Introduction

The cardiovascular responses to breath-holding have been investigated for long. Bradycardia is a well-known phenomenon, reported in almost all studies carried out so far. Its intensity is accentuated by face immersion, especially in cold water (Andersson et al., 2000), and is stronger in elite divers than in non-diver controls (Andersson and Schagatay, 1998). Cardiac output is correspondingly decreased (Bjertnæs et al., 1984; Palada et al., 2008), whereas arterial blood pressure is increased (Bjertnæs et al., 1984). Most of the studies report either values from measurements obtained before and at the end of apnoeas, or mean values at various breath-holding times. Descriptions of the beat-by-beat dynamics of cardiovascular changes during breath-holding, whether dry or on the water surface, are scanty. Observations during short apnoeas in air (Palada et al., 2007) or with face immersion (Andersson and Schagatay, 1998; Andersson et al., 2000) suggested that a transient fall in blood pressure associated with tachycardia may precede the typical cardiovascular response. In apnoeas prolonged to the breaking point, hypertension and bradycardia were described as continuously developing phenomena (Andersson and Schagatay, 1998; Andersson et al., 2000).

More recently, continuous beat-by-beat observations during apnoeas prolonged to the volitional breaking point, carried out on elite divers who could sustain apnoea for longer than 3 min (Perini et al., 2008, 2010), have demonstrated the occurrence of three distinct phases of the cardiovascular response to apnoea: i) a dynamic phase of rapid changes, lasting no more than 30 s; ii) a steady phase, lasting about 2 min, in which the blood pressure and heart rate (HR) values attained at the end of phase I are maintained invariant; and iii) a further dynamic phase, in which there are continuous decrease in HR and increase in blood pressure, linear with time, prolonged to the attainment of the volitional breaking point. The interpretation was that the end of the steady phase could represent the physiological breaking point (Hong et al., 1971). In fact the amplitude of HR and blood pressure oscillations was increased in the subsequent phase, suggesting the occurrence of a new source of fluctuation, perhaps related to the onset of diaphragmatic contractions.

During exercise, the increase of metabolism accelerates the rate at which the oxygen stores are emptied and the CO_2 stores are replenished in the body. This would shorten the time necessary to attain the O_2 and CO_2 levels characterising the physiological breaking point and raising the first diaphragmatic contractions. Therefore, we may well expect to find the three phases of cardiovascular responses to apnoea in exercise. However, if indeed the end of the steady phase represents the attainment of the physiological breaking point, its duration should be reduced in apnoeas carried out during light exercise. Moreover, the rate at which the cardiovascular variables change in the subsequent unsteady phase would be steeper in exercise than at rest. These direct experimental consequences of the above hypothesis were not tested so far. Several studies investigated the cardiovascular responses to breath-holding during exercise (Bjertnaes et al., 1984; Breskovic et al., 2011; Lindholm et al., 2002; Nishiyasu et al., 2012; Smeland et al., 1984; Tocco et al., 2012), but to the best of our knowledge no study ever described these responses on a beat-by-beat basis.

The aim of the present study was to investigate the cardiovascular responses to breath-holding during light dynamic exercise in humans on a beat-by-beat basis, in an attempt at testing some cardiovascular consequences of the general hypothesis formulated above.

Methods

The methods have been described in the global paragraph above.

Results

1. Apnoeas at rest.

In quiet rest, $V'O_2$ was 246 $\pm$ 47 ml min^{-1} and V'E was 9.0 $\pm$ 2.3 L min^{-1}. Mean duration of maximal apnoea at rest was 239.4 $\pm$ 51.6 s. An example of HR and SBP recordings obtained on one subject during maximal resting apnoea is shown in Figure 3.

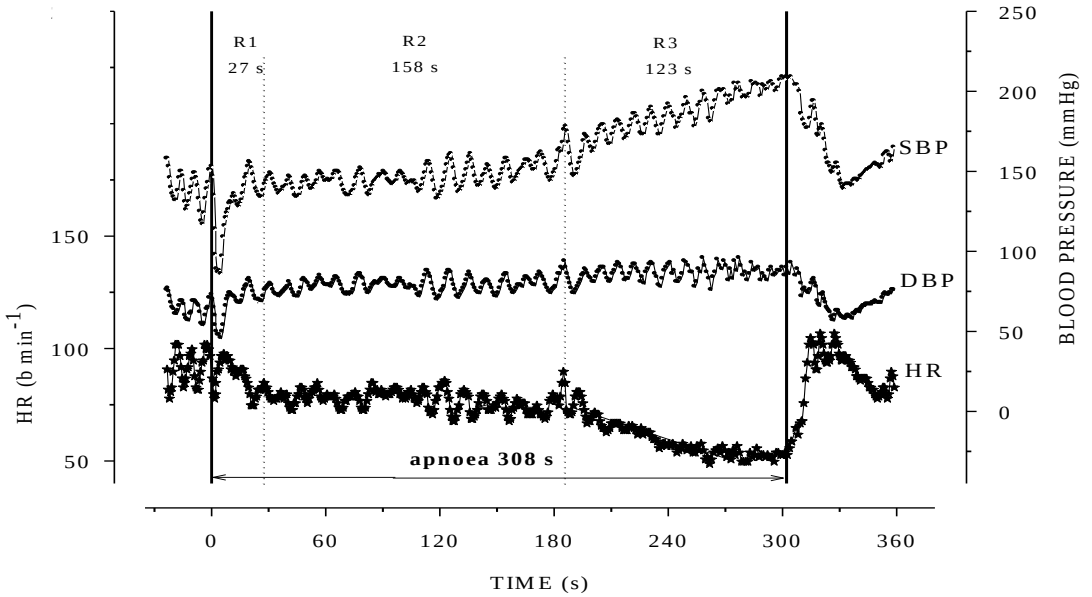

Figure 3. Heart rate (HR) and systolic blood pressure (SBP) recordings obtained on one subject during maximal resting apnoea.

The same patterns were followed by all subjects. Values of all variables obtained during quiet resting are reported in Table 1. During the hyperventilation that preceded breath-holding, HR grew to attain 96 $\pm$ 6 min^{-1} at the beginning of apnoea ($p<0.05$ with respect to control). The corresponding SBP and DBP were 154 $\pm$ 19 and 77 $\pm$ 12 mmHg, respectively (NS with respect to control). SaO_2 was 0.998 $\pm$ 0.004.

	SBP mmHg	DBP mmHg	MSP mmHg	HR b min^{-1}	SV ml	Q' L min^{-1}	TPR mmHg min L^{-1}
Resting	135$\pm$ 20	68$\pm$ 10	87$\pm$ 11	84$\pm$ 10	97$\pm$ 21	8.1$\pm$ 1.8	11.5$\pm$ 3.4
Exercise	159 $\pm$ 16	74 $\pm$ 9	97 $\pm$ 11	91 $\pm$ 9	102 $\pm$ 13	9.4 $\pm$ 1.9	10.8 $\pm$ 2.1

Table 1: Cardiovascular variables during rest and light exercise (means $\pm$ S.D.).

The evolution of all variables during maximal breath-holds at rest is reported in Figures 4 and Figures 5, and table 2.

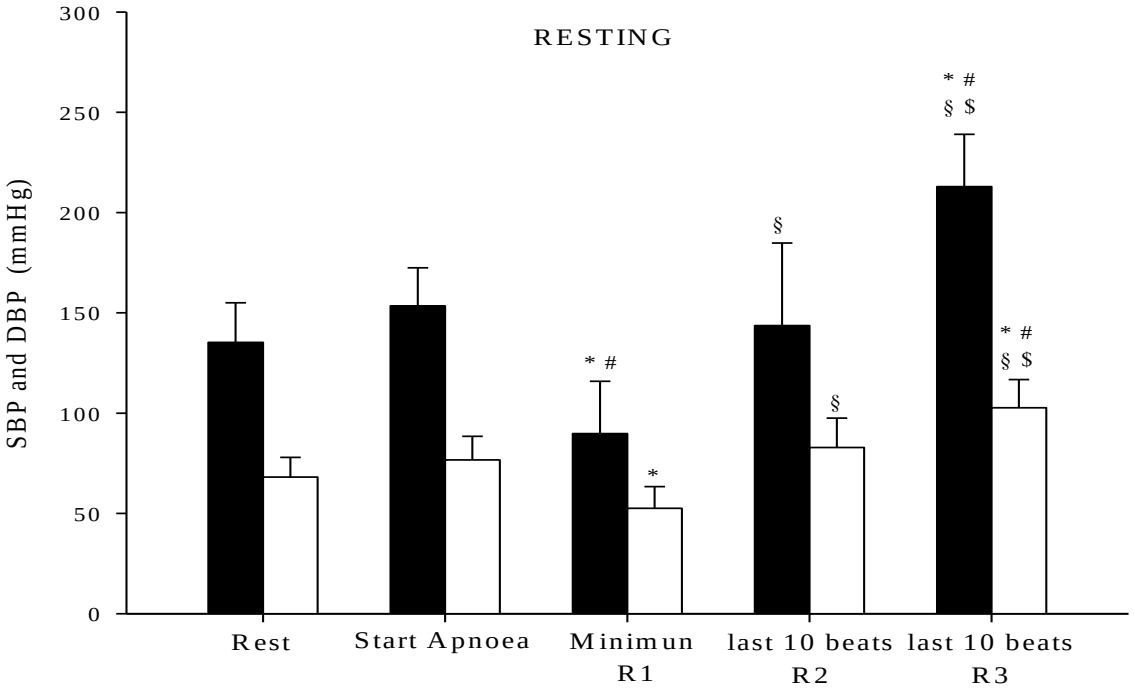

Figure 4. Evolution of systolic blood pressure (SBP) and diastolic blood pressure (DBP) during resting apnoea *: p<0.05 respect to rest; #: p<0.05 respect to start apnoea; §: p<0.05 respect to minimum R1; $: p<0.05 respect to R2.

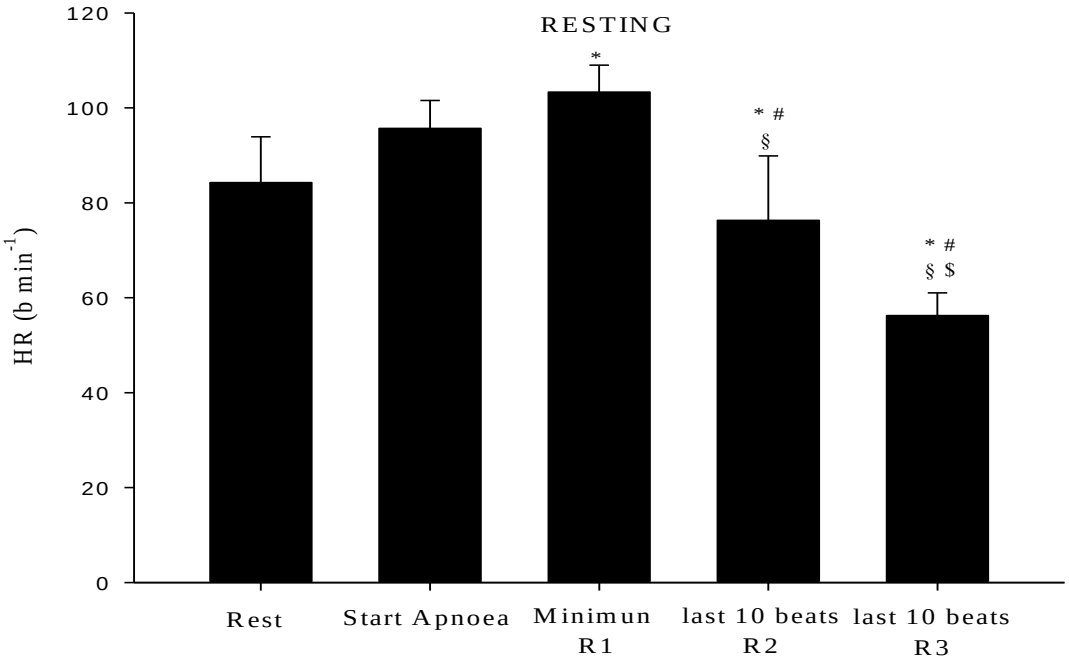

Figure 5. Evolution of heart rate (HR) and stroke volume (SV) during resting apnoea *: p<0.05 respect to rest; #: p<0.05 respect to start apnoea; §: p<0.05 respect to minimum R1; $: p<0.05 respect to R2.

a

RESTING	Rest	Start	Minimum R1	Last 10 beats R2	Last 10 beats R3
SV (ml)	97 + 21	96 + 13	53 + 18$^{*\#}$	68 $\pm$ 19$^{*\#}$	100 $\pm$ 10$^{\S\$}$
Q' (L min^{-1})	8.1 $\pm$ 1.8	9.1 $\pm$ 1.1	5.4 $\pm$ 1.9$^{*\#}$	5.1 $\pm$ 0.6$^{*\#}$	5.6 $\pm$ 0.4$^{*\#}$
TPR (mmHg min L^{-1})	11.5 $\pm$ 3.4	10.8 $\pm$ 1.7	12.6 $\pm$ 4.0	20.0 $\pm$ 4.9$^{*\#\S}$	25.1 $\pm$ 4.8$^{*\#\S\$}$

b

EXERCISE	30 W	Start	Minimum E1	Last 10 beats E2
SV (ml)	102 $\pm$ 13	92 $\pm$ 27	51 $\pm$ 18$^{*\#}$	90 $\pm$ 14§
Q' (L min^{-1})	9.4 $\pm$ 1.9	9.6 $\pm$ 2.5	5.7 $\pm$ 1.8$^{*\#}$	5.2 $\pm$ 0.9$^{*\#}$
TPR (mmHg min L^{-1})	10.8 $\pm$ 2.1	11.0 $\pm$ 3.2	12.0 $\pm$ 5.3	29.5 $\pm$ 6.2$^{*\#\S}$

Table 2: evolution of SV, Q' and TPR during resting (a) and exercise (b) apnoeas (means $\pm$ S.D.).

*: $p<0.05$ respect to rest; #: $p<0.05$ respect to start apnoea;§: $p<0.05$ respect to minimum R1 or minimum E1; $: $p<0.05$ respect to R2.

The first unsteady phase of breath-holding (R1) lasted 27.9 $\pm$ 1.6 s. During R1, SBP and DBP fell remarkably, to attain a minimum of 90 $\pm$ 26 mmHg ($p<0.05$ with respect to both quiet rest and beginning of apnoea) and 53 $\pm$ 11 mmHg ($p<0.05$ with respect to beginning of apnoea) after 5.7 + 1.9 s. Correspondingly, HR increased to a maximum of 103 $\pm$ 6 min^{-1}. The corresponding SV was 53 $\pm$ 18 ml, so that Q' at the nadir of SBP resulted equal to 5.4 $\pm$ 1.9 L min^{-1}. Since MSP was 63 $\pm$

13 mmHg, TPR turned out equal to 12.6 ± 4.0 mmHg min L^{-1}. All these values except TPR were significantly different from those observed both during quiet rest and at the beginning of apnoea.

An example of an individual relationship between HR and MSP during the initial phase of apnoea, before the attainment of the minimum of SBP, is shown in Figure 6.

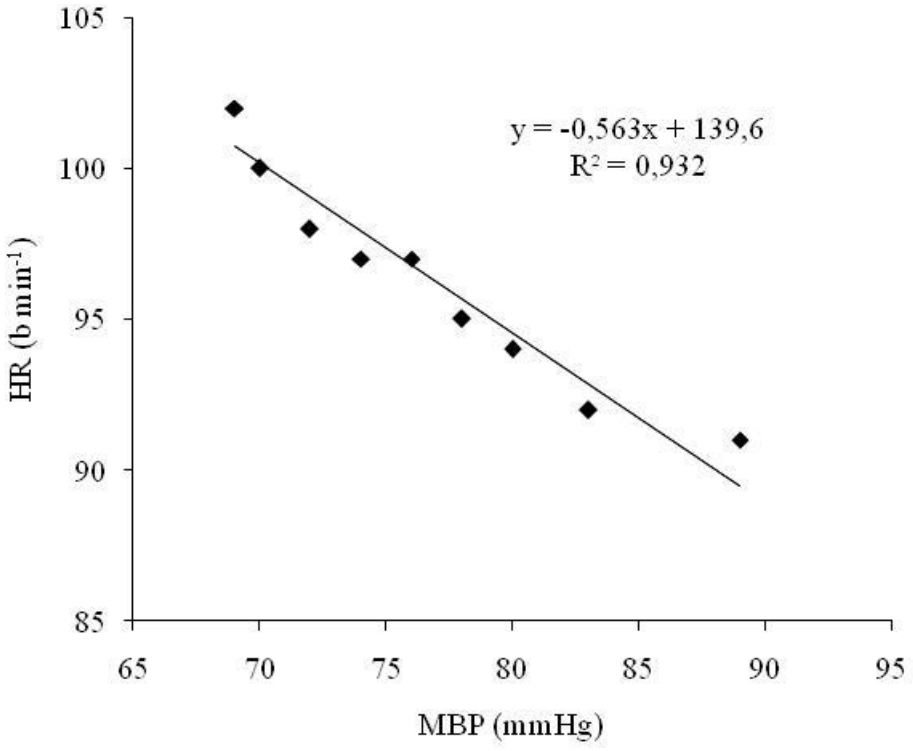

Figure 6. Mean blood pressure (MSP) and heart rate (HR) relationship obtained on one subject during R1, before the attainment of the minimum of systolic blood pressure (SBP).

The figure includes a series of consecutive beats in which MSP decreased and HR increased with respect to the immediately preceding beat. Application of linear regression analysis provided a slope of -0.563. This value was taken as indicative of the spontaneous baroreflex sensitivity for the subject at stake during apnoea R1. Similar relationships were obtained on all subjects. The slopes of all individual regression lines are reported in Table 3.

RESTING APNOEA									
	R1 (before minSBP)			**R1 (after minSBP)**			**R3**		
Subject	**Slope**	**BATT. (R^2)**	**R**	**Slope**	**BATT. (R^2)**	**R**	**Slope**	**BATT. (R^2)**	**R**
1	-	-	-	-1.098	33(0.903)	0.950	-1.575	158(0.837)	0.915
2	-0.792	8(0.905)	0.951	-0.645	7(0.730)	0.854	-0.332	88(0.265)	0.515
3	-0.520	7(0.909)	0.953	-0.175	7(0.439)	0.663	-0.729	153(0.574)	0.758
4	-0.310	5(0.915)	0.957	-1.379	13(0.898)	0.948	-0.278	139(0.245)	0.495
5	-0.563	10(0.932)	0.965	-0.726	6(0.785)	0.886	-0.294	136 (0.392)	0.626
6	-0.353	6(0.953)	0.976	-1.267	8(0.814)	0.902	-0.319	112(0.415)	0.644
Mean	**-0.508**			**-0.882**			**-0.588**		
DS	**0.192**			**0.452**			**0.513**		

EXERCISE APNOEA									
	E1 (before minSBP)			**E1 (after minSBP)**			**E2**		
Subject	**Slope**	**N**	**R**	**Slope**	**N**	**R**	**Slope**	**N**	**R**
1	**-0.840**	**6**	**0.885**	**-1.716**	**10**	**0.918**	**-1.258**	**10**	**0.944**
2	**-0.675**	**8**	**0.978**	**-0.400**	**8**	**0.687**	**-0.990**	**5**	**0.712**
3	**-0.273**	**6**	**0.861**	-	-	-	**-0.855**	**12**	**0.853**
4	**-0.300**	**7**	**0.928**	**-0.633**	**7**	**0.928**	**-1.148**	**6**	**0.946**
5	-	-	-	-	-	-	**-0.546**	**9**	**0.532**
6	**-0.663**	**6**	**0.822**	**-0.843**	**8**	**0.921**	**-0.569**	**6**	**0.782**
Mean	**-0.550**			**-0.898**			**-0.894**		
DS	**0.251**			**0.575**			**0.295**		

Table 3: relationship between heart rate (HR) and mean blood pressure (MAP) during the first phase, before and after the attainment of the minimum of systolic blood pressure (minSBP) and the last phase of resting and exercise apnoeas. N indicates the number of consecutive beats over which regression lines were computed. Data are presented as slope of all individual regression lines and relative Paerson's coefficient. -: data not available, due to insufficient (three or less) number of consecutive beats in which HR and MAP varied in opposite direction.

After attainment of the minimum of SBP, the values of the cardiovascular variables underwent partial recovery, to attain a steady level (R2) that lasted 84.9 $\pm$ 33.5 s. The re-establishment of SBP was accompanied by a drop of HR. The mean slopes of the individual relationships between HR and MSP during the recovery part of R1 are also reported in Table 3. As long as HR decreased, SV

increased, and the opposite patterns followed by HR and SV were compensatory, so that during R2, the steady Q' was not different from that found at the minimum of SBP in R1. As a consequence, R2 was characterised by higher TPR values than before apnoea or in R1. HR, SV and Q' were lower than in quiet rest.

R2 was followed by a new unsteady phase (R3), wherein HR decreased steadily and continuously, whereas SBP and DBP increased. At the end of apnoea, HR was significantly lower than in all previous conditions. The reverse was the case for SBP. The SV increase compensated for the fall of HR, so that Q' did not vary during R3. As a consequence, TPR continuously increased. The slopes of the individual relationships between HR and MSP during R3 are also reported in Table 3.

SaO_2 started to decrease in R2. It reached a value of 0.945 $\pm$ 0.067 at the end of R2. SaO_2 kept decreasing during the entire R3, to attain a minimum of 0.710 $\pm$ 0.149 a few seconds after the end of apnoea.

2. Apnoeas at exercise

Before apnoea, the steady state $V'O_2$ during exercise was 862 $\pm$ 160 ml min^{-1}, i.e. twice as high as at rest. The corresponding V'E was 20.5 $\pm$ 3.9 L min^{-1}. Mean duration of maximal apnoea during exercise was 88.2 $\pm$ 20.9 s. An example of HR and SBP recordings obtained on one subject during maximal apnoea at exercise is shown in Figure 7. The same patterns were followed by all subjects.

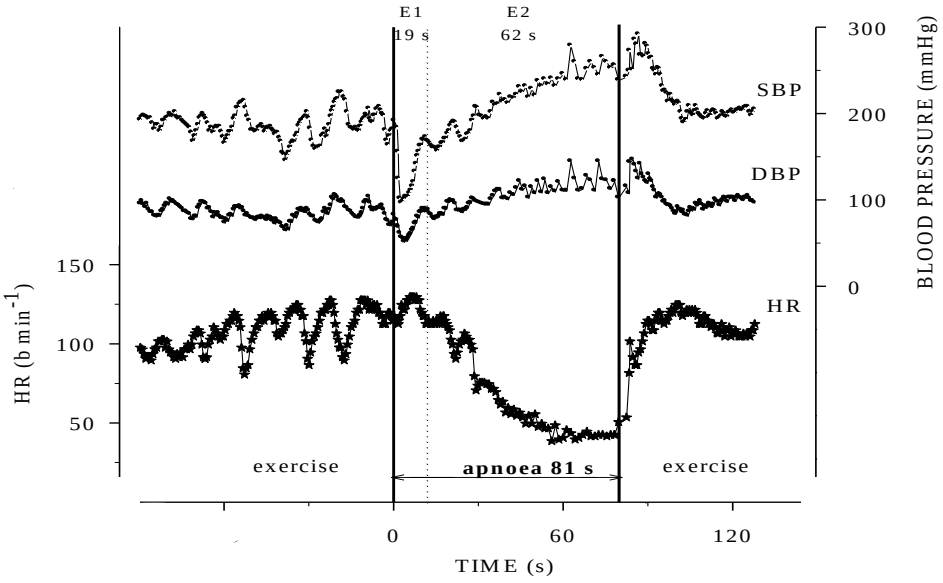

Figure 7. Heart rate (HR) and systolic blood pressure (SBP) recordings obtained on one subject during maximal exercise apnoea.

Values of all variables obtained during exercise at steady state before apnoea are reported in Table 1. In the minute that preceded the apnoea, no hyperventilation was observed. The evolution of all variables during maximal breath-holds at exercise is reported in Figures 8, Figure 9 and table 2.

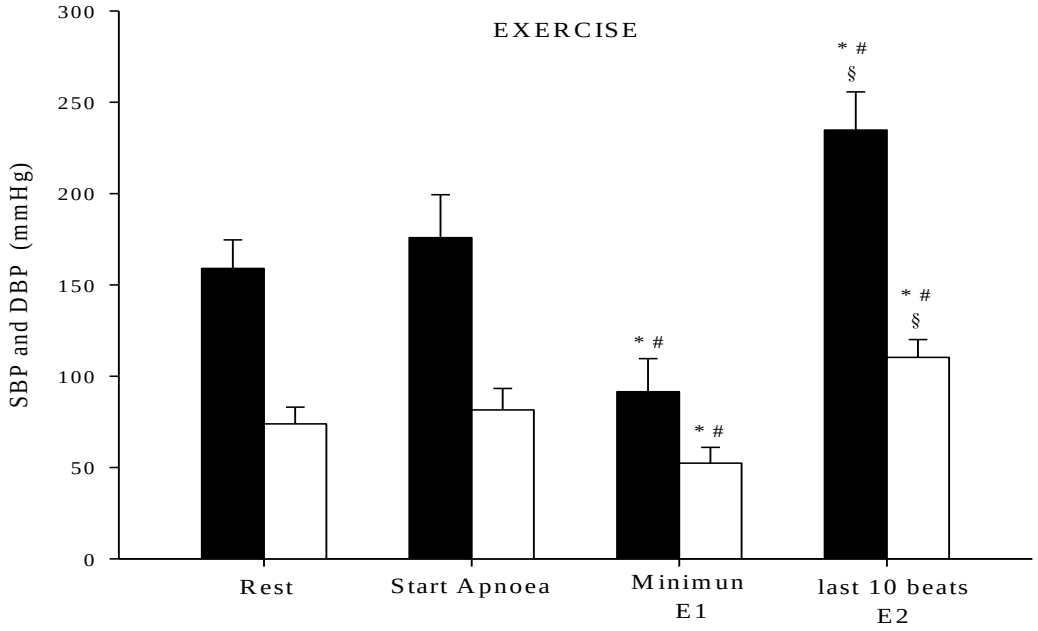

Figure 8. Evolution of systolic blood pressure (SBP) and diastolic blood pressure (DBP) during exercise apnoea. *: p<0.05 respect to rest; #: p<0.05 respect to start apnoea; §: p<0.05 respect to minimum E1; $: p<0.05 respect to R2.

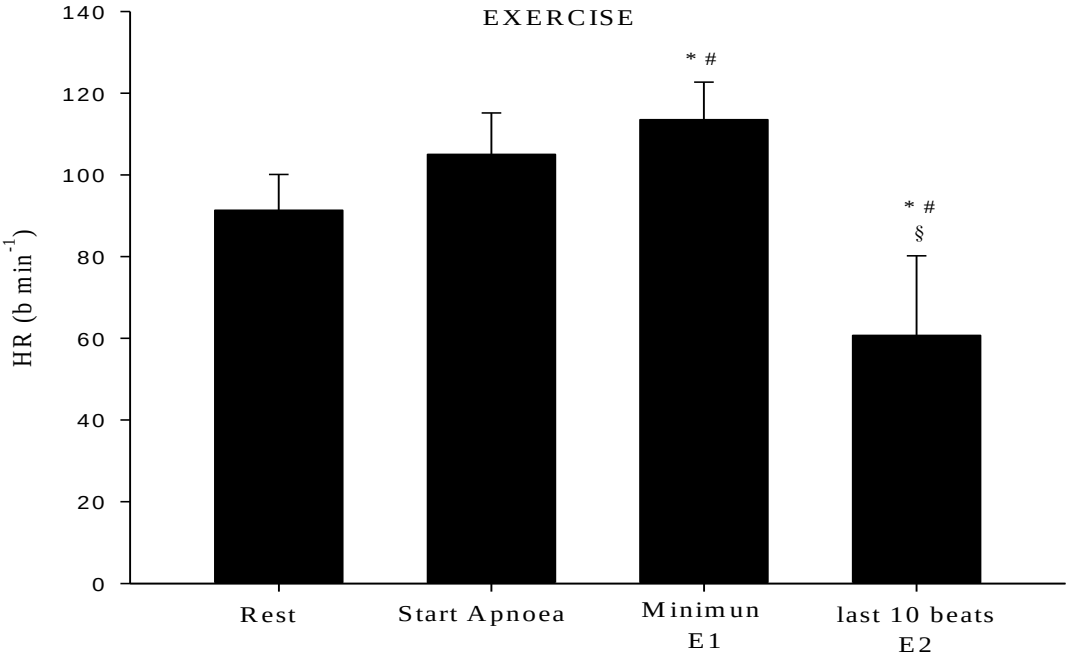

Figure 9. Evolution of heart rate (HR) and stroke volume (SV) during exercise apnoea. *: p<0.05 respect to rest; #: p<0.05 respect to start apnoea; §: p<0.05 respect to minimum E1; $: p<0.05 respect to R2.

After the beginning of apnoea, we observed a pattern comparable to that of R1 (E1),although the minimum of SBP was attained within a shorter time (3.6 + 1.1 s, $p<0.05$). In E1, SBP attained a minimum of 92 $\pm$ 18 mmHg and DBP a minimum of 52 $\pm$ 9 mmHg, whereas HR attained a maximum of 114 $\pm$ 9 min^{-1}. At the minimum of SBP, SV and *Q'* were respectively 51 $\pm$ 18 ml and 5.7 $\pm$ 1.8 L min^{-1}. Since MSP was 61 $\pm$ 10 mmHg, TPR turned out equal to 12.0 $\pm$ 5.3 mmHg min L^{-1}. E1 lasted 21.3 $\pm$ 6.9 s. The slopes of the individual relationships between HR and MSP, determined during E1 before – descending SBP the point of minimum SBP are reported in Table 2.

After completion of E1, we found no steady state phase. At the beginning of the following phase (E2), which lasted 66.8 $\pm$ 22.8 s, SBP had returned at its initial values, whereas HR was higher than in control, similar to that attained at the minimum of SBP in E1. The recovery of SV was responsible for the increase in Q', which attained values closer to yet still significantly lower than those observed before apnoea. During E2, a continuous fall of HR, and increase in SBP and DBP occurred, similar to what was found in R3. At the end of apnoea, HR was 61 $\pm$ 19 min^{-1}, SBP was 235 $\pm$ 21 mmHg and DBP 110 $\pm$ 10 mmHg. These values were significantly different from those found in all other conditions. As in R3, the SV increase compensated for the fall of HR, so that Q' did not vary during E2. As a consequence, TPR continuously increased. SaO_2 started to drop after ~30 s of apnoea to attain 74 $\pm$ 14% at the end of apnoea. The slopes of the individual relationships between HR and MSP during E2 are also given in Table 2.

The values observed at the beginning R3 and of E2 are compared in Table 4.

	SBP mmHg	DBP mmHg	MSP mmHg	HR b min^{-1}	SV ml	Q' L min^{-1}	TPR mmHg min L^{-1}
R3	148 $\pm$ 12	85 $\pm$ 7	103 $\pm$ 8	75 $\pm$ 15	69 $\pm$ 8	5.1 $\pm$ 0.8	20.5$\pm$ 3.9
E2	161 $\pm$ 12	79 $\pm$ 7	97 $\pm$ 5	111 $\pm$ 10	70 $\pm$ 16	7.7 $\pm$ 1.7	13.0 $\pm$ 2.9
Test T	p<0.05	n.s.	n.s.	p<0.05	n.s.	p<0.05	p<0.05

Table 4: cardiovascular variables (means $\pm$ S.D.) during the first 10 beats of R3 (resting apnoea) and E2 (exercise apnoea).

Due to exercise, HR and SBP were higher at start of E2 than of R3, whereas MSP and DBP did not differ significantly. If any, DBP tended to be lower at start of E2. SV was the same in both cases, so that Q' resulted more elevated due to higher heart rate. TPR were lower at start of E2 than of R3. At the end of E2, with respect to the end of R3, SBP, MSP and DBP were significantly higher,

whereas SV was significantly lower. No significant differences were found concerning HR, Q' and TPR.

Discussion

In this study, we tested a few direct experimental consequences of the general hypothesis that the end of the R2 of the cardiovascular response to apnoea at rest corresponds to the attainment of the physiological breaking point of apnoea. This point is characterised by precise combinations of alveolar gas composition, which are attained as long as during apnoea the oxygen stores of the body are emptied and the CO_2 stores correspondingly filled up. The time required to attain the physiological breaking point is thus a consequence of two factors: i) the overall amount of oxygen and CO_2 stores at the beginning of apnoea, and ii) the rate at which oxygen is consumed and CO_2 is accumulated in the body, which is related to the metabolic rate. Metabolic rate is higher at exercise than at rest, so that in the former case the time required to reach the physiological breaking point would be shorter. On this basis, we expected a reduction of R2 duration during exercise apnoeas.

In fact during exercise we did not find something comparable to R2, i.e. a steady state period for all cardiovascular variables. As soon as the initial phase of rapid changes had been completed, we observed a continuous increase in SBP and DBP, accompanied by a continuous decrease in HR, similar to what is usually observed during the R3. The ensuing changes in SV were such as to compensate for the increase in HR, so that Q' did not vary, both E2 and in R3. At a first sight, these findings seem to contradict the tested hypothesis, in the sense that exercise apnoeas seem to be characterised by different dynamic cardiovascular responses from those of resting apnoeas. However, another explanation may be put forward: the duration of R2 was reduced to zero, because the end of E1 occurs already at or beyond the physiological apnoea breaking point.

The present results at exercise are at variance with what occurs during apnoeas in water immersion, which are characterised by immediate bradycardia, both during resting (Hansel et al., 2009; Perini et al., 2010) and during exercise (Lindholm et al., 2002; Tocco et al., 2012) apnoeas. This bradycardia, which is generally considered of vagal origin (Foster and Sheel, 2005; Perini et al., 2010), undermines the concept of baroreflex responses at the beginning of apnoeas. The immediate hypertensive response during wet apnoeas was reported at rest (Perini et al., 2010), but not at exercise (Tocco et al., 2012).

The characteristics of R1 and E1 were very similar, with HR and SBP showing opposite trends. This strongly suggests that these two phases are representative of the same physiological phenomena. If this is so, the size of the cardiovascular adjustments observed in R1 is independent of the rate at which oxygen stores are emptied. The rapid fall of SBP after the beginning of apnoea, which we observed both at rest and at exercise, was coherent with previous results at rest (Andersson and Schagatay, 1998; Palada et al., 2007; Perini et al., 2008). According to Andersson and Schagatay (1998), this fall may be a consequence of an acute reduction of venous return related to the act of holding the breath at elevated lung volumes. The observed fall of stroke volume is in agreement with this concept. If this is the case, then the fall of blood pressure and of SV has a mechanical origin, and the simultaneous increase in HR, which is in agreement with previous studies (Perini et al., 2008), is an attempt at compensating for the fall of SV and/or of blood pressure. If the former is the case, the compensation failed, as demonstrated by the remarkable reduction of Q' at the minimum of SBP. The recovery of SBP, after the attainment of its minimum, was accompanied by an increase in SV and in TPR. This last is suggestive of strong peripheral vasoconstriction, compatible with peripheral sympathetic stimulation. The increase in SV does not seem to have mechanical origin, since i) the mechanical condition which may have induced the drop of blood pressure at the start of apnoea are still present, and ii) the lack of increase in Q' implies a lack of increase in venous return. We therefore postulate that after the attainment of the minimum of SBP, a general stimulation of the sympathetic branch of the autonomic nervous system might have led to higher SBP, TPR and SV. In this context, HR would be exposed to two conflicting demands: on one side, sympathetic stimulation of the heart would tend to increase it; on the other hand, baroreflex stimulation by increased blood pressure would tend to decrease it. In fact HR recovered, but at a variable rate.

In the initial phase of R1, when blood pressure decreased, the increase in HR might be the result of baroreflex control of blood pressure. According to Perini et al. (2008), however, changes in HR do not compensate for changes in SBP, suggesting that the interaction between HR and blood pressure, normally mediated by baroreflexes, is negligible in the initial phase of apnoea. To clarify this aspect, we constructed relationships between HR and MSP like those reported in Figure 6. These allowed computation of a dynamic baroreflex sensitivity from the slope of the linear regression lines, thus by application of an analogous of the sequence method (Iellamo et al., 1997) in which however the analysis can be done over a larger number of points than usually done with the sequence method. Dynamic baroreflex sensitivity (Table 3) during the early part of R1 and E1 ranged between -0.273 and -0.840. These values correspond well to those of other studies in steady-state conditions, whether with open-loop carotid cardiac baroreflex measures, or with closed-loop

spontaneous baroreflex evaluation with the sequence method (Akimoto et al., 2011; Fisher et al., 2009, 2010; Gallagher et al., 2006). They correspond well also to those obtained by similar procedure in another dynamic condition at the end of prolonged bed rest (Adami et al., 2013). On this basis, our postulate is that the increase in HR during the first seconds of apnoea (whether in R1 or in E1) tends to attenuate the SBP drop via arterial baroreflex stimulation. This may not be so after the attainment of the minimum of SBP, as shown by the much wider range of dynamic baroreflex sensitivity values at rest and by the impossibility of performing this computation at exercise.

If we admit that the present results are coherent with the tested hypothesis, as discussed above, then E2 would indeed correspond to R3. We expected it to be shorter than R3 due to higher metabolic rate at exercise than at rest, with subsequent faster depletion of oxygen stores, and this was the case. Coherently, the rate at which the cardiovascular variables changed in E2 was steeper than in R3. As R3, E2 was also characterised by a progressive reduction in HR and increase in SBP and DBP, suggesting continuous readjustment of cardiovascular haemodynamics. No differences between E2 and R3 were observed at the end of maximal breath-hold, independent of whether a subject was at exercise or at rest, except for SBP, which was significantly higher than the corresponding value observed at the end of R3. Notwithstanding E2 and R3 seemed characterised by the same phenomena, at the beginning of E2 HR, SBP and Q' were significantly higher than those at the beginning of R3, probably due to sympathetic activation during exercise apnoeas; moreover vascular recruitment during exercise apnoea led to significantly lower TPR with respect to resting apnoea.

The negative relation between HR and MSP in E2 was similar to that observed in R3 and to that observed in previous studies (Perini et al., 2008). Also in this case the slopes took values close to those usually reported for baroreflex sensitivity, independent of the procedure used for baroreflex analysis (Adami et al., 2013; Akimoto et al., 2011; Fisher et al., 2009, 2010;Gallagher et al., 2006). This is indicative of baroreflex activity not only in also in E2 and R3. We speculate that in the final phase of apnoea the baroreflex activation prevailed over exercise sympathetic activity, so that HR, Q' and TPR were similar in both resting and exercise apnoeas.

The SaO_2 values observed after the end of apnoeas suggest that changes in alveolar gas composition may be occurring during both E2 and R3. These changes may even take place more rapidly in the former than in the latter case, due to higher $V'O_2$ in exercise than in resting apnoeas. We therefore cannot exclude that also chemoreflex activation may contribute, at least in part, to the HR reduction in the final phases of apnoeas, as previously suggested (Foster and Sheel, 2005; Perini et al., 2008,

2010). This HR fall is accompanied by a further increase in SV, which we postulate may have mechanical origin. In fact, if the tested hypothesis was correct, central venous pressure may become negative during diaphragmatic contractions in the final phase of apnoeas, thus determining an increase in venous return. The increase in SV was such as to correct the drop of HR, so that Q' remained unchanged. But this carried along a further increase in TPR, suggestive of even stronger peripheral vasoconstriction.

Conclusion

In conclusion, no steady state phase occurred during exercise apnoeas, despite the lightness of the exercise carried out in this study. We postulate that in exercise apnoeas the cardio-respiratory conditions determining R3 were already attained at the end of the first phase of rapid cardiovascular changes. If this so, then E2 would indeed correspond to R3.

STUDY 2

A BEAT-BY-BEAT ANALYSIS OF CARDIOVASCULAR RESPONSES TO DRY RESTING APNOEAS IN OXYGEN

Paper in preparation

Introduction

The dynamics of cardiovascular changes during breath-holding, whether dry or on the water surface, was poorly investigated so far. Observations during short apnoeas in air (Palada et al., 2007) or with face immersion (Andersson and Schagatay, 1998; Andersson et al., 2000) suggested that a transient fall in blood pressure associated with tachycardia may precede the typical cardiovascular response. In apnoeas prolonged to the breaking point, hypertension and bradycardia were described as continuously developing phenomena (Andersson and Schagatay, 1998; Andersson et al., 2000).

A deeper insight into the cardiovascular dynamics during apnoea was attained recently, after obtention of continuous beat-by-beat observations during apnoeas prolonged to the volitional breaking point, carried out on elite divers who could sustain apnoea for longer than 3 min (Perini et al., 2008, 2010; Sivieri et al, 2014). These studies demonstrated the occurrence of three distinct phases of the cardiovascular response to apnoea at rest: i) a dynamic phase of rapid changes, lasting no more than 30 s (R1); ii) a steady phase, lasting about 2 min, in which the blood pressure and heart rate (HR) values attained at the end of phase I are maintained invariant (R2); and iii) a further dynamic phase, in which there are continuous decrease in HR and increase in blood pressure, linear with time, prolonged to the attainment of the volitional breaking point (R3). The interpretation was that the end of the steady phase could represent the physiological breaking point (Hong et al., 1971). This hypothesis was supported by indirect evidence by Sivieri et al (2014), who showed disappearance of R2 during apnoeas carried out at exercise.

If this hypothesis is correct, when pure oxygen is breathed before apnoea instead of air, because of the increase in oxygen stores in the former case, we would expect that the duration of R2 and R3 would be longer. In fact the increase of oxygen stores should prolong the time that, at a given metabolic rate, is required to empty the oxygen stores and replenish the CO_2 stores in the body. Thus, it would take a longer time to attain the oxygen and CO_2 levels characterising the physiological breaking point and raising the first diaphragmatic contractions, which would imply

longer R2 and R3. Moreover, the rate at which the cardiovascular variables change in R3 would be slower in oxygen than in air. These direct experimental consequences of the above hypothesis were not tested so far. We expected to find the same values for cardiovascular variables at end of R2 in the same conditions, with similar patterns during R3.

The aim of the present study was to investigate the cardiovascular responses to breath-holding during light dynamic exercise in humans on a beat-by-beat basis, in an attempt at testing some cardiovascular consequences of the general hypothesis formulated above. This is the object of the second article of this thesis, which is currently under preparation.

Methods

The methods have been described in the global paragraph above.

Results

In all subjects, both in air and in oxygen, SBP decreased abruptly at the beginning of apnoea, reaching a minimum (minimum of blood pressure, MSP) within 6.0 ± 1.2 and 7.5 ± 3.2 s in supine and sitting posture in air, respectively, and in 7.8 ± 2.7 and 6.8 ± 1.4 s in the same postures in oxygen (NS). At the same time, HR tended to rise, reaching a peak at the MSP. The peaks of SBP and HR were significantly lower and higher, respectively, than the corresponding values pre-apnoea. After the attainment of the MSP, these values tended to recover, so that at the beginning of R2 the values of HR and SBP had returned to the levels that they had before apnoea. These values remained stable during the entire R2. In R3, SBP grew steadily, while HR decreased steadily. This general trend was evident in all conditions, which differed between them only for the different duration of the three phases of apnoea, as shown in table 5.

Table 5 shows that in both positions there was a significant increase of the total duration of apnoea in oxygen with respect to air. There was also a trend toward a detectable increase in apnoea duration in sitting position with respect to supine, although statistical significance was not reached, because of the large interindividual variability. By contrast, the duration of R1 resulted unchanged in all investigated conditions.

DURATION (s)	Sitting air	Supine air	Sitting oxygen	Supine oxygen
R1	29.7 ± 5.9	26.4 ± 3.4	33.3 ± 5.5	30.0 ± 6.1
R2	110.6 ± 21.8	117.6 ± 39.2	166.3 ± 59.0 *	190.6 ± 47.8 *+
R3	99.3 ± 12.6	107.9 ± 27.3	255.4 ± 139.0 *+	329.9 ± 176.7 *+
total	239.6 ± 31.9	251.9 ± 47.4	455.0 ± 155.2 *+	549.4 ± 188.9 *+

Table 5. Total and average duration of the three phases of apnoea carried out under the indicated conditions. The symbol * represents a significant difference with respect to sitting in air. The symbol + represents a significant difference with respect to supine in air.

From the data obtained in each condition, for each of the recorded variables, we calculated some reference values, namely, the average values of the first registration period of quiet resting (control, CTRL); the mean of the first and of the last 10 beats of R2, and of the last 10 beats of R3. We identified two single-beat values of primary interest, namely the value in corresponding to the MSP, and the value corresponding to the minimum peak of HR in R3. Table 6 shows the average HR values obtained during apnoea in the different conditions.

HR (bpm)	CTRL	MSP	START R2	END R2	MHR	END R3
Sitting air	83.1 ± 20.0	94.4 ± 20.5	71.7 ± 13.1	73.9 ± 14.7	56.3 ± 10.4*	65.7 ± 13.5
Supine air	77.9 ± 16.7	92.3 ± 14.3	72.6 ± 15.7	77.0 ± 15.7	58.6 ± 16.6*	66.9 ± 16.8
Sitting oxygen	77.7 ± 13.0	89.9 ± 15.8	71.3 ± 13.0	71.7 ± 10.9	59.3 ± 3.9*	71.0 ± 9.7
Supine oxygen	72.4 ± 11.0	84.7 ± 18.4	69.0 ± 16.0	72.6 ± 15.7	53.7 ± 6.2*	71.0 ± 11.6

Table 6. Heart rate (HR, mean ± SD) during apnoeas performed in the indicated conditions. CTRL, control in calm situation; MSP, single value at the minimum of systolic blood pressure in R1; MHR, single minimum value of HR in R3. The symbol * represents a significant difference with respect to MSP.

The HR showed a tendency to increase before the start of apnoea. It reached a peak at the MSP, to decrease in the second part of R1, attaining lower values than in CTRL at the beginning of R2. The values remained stable during the entire R2. A further decrease in HR occurred in R3, so that the minimum value attained in R3 was significantly lower than the peak observed at the MSP. These trends were observed in all investigated conditions: no significant differences between the various conditions were observed.

Table 7 shows the average values of the SBP and DBP calculated at different times in different conditions. SBP remained substantially unchanged before the start of apnoea. The sudden decrease

of SBP at the beginning of R1 was such that at the MSP it became significantly lower than before apnoea. Then it recovered, so that at the beginning of R2, SBP was again similar to that before apnoea. This value remained unchanged for the entire R2. With the start of R3, SBP further increased in a steady way until the end of apnoea, with values at the end of R3 that were significantly higher than those recorded during R2, at the MSP, in CTRL and immediately before the start of apnoea. The DBP followed a trend similar to that of SBP, but without reaching statistical significance. Only at the end of R3 is a significant difference from the value at MSP. There were no significant differences in the pattern of blood pressure values in the different conditions in which apnoea was performed.

	SBP (mmHg)					
	CTRL	**MSP**	**START R2**	**END R2**	**MHR**	**END R3**
Sitting air	139.7 ± 18.7	99.4 ± 31.2	138.6 ± 22.3	141.1 ± 18.3	194.4 ± 24.7	194.1 ± 22.4
Supine air	127.0 ± 7.6	102.9 ± 11.3	131.7 ± 9.1	133.7 ± 11.2	188.3 ± 28.8	186.0 ± 23.8
Sitting oxygen	123.7 ± 17.4	98.0 ± 23.1	127.7 ± 19.5	132.6 ± 18.2	180.4 ± 27.3	183.4 ± 18.3
Supine oxygen	123.9 ± 18.6	105.6 ± 20.9	133.3 ± 18.0	135.7 ± 14.6	173.3 ± 14.2	186.6 ± 26.6
	DBP (mmHg)					
Sitting air	68.4 ± 12.4	57.4 ± 12.8	75.9 ± 10.1	76.1 ± 11.4	93.0 ± 18.5	93.0 ± 15.4
Supine air	65.4 ± 10.6	58.0 ± 10.1	73.7 ± 13.7	76.3 ± 15.0	92.9 ± 16.8	92.6 ± 16.0
Sitting oxygen	69.0 ± 10.6	57.0 ± 14.1	75.9 ± 13.8	75.7 ± 12.8	93.7 ± 16.1	93.1 ± 13.8
Supine oxygen	70.4 ± 9.5	61.4 ± 8.1	77.3 ± 11.5	79.4 ± 11.1	97.4 ± 12.7	97.7 ± 14.0

Table 7. Systolic blood pressure (SBP) and diastolic (DBP) (mean and SD) during an apnea in different conditions. CTRL, control in calm situation, PRE, mean value prior to the apnea; MSP, the minimum value of systolic blood pressure in stage I; MHR single value at the minimum of HR in R3.

An overview of the results of statistical analysis for SBP and DBP, with indications of all the cases in which a significant difference was found at various times in each conditions, are reported in Tables 8, 9,10 and 11.

Sitting air						
SBP						
	CTRL	MSP	START R2	END R2	MHR	END R3
CTRL	x					
PRE	n.s.					
MBP	*p*<0.05	x				
START R2	n.s.	*p*<0.05	x			
END R2	n.s.	*p*<0.05	n.s.	x		
MHR	*p*<0.05	*p*<0.05	*p*<0.05	*p*<0.05	x	
END R3	*p*<0.05	*p*<0.05	*p*<0.05	*p*<0.05	n.s.	x
DBP						
CTRL	x					
PRE	n.s.					
MBP	n.s.	x				
START R2	n.s.	n.s.	x			
END R2	n.s.	n.s.	n.s.	x		
MHR	*p*<0.05	*p*<0.05	n.s.	n.s.	x	
END R3	*p*<0.05	*p*<0.05	n.s.	n.s.	n.s.	x

Table 8 : Results of statistical analysis for systolic blood pressure (SBP) and diastolic blood pressure (DBP) during sitting in air.

Supine air						
SBP						
	CTRL	MSP	START R2	END R2	MHR	END R3
CTRL	x					
PRE	n.s.					
MBP	n.s.	x				
START R2	n.s.	n.s.	x			
END R2	n.s.	*p*<0.05	n.s.	x		
MHR	*p*<0.05	*p*<0.05	*p*<0.05	*p*<0.05	x	
END R3	*p*<0.05	*p*<0.05	*p*<0.05	*p*<0.05	n.s.	x
DBP						
CTRL	x					
PRE	n.s.					
MBP	n.s.	x				
START R2	n.s.	n.s.	x			
END R2	n.s.	n.s.	n.s.	x		
MHR	*p*<0.05	*p*<0.05	n.s.	n.s.	x	
END R3	*p*<0.05	*p*<0.05	n.s.	n.s.	n.s.	x

Table 9. Results of statistical analysis for systolic blood pressure (SBP) and diastolic blood pressure (DBP) in supine posture in air.

Sitting oxygen						
SBP						
	CTRL	MSP	START R2	END R2	MHR	END R3
CTRL	x					
PRE	n.s.					
MBP	n.s.	x				
START R2	n.s.	n.s.	x			
END R2	n.s.	*p*<0.05	n.s.	x		
MHR	*p*<0.05	*p*<0.05	*p*<0.05	*p*<0.05	x	
END R3	*p*<0.05	*p*<0.05	*p*<0.05	*p*<0.05	n.s.	x
DBP						
CTRL	x					
PRE	n.s.					
MBP	n.s.	x				
START R2	n.s.	n.s.	x			
END R2	n.s.	n.s.	n.s.	x		
MHR	*p*<0.05	*p*<0.05	n.s.	n.s.	x	
END R3	*p*<0.05	*p*<0.05	n.s.	n.s.	n.s.	x

Table 10 : Results of statistical analysis for systolic blood pressure (SBP) and diastolic blood pressure (DBP) during sitting in O_2.

Supine oxygen						
SBP						
	CTRL	MSP	START R2	END R2	MHR	END R3
CTRL	x					
PRE	n.s.					
MBP	n.s.	x				
START R2	n.s.	n.s.	x			
END R2	n.s.	n.s.	n.s.	x		
MHR	*p*<0.05	*p*<0.05	*p*<0.05	*p*<0.05	x	
END R3	*p*<0.05	*p*<0.05	*p*<0.05	*p*<0.05	n.s.	x
DBP						
CTRL	x					
PRE	n.s.					
MBP	n.s.	x				
START R2	n.s.	n.s.	x			
END R2	n.s.	n.s.	n.s.	x		
MHR	*p*<0.05	*p*<0.05	n.s.	n.s.	x	
END R3	*p*<0.05	*p*<0.05	*p*<0.05	n.s.	n.s.	x

Table 11: Results of statistical analysis for systolic blood pressure (SBP) and diastolic blood pressure (DBP) during in supine posture in O_2.

Table 12 reports the SV values in the various investigated conditions. In all cases, SV showed a significant decrease during R1. Then it maintained its values stable during the entire R2, tending to rise again during R3. The values observed at the MSP and at the beginning of R2 were significantly

lower than the corresponding values measured before apnoeas and at the end of R3. There were no statistically significant differences between the different conditions.

SV (ml)	CTRL	MSP	START R2	END R2	MHR	END R3
Sitting air	102.6 ± 17.4*	61.3 ± 21	74.9 ± 15.3	82.1 ± 16.1	104.9 ± 19.8*#	103.0 ± 15.4*
Supine air	98.1 ± 21.7	68.3 ± 22.6	74.9 ± 27.0	73.9 ± 25.5	99.1 ± 27.5	97.9 ± 19.6
Sitting oxygen	88.0 ± 19.1	66.4 ± 20.0$	68.1 ± 22.1$	79.1 ± 20.0	94.6 ± 11.1	97.4 ± 11.8
Supine oxygen	86.1 ± 28.6	71.7 ± 42.9	74.0 ± 37.3	77.4 ± 29.7	83.6 ± 18.4	91.1 ± 19.9

Table 12. Stroke volume (SV, mean and SD) during apnoea in the four investigated conditions. CTRL, control; MSP, minimum systolic blood pressure value in R1; MHR, single-beat minimum of HR during R3. * significantly different from MSP. # significantly START R2.

Table 13 reports the average values of Q'. Q' tended to increase before the start of apnoea (NS). Then it decreased during R1 below the CTRL value, but without attaining significant changes. During R2, the values stabilized and only at the end of apnoea we could observe a slight trend upward. Yet Q' remained significantly lower than the values observed in PRE and CTRL over the remaining duration of apnoea. There were no significant differences between the different conditions for this variable.

Q' (l/min)	CTRL	MSP	START R2	END R2	MHR	END R3
Sitting air	8.4 ± .3	5.7 ± 2.3	5.3 ± 1.4	6.0 ± 1.6	6.0 ± 2.0	6.8 ± 1.9
Supine air	7.6 ± 1.9	6.3 ± 2.2	5.2 ± 1.5	5.5 ± 1.6	5.6 ± 1.7	6.5 ± 1.8
Sitting oxygen	6.8 ± 2.0	5.8 ± 1.6	4.8 ± 1.5	5.7 ± 1.8	5.6 ± 0.9	6.9 ± 1.4
Supine oxygen	6.1 ± 2.0	5.5 ± 2.4	4.8 ± 2.0	5.4 ± 1.8	4.5 ± 1.1	6.4 ± 1.8

Table 13. Cardiac output (Q', mean and SD) during apnoea in the four investigated conditions.. CTRL, rest; MSP, minimum sistolic blood pressure during R1; MHR, minimum value during R3.

TPR is shown in Table 14. In R1, at the MSP, TPR was similar to the control value. In R2, TPR tended to be higher than in CTRL and PRE, reaching a peak in R3 at the minimum of HR. However, the fact that TPR was calculated from values that were subject to strong variability

prevented the attainment of statistically significant differences. No significant differences among conditions were found.

TPR (URP)	CTRL	MSP	START R2	END R2	MHR	END R3
Sitting air	0.7 ± 0.2	0.8 ± 0.2	1.1 ± 0.3	1.0 ± 0.3	1.3 ± 0.5	1.2 ± 0.4
Supine air	0.7 ± 0.3	0.8 ± 0.4	1.2 ± 0.5	1.1 ± 0.6	1.4 ± 0.6	1.2 ± 0.5
Sitting oxygen	0.8 ± 0.3	0.7 ± 0.3	1.3 ± 0.7	1.1 ± 0.4	1.3 ± 0.4	1.1 ± 0.3
Supine oxygen	0.9 ± 0.4	1.1 ± 0.8	1.5 ± 0.9	1.2 ± 0.5	1.6 ± 1.4	1.3 ± 0.4

Table 14. Total peripheral resistance (TPR, mean and SD) during apnoea in the four investigated conditions. CTRL, control value; MSP, minimum sistolic blood pressure during R1; MHR, minimum value during R3.

The SaO_2 values attained during apnoea in oxygen was in all cases equal to 1.0. Table 15 shows the SaO_2 values observed during apnoea in air. At the end of R3, as well as at the minimum observed after the end of the apnoea (this was attained between 4 and 10 s after the end of apnoea), SaO_2 was significantly lower than in CTRL. Statistically significant differences were not detected between the two positions.

SaO_2 (%)	CTRL	END R2	END R2 *	END R3	MINIMUM
Sitting air	100 ± 0	94.0 ± 1.9	92.6 ± 2.4	75.3 ± 6.3	72.9 ± 6.8
Supine air	100 ± 0	94.6 ± 3.6	94.1 ± 4.1	73.9 ± 8.0	71.9 ± 7.9

Table 15. Arterial oxygen saturation (SaO_2 ,mean and SD) during apnoea in air. CTRL, control value; MINIMUM, minimum value after the end apnoea. The data at end of R3 and at MINIMUM were significantly lower than in CTRL.

Discussion

The main result of this study is that the three phases of the cardiovascular responses to apnoea that were firstly identified by Perini et al (2008) during resting apnoeas in air, and subsequently confirmed by other studies (Costalat et al, 2013; Perini et al, 2010; Sivieri et al, 2014) in air and in water, could be recognized in all investigated conditions, independent of the metabolic rate and of the inspired gas mixture. The patterns followed by the various measured variables were the same in all cases. No significant differences were found among conditions for any of the investigated variables at any time of maximal apnoeas in any of the three phases. The only significant differences that we found concerned the duration of the three phases. Whereas R1 had the same duration in air as in oxygen, compatibly with a stable reflex response at the onset of apnoea, the duration of R2 and R3 was very variable, being longer in oxygen than in air, whereas it tended to be shorter when the metabolic rate was increased, as was the case in sitting posture, even if by a small amount.

Making abstraction of R1, whose duration may be considered invariant, the time t, which we thus may define as the sum of R2 plus R3 durations, turns out directly proportional to the amount of energy available in the body at the beginning of apnoea (E) and inversely proportional to the rate of metabolic power (E'). In the present case, since in resting dry apnoeas there is no full diving response comparable to that described by Ferrigno et al (1997), and thus there is no net blood lactate accumulation indicative of ongoing anaerobic metabolism during apnoea, E corresponds to the amount of energy generated by aerobic sources, thus to the oxygen stores at the beginning of apnoea, and E' to the rate of oxygen consumption during the apnoea. Thus, the higher is E at any given E', the longer would be the duration of R2 and R3, whereas the higher is E' at any given E, the shorter would be the duration of R2 and R3. This is what was found in this study.

The general hypothesis of this study, which was originally put forward by Perini et al (2010), assumes that the end of R2 occurs when an alveolar gas composition appears that corresponds to the one that we may observe at the physiological breaking point of apnoea (Lin et al, 1974). If this hypothesis was correct, one could expect the duration of R3 to be unchanged in oxygen. In fact the duration of R3 should correspond to the time that is necessary to pass from the physiological to the physical breath-hold breaking point, represented by the attainment of an alveolar and blood gas composition incompatible with a further prolongation of apnoea: for a given metabolic rate, this time could be expected to be the same. However, this was not so. The present results show that,

when apnoeas are carried out in oxygen, SaO_2 is still equal to 1 at the end of maximal breath-holds, which means that apnoea ended at a time when oxygen stores were still elevated and there was no hypoxic stimulus. The only respiratory stimulus was that mediated by CO_2. Since the combined action of oxygen and CO_2 as respiratory stimulus is synergic (Cunningham et al, 1986), we speculate that, during apnoeas in oxygen, higher CO_2 levels in alveolar air or in arterial blood are to be attained than in air in order for the subject to be unable to further sustain apnoea, because of the lack of this synergy. As a consequence, the duration of R3 would be prolonged.

The characteristics of R1 are independent of the state of oxygen stores at the beginning of apnoea. The initial bradycardia, which is known to occur during apnoeas with face immersion (Hansel et al, 2009; Perini et al, 2010) was not found in this study. Conversely, the rapid fall of SBP at the beginning of apnoea is coherent with previous observations during dry apnoeas in air (Costalat et al, 2013; Palada et al, 2007; Perini et al, 2008; Sivieri et al, 2014). According to Andersson and Schagatay (1998), such a rapid drop of SBP is a consequence of a decrease in venous return caused by the increase in central venous pressure. This in turn is due to the high lung volumes at which the apnoeas are carried out. The simultaneous fall of SV in R1 is coherent with this notion. The fall of SBP was such that at the minimum of SBP in R1 SBP was significantly lower than before apnoea. The simultaneous increase in HR, which attains a peak at the minimum of SBP, and which might be mediated by baroreflex inhibition, corrects the drop of SBP, such that i) at the beginning of phase II SBP has returned to the levels before apnoea, and ii) despite a persistently low SV, an excessive drop of Q' may be prevented. Also a remarkable peripheral vasoconstriction contributes to the recovery of SBP, as demonstrated by the tendency toward an increase in TPR. The patterns followed by DBP and MSP were similar, although less marked. The characteristics of R1 were the same in all experimental conditions, as was R1 duration. So we conclude that R1 is a fix phase, likely of reflex origin, characterised by the attempt at correcting the sudden drop of blood pressure at the onset of apnoea: this attempt includes at least baroreflex-mediated tachycardia (Sivieri et al, 2014) and peripheral vasoconstriction.

The HR and SBP values attained at the end of R1 remained stable for the entire R2 duration. Since also SV stays stable, no changes in Q' occurred in R2. The duration of R2 was longer in oxygen than in air, suggesting that the perturbation that leads to the onset of R3 may indeed have respiratory origin. The cardiovascular patterns observed in R3 were similar to those reported in previous studies in air (Perini et al, 2008; Sivieri et al, 2014). They consisted of increased SBP accompanied by a reduction of HR and of Q'. Beat-by-beat cardiovascular signals underwent

oscillations whose amplitude increased with time. These oscillations are suggestive of rhythmic activities inside the thorax implying cyclic variations of central venous pressure and venous return. According to Perini et al (2008), these rhythmic patterns are a consequence of the appearance of diaphragmatic contractions, whose intensity after their onset increases with time (Breskovic et al, 2012). The apparent negative relations between SBP and HR, similar to those previously reported in air (Sivieri et al, 2014), suggest that arterial baroreflexes try to mitigate blood pressure oscillations in R3.

The presence of SBP and HR oscillations in R3 is compatible with the hypothesis that the onset of diaphragmatic contractions is the cause of the cardiovascular changes observed in R3. In air, the combined action of hypoxia and hypercapnia can certainly determine chemoreceptor stimulations such to induce diaphragmatic contractions (Hong et al, 1971; Lin et al, 1974). According to Lin et al (1974), these may already appear after just 2 min of apnoea, a time only slightly lower than the sum of the durations of R1 and R2 in air (See table 5). In oxygen, R3 duration was longer and the rate at which hypertension developed was slower than in air. In oxygen, the only respiratory stimulus that develops during apnoea is related to hypercapnia, since the synergic action of hypoxia is missing (SaO_2 was never below 1 in oxygen): this is sufficient to explain the mitigation of the cardiovascular changes in R3. If this is so, it sounds logical to maintain that the passage from R2 to R3 is a consequence of the onset of diaphragmatic contractions, as postulated (Lin et al, 1974; Palada et al 2008). In this context we note also that the activation of respiratory chemoreflexes may also contribute to the continuous elevation of SBP and to the maintenance of TPR in R3, as they have been proposed as a further potential mechanism sustaining peripheral vasoconstriction (Leuenberger et al, 2001; Lindholm et al, 2002).

Conclusions

The present results, on one side, identify R1 as a pure phenomenon of short-term cardiovascular regulation; on the other side, they clearly associate R2 and R3, and especially their duration, to the state of aerobic energy stores in the organism. The similar patterns followed by all cardiovascular variables in any investigated condition, but with phenomena of different duration and intensity according to the size of oxygen stores and to the overall metabolic rate, suggest a key role of respiratory chemoreflexes in the shift from R2 to R3. We therefore speculate that at the end of R2 there may be the following sequence of events: i) either the combination of hypoxia and hypercapnia in air apnoeas, or the single strong hypercapnia in oxygen apnoeas, induces the first diaphragmatic contractions, whose strength increases with time during R3; ii) the stronger and stronger chemoreflex stimulation may induce the further continuous increase in SBP; iii) the subsequent bradycardia, of baroreflex origin, does not correct hypertension completely; iv) the variations of intrathoracic pressure generate the blood pressure oscillations observed in R3. The physical break point of apnoea is attained when the combination of stimuli lead to an unsustainable respiratory situation.

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