

Stress biomarker changes following a series of repeated static and dynamic apneas in non-divers

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ABSTRACT

Purpose: This study examined the magnitude of physiological strain imposed by repeated maximal static and dynamic apneas through assessing a panel of stress-related biomarkers.

Methods: Eleven healthy men performed on three separate occasions (≥ 72 -h apart): a series of five repeated maximal (i) static (STA) or (ii) dynamic apneas (DYN) or (iii) a static eupneic protocol (CTL). Venous blood samples were drawn at 30, 90, and 180-min after each protocol to determine ischaemia modified albumin (IMA), neuron-specific enolase (NSE), myoglobin, and high sensitivity cardiac troponin T (hscTnT) concentrations.

Results: IMA was elevated after the apnoeic interventions (STA, +86%; DYN, +332%, $p \leq 0.047$) but not CTL ($p = 0.385$). Myoglobin was higher than baseline (23.6 ± 3.9 ng/mL) 30-min post DYN (+70%, 38.8 ± 13.3 ng/mL, $p = 0.030$). A greater myoglobin release was recorded in DYN compared with STA and CTL ($p \leq 0.035$). No changes were observed in NSE ($p = 0.207$) or hscTnT ($p = 0.274$).

Conclusions: Five repeated maximal DYN led to a greater muscle injury compared with STA but neither elicited myocardial injury or neuronal-parenchymal damage.

1. Introduction

Breath-hold-related activities have been practiced by humans throughout history; primarily as a means of sustenance (Davis, 1934). In recent years, breath-holding has gained prominence as a competitive endeavour, with regular indoor and outdoor national/international competitions being held across the calendar year. During these events, athletes can choose to compete across a number of different disciplines, including: the longest breath-hold they can attain whilst remaining motionless (static apnea) and the furthest distance they can cover horizontally (dynamic apnea) or vertically (depth diving) on a single breath of air (AIDA, 2023). The extreme physiological (hypoxic hypercapnia) and environmental (increased hydrostatic pressures) strains these athletes endure during their maximal attempts have fuelled a surge of researchers in pursuing to examine the physiological characteristics and responses arising during and shortly after apneic bouts (Elia et al., 2021). However, information pertaining to the possible health

implications associated with such activities is limited at present.

Previous studies have shown that, in competitive breath-hold divers (BHDs), a single, maximal static apneic bout was associated with a transient disruption of the blood-brain barrier (Andersson et al., 2009b; Bain et al., 2018) and minor gliovascular damage (Gren et al., 2016; Bailey et al., 2022), while no changes were denoted in biomarkers of striatal muscle nor myocardial injury (Andersson et al., 2009a; Marlinge et al., 2019). Collectively, these studies highlighted the profound haemodynamic stress to which the apneic brain is exposed during a single maximal attempt, with longitudinal studies posing concerns about the possible long-term ramifications of habitual apneic activities on neuro-cognitive health (see review by Elia et al., 2021). Notwithstanding, in contrast to a competition setting whereby one maximal attempt per discipline of choice is performed, during training athletes commonly perform a series of repeated maximal and/or submaximal attempts interspersed with short rest periods. Similarly, during recreational and competitive spearfishing sessions BHDs dive in a serial manner to

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varying depths. Yet, at present, only a small number of studies have explored the physiological consequences of serial apneic bouts (Marlinge et al., 2019; Elia et al., 2022b).

Following a 5-h spearfishing competition, during which BHDs performed a series of repeated dives (65 ± 10) at varied depths (15 ± 10 m), both cardiac troponin I (+275%) and brain natriuretic peptide (+229%) were elevated. Such increases, however, were not evident following a single maximal static apneic effort (Marlinge et al., 2019). Subsequent work by Elia et al. (2022b) demonstrated that, in elite BHDs, a series of repeated maximal static and dynamic apneic attempts instigated muscle injury (myoglobin +64% and +63%, respectively) as well as a transient, albeit minor, disruption of the blood-brain barrier (S100 β +149% and +166%, respectively). Notably, a retrospective analysis of these data indicates that these increases were substantially greater than those previously reported after one maximal static apnea (Andersson et al., 2009a; Andersson et al., 2009b; Bain et al., 2018). Still, neither of the protocols led to neuronal-parenchymal nor myocardial damage (Elia et al., 2022b). To the best of our knowledge, no study exists that investigated these in a non-diving cohort. It is, thus, currently unclear whether the aforementioned physiological responses are coherently expressed in non-divers.

Accordingly, this study aimed to evaluate, in a group of non-divers, a panel of stress biomarkers (i.e., cardiac, cerebral, skeletal muscle and oxidative stress) after a series of repeated maximal static and dynamic apneic bouts in a group of non-divers. It was hypothesised that, compared to static apneas, serial dynamic apnoeas would provoke augmented physiological stress, leading to greater elevations in stress biomarkers.

2. Materials & methods

2.1. Participants

Eleven healthy men volunteered for this study. All participants were non-smoking, physically active individuals and had no prior breath-hold diving experience. Participants were habitual sea-level residents, and provided written informed consent before the study. The study received institutional ethics approval (ethics approval number: 86001) and all experimental procedures were completed in accordance with the Declaration of Helsinki.

2.2. Experimental protocol

During each testing session participants reported to the testing site after a 12 h fast and refrained from consuming caffeine- and alcohol-containing beverages (Elia et al., 2022a). Participants were also instructed to avoid strenuous exercise for at least 24 h before each trial.

2.3. Baseline measurements

Following arrival at the laboratory the participants' height and body mass were assessed ([mean \pm standard deviation (SD); age, 24 ± 8 years; body mass, 78 ± 10 kg; height, 1.83 ± 0.06 m). Thereafter, they underwent a 20-min seated resting period followed by monitoring of their peripheral oxygen saturation (SpO₂) through a finger pulse oximeter placed on the left-hand index finger (Radical-7, Masimo, Irvine, CA). Thereafter, a finger capillary blood sample was collected to evaluate blood lactate concentrations (Accutrend Plus, Roche, Boehringer Mannheim, Indianapolis, IN). Subsequently, four venous blood samples (3×5 mL and 1×4 mL) were drawn to assess for resting serum ischemia modified albumin (IMA), neuron-specific enolase (NSE), myoglobin (BD Vacutainer SST II Advance, 366444), and high sensitivity cardiac troponin T (hscTnT) (BD Vacutainer Lithium Heparin, 368884) concentrations.

2.4. Familiarisation session

Participants underwent a familiarisation session within 24 h of completing the baseline measurements to introduce them to the testing environment, trial conditions and requirements.

2.5. Apnea protocols

Within a week of completing the familiarisation session, participants reported, on two separate days (i.e., ≥ 72 h interval), to the swimming pool facilities (water temperature: 27 ± 0.7 °C). Under the supervision of a qualified safety diver, they performed, in a Latin-Square fashion and randomized order one of the following protocols: five repeated maximal (i) static (i.e., breath-holding performed in a prone/semi-seated position on the water surface) or (ii) dynamic (i.e., horizontal underwater swimming without fins) apneic attempts. To ensure that diurnal oscillations of the measured variables were similar, both apneic protocols were performed at the same time of day.

Participants were instructed to hold their breath after a deep but not maximal inspiration, without prior hyperventilation or glossopharyngeal insufflation. After each apneic attempt, participants underwent a two-minute resting period whereby they were allowed to, in a seated position, relax and breathe normally, while remaining immersed to the level of the xiphoid process. This procedure was repeated five times for each apneic protocol.

SpO₂ levels were monitored from, and up to 30 s after each maximal apneic effort. Finger capillary blood lactate concentrations were assessed immediately after and 30-min post the last apneic (i.e., fifth) repetition.

2.6. Control protocol

To isolate any potential confounding effects of whole-body water immersion per se, the participants completed a static eupneic (i.e., control) protocol. This protocol, which replaced the apneic bouts with normal breathing periods, replicated the water immersion and exposure times, resting periods and data collection timepoints (i.e., whole-blood samples) of the static apnea protocol (since the water exposures were longer in the static versus the dynamic apnea protocol). Participants reported to the swimming pool facilities at the same time of day as during the apneic protocols.

2.7. Post-apnea whole blood sampling

At completion of the apneic and control protocols, a cannula was inserted into a suitable median cubital or basilic vein of the participant's arm and three venous blood samples were drawn at 30, 90, and 180 min after the last apneic/eupneic repetition to determine the serum concentrations of circulating IMA, NSE and myoglobin, and plasma concentrations of hscTnT.

2.8. Blood sample treatment

Samples were gently inverted. hscTnT samples were centrifuged immediately at $1300 \times g$ for 10 min at room temperature. NSE and myoglobin samples were allowed to coagulate for 30 min and IMA samples for 2 h prior to being centrifuged [$1000 \times g$ for 15 min (NSE and IMA) or $2000 \times g$ for 10 min (myoglobin)] at room temperature, and the supernatants were frozen at -80 °C for subsequent analyses.

2.9. Blood analyses

hscTnT was quantified by electro-chemiluminescence immunoassay (Cobas E 411 Analyzer; Roche Diagnostics, Mannheim, Germany; intra-assay variability $\sim 4.3\%$). Enzyme-linked immunosorbent assay (ELISA) was performed to assess serum concentrations of NSE (R&D systems,

Quantikine IVD ELISA, Human Enolase 2/Neuron-Specific Enolase immunoassay, DENL20; intra-assay variability $\sim 3.1\%$), IMA (BIOMATIK, Ischemia Modified Albumin ELISA, EKU08701; intra-assay variability $\sim 6\%$), and myoglobin (Abcam, Human Myoglobin ELISA, ab171580; intra-assay variability $\sim 4.2\%$).

3. Statistical Analysis

All data were statistically analysed using IBM SPSS Statistics software version 28 (IBM Corp., Armonk, NY, USA). The Shapiro–Wilk test was used to assess whether data were normally distributed ($p < 0.05$). Two-way repeated measures ANOVA were used to compare differences amongst the apneic and eupneic protocols. Sphericity was assessed using Mauchly's test of sphericity; where the assumption of sphericity was violated, the Greenhouse–Geisser correction was applied. Pearson's correlation was used to assess for a potential relationship between the absolute change in IMA and the total distance covered during the dynamic apnoeic protocol. Unless otherwise stated, data are reported as means \pm SD and significance was accepted at $p < 0.05$. GraphPad Prism version 9.5.1 (GraphPad Software Inc., La Jolla, CA, USA) was used to construct figures.

4. Results

All participants completed all protocols successfully. None suffered nor exhibited any signs or symptoms associated with loss of motor control.

4.1. Apneic performances

Mean distance covered was 26 ± 10 m (range 15–50 m) and mean breath-hold duration was 112 ± 66 s (range 40–245 s) during the dynamic and static apneic protocols, respectively.

4.2. Peripheral oxygen saturation

A gradual reduction was observed in mean end-apnoeic SpO₂ nadir during both apneic protocols ($p = 0.022$), with lower SpO₂ levels being registered after the dynamic compared with the static apnea protocol (Fig. 1). Specifically, end-apnoeic SpO₂ levels were lower after the first (dynamic, $86 \pm 8\%$; static, $94 \pm 6\%$, $p = 0.021$) and third repetition (dynamic, $80 \pm 10\%$; static, $91 \pm 9\%$, $p = 0.007$).

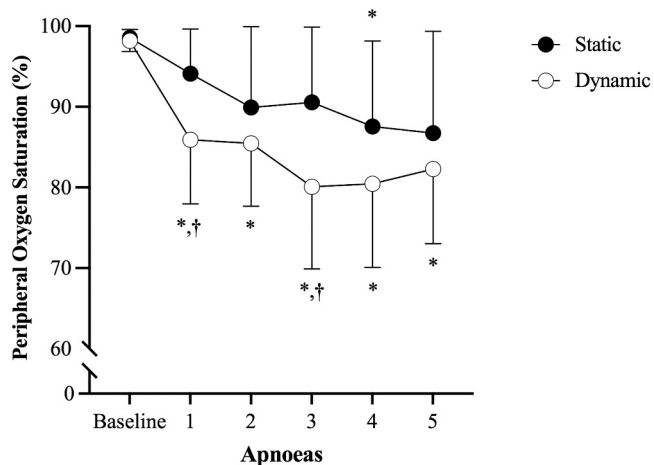


Fig. 1. End-apnoeic SpO₂ nadir after each maximal apneic attempt. * denotes significant ($p < 0.05$) difference from baseline; † indicates significant differences ($p < 0.05$) between protocols.

4.3. Blood lactate

Following the fifth maximal apneic bout, blood lactate concentrations were significantly elevated both after the static (1.7 ± 0.5 mmol/L; $p < 0.001$) and dynamic apnea protocols (4.8 ± 1.7 mmol/L; $p < 0.001$), with values being restored back to baseline (0.7 ± 0.2 mmol/L) 30 mins post the apneic interventions (static, 0.6 ± 0.2 mmol/L, $p = 1$; dynamic, 0.7 ± 0.2 mmol/L, $p = 1$). Blood lactate concentrations were higher after the dynamic in comparison to the static apnea protocol ($p < 0.001$) immediately after each protocol but no differences were discerned at the 30-min time point ($p = 0.341$).

4.4. Ischemia modified albumin

There was a significant effect over time for IMA ($p = 0.032$) (Fig. 2). Both the static and dynamic apneas increased IMA (837 ± 595 ng/mL, +86%, $p = 0.030$; 1816 ± 2043 ng/mL, +332%, $p = 0.047$, respectively) from baseline (441 ± 141 ng/mL), whereas no differences were detected in the control (pre, 453 ± 188 ng/mL; post, 448 ± 188 ng/mL, $p = 0.385$). There were significant between protocol differences ($p = 0.039$). A higher post-apnoeic IMA was denoted in the dynamic compared with control ($p = 0.037$). No differences were discerned between the apneic protocols ($p = 0.167$).

There was a strong, positive correlation ($r = 0.80$, $R^2 = 0.63$, $p = 0.004$) between the absolute change in IMA and the total distance covered during the dynamic apnea protocol.

4.5. Myoglobin

There was a statistically significant interaction between treatment and time on serum myoglobin ($p < 0.001$) (Table 1). Specifically, myoglobin was higher (+70%, $p = 0.030$) than baseline 30 mins post the last dynamic repetition (Table 1), while no differences over time were identified in the rest of the interventions. In addition, there were significant between protocol differences ($p = 0.005$), with dynamic apneas eliciting a greater myoglobin release compared with static ($p = 0.023$) and control ($p \leq 0.035$) (Table 1). No differences were denoted between the two latter interventions at any time point ($p \geq 0.619$) (Table 1).

4.6. hscTnT & neuron-specific enolase

There was no effect of the apneic or control interventions on hscTnT ($p = 0.274$) and NSE concentrations ($p = 0.207$) (Table 1).

5. Discussion

This study aimed to evaluate the magnitude of the physiological

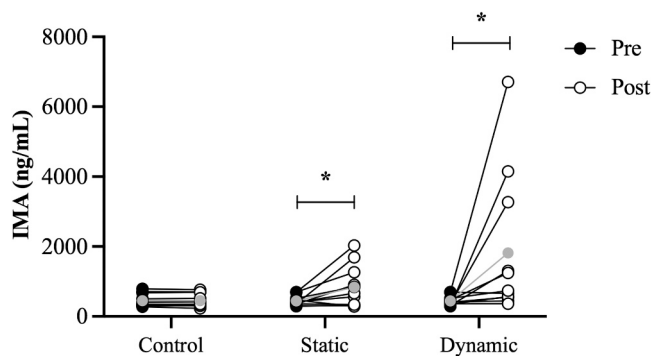


Fig. 2. Individual (black and white dots) and mean (grey dots) ischemia modified albumin (IMA) concentrations before and 30-min after the control and apneic protocols. * denotes significant ($p < 0.05$) difference from baseline.

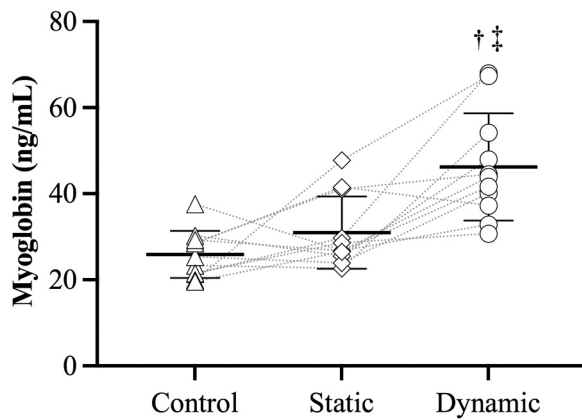


Fig. 3. Peak individual (symbols) and mean \pm SD myoglobin concentrations following the apneic and control protocols. † indicates significant differences ($p < 0.05$) between dynamic and static apneas; ‡ denotes significant ($p < 0.05$) differences between dynamic and control.

strain imposed by a series of repeated apneas in a group of non-divers through assessing stress-related biomarkers. Our findings signify that a series of dynamic, as opposed to static apneas, instigate a stronger hypoxic stress, and concomitantly lead to greater transient releases of blood lactate, IMA and myoglobin. However, we did not observe any differences in NSE or hscTnT concentrations. Taken together, our data suggest that, in a non-diving population, a series of five repeated maximal dynamic apneas are capable of inducing muscle injury and may incur a more pronounced state of oxidative stress. However, similarly to static apneas they do not induce neuronal-parenchymal nor myocardial injury.

In line with the literature, the present study demonstrates that dynamic apneas are associated with a greater degree of hypoxic stress compared with static apneas as evidenced by both the lower end-apneic SpO_2 nadirs and the higher post-apneic blood lactate accumulation (see Fig. 1) (Rodríguez-Zamora et al., 2018; Elia et al., 2020). Over the series of the repeated attempts, we denoted progressive decrements in SpO_2 nadirs with latter bouts being terminated at lower SpO_2 levels than the preceding ones – a response that has been well documented across the literature (Heath and Irwin, 1968; Elia et al., 2020). Of notable interest, however, is the net blood lactate accumulation, which is somewhat akin to those observed in elite BHDs, despite substantially lower apneic performances being recorded herein (Joulia et al., 2002; Rodríguez-Zamora et al., 2018; Elia et al., 2020); an observation that likely relates to training-induced adaptations (Joulia et al., 2003). Therefore, our findings provide further evidence in support of the considerably greater physiological strain encountered by apneist during dynamic as opposed to static apneas.

IMA, a marker of reactive oxygen species (ROS) release and tissue ischemia (Bar-Or et al., 2001; Pellegrino et al., 2011), was significantly elevated 30 mins after the static and dynamic apnea interventions; with the increases being greater (+117%), albeit not statistically different, in the latter protocol. Our findings concur with those of Marlinge et al.

(2019), who also recorded increases in IMA following sequential breath-hold dives. Intermittent hypoxemia, as posed by our experimental design (i.e., repeated hypoxic bouts interspersed with short normoxic periods) is well known to initiate the generation of ROS and, in the absence of adequate antioxidant defences, bring about a state of oxidative stress (Li and Jackson, 2002; Rousseau et al., 2006; Theunissen et al., 2013; Mrakic-Spota et al., 2019). Since ROS scavengers inhibit the elevation of IMA in experimental Fenton reaction-induced ROS production (Pellegrino et al., 2011), it is reasonable to conject that the greater IMA elevations observed after the dynamic apneas might reflect a more pronounced ROS generation in this protocol. This supposition is partly substantiated by the lower end-apneic SpO_2 nadirs and the higher blood lactate levels recorded in this discipline.

Thirty minutes after the last maximal dynamic attempt, circulating myoglobin concentrations were markedly elevated from basal levels; such increases were not manifested neither following the static apnea nor the eupneic protocols (Table 1). Similar observations have previously been made in a group of elite BHDs after a series of five maximal dynamic apneas (Elia et al., 2022b), conjointly demonstrating that repeated apneas are capable of inciting myoglobin release, even in the absence of a hypoxic blackout (Andersson et al., 2009a). Increases in serum myoglobin are widely accepted to be indicative of muscle cell damage and reflective of changes in sarcolemma permeability (Vanholder et al., 2000; Giannoglou et al., 2007). Notably, intense/unaccustomed physical activity and excessive free radical accumulation compromise sodium-calcium channel functioning and, consequently, give rise to free cytosolic ionized calcium levels (Ritter et al., 1979; Vanholder et al., 2000). This in turn triggers a cascade of events including the activation of calcium-dependent enzymes, which go on to induce muscle cell membrane damage and, subsequently, lead to the extravasation of intracellular protein contents (i.e., myoglobin and creatine kinase) into the systemic circulation (Vanholder et al., 2000). Thus, present findings signify that a series of five dynamic but not static apneas are capable of inducing muscle injury.

Despite never reaching pathological limits (> 85 ng/mL), current increases in serum myoglobin are higher than those recorded previously in elite BHDs after a similar dynamic apnea intervention (Elia et al., 2022b). It is noteworthy that the increases herein were induced after substantially lower apneic performances (26 ± 2 m vs. 66 ± 5 m). These differences likely stem from apnea-related training-induced adaptations (see review by Elia et al., 2021). In this context, cross-sectional studies that examined the acute effects of apneic modalities on redox balance unveiled both a lower production of ROS (Joulia et al., 2002) and a greater superoxide dismutase activity (Bulmer et al., 2008) in elite BHDs compared with controls. More importantly, three months of simulated dynamic apnea training (i.e., repeated cycles of 20 s apneas separated by 40 s of normoxic breathing over a 1-h steady state cycling period) blunted post-apneic ROS and oxidative stress levels (Joulia et al., 2003). In keeping with this line of reasoning and considering that oxidant-dominant redox imbalances instigate a greater degree of muscle cell membrane damage and myoglobin release (Vanholder et al., 2000; Giannoglou et al., 2007), then present increases may have originated from either a greater production of oxidants and/or insufficient

Table 1

Mean hscTnT, NSE and myoglobin concentrations from baseline to 180 min after the apneic and eupneic protocols.

Timepoint	hscTnT (ng/L)			NSE (pg/mL)			Myoglobin (ng/mL)		
	STA	DYN	CTL	STA	DYN	CTL	STA	DYN	CTL
Baseline	5 \pm 2	5 \pm 2	5 \pm 2	3.04 \pm 0.79	3.03 \pm 0.84	3.03 \pm 0.84	23.6 \pm 3.9	24.4 \pm 5.2	24.4 \pm 5.2
30 min	5 \pm 2	6 \pm 2	5 \pm 2	3.76 \pm 1.33	3.82 \pm 1.51	3.07 \pm 0.80	26.7 \pm 4.5	38.8 \pm 13.3 * , †, ‡	24.8 \pm 4.2
90 min	5 \pm 2	6 \pm 3	5 \pm 2	3.18 \pm 1.02	3.16 \pm 0.75	3.10 \pm 0.89	27.6 \pm 7.8	39.7 \pm 16.5 †, ‡	23.6 \pm 5.5
180 min	5 \pm 2	6 \pm 2	5 \pm 2	3.26 \pm 1.05	3.94 \pm 1.81	3.06 \pm 0.85	28.5 \pm 8.6	34.6 \pm 10.5	23.9 \pm 6.1

Data are presented as mean \pm standard deviation. * denotes significant difference ($p < 0.05$) compared with baseline; † represents significant difference between apneic protocols, ‡ demonstrates significant difference between the dynamic apnea with control. hscTnT, high sensitivity cardiac troponin T; ng/L, nanograms per litre; NSE, neuron-specific enolase; pg/mL, picograms per millilitre.

antioxidant enzyme defences. Further measures of redox-related enzymes would have certainly provided additional insights to the mechanistic basis of our findings.

The experimental protocol utilised herein and the performances attained by our non-diving cohort did not elicit any detectable changes in circulating hscTnT or NSE levels, attesting against any form of myocardial injury (Kemp et al., 2004) and neuronal-parenchymal damage (Marchi et al., 2003; Cheng et al., 2014). Our data align well with earlier observations made in elite BHDs that showed no changes in hscTnT and/or NSE following a series of repeated apneic bouts (Elia et al., 2022b) and after a solitary dry maximal static apneic attempt (Bain et al., 2018; Marlinge et al., 2019). Presently, only one study by Kjeld et al. (Kjeld et al., 2015) has reported increases in NSE (+70%), ~3 h after a combined bout of static and dynamic apneas. However, out of the 17 competitive breath-hold divers examined within that study, nine of them suffered a hypoxemic blackout (Kjeld et al., 2015). An accumulation of congruent findings since, including those derived from this study, eventuate that in the absence of a blackout incident neither single (Bain et al., 2018; Bailey et al., 2022) nor repeated bouts of apneas (Elia et al., 2022b) seem to be associated with neuronal-parenchymal damage. Nonetheless, the distinct differences in experimental design and analytical approaches employed across studies make it evident that additional work is necessary to fully elucidate the effects of apneas on neuronal stress.

In summation, in a group of non-divers, a series of five repeated maximal dynamic as opposed to static apneas are associated with a stronger hypoxemic stress and greater muscle injury but neither appear to elicit any detectable myocardial injury nor neuronal-parenchymal damage.

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CRediT authorship contribution statement

Elia Antonis: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Barlow Matthew:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Petri Georgios:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis. **Keramidas Michail E:** Writing – review & editing, Project administration, Methodology, Investigation.

Declaration of Competing Interest

The authors report there are no competing interests to declare.

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