PHYSIOLOGY AND PHYSIOPATHOLOGY OF BREATH-HOLDING ACTIVITY

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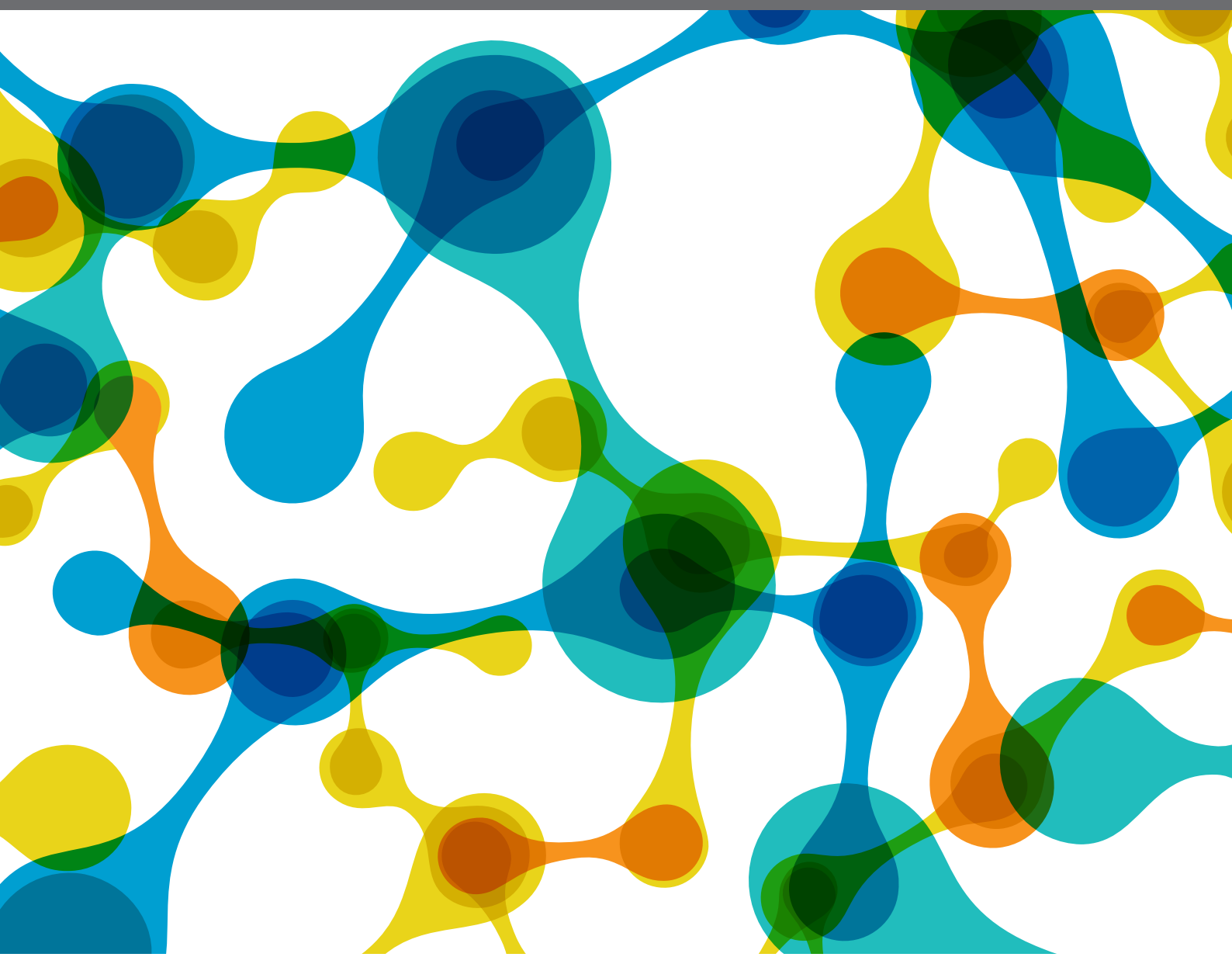

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PHYSIOLOGY AND PHYSIOPATHOLOGY OF BREATH-HOLDING ACTIVITY

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Table of Contents

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EDITORIAL
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Editorial: Physiology and Physiopathology of Breath-Holding Activity

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Keywords: hypoxia, cardiovascular, pulmonary, training, sport

Editorial on Research Topic

Editorial: Physiology and Physiopathology of Breath-Holding Activity

Research into voluntary apnea is becoming increasingly popular in varied laboratories and in the field around the world. In the last 10 years, as many articles have been published than between 1954 and 2011. Breath-hold diving is truly an antique practice; for example, Alexandre Legrand employed professional breath hold divers called "Urinator" in his army, and Japanese Ama and Philippine Bajau people have been known to dive for hundreds of years to gather food. More recently, apnea diving became a high-level competitive sport with impressive performances (e.g., world record for static apnea of 11 mins 35 s and for depth No-Limit diving −214 m), but although constant progress is made, human performance is still far from the prowess of marine mammals (e.g., *Ziphius cavirostris*, apnea duration: 137 mins and 2,992 m depths). Despite major anatomical and physiological differences between humans and marine mammals, humans present with an interesting physiological response to apnea to limit the effects of hypoxia and/or pressure increase, in which the main physiological functions such as pulmonary, cardiovascular, and nervous functions can be modulated acutely and chronically. The Research Topic focuses on these varied facets but also their trainability and their consequences on health in relation to some hypoxia-related pathologies such as sleep apnea syndrome, pulmonary edema and decompression sickness.

THE RESEARCH TOPIC ARTICLES

Freedivers (Tetzlaff et al.) and spearfishermen (Diniz et al., 2014) present an increase in total lung capacity and a decrease in residual volume because of their specific ventilatory training. This allows them to increase their performance in duration and depth, pushing back the physiological breaking point and favouriting the equalization (McCulloch et al.). Apnea training is therefore a relevant hypoxia training for several physical activities since it induces hypoxia and hypercapnia despite a low intensity of exercise (Joulia et al., 2003; Lemaître et al., 2009; Guimard et al.; Pla et al.) Grossman et al. reported how swimmers should better manage their ventilation per arm cycle

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during aerobic events to avoid consuming too much oxygen (Grossman et al.). Moreover, Guimard et al. demonstrated that performing dynamic apnea at 30% of maximal aerobic power is the best compromise between exercise intensity and apnea duration to induce a sufficient diving reflex in non-apnea trained participants. In fact, as long as the exercise intensity is mild, the diving reflex can overwhelm the muscle metaboreflex (Di Giacomo et al.). Dynamic apnea can therefore be used in other physical activities to induce hypercapnia, hypoxia and increased lactatemia even during low intensity efforts (Guimard et al.). During static and dynamic apneas, the dive reflex decreases oxygen consumption through bradycardia and peripheral vasoconstriction, and preserves oxygen-dependent organs through increased cerebral and cardiac perfusion (Joulia et al., 2009; Elia et al., 2021). The diving reflex is accentuated with face immersion, but there is no significant difference when apnea was performed only with the face immersed (dry condition) or the total body immersed (Nordine et al.). Those adaptive responses have been described in both spearfishermen and freedivers, and are correlated with a high adenosine release which exerts powerful bradycardia and vasodilation in coronary and cerebral blood vessels (Marlinge et al.). Moreover, an increase in serum heat shock protein levels in response to higher nitric oxide levels could improve cardiovascular adaptation to hypoxia in elite freedivers (Solich-Talanda et al.). Despite a low production of free radicals observed in freedivers during exercise and dynamic apnea (Joulia et al., 2002), it seems that there are no genetic predispositions to limit the oxidative stress in freedivers (Cialoni et al.). Joulia et al. (2002) had previously shown that the blood lactate concentrations recorded during dynamic apneas were lower in freedivers compared to non-divers despite the same exercise intensity. In addition, it has been shown that this increase in lactate concentration are accentuated when apneas were performed with face immersion (Bouten et al.). Despite diving reflex allows the freedivers to be more economical with oxygen, they become hypoxic depending on the duration of apnea and the intensity of exercise performed during dynamic apneas. These hypoxias can induce arrhythmias in both humans and marine mammals (Kjeld et al.; Costalat et al., 2021). Even if these cardiac rhythm disorders are reversed after the end of apnea their long-term effects remained unknown. Repetitive dives in apnea are not without risk since they can contribute to an increase in nitrogen dissolution in the tissues and then favouriting decompression sickness (Kohshi et al.). Interestingly, for freedivers, decompression illness is poorly understood and unlike in scuba diving, it is only cerebral in nature (Kohshi et al.). This suggests different mechanisms may occur within the spinal cord or skin (Edge and Wilmshurst, 2021) or in distal arteries (Arieli, 2021). It is also possible that a pulmonary shunt is created artificially due to the movement of blood during diving which increases the pulmonary artery pressure (Kohshi et al.). Both deep and repetitive breath-hold diving can lead to endothelial dysfunction (Eichhorn et al., 2017) that may play an important role in the genesis of neurological decompression sickness (Barak et al., 2020). The freediver "narcosis" has also recently been hypothesized but is still very poorly understood today (Tetzlaff et al.). The mechanisms put forward appear to favor a mechanical hypothesis via increases in cerebral blood flow and disturbances in cerebral autoregulation as described in elite freedivers (Moir et al., 2019). Depth induces haemoptysis in freedivers (Barković et al., 2021) and increases the extravascular lung water (Boussuges et al., 2011). Some freedivers present a genetic predisposition to develop haemoptysis during breath hold diving (Cialoni et al., 2015). Haemoptysis affects up to 25% of freedivers during championships, and in addition to its physiopathology of barotraumatic origin, this phenomenon may reduce oxygenation and increase the risk of syncope. Its characterization and prevention are therefore essential for the safety of freedivers. Beyond all these concepts, the use of apnea as a physiological model also appears relevant in the context of other pathologies such as, for example, sleep apnoea (Taylor et al.). Taylor et al. tested the hypothesis that individuals with documented obstructive sleep apnea (OSA) exhibit greater muscle sympathetic nerve discharge and synchronous blood oxygen level-dependent signal responses during volitional simulation of central or OSA by Mueller Maneuvers (MM) and apneas. The hemodynamic and neural responses to both apneas and MMs were in fact essentially identical in participants with and without OSA, indicating that recurring episodes of cyclical apnea during sleep does not alter cortical or peripheral neural responsiveness.

CONCLUSIONS

Past research on apnea as well as the recent advances published in the current Research Topic really highlight breath-holding as an excellent model for studying the interactions between key physiological functions. It allows understanding how our body responds and adjusts to different chemical (*via* chemoreceptors) and mechanical (*via* the baroreflex) stimuli to maintain its homeostasis. Breath-holding is also an excellent stimulus to generate natural hypoxia. This poikilocapnic hypoxia may be incorporated into training periodization to prepare the organism for the lack of oxygen during a trip to altitude or simply to improve performance at sea level. Apnea may also be considered as preconditioning stimulus to better withstand health-damaging hypoxic situations, for example during a heart attack or stroke. Future studies are warranted in this area to refine the exercise characteristics and hypoxic dose of such apneic training for achieving the desired physiological outcome.

AUTHOR CONTRIBUTIONS

FL, FB, and FJ wrote together the editorial. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Comparison of Cortical Autonomic Network-Linked Sympathetic Excitation by Mueller Maneuvers and Breath-Holds in Subjects With and Without Obstructive Sleep Apnea

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In healthy young volunteers, acquisition of blood oxygen level-dependent (BOLD) magnetic resonance (MR) and muscle sympathetic nerve (MSNA) signals during simulation of obstructive or central sleep apnea identified cortical cardiovascular autonomic regions in which the BOLD signal changed synchronously with acute noradrenergic excitation. In the present work, we tested the hypothesis that such Mueller maneuvers (MM) and breath-holds (BH) would elicit greater concomitant changes in mean efferent nerve firing and BOLD signal intensity in patients with moderate to severe obstructive sleep apnea (OSA) relative to age- and sex-matched individuals with no or only mild OSA (Apnea Hypopnea Index, AHI, $<$15 events/h). Forty-six participants, 24 with OSA [59 $\pm$ 8 years; AHI 31 $\pm$ 18 events/h (mean $\pm$ SD); seven women] and 22 without (58 $\pm$ 11 years; AHI 7 $\pm$ 4; nine women), performed a series of three MM and three BH, in randomly assigned order, twice: during continuous recording of MSNA from the right fibular nerve and, on a separate day, during T2*-weighted echo planar functional MR imaging. MSNA at rest was greater in those with OSA (65 $\pm$ 19 vs. 48 $\pm$ 17 bursts per 100 heart beats; $p < 0.01$). MM and BH elicited similar heart rate, blood pressure, and MSNA responses in the two cohorts; group mean BOLD data were concordant, detecting no between-group differences in cortical autonomic region signal activities. The present findings do not support the concept that recurring episodes of cyclical apnea during sleep alter cortical or peripheral neural responsiveness to their simulation during wakefulness by volitional Mueller maneuvers or breath-holds.

Keywords: breath hold, sleep apnea, sympathetic nerve activity, Mueller maneuver, functional magnetic resonance imaging (fMRI)

INTRODUCTION

Initiated by occlusion of the upper airway, obstructive sleep apnea (OSA) is characterized by recurring pauses in breathing, which, if sustained, beget progressive hypoxia and hypercapnia until arousal from sleep causing ventilation to resume. Each of apnea, hypoxia, hypercapnia, and arousal from sleep stimulates, reflexively and additively, synchronous clusters of increased sympathetic nerve firing. The latter interrupt, cyclically, the reductions in blood pressure and heart rate typical of normal sleep (Somers et al., 1995; Bradley and Floras, 2003; Floras, 2018). These acute disturbances induce, over time, increased peripheral chemoreceptor reflex sensitivity (Mateika et al., 2004; Mahamed et al., 2005), upward resetting of central sympathetic outflow during wakefulness, and thickening and thinning of specific gray matter constituents of a cortical autonomic network (CAN) engaged in the generation or modulation of cardiovascular autonomic tone (Taylor et al., 2016a, 2018; Floras, 2018). The latter can be considered one element of a broader human blood pressure regulating "connectome" converging on brainstem sympathetic motor units (Macefield and Henderson, 2019). The magnitude of efferent post-ganglionic muscle sympathetic nerve firing recorded during wakefulness is directly proportional to the frequencies of nocturnal breathing cessation and arousal from sleep; the magnitude of subsequent arterial oxygen desaturation; and structural changes within the CAN, specifically, left mid-cingulate cortex thickness and thalamic volume (Taylor et al., 2016a,b, 2018; Floras, 2018).

Application of independent component analysis to blood oxygen level-dependent (BOLD) contrast functional magnetic resonance imaging (fMRI) during wakefulness, during documented spontaneous normal breathing, identified spatially large and strong correlations between the strength of resting-state connectivity and the magnitude of muscle sympathetic nerve firing, expressed as burst incidence, i.e., the percentage of cardiac cycles accompanied by a pulse-synchronous discharge within several CAN nodes of the salience network, which is a cluster of spatially distinct brain regions including the left insular cortex, the right pregenual anterior cingulate cortex, the left temporo-parietal junction, the thalamus, and the cerebellum with temporally correlated spontaneous BOLD oscillations at <0.1 Hz that is engaged by autonomic challenges and homeostatic threats (Taylor et al., 2009). Connectivity within the paraventricular nucleus of the hypothalamus, periaqueductal gray, pons, and rostral ventral lateral medulla also correlated with sympathetic burst incidence. However, despite evident structural differences (Taylor et al., 2018), in this investigation, dual-regression analysis discerned no difference with respect to the strength of resting-state connectivity within these salience network regions between study participants with moderate or severe OSA, relative to matched subjects with no or only mild OSA (Taylor et al., 2016a). Importantly, these signals were acquired during wakefulness, in the absence of apnea.

In earlier experiments, in which BOLD fMR images and MSNA recordings were acquired in healthy young volunteers at rest and during two volitional interventions selected to simulate obstructive [Mueller maneuvers (MM)] or central sleep apnea [breath-holds (BH)] (Bradley et al., 2003; Kimmerly et al., 2013), we identified cortical and cerebellar cardiovascular autonomic regions in which the BOLD signal either increased (activation) or decreased (deactivation) profoundly and synchronously with acute sympathetic excitation. The greatest increases in such neural activity were evident in the right posterior and anterior insular cortices and in the dorsal anterior cingulate, fastigial, and dentate cerebellar nuclei, whereas signal intensity decreased in the left posterior insula and ventral anterior cingulate (Kimmerly et al., 2013).

The present purpose was to determine whether such acute neuroanatomical substrate-adrenergic outflow coupling differs quantitatively or qualitatively between cohorts of otherwise similar individuals documented to have, or not have, OSA. We tested the hypothesis that the simulation of obstructive or central sleep apnea by these two volitional interventions would elicit greater changes in efferent muscle sympathetic firing and BOLD signal activation or deactivation in those subjects with OSA.

MATERIALS AND METHODS

Participants

We recruited, primarily by advertisement, middle-aged men and women, self-identified as being in good health, who were agreeable to participating in research requiring overnight polysomnography, a daytime physiological recording session, and a functional MRI session. These investigations were approved by the Research Ethics Board of the University Health Network, in accordance with principles articulated in the Declaration of Helsinki. Informed written consent was obtained from all subjects in advance of their participation.

Cognizant of the high prevalence of asymptomatic and therefore unrecognized OSA in the general population (Kasai et al., 2012; Franklin and Lindberg, 2015) and to mitigate the commonplace biases risked by selecting as OSA patients only those identified as such after symptoms prompted referral for sleep studies and recruiting as control subjects individuals who self-reported freedom from OSA, we performed polysomnography first, then categorized participants as having either moderate to severe OSA (Apnea–Hypopnea Index; AHI $\geq$ 15 events/h) or no or mild OSA (AHI $<$ 15 events/h) (Berry et al., 2012).

Each individual completed a standard MRI prescreening questionnaire, to ensure they could be exposed safely to a high magnetic field. Screening also excluded from study smokers, pregnant women, or patients treated for OSA or known to have central sleep apnea, as well as individuals with atrial fibrillation or a medical history of heart failure, myocardial infarction, frequent atrial or ventricular ectopy, kidney disease, Raynaud's disease, autonomic neuropathy, drug-resistant hypertension, neurological disorders, or chronic back pain. Prescribed medications were continued to maintain clinical stability.

Two experimental sessions (physiological and MRI) were performed at the same time of day, within 2 weeks of each other, 2–3 h after a standard light meal. Subjects were instructed

to abstain from alcohol and caffeine for at least 12 h before these sessions. Resting-state connectivity data, acquired in the majority of these participants, has been previously reported (Taylor et al., 2016a).

Polysomnography

On the evening before the sleep laboratory study, the Epworth Sleepiness Scale (ESS) questionnaire (Johns, 1994) was administered by a technician unaware of this protocol's purpose. Subjects then underwent overnight polysomnography using standard techniques and scoring criteria for sleep stages and arousals from sleep (Bonnet et al., 1992). Thoraco-abdominal movements and tidal volume were measured by respiratory inductance plethysmography. Airflow was measured by nasal pressure cannulae and arterial oxyhemoglobin saturation (SaO_2) by oximetry. Mean and minimum SaO_2 ($minSaO_2$) were recorded. The oxygen desaturation index (ODI) was calculated as the frequency with which SaO_2 fell by $\geq$3% during each hour of sleep (American Academy of Sleep Medicine., 1999). Apneas and hypopneas were scored according to the American Academy of Sleep Medicine criteria (Berry et al., 2012). OSA severity was graded by a continuum with an AHI < 5 events/h of sleep classified as no sleep apnea; AHI 5–15, mild OSA; AHI 15–30, moderate OSA; and AHI > 30, severe OSA (American Academy of Sleep Medicine., 1999; Berry et al., 2012).

Sympathetic and Hemodynamic Data Acquisition and Analysis

Physiological recordings were acquired in a quiet, temperature-controlled room. Heart rate (HR) was derived from Lead II of an electrocardiogram. Blood pressure was recorded continuously from the right hand index finger (Portapres, Finapres Medical Systems B.V., Netherlands) and at each minute from an upper arm cuff (Dinamap Pro 100, Critikon, Tampa, FL, United States; standard 23–33-cm adult cuff). Breathing was recorded using a pneumobelt connected to a pressure transducer. Multiunit recordings of post-ganglionic muscle sympathetic nerve activity were acquired using a unipolar tungsten microelectrode inserted percutaneously into a fascicle of the right common fibular nerve (Kimmerly et al., 2013; Taylor et al., 2016b). MSNA, blood pressure, and HR were acquired over two separate 5-min "Apnea Protocol" periods. Using the LabVIEW® software platform (National Instruments, Austin, TX, United States), signals underwent analog-to-digital conversion for storage and analysis on a standard PC desktop. To evaluate task-stimulated efferent sympathetic outflow and to control for any between-subject variation in HR, MSNA was expressed conventionally as the cardiac frequency-independent measure of sympathetic discharge intensity, bursts per 100 heart beats, or burst incidence (BI).

Apnea Protocol

Before data were collected, each subject participated in a standardized practice run to ensure familiarity with the protocol and the ability to follow verbal instructions (i.e., "the next BH will begin in 10 s. . .0.5, 4, 3, 2, 1, hold") and then meet the target negative inspiratory pressure and maintain this over the required time period (Kimmerly et al., 2013).

The identical apnea protocol was performed on 1 day within the physiology laboratory and on a second within the MR suite. On both occasions, participants breathed through a mouthpiece connected to a transducer that measured airway load pressure. To acquire baseline data, signals were recorded over at least 10 min of quiet supine rest with documented spontaneous breathing. The 5-min apnea sequence began with 30 s of rest, followed by 15 s of either the MM or 15 s of end-expiratory BH, assigned at random (**Figure 1**). For each participant, the task order during the two (MR and physiological) sessions was identical. Each sequence consisted of three 15-s MMs and three 15-s BHs. After recovery to baseline, the sequence was duplicated. For the MM, a stopper with a small air leak was inserted into the end of the mouthpiece. Subjects generated then sustained a negative pressure of −30 mmHg for 15 s. For the BH, task subjects were instructed to hold their breath for 15 s following a normal expiration. To ensure adherence with these endpoints and that the target inspiratory pressure was achieved and sustained throughout the MM, generated pressures were displayed continuously on a computer screen within subjects' line of sight.

Muscle Sympathetic Nerve Activity Acquisition Session: Statistical Analysis

Physiological data are expressed as means ± SD. Group differences were compared by unpaired Student's *t*-test. BH and MM-stimulated changes were analyzed and compared, between cohorts, using a repeated-measure two-way ANOVA using SigmaStat 3.5 (Systat Software Inc., Chicago, IL, United States). For BHs, data acquired during the 15-s task were compared with baseline values. For MMs, due to the time delay preceding sympathetic excitation, and the carryover of such excitation after the end of the intervention, data during the 15 s of the task plus the first 5-s interval following this task were compared with baseline values.

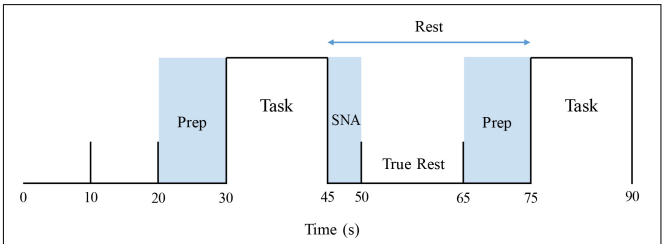

FIGURE 1 | Stimulus paradigm, covering the first 1.5 min of an apnea protocol. Prep: verbal instruction period. Task: Mueller maneuver or breath-hold (order for each participant allocated at random). SNA: "Spill-over" period during which elicited sympathetic excitation returned to baseline. True rest: the 15-s period between these two intervals that was used to determine pre-intervention resting baseline values.

Tukey's *post hoc* analysis was performed to estimate differences among means. A probability threshold of <0.05 was set for statistical significance.

Magnetic Resonance Data Acquisition and Analysis

Magnetic resonance imaging (MRI) data were obtained while subjects lay supine within a 3-Tesla whole body scanner (HDxt 16.0 General Electric Health Care, Milwaukee, WI, United States) fitted with an eight-channel phased-array head coil. Foam pads were positioned on either side of the head to minimize its movement during the breathing tasks, and under the elbows and knees for participant comfort. An MRI-compatible vital sign monitor (Veris, Medrad Inc., Warrendale, PA, United States) recorded the ECG at rest and during anatomical data collection and end-tidal CO_2 during the assigned breathing tasks. Headphones and MR-compatible goggles were worn to transmit instructions (i.e., "begin breath hold") and to feedback visually adherence to the MM protocol, respectively. Gradient-echo echo-planar imaging-sensitive BOLD contrast, reflecting local field potentials (Ogawa et al., 1990), was used to identify cortical and cerebellar regions in which blood flow and/or metabolism changed from baseline during assigned tasks. Brain stem regions were excluded from study; it was assumed, from prior experience (Kimmerly et al., 2013), that movement artifact therein would confound precise localization of any induced BOLD signal changes.

A three-dimensional (3D) high-resolution anatomical scan of the whole brain (120 slices, 24_24-cm FOV, 256_256 matrix, 1.5_0.859_0.859 voxels) was acquired with a T1-weighted 3D spoiled gradient echo sequence (flip angle_45°, TE_5 ms, TR_25 ms). Functional MRI data were acquired with a T2*-weighted echo planar imaging sequence (32 interleaved contiguous axial slices, FOV_20_20 cm, 64_64 matrix, 3.75_3.75_4.4-mm voxels, TE_40 ms, TR_2,000 ms). The scan was 5 min long, for a total of 150 frames. Each functional scanning period utilized the same apnea protocol described for the physiological testing session [i.e., two 5-min runs consisting of a 30-s rest followed by a 15-s apnea with a total of six blocks of apnea (three BH and three MM) and eight blocks of rest].

Magnetic resonance imaging data were analyzed with statistical parametric mapping software (SPM12, Wellcome Department of Cognitive Neurology) (Friston et al., 1995). The first three frames of each run (6 s) were discarded to allow for T1 equilibration effects. Next, subject data were corrected for slice timing collection and motion-related artifacts using SPMs Realign and Unwarp (which has been recommended when data are subject to task-correlated motion) (Jezzard and Clare, 1999; Andersson et al., 2001). Data were then co-registered to their anatomical scans and normalized to Montreal Neurological Institute (MNI) coordinates so that image volumes for all subjects were in the same three-dimensional space (Friston et al., 1995). A Gaussian smoothing kernel, set at 8 mm, was applied to allow better alignment of cortical anatomy across individuals. Data were high-pass filtered with a 90-s time constant to minimize low-frequency noise. Smoothed images then were de-trended for global signal intensity correction, to account for changes due to CO_2 and/or perfusion variations.

Despite their practice sessions, task-correlated head motion was evident nonetheless in most subjects, regardless of cohort. To reduce confounding of image interpretation by such movement, any subjects with greater than 4 mm of translational (x, y, or z) and 4° of rotational (pitch, roll or yaw) motion were excluded.

Resulting data were analyzed utilizing a two-level statistical paradigm. The first-level analysis was used to identify within-subject differences in signal intensity between the particular apnea stimulus (MM or BH) and baseline. To accomplish this, a time course was constructed individually for each subject based on their MSNA response, as described in detail by Kimmerly (2013). For each apnea, the following measurements were acquired from the subjects' physiological testing session: (1) MSNA delay: time from the beginning of each apnea (MM or BH) to the initiation of the MSNA burst and (2) MSNA duration. These values were measured for each BH and MM, across both 5-min runs of the apnea protocol (thus there were six MM and six BH measurements for delay and duration), in each individual subject. From these measurements, the average MSNA delay and duration were calculated for each subject for MM and BH separately. As the MSNA response persisted after the end of the apnea, the first 5 s of each subsequent period were not included when calculating pre-intervention rest data for the next task. Furthermore, as task instructions were given during the last 10 s of each rest period, that time also was excluded from resting value calculations. Thus, baseline, pre-intervention calculations were restricted to those "true rest" data acquired over the 15 s between the dissipation of prior sympathetic excitation and the verbal instructions for the next apnea (**Figure 1**).

Average delay and duration values combined with the true rest time periods were utilized to create individual fMRI regressors for each subject. These regressors were then convolved with the canonical hemodynamic response function and correlated with each subject's fMRI time series for each voxel of the brain. Subject-specific contrast images containing whole-brain information related to both increase and decrease in BOLD signal for MM versus baseline and BH versus Baseline were constructed. These contrast images were utilized at the second level in a two-sample *t*-test to compare differences between groups (healthy control vs. OSA) for BH and MM separately. Contrasts were generated to examine BH $>$ rest, rest $>$ BH, MM $>$ rest, and rest $>$ MM. An uncorrected threshold of $p < 0.001$ and a minimum cluster size of 10 voxels were required to refute the null hypothesis.

RESULTS

Participant Characteristics

Forty-six such individuals (average age 59), of whom 16 were women, completed all three tasks. Of these, 24 (seven women) were classified, following polysomnography, as having moderate to severe OSA [AHI, 31 $\pm$ 18 events per hour (mean $\pm$ SD)] and 22 (9 women) who had either no or mild sleep apnea (AHI,

7 ± 4 events per hour) were categorized as controls. Participants' characteristics are presented in **Table 1**. As anticipated, mean values for both MSNA BI (65 ± 19 vs. 48 ± 17 bursts per 100 heart beats; $p < 0.01$) and the ODI (30 ± 21 vs. 11 ± 11 events per hour; $p < 0.01$) were greater in those with moderate to severe OSA, as compared with individuals with no or only mild OSA. Mean age, BMI, HR, and systolic blood pressure were similar, whereas diastolic pressure was significantly higher in those with OSA.

After blinded analysis and extraction of those BOLD signal recordings that did not meet prespecified stringent head movement artifact exclusion criteria during MM and BHs, acceptable data were collated in 10 participants (five women) classified as having OSA and in 14 control subjects (three women) for comparative analysis. Group mean values for translational and rotational movement during simulated apneas are presented in **Table 2**. High-quality mean voltage neurograms of multiunit MSNA were acquired in all 24 individuals. Characteristics of this subgroup of participants (**Table 3**) were broadly similar to and thus representative of the group as a whole (**Table 1**).

TABLE 1 | Participant characteristics.

Characteristics	Control (No or mild apnea) *n* = 22	OSA (Moderate or severe apnea) *n* = 24	*p*-value
Age (years)	59 ± 8	58 ± 11	0.867
Men (*n*)	13	17	
AHI (events/h)	7 ± 4	31 ± 18	<0.001
BMI (kg/m^2)	28 ± 5	31 ± 5	0.117
MSNA (bursts/100 HB)	48 ± 17	65 ± 19	<0.01
MSNA (bursts/min)	30 ± 11	42 ± 12	<0.001
HR (bpm)	59 ± 10	64 ± 9	0.240
SBP (mmHg)	122 ± 17	127 ± 13	0.179
DBP (mmHg)	64 ± 8	71 ± 10	<0.01
ODI	11 ± 11	30 ± 21	<0.01

Mean ± SD.
AHI, apnea–hypopnea index; BMI, body mass index; DBP, diastolic blood pressure; HB, heart beats; HR, heart rate; MSNA, muscle sympathetic nerve activity; ODI, oxygen desaturation index; OSA, obstructive sleep apnea; SBP, systolic blood pressure.

TABLE 2 | Translational and rotational head movement during simulated apnea.

Direction	Control (No or mild apnea) *n* = 10	OSA (Moderate–severe apnea) *n* = 14
X (mm)	0.97 ± 0.12	1.25 ± 0.14
Y (mm)	1.06 ± 0.16	1.27 ± 0.17
Z (mm)	2.29 ± 0.25	2.17 ± 0.20
Pitch (°)	2.36 ± 0.38	2.56 ± 0.29
Roll (°)	1.10 ± 0.16	1.11 ± 0.19
Yaw (°)	0.85 ± 0.16	0.96 ± 0.08

Group mean values for translational (x, y, or z) and rotational (pitch, roll, or yaw) head motion (mean ± s.e) documented during Mueller maneuvers and breath-holds. OSA: obstructive sleep apnea.

TABLE 3 | Participant characteristics: subgroup meeting fMRI analysis criteria.

Characteristics	Control (No or mild apnea) *n* = 10	OSA (Moderate or severe apnea) *n* = 14	*p*-value
Age (years)	59 ± 9	57 ± 13	0.722
Men (*n*)	5	11	
AHI (events/h)	5 ± 3	34 ± 23	<0.001
BMI (kg/m^2)	27 ± 3	31 ± 5	<0.05
MSNA (bursts/100 HB)	48 ± 17	66 ± 19	<0.05
MSNA (bursts/min)	27 ± 11	45 ± 14	<0.01
HR (bpm)	58 ± 11	65 ± 8	0.164
SBP (mmHg)	119 ± 9	127 ± 16	0.118
DBP (mmHg)	64 ± 10	71 ± 11	0.107
ODI	9 ± 9	31 ± 23	<0.01

Mean ± SD.
AHI, apnea–hypopnea index; BMI, body mass index; DBP, diastolic blood pressure; HB, heart beats; HR, heart rate; MSNA, muscle sympathetic nerve activity; ODI, oxygen desaturation index; OSA, obstructive sleep apnea; SBP, systolic blood pressure.

Physiological Responses to Simulated Apneas

Figure 2 illustrates typical changes in MSNA, arterial blood pressure and HR of a participant before, during, and after a 15-s end expiratory BH, and a MM of similar duration. **Figure 3**, encompassing the entire study population, depicts mean changes in HR, blood pressure, and MSNA burst frequency and incidence for those with and without moderate to severe OSA. BHs and MMs had similar effects on HRs and blood pressures of these two cohorts. As anticipated (Bradley et al., 2003; Kimmerly et al., 2013), MSNA BI in both groups increased from baseline within the first 5 s of BHs ($p < 0.001$), reaching peak sympathetic excitation in the final 15 s ($p < 0.001$), and remained elevated until breathing resumed; thereafter, MSNA returned to baseline levels ($p = 0.89$). Consistent with previous observations demonstrating a reflex inhibitory response to acute stimulation of aortic baroreceptors by increased intra-thoracic transmural pressure (Bradley et al., 2003), BI fell ($p < 0.005$) within the first 5 s of the MM then returned to baseline levels at 10 s ($p = 0.99$) and rose above baseline during the final 15 s of MM ($p < 0.005$) and the first 5 s of recovery ($p < 0.001$). Although MSNA burst frequency was consistently higher, in participants with OSA, throughout both breathing tasks ($p < 0.01$ for BHs; $p < 0.05$ for MMs), the reflex sympathetic excitation induced by each task did not differ between those with OSA and the control participants (group × time interaction terms ≥0.08). Importantly, within this population as a whole, there were no between-group differences with respect to MSNA BI during either the BHs ($p = 0.13$) or the MMs ($p = 0.12$), and when expressed as BI, reflex sympathetic excitation induced by each task did not differ between those with OSA and the control participants (group × time interaction terms ≥0.13).

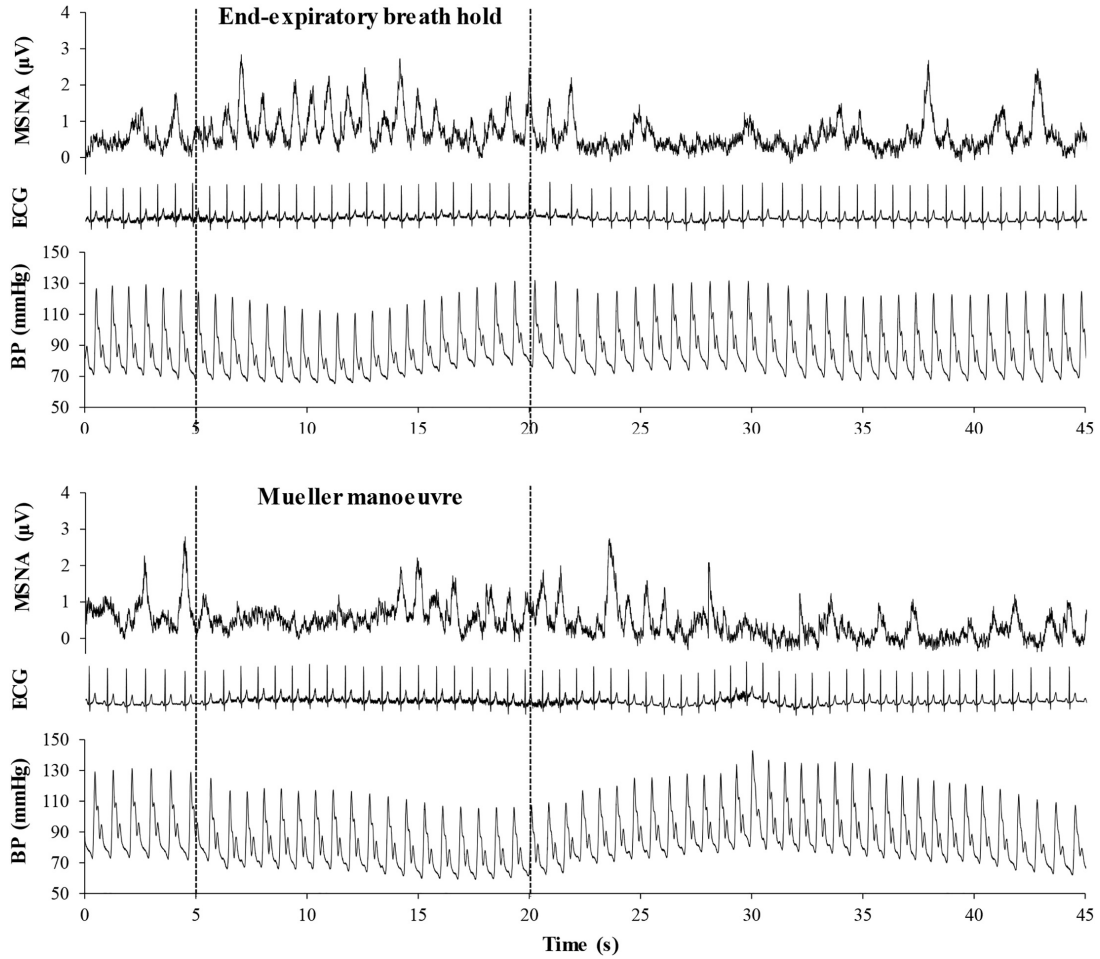

FIGURE 2 | Mean voltage neurogram of muscle sympathetic nerve activity (MSNA), arterial blood pressure (BP), and the electrocardiogram (ECG), illustrating heart rate in a representative study participant before, during, and after a 15-s end expiratory breath-hold (*upper panel*) and a Mueller maneuver (*lower panel*). Note that the time delay between the initiation of the breathing task and the onset of sympathetic excitation was longer for the Mueller maneuver than for the breath-hold, due to initial aortic baroreceptor stimulation by the increase in intra-thoracic transmural pressure. However, the sympatho-excitatory responses to the two breathing tasks were of similar duration.

Figure 4 illustrates the changes in HR, blood pressure, and MSNA burst frequency and incidence of the study subjects with and without OSA whose movement artifact during simulated apnea did not exceed the defined exclusion thresholds. BHs and MMs had similar effects, in the two cohorts, on blood pressure ($p \geq 0.69$), whereas it was higher in those with OSA HR over the course of the MM ($p < 0.01$ for group × time interaction). As with the participants as a whole, there were no between-group differences within this subset with respect to MSNA responses expressed as burst frequency during either the BHs ($p = 0.23$) or MMs ($p = 0.10$). When expressed as MSNA BI, responses to BHs in the two cohorts were similar ($p = 0.34$); there was a trend toward greater sympathetic excitation in those with OSA ($p = 0.05$).

Group mean BOLD fRMI image data were concordant with these neutral findings; second-level group comparison of MR images, synchronous with the peripheral neural responses depicted in **Figure 4**, detected nil after subtraction, indicating no between-group differences with respect to activity within cortical and cerebellar autonomic regions.

DISCUSSION

The acute, brief, mechanical, autonomic, chemical, and hemodynamic turbulence that is characteristic of obstructive apnea is exclusive to sleep, but when repeated nightly over months and years these induce an array of systemic after-effects evident during wakefulness (Floras, 2018; Taylor et al., 2018). One such consequence is long-term facilitation of central sympathetic outflow (Taylor et al., 2016a, 2018; Floras, 2018). The presumed stimulus to such autonomic neuroplasticity is recurrent hypoxia, which in experimental models increases the sensitivity of the afferent limb of the peripheral chemo-reflex to reductions in oxygen tension (Mahamed et al., 2005; Abboud and Kumar, 2014; Prabhakar, 2016); amplification of this neural input, if

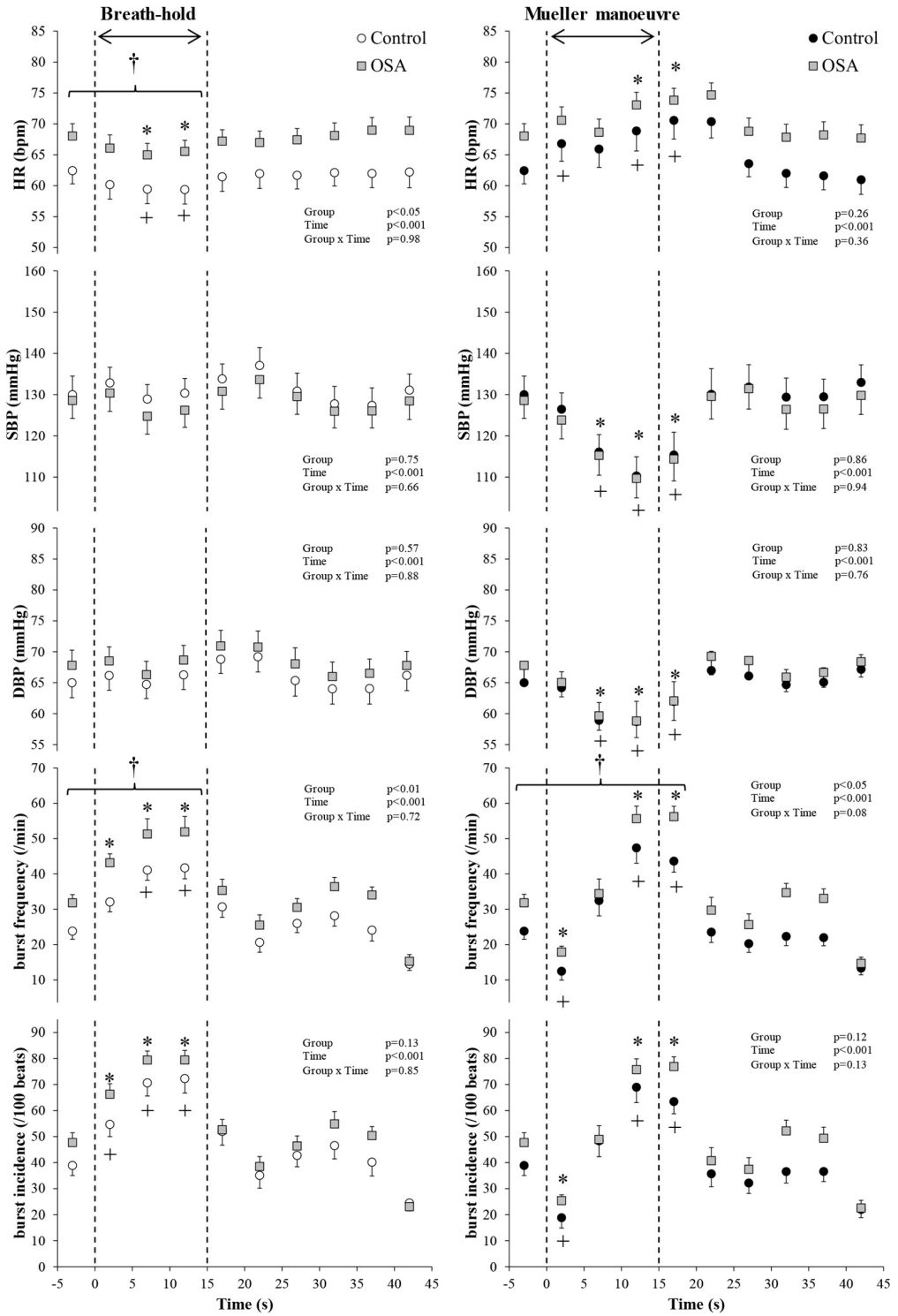

FIGURE 3 | All study participants. Mean (±95% confidence limits) heart rate (HR), systolic and diastolic blood pressure (SBP and DBP), and muscle sympathetic nerve activity (MSNA) responses before, during, and after the series of 15 s of end-expiratory breath-holds (*left*) and 15 s of Mueller maneuvers (*right*) in 24 individuals with obstructive sleep apnea (OSA, *gray* squares) and 22 healthy controls (*white* and *black* circles) are displayed. The dashed vertical lines indicate the beginning (time = zero) and end (time = 15 s) of both maneuvers. Continuous responses were bin-averaged at 5-s intervals. For breath-holds, data during the 15 s of the task were compared with baseline values. For Mueller maneuvers, due to the time delay preceding sympathetic excitation, data during the 15 s of the task plus the first 5-s interval following the task were compared with baseline values. $^{\dagger}p < 0.05$ compared to control group; $^{*}p < 0.05$ compared to pre-maneuver (i.e., −2 s) in healthy controls; $^{+}p < 0.05$ compared to pre-maneuver (i.e., −2 s) in OSA.

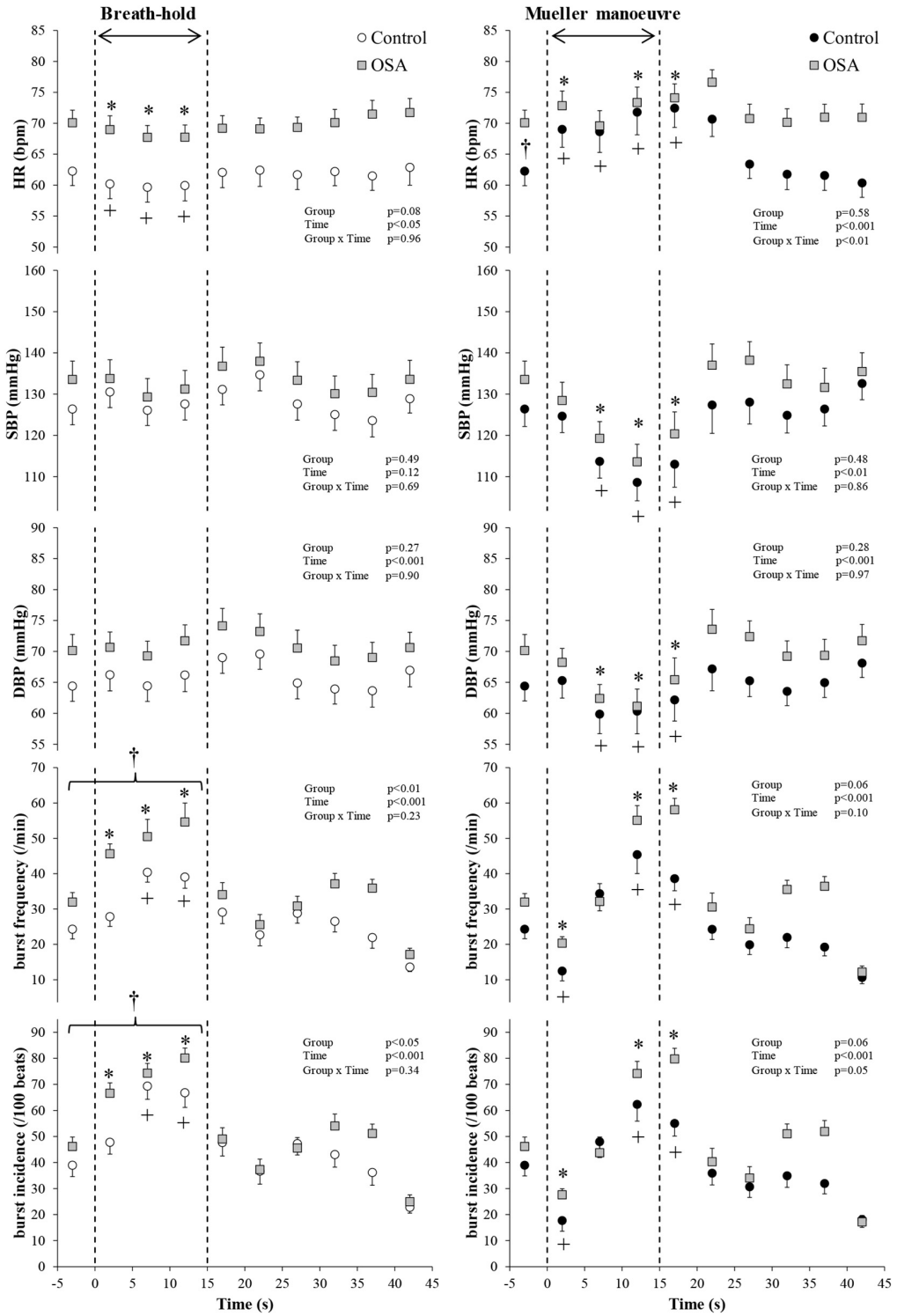

FIGURE 4 | Participants with both blood oxygen level-dependent (BOLD) functional magnetic resonance signals meeting inclusion thresholds (see text) and high-quality mean voltage neurograms. Mean (±95% confidence limits) HR, SBP, DBP, and MSNA responses before, during, and after the series of 15 s of end-expiratory breath-holds (*left*) and 15 s of Mueller maneuvers (*right*) in 14 individuals with obstructive sleep apnea (OSA, *gray* squares) and 10 healthy controls (*white* and *black* circles) are displayed. The dashed vertical lines indicate the beginning (time = zero) and end (time = 15 s) of both maneuvers. Continuous responses were bin-averaged at 5-s intervals. For breath-holds, data during the 15 s of the task were compared with baseline values. For Mueller maneuvers, due to the time delay preceding sympathetic excitation, data during the 15 s of the task plus the first 5-s interval following the task were compared with baseline values. $^{\dagger}p < 0.05$ compared to the control group; $^{*}p < 0.05$ compared to pre-maneuver (i.e., −2 s) in healthy controls; $^{+}p < 0.05$ compared to pre-maneuver (i.e., −2 s) in OSA.

sustained, has been posited to induce trophic changes, such as the thickening documented, in patients with OSA, in cortical regions participating in cardiovascular autonomic regulation (Taylor et al., 2018), cognition, and other brain functions (Baril et al., 2017). Concurrently, hypoxia-mediated cellular trimming appears to thin other CAN regions, such as the left and right dorsal posterior insular cortices and the left posterior cingulate or precuneus (Taylor et al., 2018).

The present experiments considered an hypothesis inspired by three novel prior findings by our group. The first, involving healthy young volunteers invited to perform a volitional protocol replicated in the present series, was the identification of cortical and cerebellar regions participating in cardiovascular autonomic regulation in which BOLD contrast signaling either increased (activation) or decreased (deactivation) in temporal concordance with excitation of efferent post-ganglionic sympathetic discharge to skeletal muscle (Kimmerly et al., 2013). The second, revealed by a comparison of cortical autonomic regions of age-matched participants with and without polysomnography-classified OSA, was thinning of the left dorsal posterior insular cortex, proportionate to the ODI, and thickening of the left-mid cingulate cortex and thalami in those whose AHI was $\geq$15 events/h (Taylor et al., 2018). The third was the detection of positive correlations between thickening within these specific regions and muscle sympathetic BI, recorded during wakefulness (Taylor et al., 2018). The purpose of the present work was to test the hypothesis that individuals with documented OSA and such structural changes exhibit also greater muscle sympathetic nerve discharge and synchronous BOLD signal responses during volitional simulation of central or OSA by BHs and by MMs.

The results of these experiments do not support this hypothesis. Our principal findings with respect to MSNA and the concurrent BOLD contrast signal were both neutral and concordant; there were no between-group differences in response patterns of either variable to BHs or to MMs. These observations therefore are consistent with the results of prior dual-regression analyses, which found no between-group differences within this cohort with respect to the strength of resting-state connectivity within cortical and cerebellar CAN nodes of the salience network between those with moderate to severe OSA, relative to age- and sex-matched volunteers with no or only mild OSA (Taylor et al., 2016a).

A key strength of the present work is the classification of participants into cohorts with and without moderate to severe OSA on the basis of polysomnography, rather than unsubstantiated self-report. Indeed, several volunteers who professed normal breathing during sleep were discovered to have moderate or severe apnea; conversely, several who assumed that they did, because of loud snoring, had no or only mild airway obstruction during sleep. A second is the use of the MM, a stimulus to sympathetic activation that persists beyond its termination (**Figure 2**) (Bradley et al., 2003). This temporal discordance removes the potential confounding influences of volitional motor effort and baroreceptor- and chemoreflex-mediated, emotional, and sensory responses on cortical neural patterns generated by such simulation of obstructive apnea.

The MR incompatibility of the conventional microneurography apparatus deployed in our laboratory necessitated replication of these apnea-simulating tasks on different days; the assumption that the magnitude and duration of MSNA responses to apnea are consistent when repeated over such short time frame is reasonable (Kimmerly et al., 2004; Badrov et al., 2016). Using a pre-amplifier encased in stainless-steel Macefield's group acquired 4-s neurograms interpolated between scanning periods; they identified, during spontaneous breathing, MSNA-coupled increases in BOLD signal intensity bilaterally, in the dorsolateral prefrontal cortex, posterior cingulate cortex, precuneus, ventromedial hypothalamus, and rostral ventrolateral medulla and on the left, within the mid-insular cortex and dorsomedial hypothalamus (Macefield and Henderson, 2019).

We considered several possible explanations for the absence of between-group differences. The duration of these volitional stimuli (15 s) was brief, relative to that of spontaneous apnea evident in the majority of patients with OSA (Bradley and Floras, 2003; Kasai et al., 2012), and thus may not have been sufficient to replicate the full intensity of sympathetic discharge elicited by the confluence of apnea, hypoxia, hypercapnia, and the arousal from sleep that terminates spontaneous obstructive or central events. In experiments by Morgan et al. (1993), supplemental oxygen (inspired fraction 0.30) attenuated markedly the time-dependent increases in MSNA, accompanied by 2% reductions in O_2 saturation, elicited by BHs and MMs of greater duration (20 s). Also, patients with moderate to severe OSA will be exposed to scores or hundreds of apneic events over the course of the night, as compared with the six simulated events in the present protocol. Nonetheless, since longstanding OSA is accompanied by altered cortical autonomic gray matter thickness (Taylor et al., 2018) and, during wakefulness, both upward resetting of central sympathetic outflow (Taylor et al., 2016a,b) and augmented peripheral chemoreflex sensitivity (Mateika et al., 2004; Mahamed et al., 2005; Abboud and Kumar, 2014; Prabhakar, 2016), a single volitional apnea should be sufficient to detect between-group evidence for heightened central and peripheral autonomic responses to this stimulus–as has been shown, with MSNA recordings, for patients with heart failure (Bradley et al., 2003). For the purpose of between-cohort comparison, we classified participants conventionally, on the basis of their AHI, rather than the ODI (Linz et al., 2018). Importantly, in the present series, ODI tracked AHI and was three-fold higher in those with OSA. Present experiments were conducted during wakefulness, which resets upward, from sleep, the threshold eliciting PCO_2-stimulated ventilation (Ainslie and Duffin, 2009) [and, by inference, chemo-reflex-mediated sympathetic discharge (Keir et al., 2019)]. Reflex sympathetic excitation would be anticipated earlier in the course of apneas occurring during sleep. An additional conjecture is that cortical autonomic neural activity in these study participants, who on average were twice as old as our previously studied healthy volunteers (Kimmerly et al., 2013), may be at or near ceiling under resting conditions. Thus, dynamic range constraint may preclude detection of material BOLD signal changes. In contrast

to the stability of the head and neck of healthy young subjects when executing these instructed breathing maneuvers in our previous experiments (Kimmerly et al., 2013), and despite our training and rigorous attempts to ensure these middle-aged individuals could perform these interventions with minimal head movement, only 24 of our 46 participants (whose characteristics were similar to the group as a whole) were capable of doing so, suggesting subtle age-related sarcopenic loss of muscle strength. Such attrition raises the question as to whether subject numbers in this residual cohort were insufficient to detect between-group BOLD image differences. Notwithstanding, this principal study finding of neutrality was consistent across all endpoints measured: the hemodynamic and neural responses to both BHs and MMs were essentially identical in participants with and without OSA, within both the study population as a whole (**Figure 3**) and in the subgroup whose translational and rotational head motion permitted reliable image interpretation (**Figure 4**); consequently, if the MSNA responses were modulated by changes in neural activity within elements of the CAN, similar BOLD image data, as observed, would be anticipated. We nonetheless acknowledge that the range-of-motion artifact permitted for this subgroup analysis (about 2.2-mm motion and 2.5° rotation that is TASK correlated) may have been insufficiently conservative. Future investigations of this present pathophysiological construct in individuals with sleep-related breathing disorders should ensure a more robust head stabilization protocol.

In sum, the present investigation does not support the concept that recurring episodes of cyclical apnea during sleep alter cortical or peripheral neural responsiveness to their simulation during wakefulness by volitional MMs or BHs.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Research Ethics Board of the University Health Network. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KT and JF contributed to the conception, design of the experiments, and secured grant funding. KT, NH, HM, and PM contributed to data collection. KT and DKe analyzed data and prepared figures. KT, DKe, and JF drafted and revised the manuscript. All authors contributed to the interpretation of data, approved the manuscript, and agreed to be accountable for all aspects of the work.

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REVIEW
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Going to Extremes of Lung Physiology–Deep Breath-Hold Diving

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Breath-hold diving involves environmental challenges, such as water immersion, hydrostatic pressure, and asphyxia, that put the respiratory system under stress. While training and inherent individual factors may increase tolerance to these challenges, the limits of human respiratory physiology will be reached quickly during deep breath-hold dives. Nonetheless, world records in deep breath-hold diving of more than 214 m of seawater have considerably exceeded predictions from human physiology. Investigations of elite breath-hold divers and their achievements revised our understanding of possible physiological adaptations in humans and revealed techniques such as glossopharyngeal breathing as being essential to achieve extremes in breath-hold diving performance. These techniques allow elite athletes to increase total lung capacity and minimize residual volume, thereby reducing thoracic squeeze. However, the inability of human lungs to collapse early during descent enables respiratory gas exchange to continue at greater depths, forcing nitrogen (N_2) out of the alveolar space to dissolve in body tissues. This will increase risk of N_2 narcosis and decompression stress. Clinical cases of stroke-like syndromes after single deep breath-hold dives point to possible mechanisms of decompression stress, caused by N_2 entering the vasculature upon ascent from these deep dives. Mechanisms of neurological injury and inert gas narcosis during deep breath-hold dives are still incompletely understood. This review addresses possible hypotheses and elucidates factors that may contribute to pathophysiology of deep freediving accidents. Awareness of the unique challenges to pulmonary physiology at depth is paramount to assess medical risks of deep breath-hold diving.

Keywords: breath-hold diving, apnea, glossopharyngeal insufflation, decompression, narcosis, lung

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INTRODUCTION

Breath-hold diving (synonyms: apnea diving, freediving, and skin diving) has been practiced by humans throughout history for leisure and commercial purposes, e.g., harvesting sea food or spearfishing. More recently breath-hold diving also became a competitive sports activity, inspired by the famous competition between Italian freediver *Enzo Maiorca*, who breached the 50 m depth in 1960, and French freediver *Jacques Mayol*, who was the first to reach 100 m in 1976. These achievements also raised interest by the scientific community, as they challenged the pertinent

understanding of the depth limits of breath-hold diving. This review aims at elucidating the particular challenges of deep human breath-hold diving to respiratory physiology, and to highlight the serious medical risks associated with competitive freediving to great depths.

THE DIVING RESPONSE

A major challenge for humans inherent to the aquatic environment is the lack of oxygen, as mammals cannot breathe under water. Thus, oxygen depletion relies on air inhaled into the lungs before breath-holding, and intrinsic oxygen stores bound in blood to hemoglobin, in muscle to myoglobin and in smaller amounts also physically dissolved in plasma. Diving mammals have developed a major physiological adaptation to withstand asphyxia for prolonged dive duration called the diving response. This fundamental mechanism enabling organisms to maintain life during asphyxia has been demonstrated in many terrestrial species and involves both bradycardia and peripheral vasoconstriction. In humans, the diving response is characterized by a synergistic sympathetic and parasympathetic activiation and catecholamine increase (Heusser et al., 2009; Eichhorn et al., 2017), redistribution of blood flow to maintain adequate oxygen supply to hypoxia sensible organs (Joulia et al., 2009; Eichhorn et al., 2018), and reduced heart rate (Perini et al., 2008; Costalat et al., 2021). More recently, active contraction of the spleen has also been attributed to the human diving response without clear relationship.

The human diving response is highly variable and can be modified by physiological as well as emotional factors. Elite breath-hold divers have developed certain adaptions to repeated apnea training, such as greater tolerance to hypoxia and hypercapnia, and a more pronounced diving response. For some factors like the blunted chemosensitivity to hypercapnia a possible genetic predisposition has been discussed while apnea training may modify these or other physiologic and psychophysical factors (Bain et al., 2018).

Trained freedivers achieve average breath-hold times of 5 min under resting conditions (Hansel et al., 2009), allowing them in theory to reach great depths with assisted descent using a ballast, and ascending with lifting bags. In fact, total dive time for documented world record dives were less than 5 min. Maximum achievable breath hold time is not determining the maximal diving depth, but physiological factors (lung size and compliance, cardiac fitness), blood redistribution, economization of diving movements, equalization techniques, mental strength and other factors influence the risk for serious hypoxemia upon ascent and are therefore affecting the maximum diving depth.

THE RV/TLC RATIO

It had been generally believed that the maximum depth to which a breath-hold diver could descend would be determined by the individual ratio of residual volume (RV), i.e., the lung volume remaining in the lungs after complete exhalation, to total lung capacity (TLC) (Carey et al., 1956; Agostoni, 1965). As intrathoracic gas will be compressed during a breath-hold dive with increasing depth, volume halves at 10 m depth (2 atm absolute pressure), and will be reduced to one third of its initial volume at 20 m depth. Thus, in a subject with an RV of 20% of TLC, the critical lung volume will be reached at 40 m depth (5 atm absolute pressure) when the TLC is compressed to one fifth of its initial volume. Intrathoracic pressure will become lower than ambient pressure and therefore pressure of body tissues and blood. With further descent beyond RV the distensibility limit of the blood-containing structures in the chest may be reached, resulting in possible fluid extravasation and pulmonary trauma including alveolar damage and intra-alveolar bleeding.

The classical concept that the RV/TLC ratio determined the maximum breath-holding diving depth had been consistent with early research in professional Japanese and Korean diving women harvesting seafood in the Sea of Japan and Yellow Sea. Teruoka (1932) was the first to extensively study the so called Japanese "Ama" women divers and documented maximum diving depths of up to 25 m, although anecdotally reported dives were up to 45 m. A study in US Navy submarine escape training tank instructors reported larger TLC and vital capacity compared to laboratory control personnel, supporting the concept that a lower RV/TLC ratio was beneficial to achieve greater freediving depths (Carey et al., 1956). Divers had 14.6% higher than predicted vital capacity; a longitudinal follow-up over 1 year attributed the higher VC to training adaptation rather than a selection effect. Song et al. (1963) measured RV and TLC in Korean diving woman and controls and determined an average ratio of 25% that allowed dives to approx. 30 m. There was no significant difference between divers and controls, however, diving women tended to have a lower RV/TLC ratio per corresponding age group.

Subsequent reports confirmed that subjects achieving record depths had favorable individual RV/TLC ratios, allowing them to go deeper than predicted. US Navy diver *Robert Croft* had a TLC of 9.1 L and an RV of 1.3 L providing a ratio that would enable him to dive safely up to 60 m depth (Schaefer et al., 1968). In fact, the predicted values according to his age, height and sex would have amounted to 6.9 and 2 L for TLC and RV, respectively, allowing him to hardly reach 30 m. Thus, favorably low RV/TLC ratios enabled certain individuals to reach record depths that exceeded the depth range of 30–40 m known from professional breath-hold divers.

However, *Robert Croft* reached 66 m depth in 1968 and French freediver *Jacques Mayol* became the first to break the 70 m limit in the same year. These achievements alerted physiologists that the concept of RV/TLC determining maximum breath-hold diving depth needed to be refined.

THORACIC BLOOD SHIFT

Although the anatomy of the human lung, i.e., size and properties of lung parenchyma and surrounding organs and tissues, will primarily determine individual gas volumes, physiologic factors such as the force exerted by respiratory muscles, lung reflexes, properties of airways etc. will affect volume changes. RV of the

lungs varies markedly with pressure, being greater at positive pressures and smaller at negative pressures (Rahn et al., 1946). Seminal work by Fenn et al. (1947) showed that an increase of pulmonary pressure of 30 cm H_2O could displace 500 mL or about half of the blood contained in the lungs. Conversely, submersion of the chest reduces lung volumes, equaling an effect of negative pressure breathing (Hong et al., 1960). It was shown that the change in lung volume during submersion was mainly due to the abdomen with the diaphragm displaced cranially (Agostoni et al., 1966). Measuring the diffusion capacity of the lungs for carbon monoxide showed a marked increase during immersion attributable directly to storage in the lung of blood displaced by pressure from the vessels of immersed parts of the body (Guyatt et al., 1965).

Schaefer et al. (1968) measured thoracic blood volume displacements at various depths during breath-hold dives by use of impedance plethysmography. At 30 m depth in the US Navy escape training tank they could show that 1047 mL of blood were shifted into the thorax. Thus, a significant amount of blood shift due to body immersion will replace intrathoracic air and consequently reduce RV. The authors reported that record freediver *Jacques Mayol* who reached 70 m depth had an RV of 1.88 L and a TLC of 7.22 L that would have allowed him to dive to 28 m only. It could be calculated that a blood shift of 980 mL into the thorax was necessary in order to not suffer lung squeeze at 70 m.

The authors also determined oxygen costs of breath-hold dives, showing that oxygen requirement was lower than oxygen consumption during exercise and neither hypoxia nor hypercapnia determined dive limits during these breath-hold dives. The authors had measured gas tensions of mixed expired and alveolar air at various depths during descent and ascent of breath-hold dives (Schaefer and Carey, 1963). Their results indicated that at depth carbon dioxide is transferred back from lung alveoli into the pulmonary capillaries and oxygen (O_2) content of the lungs does not change linearly, with decreased O_2 transfer at depth and steep decrease during ascent.

Craig (1968) postulated that during descent the size of the thoracic volume would change with compression of the thoracic cage and elevation of the diaphragm but that this mechanism alone could not account for the depths reached. As the intrathoracic pressure at depth would become less than ambient pressure, there would be a pressure gradient along which blood would shift into the thorax. In an interesting experiment he measured thoracic pressure by esophageal balloon during a breath-hold dive to 4.75 m which the subject started at surface exhaling to RV of 2 L, confirming that 600 mL of blood must have shifted into the thorax to compensate for the compression to 1.4 L. The author speculated "that the capacity of a diver to compress the air volume by fluid changes might be the major limiting factor in the depth of a dive. If as much as 1 L of blood could move from the peripheral to the central circulation, a diver might go as deep as 140 m".

While these studies elucidated thoracic blood shift during (deep) breath-hold diving as an important physiologic mechanism to prevent lung squeeze, athletes continued to compete for ever increasing record depths (Sharp, 2003). *Umberto Pelizzari* reached 150 m depth in 1999 and *Loic Leferme* set a record depth at 162 m in 2002[1]. These depths could only be achieved using technical assistance such as weight sleds upon descent and balloons to assist ascent from depth, in order to keep breath-hold time to a tolerable minimum. However, consideration of RV/TLC ratio and thoracic blood shift alone would not allow subjects to survive exposures to these depths. An increased awareness by the scientific community of the record depths achieved by some elite breath-hold divers prompted early case studies of elite athletes who used a unique breathing technique to further increase their TLC (Örnhagen et al., 1998; Muth et al., 2003; Simpson et al., 2003; Lemaitre et al., 2010).

GLOSSOPHARYNGEAL BREATHING

Glossopharyngeal breathing was first described as a method of independent breathing in patients with post-poliomyelitic syndrome (Collier et al., 1955). In these patients with pronounced impairment of the respiratory muscles and a consecutively low vital capacity, the technique allowed an increase in VC of 5.4 times, thereby prolonging breathing time without mechanical aid by 184 times. With technical progress in ventilation the technique got somewhat forgotten, however, more recently was recognized as a method used by elite breath-hold divers to improve performance. Increasing the volume of intrathoracic air will further reduce the RV/TLC ratio, thus, allowing athletes to dive deeper before reaching the critical lung compression point for lung squeeze. Moreover, it will increase pulmonary oxygen stores and thereby breath-hold duration, and dispose of greater air volume to equalize cranial air cavities (ears, sinuses) at depth. It must be acknowledged that TLC is not equivalent to maximum lung volume, and due to its enormous distensibility the lung can stretch to above normal volumes.

Glossopharyngeal insufflation is started after filling the lungs to TLC. Then a mouthful of air with the glottis closed is compressed by the oropharyngeal muscles and then forced into the lungs. Before the pharyngeal muscles are relaxed the glottis is closed to allow new air entering the oral cavity. This buccal pumping cycle is repeated several times until a sensation of fullness occurs (see **Supplementary Video**). By applying this technique, elite breath-hold divers may increase their TLC by up to 47% (Loring et al., 2007); mean increases in TLC were reported in several larger samples of elite breath-hold divers to average 15–25% (Lindhom and Nyren, 2005; Overgaard et al., 2006; Seccombe et al., 2006; Tetzlaff et al., 2008). The number of buccal pumping cycles employed to reach the individual sensation of fullness varies greatly across individuals; a median of 19 (range 13–75) were reported by Tetzlaff et al. (2008).

Seccombe et al. (2006) recorded mouth relaxation pressure immediately and 5 min after glossopharyngeal insufflation and plotted this vs. plethysmographic volume change. They calculated that the mean increase in TLC attributable to overdistension of the lung was 69%, while the remainder was attributable to air compression. Since glossopharyngeal insufflation must overcome

[1]https://worldrecords.aidainternational.org/NoLimit

lung recoil pressure to increase lung volume above TLC, the authors speculated that recoil pressure at high lung volumes may be altered transiently, supported by the fact that TLC 5 min after glossopharyngeal insufflation still was above expected normal variation. Indeed, static lung compliance was measured during and up to 3 min after glossopharyngeal insufflation in five elite breath-hold divers (Tetzlaff et al., 2008). A transient decline of lung elastic recoil could be confirmed as compliance was still elevated when subjects had exhaled and returned to tidal breathing.

Lindhom and Nyren (2005) also investigated magnetic resonance imaging (MRI) during glossopharyngeal insufflation and described a reduction of thoracic blood volume with emptying of the vessels and heart, and a downward shift of the diaphragm. In a study employing dynamic MRI and spirometry, Eichinger et al. (2008) demonstrated that the shape of the thorax was primarily preserved despite the marked increase in total lung volume as measured by MR-spirometry. Herniation of the lung underneath the sternum and enlargement of the costodiaphragmatic angle furthermore demonstrated the distensibility and high performance of trained lungs (see **Figure 1**). Seccombe et al. (2010) measured single photon emission computed tomography (CT) of the chest after labeled albumin injection and showed markedly diminished or even absent lung perfusion in areas of expanded lung. Lung hyperinflation induced by glossopharyngeal insufflation elicits significant hemodynamic effects, including a decrease in systemic arterial blood pressure as well as decrease in cardiac output (Potkin et al., 2007). Hypotension with glossopharyngeal breathing is associated with acute biventricular systolic dysfunction (see **Figure 2**, video axial sequence 113–293). Cardiac MRI revealed changes resembling pulmonary arterial hypertension which however were reversible shortly after cessation of voluntary lung hyperinflation (Eichinger et al., 2010). Quantitative analyses of pulmonary and central blood volume changes revealed an almost 50% decrease of central blood volume after lung hyperinflation in the non-immersed state (Mijacika et al., 2017a), and it could be shown that this lung hyperinflation results in translocation of blood from the central blood volume into abdominal, pelvic, and peripheral veins (Mijacika et al., 2017b).

Obviously, glossopharyngeal insufflation exerts considerable mechanical stress on lung elastic properties. Increases in intrapulmonary and transpulmonary pressures up to 109 cmH_2O and 80 cmH_2O, respectively, have been measured after glossopharyngeal insufflation (Loring et al., 2007). These data indicate that some individuals can withstand transpulmonary pressures and volumes far greater than those to which lungs would normally be exposed to. However, in one subject the authors reported evidence of asymptomatic mediastinal emphysema by CT of the chest (Jacobson et al., 2006), suggesting that forceful glossopharyngeal insufflation at volumes above TLC can lead to lung rupture with air dissecting centrally along the vessels into the mediastinum. Chung et al. (2010) subsequently studied glossopharyngeal insufflation in a sample of six elite breath-hold divers who performed routine maneuvers with CT scans obtained after a maximal glossopharyngeal breathing maneuver and a control CT scan at least 3 days later at TLC. A pneumomediastinum was revealed in five subjects that were able to increase thoracic gas volume above TLC. In two subjects mediastinal air was still present after 3 days although the total amount was reduced. None of the subjects at any time reported symptoms of chest discomfort, dyspnea, neck pain, or difficulty swallowing or voice change. Based on these results, the authors speculated whether glossopharyngeal insufflation may lead to long-term damage to the lungs.

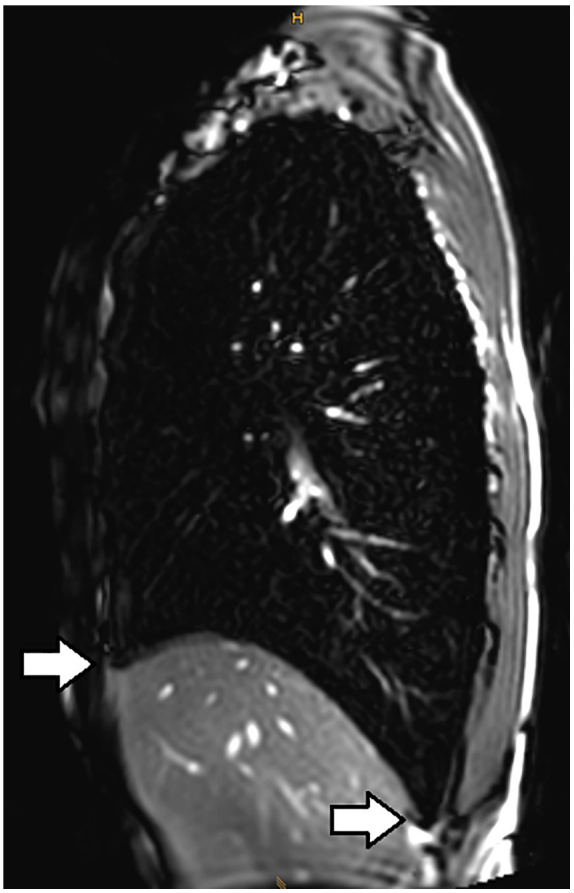

FIGURE 1 | Subxiphoidal and costodiaphragmatic recessus (marked with white arrows) filled with air during glossopharyngeal insufflation.

Pulmonary function testing of elite breath-hold divers consistently showed higher vital capacity than predicted from population derived equations (Overgaard et al., 2006; Tetzlaff et al., 2008; Lemaitre et al., 2010). Elite breath-hold divers consistently had higher VC compared to age-matched controls (Tetzlaff et al., 2008; Lemaitre et al., 2010). There is only scarce information on longitudinal lung function changes with continuously practiced glossopharyngeal breathing. Walterspacher et al. (2011) reported an increase in ventilatory volumes and normal compliance in four elite breath-hold divers that had been available for a 3-year follow-up. At that time the mean breath-hold diving performance in the subjects amounted to 6.6 years, indicating that the stress on the pulmonary

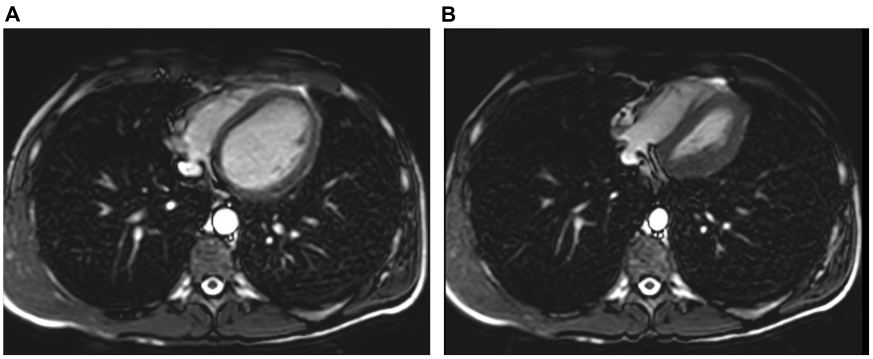

FIGURE 2 | Pulmonary hyperinflation disrupts cardiac filling and leads to cardiac dysfunction. Panel **(A)** axial slice shows normal filling whereas Panel **(B)** demonstrates cardiac dysfunction with impaired left ventricular filling and right ventricular overload (D-shaped configuration) and reduced aortic diameter.

parenchyma imposed by glossopharyngeal insufflation may be transient and reversible. Seccombe et al. (2013) interpreted an increase in VC, TLC, and functional residual capacity in a breath-hold diver over 8 years as a reduction of chest wall recoil at higher lung volumes, in consequence of repeated glossopharyngeal insufflation. These results should be interpreted with caution due to small sample size and only limited follow-up. However, the follow-up supports the cross-sectional findings of large vital capacity, probably due to training due to structural adaptation to repeated lung extension.

Lindhom and Nyren (2005) also reported a breathing method similar to glossopharyngeal insufflation but reversing the pumping maneuver, called glossopharyngeal exsufflation. The procedure is used to by breath-hold divers to suck air into the mouth at great depth as respiratory muscles will not be able to provide the force needed against the pressure difference. This air will be needed to equalize middle ear pressure when going deeper. The method is also employed for training purposes as divers can simulate greater depth when exhaling beyond RV and diving in a swimming pool. Lindhom and Nyren (2005) reported that RV could be reduced by 21%. Loring et al. (2007) confirmed this magnitude of reduction in RV and measured intrapulmonary pressures of −10 to −30 cmH_2O, probably indicating the point of complete airway closure. The authors used MRI lung imaging with inhaled hyperpolarized ^{129}Xe to characterize lung inflation from sub-RV volumes after glossopharyngeal exsufflation (Muradyan et al., 2010). MRI demonstrated sharply demarcated ventilated vs. non-ventilated regions of the lungs of the divers, indicating punctate opening, secondary to frank airway closure during the preceding glossopharyngeal exsufflation used to reach those low volumes.

Is there a limit to deep breath-hold diving?

Summarizing the above, it becomes obvious that prior physiologic concepts for the depth limits of breath-hold diving have been refuted. The RV/TLC ratio is an important determinant of the depth a breath-hold diver may descend to without harm, but thoracic blood shift, and individual adaptations such as glossopharyngeal insufflation and exsufflation allow to reach considerably greater depths than previously assumed. For example, an elite breath-hold diver with an RV of 1.7 L and TLC of 10 L (measured) would be able to descend to 49 m depth according to RV/TLC ratio. With thoracic blood shift of 1000 mL (assumed) the depth limit would be at 134 m, because RV would be reduced to 0.7 L. Increasing TLC with glossopharyngeal insufflation to 13.2 L (measured) he could go to 179 m, and with glossopharyngeal exsufflation of 0.4 L (measured) maximal depth would be at 320 m (personal communication, Dr. C.M. Muth, Ulm, Germany). Accordingly, there had been a fast evolution of depth records with ever increasing depths up to the beginning of the new millenium (Ferretti, 2001). Austrian freediver *Herbert Nitsch* set the official world record at 214 m depth in 2007[2]. Thus, pulmonary anatomy and physiology could not be accounted to limit the breath-hold diving depths achieved by competitive athletes.

Herbert Nitsch tried to surpass his own record in 2012 and reached a depth of 244 m; however, when surfacing he presented with a stroke-like clinical syndrome with loss of consciousness and needed immediate medical support. Permanent disability caused him to end his career. His medical condition was attributed to decompression illness.

DECOMPRESSION STRESS

Another area where previous concepts of physiology needed to be revised more recently is the possibility of decompression stress in breath-hold diving. Historically it had been widely believed that diving mammals and human freedivers alike were immune to decompression illness, since nitrogen (N_2) uptake from alveolar air is restricted to the available amount of gas in the lung at depth (Lanphier, 1965; Kooyman and Ponganis, 1997). Marine mammals dive routinely and repeatedly to impressive depths without obvious injury, and the only inert gas added is the N_2 that remains in the lungs from the inhalation before submerging. Studies have suggested that behavioral and physiological adaptations such as the reduction in blood flow to non-essential tissues and a progressive collapse of alveoli would prevent them from N_2 uptake and thereby

[2]https://www.guinnessworldrecords.com/world-records/freediving-no-limit-(men)

minimize decompression stress. However, recent postmortem studies in stranded beaked whales and sperm whales pointed to lesions consistent with acute trauma due to *in vivo* bubble formation resulting from rapid decompression and adverse long-term sequelae, respectively, in these species (Jepson et al., 2003; Moore and Early, 2004). These recent observations led to the understanding that diving mammals are in fact prone to decompression stress during ascent. They obviously manage their inert gas load by physiological responses that are still poorly understood, yet in certain situations may suffer decompression illness if they ascend from depth too quickly, e.g., by irritation through sonar noise (Hooker et al., 2012).

An important physiological adaptation of diving mammals to minimize decompression stress is lung collapse. The early occurrence of lung collapse at relative shallow depths of around 50 m of seawater will entirely curtail any gas exchange between the lungs and the blood and, thus, restrict N_2 absorption during the dive. In contrast, the human chest and lungs are much less compressible. Human airway and alveolar compression and re-expansion during deep breath-hold dives were studied using a computational model of the human respiratory tract (Fitz-Clarke, 2007). The model predicted total lung collapse with degassing of all alveoli at a depth of approximately 235 m, which is considerably deeper compared to the collapse depth in diving mammals. It has also been calculated that human lungs are highly efficient at gas exchange just prior to total collapse (Fitz-Clarke, 2009) and N_2 uptake will increase linearly during descent up to that point. Thus, in humans, there will still be N_2 absorption at greater depths, and with glossopharyngeal insufflation significantly more N_2 will be available for uptake.

The evidence that decompression sickness from repetitive breath-hold diving may occur in humans has meanwhile been established (Schipke et al., 2006; Lemaitre et al., 2009; Schipke et al., 2019). Apart from clinical pathology findings there has also been proof of intravascular gas bubbles by Doppler measurements in professional Japanese breath-hold divers after a series of repetitive dives to depths of 20 m (Lemaitre et al., 2014), and tear film bubble formation has been reported in submarine escape tank divers after repeated breath-hold dives to 30 m (Sheard, 2008). However, it has been considered unlikely that breath-hold divers would be submersed for sufficient time and sufficient depth to suffer decompression stress after single deep dives.

There is increasing evidence in humans that decompression stress may apply to single deep breath-hold dives: Schagatay (2011) reported cases of repeated breath-hold dives to 45 m depth presenting with paralysis of the limbs, and a case of speech disturbance and amnesia after a final single free dive to 90 m. In an ultrasound study after single deep dives up to 70 m there were a couple of subjects with confirmed low grade gas bubbles in cardiac chambers. Finally, an increasing number of serious incidents during deep breath-hold dives indicated the possibility of neurological insult, e.g., stroke, due to cerebral arterial gas embolism as a consequence of decompression stress (Tetzlaff et al., 2017). Remarkably, these cases occurred in young healthy subjects without a history of cardiovascular disease or other pertinent risk factors.

PULMONARY ARTERIOVENOUS SHUNTS

Usually the capillary bed of the pulmonary vasculature is an effective filter for venous N_2 microbubbles (Butler and Hills, 1985). However, bubbles may bypass this filter through a right-to-left shunt, e.g., patent foramen ovale. The presence of a persistent patent foramen ovale has indeed been associated with the risk of decompression illness in self-contained underwater breathing apparatus (SCUBA) divers (Wilmshurst et al., 1989). However, arterial gas bubbles have been reported in SCUBA divers without a patent foramen, indicating the possibility of microbubble transfer through extracardiac shunts (Gerriets et al., 2000). In fact, intrapulmonary arteriovenous anastomoses may offer another opportunity for venous gas bubbles to bypass the capillary filter and become arterialized (Madden et al., 2015; Schipke and Tetzlaff, 2016). The existence of these anastomoses in human lungs has been shown in morphological studies (Tobin, 1966).

Several factors may facilitate the opening of intrapulmonary shunts, including body positioning (Stickland et al., 2004), physical exercise (Eldridge et al., 2004), and hypoxia (Lovering et al., 2008). It appears unlikely that body position or exercise would be relevant factors during deep breath-hold dives where the diver is using a weighted sled to descend down a rope, and ascend with lifting bags. Hypoxia, however, is increasing with breath-hold duration and alveolar pO_2 may reach values as low as 30 mmHg after surfacing from deep breath-hold dives (Ferretti et al., 1991). Thus, hypoxia may allow transpulmonary passage of venous N_2 microbubbles, although it is yet unclear whether acute severe hyperbaric hypoxia would have the same effect on intrapulmonary arteriovenous anastomoses as prolonged normobaric hypoxia. As the N_2 gas bubble will increase in size during ascent and probably coalesce with other bubbles, greater sized bubbles may be present on the outflow side than what originated on the inflow, and hence not be restricted by the diameter of the shunting vessel (Kerut et al., 2014). As airway closure during atelectasis or lung collapse prevents the ventilation of alveoli supplied by that airway, there will be right-to-left shunt of blood perfusing those alveoli. However, this would require the existence of venous N_2 microbubbles during the descent of the dive already which is unlikely to occur. As gas exchange in the human lung is still occurring at greater depths, venous gas bubbles may be formed along the extreme N_2 gradient between tissues and the alveolar space, and eventually these bubbles may arterialize through intrapulmonary arteriovenous anastomoses that are opening during ascent from a deep breath-hold dive triggered by severe hypoxia.

NITROGEN NARCOSIS: MYTH OR REALITY?

Inert gas narcosis (IGN) is a well-described phenomenon of subjective and objective mental disturbance due to the narcotic effects of certain inert gases at increased pressure. In SCUBA diving, IGN is mainly related to nitrogen (Clark, 2015). One of

the first reports of what is now known as nitrogen narcosis was by *Colladon*, a French physician who, in 1826, descended to 20 m in a diving bell. He described "a state of excitement as if I had drunk some alcoholic liquor" (Unsworth, 1966). In 1935, Behnke first suggested that nitrogen gas might have been the mediator of the observed behavior, by utilizing different breathing gas mixtures in their experiments (Behnke et al., 1935; Grover and Grover, 2014). Nitrogen narcosis can impede cognitive functions and physical performance from depths as low as 10 m, and will be apparent around 30–40 m depth (Rostain and Lavoute, 2016). Symptoms include spatial and temporal disorientation, memory impairments, euphoria, hallucinations, mood changes, impaired neuromuscular coordination, psychomotor and intellectual decrements and unconsciousness (Rostain et al., 2011; Rostain and Lavoute, 2016; Jain, 2017). Loss of consciousness occurs at pressures higher than 11 ATA (Rostain and Lavoute, 2016). These symptoms may affect diver's safety under water, and contribute directly to up to 6% of deaths in SCUBA divers and/or can be indirectly associated with other diving incidents at depth (Clark, 2015).

During deep breath-hold dives, elite freedivers quite often describe narcosis-like symptoms (personal observation and testimonials from freediving champions *Guignes*, *Zucchari*, *Boudhiaf*, *Streeter*, and *Néry*). Among the symptoms regularly reported by freedivers are hallucinations, the sensation of dreaming or being in a dream, altered perception of time, the sensation of numbness in the mouth and tingling in the fingers and toes, blurred vision, loss of memory, resulting in confusion and panic. The confusion is such that some freedivers practising No-Limit freediving (i.e., assisted freediving with a sled) say they can't remember what they did on the bottom and on part of their ascent from depth. Schagatay (2011) reported that among 24 divers during a competition, 12 experienced symptoms that could be associated with narcosis including dizziness and confusion, and these were all evident at depths greater than 40 m. Others freedivers report depths ranging from 50 to 60 m during training "shallow" dives with hangs at the bottom. The symptoms therefore seem to be quite similar to those of SCUBA divers. However, one of the main differences between SCUBA diving and freediving is the onset of narcosis. While it ususually occurs during the descent of a SCUBA diver using air as breathing gas, it occurs mainly on the way up and sometimes all the way to the surface in freedivers (personal observation and observation reported from *Guillaume Néry*). Thus, it appears that presentation of narcosis in breath-hold diving is distinct from SCUBA diving, and mechanisms of narcosis may be altered.

While the exact mechanisms of IGN remain to be poorly understood, biochemical theories based on gas toxicity and dissolution are the assumptions generally reported for air diving. The anesthetic potency of a narcotic gas has been commonly found to be correlated with the lipid solubility (Meyer-Overton theory). Thus, the site of the IGN is on a lipophilic (fat-soluble) portion of the cell membrane. Due to their lipophilicity, N_2 and other inert gases bind to membranes. This can cause the membrane to swell beyond a certain critical volume and cause narcotic effects (Rostain et al., 2011; Clark, 2015; Rostain and Lavoute, 2016) with an individual susceptibility, i.e., concentration threshold. Since pressure affects narcotic potency in a linear fashion and nitrogen narcosis does not disappear with decreasing pressure (during ascent) in freediving, it seems that the effects of IGN are more complex in the freediver.

In contrast to SCUBA diving, the intrapulmonary gas of the freediver is not renewed since he is apneic throughout the dive. Thus, the containing air and its composite gases (nitrogen, oxygen, and carbon dioxide) are subject to the effects of (substantial) environmental pressure change while being consumed (oxygen, O_2) or produced (carbon dioxide, CO_2) during apnea. On descent, the alveolar and arterial pressures of oxygen increase and can lead to transient hyperoxia at the bottom (Bosco et al., 2018). While the arterial pressure of O_2 (PaO_2) can increase during descent, the arterial pressure of CO_2 ($PaCO_2$) remains almost stable because of its markedly greater solubility in blood and tissue and the buffer capacity of the human body for CO_2. During the descent hypercapnia does not appear to be present even during a constant weight dive to 60 m. Also in case of constant weight apnea (therefore with muscular effort) or assisted apnea (with a sled for example), hypercapnia seems moderate or even non-existent on ascent (Bosco et al., 2018; Scott et al., 2021); thus, it's contribution to the narcotic effect seems negligible. Due to the complex interaction of gases, activities, and environmental conditions during a dive, nitrogen, oxygen and carbon dioxide may modify the cognitive performance for different partial pressures. It is therefore possible that there is an interplay between O_2 and CO_2 that can increase narcosis. However, these data have been established for divers with equipment breathing different gas mixtures (Freiberger et al., 2016; Rocco et al., 2019); no data are available from breath-hold diving where hypercapnia and hypoxia are prevailing. Since PaO_2 increases during descent there is transient hyperoxia, however, this does not seem to generate oxidative stress in the freediver (Joulia et al., 2003). Conversely, animal studies in rats showed, that hypercapnic hyperoxia stimulates reactive oxygen species (ROS) production in the caudal solitary complex of rat brain but does not induce oxidative stress (Ciarlone and Dean, 2016a). This is probably due to antioxidant and proteosomal removal of damaged lipids and proteins to maintain cell viability and avoid death during protracted hyperoxia (Ciarlone and Dean, 2016b). After a dive, overproduction of ROS and consequent oxidative damage to lipids of membrane and antioxidant capacity decreasing may therefore reflect a occcurrence of hypoxic conditions (Mrakic-Sposta et al., 2019). More studies are needed to determine the impact of ROS-producition on free-divers. On ascent, total pressure decreases, which can accentuate the drop in O_2 in the blood during muscular effort to dramatic levels. The freedivers become hypoxic to the point of unconsciousness (Muth et al., 2003; Lindholm and Lundgren, 2009; Costalat et al., 2017). Even in the absence of symptoms, these repeated hypoxias seem to impact the cognitive functions of freedivers. Billaut et al. (2018) showed that trained freedivers had alterations in episodic memory in relation to their level of training and the number of syncopes. Finally, several studies show that these hypoxias may have deleterious effects on the brain health of freedivers, although it is not yet possible to assess the long-term consequences (Billaut et al., 2018).

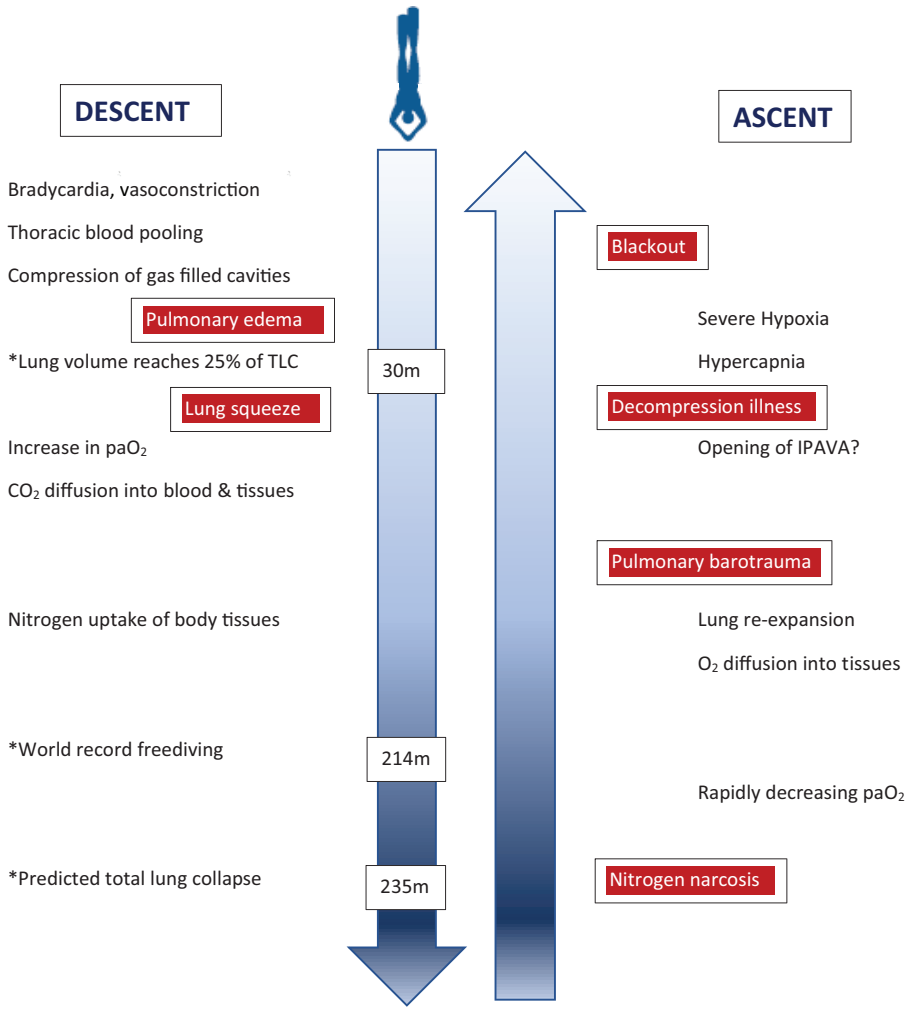

FIGURE 3 | Illustration of physiological changes and health risks (boxed red) during the descent and ascent of a deep breath-hold dive. Some examplary depths are marked (*) to indicate critical physiological challenges or records achieved. Of note, health risks are not related to certain depths but rather depth ranges during descent or ascent, respectively. paO_2 = arterial oxygen pressure; IPAVA = intrapulmonary arteriovenous anastomoses.

As described above, a certain analogy exists between the narcotic effect of nitrogen in deep diving and the pharmacokinetics of conventional anesthetic gases. The physiology of anesthetic gases has been adequately studied (Kety, 1950; Tanner, 1982), and modern computer programs can predict the uptake and onset of action of anesthetic gases with reasonable accuracy (Athiraman et al., 2016). During induction of narcosis, high partial pressures of anesthetic gases are inhaled to achieve a fast flooding at the target organ. The anesthetic gases dissolved in the blood diffuse to their site of action driven by the concentration gradient. After the induction-phase, the inspiratory gas fraction of the anesthetics can be significantly reduced because the partial pressure in the tissue is approximately equal to the partial pressure in the blood. The speed of partial pressure changes in the different compounds depends, amongst other factors, on cardiac output (Munson et al., 1973), the solubility coefficient and the type of anesthetic gas. If the gas partial pressure is reduced at the end of surgery, the effect of the anesthetic gas persists until the tissue partial pressure at the target organ has dropped. Thus, there is a time delay in both the initial flooding and the descent of the gas. Similar time-course effects may apply to narcotic effects in deep breath-hold diving. During the diver's descent there is an increase in the N_2 partial pressure due to the elevation in the ambient pressure. Since the apnea dive generally lasts only a few minutes, only a relatively short time window is available for the saturation phase. The temporal latency of the narcotic nitrogen effects, which coincides with the ascent process, could therefore also be explained by delayed tissue kinetics.

Lastly, there are possible implications of the diving reflex in breath-hold diving narcosis. Apnea induces a reflex mechanism known as the diving reflex, which is mainly characterized by bradycardia and peripheral vasoconstriction that allows blood to be redirected to oxygen-dependent organs such as the heart and brain (Joulia et al., 2009; Lindholm and Lundgren, 2009; Dujic and Breskovic, 2012; Eichhorn et al., 2017; Bain et al., 2018;

Elia et al., 2021). This mechanism is more or less present depending on the intensity of the effort, but it seems to be persistent during ascent of constant weight diving (Lemaître et al., 2013). Conversely, this vasoconstriction progressively leads to increases in mean arterial pressure (Breskovic et al., 2011). We have just shown that at the end of a deep apnea, the freediver is hypercapnic, hypoxic, and hypertensive with a significant increase in cerebral blood flow (Joulia et al., 2009; Bain et al., 2018; Eichhorn et al., 2018). Hypoxia, but especially hypercapnia, increases this cerebral blood flow, which can disrupt neuronal activity or at least dynamic brain autoregulation. Indeed, it has also been shown that, in elite freedivers, dynamic brain autoregulation can be severely impaired during maximal apneas (Moir et al., 2019), and that cerebral oxidative metabolism can be decreased. A disruption of the blood–brain barrier has even been suggested (Bain et al., 2018). It may be speculated that the mechanism of central blood shift during descent probably reverses on ascent although this has not been investigated in detail. The diving reflex may contribute to maintained cerebral blood flow during ascent which can be increased by diaphragmatic contractions (Fagoni et al., 2017). Of note, also speed of descent and ascent may influence the risk of syncope (Poiret et al., 2020). Indeed, in constant weight divers using fins, divers with a syncope had longer dive times (197 s vs. 167 s) with a faster first diving phase (the active one) (1.24 $m.s^{-1}$ vs. 1.06 $m.s^{-1}$). It is therefore possible that this higher speed leads to a greater consumption of air from the beginning of the apnea, which will have the effect of reducing the freediver lung volume, accentuating his blood shift and further increasing his cerebral blood flow throughout the dive. These mechanisms all together may contribute to disturbances of cerebral blood flow during ascent, which may impair neuronal activity and cause signs of narcosis experienced by freedivers.

CONCLUSION

Previous physiologic concepts for the depth limits of breath-hold diving have been refuted more recently. The RV/TLC ratio is an important determinant of the depth a breath-hold diver may descend to without harm, but thoracic blood shift, and individual adaptations such as glossopharyngeal insufflation and exsufflation allow to reach considerably greater depths than previously deemed possible. However, the physiology of the human lung is not adapted to deep breath-hold diving which is associated with a substantial increase in risk of serious neurological injury with increasing depth (**Figure 3**), rather than actually reaching depth limits defined by individual lung volumes. Recent evidence is increasing that there is significant decompression stress even from single deep breath-hold dives, and it seems that possible adverse effects of pulmonary N_2 gas exchange during deep breath-hold dives have been largely underestimated in the past. Narcosis is adding to the risk of injury, and its mechanisms in breath-hold diving are only incompletely understood.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.710429/full#supplementary-material

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Endothelial Nitric Oxide Production and Antioxidant Response in Breath-Hold Diving: Genetic Predisposition or Environment Related?

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Introduction: Nitric oxide (NO) is an essential signaling molecule modulating the endothelial adaptation during breath-hold diving (BH-diving). This study aimed to investigate changes in NO derivatives (NOx) and total antioxidant capacity (TAC), searching for correlations with different environmental and hyperbaric exposure.

Materials and methods: Blood samples were obtained from 50 breath-hold divers (BH-divers) before, and 30 and 60 min after the end of training sessions performed both in a swimming pool or the sea. Samples were tested for NOx and TAC differences in different groups related to their hyperbaric exposure, experience, and additional genetic polymorphism.

Results: We found statistically significant differences in NOx plasma concentration during the follow-up (decrease at T30 and increase at T60) compared with the pre-dive values. At T30, we found a significantly lower decrease of NOx in subjects with a higher diving experience, but no difference was detected between the swimming pool and Sea. No significant difference was found in TAC levels, as well as between NOx and TAC levels and the genetic variants.

Conclusion: These data showed how NO consumption in BH-diving is significantly lower in the expert group, indicating a possible training-related adaptation process. Data confirm a significant NO use during BH-diving, compatible with the well-known BH-diving related circulatory adaptation suggesting that the reduction in NOx 30 min after diving can be ascribed to the lower NO availability in the first few minutes after the dives. Expert BH-divers suffered higher oxidative stress. A preliminary genetic investigation seems to indicate a less significant influence of genetic predisposition.

Keywords: nitric oxide, oxidative stress, breath hold diving, genetic prone, diving

INTRODUCTION

Nitric oxide (NO) is considered as one of the most important molecules regulating vascular adaptations during breath-hold diving (BH-diving; Lundberg et al., 2015) because of its action in the control of the cardiovascular system, blood flow, and blood pressure.

NO is involved in many physiological and pathological processes (Rand, 1992; Hou et al., 1999). Due to its short half-life (0.05–1.8 ms) (Liu et al., 1998), NO availability is ensured by a family of NO synthases (NOS), composed of at least three different isoforms (Moncada and Higgs, 1993).

The endothelial isoform (e-NOS) is mainly involved in modulating the vasodilator tone, vascular integrity preservation, and regulation of arterial blood pressure (Rand, 1992). eNOS also inhibits platelet aggregation and adhesion and enhances vascular permeability (Behrendt and Ganz, 2002). e-NOS levels are regulated by many factors, such as hypoxia and local substrate availability (Ostergaard et al., 2007), vascular shear stress (Chatterjee et al., 2008), and, most important, by different genetic variants (polymorphisms) (Ahsan et al., 2006; Wang et al., 2010). Indeed, functional variants in the endothelial NOS3 gene might alter the expression of the enzyme (Senthil et al., 2005).

Since NO is a radical, its levels are difficult to quantify (Liu et al., 1998), and it is preferable to measure stable NO derivates such as Nitrate and nitrites (NOx) (van Vliet et al., 1997) and byproducts of NO oxidation in blood and tissues (Moncada and Higgs, 1993). Specifically, NO is oxidated to nitrite (NO_2) or, when oxyhemoglobin is available, to nitrate (NO_3), with NO_3 being predominant in blood circulation (Lundberg et al., 2008). Healthy individuals produce approximately 1 mmol of NO_3 daily due to the oxidation of endogenously synthesized NO (Macallan et al., 1997). If necessary, NO_3 can be reduced to NO_2 by several enzymes, such as xanthine oxidase (Li et al., 2003) and xanthine oxidoreductase (Jansson et al., 2008). NO_2 is further reduced to NO by different pathways, including hemoglobin (Cosby et al., 2003), myoglobin (Rassaf et al., 2007; Shiva et al., 2007a), xanthine oxidoreductase (Godber et al., 2000), and ascorbic acid (Carlsson et al., 2001). These pathways are significantly enhanced during hypoxia and acidosis to ensure NO production when the oxygen-dependent NOS enzyme activities are compromised (Giraldez et al., 1997; Ostergaard et al., 2007). In addition, NO_2 reduction to NO during physiological hypoxia seems to contribute to physiological hypoxic signaling, vasodilation, and modulation of cellular respiration (Modin et al., 2001; Cosby et al., 2003; Shiva et al., 2007a,b).

Some studies have demonstrated an increase in circulating NO in breath-hold divers (BH-divers) after repetitive diving up to 20 m over 25 min in a pool and suggested possible correlations with physical exercise (Theunissen et al., 2013a). As BH-divers, marine mammals (Elsner et al., 1998) are subjected to post-diving ischemia-reperfusion when arterial and tissue oxygen levels are restored after reaching the surface, thus leading to an increase in reactive oxygen species (ROS) production (Dhaliwal et al., 1991; Bosco et al., 2010, 2020). Higher ROS levels can be harmful (Forkner et al., 2007; Diringer, 2008) by exacerbating the redox imbalance, increasing oxidative stress, and depleting acutely the antioxidant defenses of the body (Terraneo and Samaja, 2017).

Endothelial dysfunction has also been demonstrated in BH-diving (Brubakk et al., 2005; Obad et al., 2010) and can be explained by two hypotheses. First, the BH-diving-related transient hypoxia and accumulation of CO_2 induce an increase in ROS levels, causing higher oxidative stress (Theunissen et al., 2013b; Mrakic-Sposta et al., 2019) and NO-related endothelial changes (Theunissen et al., 2013a). Second, the development of venous gas embolism, frequently observed in self-contained underwater breathing apparatus diving (SCUBA) despite correct decompression procedures (Valic et al., 2005), has also been recently demonstrated in BH-divers (Cialoni et al., 2016) and could play a role in the pathogenesis of BH-diving-related endothelial dysfunction.

Since NO is primarily released from arterial endothelium along with many other regulatory substances (Heitzer et al., 2001), any condition causing endothelial dysfunction inevitably affects NO levels and leads to cardiovascular diseases, such as coronary artery disease, peripheral arteriopathy (Drexler, 1997; Pepine, 1998), and atherosclerosis (Keaney and Vita, 1995; Tousoulis et al., 2010). Some studies have confirmed a primary role in increased oxidative stress caused by endothelium dysfunction in cardiovascular disease (Cai and Harrison, 2000). Thus, the investigation of hidden mechanisms predisposing BH-divers to the development of cardiovascular diseases is of paramount importance.

On the other hand, recent observations seem to indicate the existence of a genetic predisposition in developing BH-diving-related injuries (Cialoni et al., 2015) or diving reflex related adjustments (Baranova et al., 2017), but there is no clear evidence in the published literature whether NO availability and oxidative stress occurring in BH-diving are related to extreme environmental conditions (such as ambient pressure, salinity, and water temperature) rather than genetic predisposition. However, we also need to take into account that in niche sectors, such as diving, in which it is very difficult to plan genetic protocols in higher numbers of subjects, the recommendation in these conditions is to use biallelic markers [such as single nucleotide polymorphisms (SNPs)] to obtain indicative data even in lower numbers of subjects (Chandrika, 2001).

The study aims to investigate the changes in NO_x and antioxidant response [plasma total antioxidant capacity (TAC)] after a series of BH-dives in different environmental and hyperbaric exposure conditions. In the **Supplementary Material**, we also show the preliminary results related to the NO and oxidative stress response in BH-divers with different genetic variants of 10 selected polymorphisms.

MATERIALS AND METHODS

Subjects and Dives

50 Expert healthy BH-Divers were studied in two settings: the first group during a series of deep dives at the swimming pool

Y-40 "The Deep Joy®" (Montegrotto Terme, Italy) (42-m-deep); the second group during an open water training session at the Elba Island (Italy).

All the divers were informed about the risks and benefits of this study and read and signed a specific, informed consent form before the experiment. All the participants also signed a dedicated genetic informed consent allowing the genetic analysis. The study was conducted as per the Helsinki Declaration and was approved by the Ethical Committee of Università degli Studi di Milano, Italy (Aut. No. 37/17).

Subjects aged >18 years and non-pregnant women were included in the study. None of the subjects had previous or clinical evidence of arterial hypertension, cardio-pulmonary diseases, Taravana episodes (BH-diving-related loss of consciousness or seizure), or any other significant disease.

Subjects were asked to avoid food rich in NO_3, such as red meat (Lundberg, 2009) and leafy green vegetables (Lundberg and Govoni, 2004). None of them took prescription drugs, suffered from any acute disease during the 15 days before the experiment, or reported assumption of anti-inflammatory medications, exposure to high altitude in the 7 days, or intense exercise during the 48 h before the investigation. None of the BH-divers performed any compressed-gas diving during the 30 days before the experiment.

All the subjects were affiliated to the "Apnea Academy" training agency as instructors or high-level divers; however, all the divers could easily reach a minimum of the following criteria:

- 20 m depth in constant weight;
- 3 min of static breath-hold (at the surface); and
- 75 m of dynamic BH-diving in a swimming pool (distance).

All the divers performed their usual training with a freely determined number and time of warm-up dives, bottom time, and surface intervals.

As per the "Apnea Academy" standard procedures, the training session involved a gradual approach to the maximum daily personal depth and an unrestricted number of deep dives, at the end of which all the divers returned to the laboratory for the post diving test protocol.

Diving profiles were recorded using a UP-X1 free-diving computer (Omersub S.p.a., Sovico, Italy), including mean depth, maximum depth (MD), and number of dives (ND). The free-diving computers measured and recorded data every 2 s. The included divers were stratified into several groups to be analyzed. First, divers were interviewed and divided by diving level (LD) in medium or high experience (ME vs. HE) considering their BH-diving skills, defined by years of activity, personal depth record, number of weekly training sessions, and certification level. Another stratification considered three parameters achieved on the day of the experiment, namely: average depth (AD), an average of MD reached, and an average ND; subjects were then divided into those who dived above (AD-above, MD-above, and ND-above) and those who dived below the calculated averages (AD-below, MD-below, and ND-below).

Experimental Protocol

The protocol was the same in both the swimming pool and the sea tests.

Venous peripheral access was obtained from the antecubital vein of each subject, and blood samples were collected 30 min before the start of the diving series (basal). Blood samples were then collected 30 min (T30) and 60 min (T60) after the end of the BH-diving session, after discarding 5 ml of the blood to remove any clots. Plasma was obtained by centrifugation (3,000 rpm for 10 min) and was refrigerated at −20°C. Plasma samples were then delivered to the Laboratory of Biochemistry of the Department of Health Sciences (DISS) of the Università degli Studi di Milano for analysis.

Epithelial oral cells were also obtained using two buccal swabs from each volunteer. DNA was isolated using the ChargeSwitch kit (Invitrogen Corp., Carlsbad, CA, United States), following the instructions of the manufacturer, and both buccal swabs were suspended in 100 μl of elution buffer.

We investigated for the following:

- ✓ Differences in plasma concentration of NOx and TAC for the following diving risk factors:
  - BH-LD (ME vs. HE)
  - Environmental (swimming pool vs. sea)
- ✓ Differences in plasma concentration of NOx and TAC in the following recorded diving risk factors, between those above and those below the calculated average:
  - AD (AD-above vs. AD-below)
  - MD (MD-above vs. MD-below)
  - ND (ND-above vs. ND below)
- ✓ Differences in plasma concentration of NOx and TAC (before and after the dives) in genetic variants of 10 investigated polymorphisms (as explained in the following sections).

Plasma NOx Measurement

All 50 subjects were investigated for the NOx plasma level repeated for the three times specified in the protocol. Before the analysis, plasma was deproteinized. Briefly, 400 μl of the sample was treated with 400 μl of acetonitrile (Romitelli et al., 2007) to precipitate the proteins and centrifuged at 12,000 rpm for 10 min. NOx was measured in the deproteinized plasma using a method based on Griess's reaction as an index of NO concentration (Green et al., 1982), according to Cialoni et al. (2019). Plasma NOx levels were obtained by interpolation of standard $NaNO_3$ curves (Tsikas, 2005). All the samples were analyzed two times. Results were expressed as a percentage of difference of the control value (basal).

Plasma TAC

A total of 38 subjects out of 50 were also investigated for TAC, using the ferric reducing antioxidant capacity (FRAP) assay (Benzie and Strain, 1996), with some modifications. Briefly, 45 μl of plasma was added to 1.5 ml of freshly prepared FRAP reactive

in plastic tubes. After 5 min of incubation at 37°C, absorbance was read at 593 nm in a Uvikon 931 UV-VIS spectrophotometer (Northstar Scientific, Bardsey, United Kingdom). Aqueous solutions of $FeSO_4$ $7H_2O$ (100–1,000 μM) were used for the calibration curve. TAC values were obtained by interpolation of the $FeSO_4$ calibration curve. All the samples were analyzed two times, and the results were expressed as FRAP value [μM Fe (II)] of the samples (Zarban et al., 2009).

DNA Polymorphisms

About, 10 different genetic polymorphisms related to the investigated risk factors (available NO and oxidative stress) were analyzed, especially, 2 involved with NO availability, 4 with the anti-inflammatory activity, and 3 with the antioxidant capacity. Also, angiotensin-converting enzyme polymorphisms were analyzed.

The polymorphisms were analyzed using a real-time PCR (RT-PCR) technique. Specific primers and probes for the SNP rs1799983 were designed according to the TaqMan genotyping assay (Applied Biosystems, Foster City, CA, United States), while SNP rs2070744 was analyzed using primers and probes designed according to the Kaspar genotype assay (KBIoscience [B]). Both SNPs were analyzed on ABI 7900 following the instructions of the manufacturer.

In all the investigated polymorphisms, the NOx and TAC levels before the diving and at follow-up (T30 and T60) for the different genetic variants were analyzed.

Statistical Analysis

Data are presented as mean and SD for parametric data and median and range for non-parametric data. To minimize the subject-to-subject variability, data were normalized against the basal value. The Shapiro–Wilk normality test was used to verify a Gaussian distribution. Genetic data were compared using a one-way ANOVA for multiple comparisons or the Friedman test for multiple comparisons for parametric and non-parametric data, respectively. NOx and TAC were compared using a Mann-Whitney test or an unpaired *t*-test.

A probability lower than 5% was assumed as the threshold to reject the null hypothesis ($p < 0.05$).

RESULTS

A total of 50 healthy BH-Divers (40 male and 10 female; mean age 43.24 ± 9.8; mean height 176.3 cm ± 7.1; mean weight 74.4 kg ± 10.4; and BMI 23.8 ± 2.4) were studied in two different environmental conditions: 22 at the swimming pool and 28 at sea (**Supplementary Table 1A**).

The overall diving profiles showed an AD of 22.2 ± 8.5 m, $n = 23$ AD-above, and $n = 27$ AD-below; MD of 33.2 ± 8.2 m, $n = 24$ AM-above, and $n = 26$ AM-below; and an ND of 16.5 ± 5.8, ($n = 26$ ND-above vs. $n = 24$ ND-below) (**Supplementary Table 1B**).

Subjects were also classified as HE of $n = 28$ and ME of $n = 22$ (**Supplementary Table 1A**).

The groups obtained by dividing the sample in above and below the average (AD, MD, ND-above vs. AD, MD, ND-below, respectively) showed significant differences between the more performant subjects as compared with the less performing ones in terms of AD(AD-above vs. AD-below); MD (MD-above vs. MD-below) and ND (ND-above vs. ND-below) confirming a different diving exposure in the two groups (above vs. below).

Similar significant differences were also found between more experienced and less experienced divers [ME vs. HE and (**Supplementary Table 1B**)].

We did not find any statistical differences in BMI and age in the groups selected (**Supplementary Table 1C**) as the MD and ND were not statistically different in the two investigated environments (swimming pool vs. sea). We only found a higher mean of depth in the swimming pool group as compared with the sea group (**Supplementary Table 1C**).

Regarding overall plasma NOx concentration, a significant decrease of –27.6% at T30 (73.5% of the control value, $p < 0.0001$) and a significant increase of +24.1 at T60 (124.1%, of the control value, $p < 0.012$) were found. All these differences (decrease at T30 and increase at T60) were statistically significant in terms of the percent of the pre-diving control value (**Figure 1**).

Regarding the differences in blood NOx concentration among the groups, a significantly lower decrease was found at T30 in experts (AD, $p = 0.002$; MD, $p = 0.01$; ND, $p = 0.01$; DL, $p = 0.03$) (**Figure 2**).

The difference, if any, in NOx plasma concentration was detected comparing the swimming pool vs. the sea setting ($p = 0.81$) (**Figure 2**).

At T60, a higher increase of NOx value was found in subjects with higher diving exposure in terms of MD ($p = 0.018$) and LD ($p = 0.006$). In contrast, the mean depth and the ND were not associated with significant changes in NOx levels. Finally, a higher NOx increase at T60 was found in the sea group than in the swimming pool group ($p = 0.014$) (**Figure 3**).

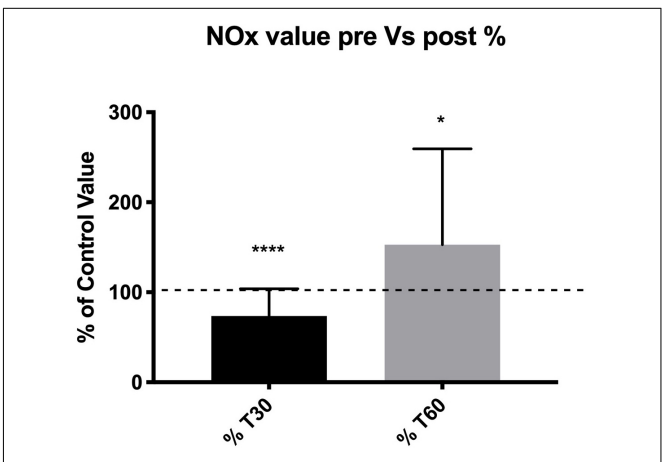

FIGURE 1 | Subjects showed a lower NOx concentration at T30 suggesting a lower NO availability after dives, and a higher NOx concentration at T60 suggesting a NO availability re-balance effect. *$p < 0.05$, ***$p < 0.001$.

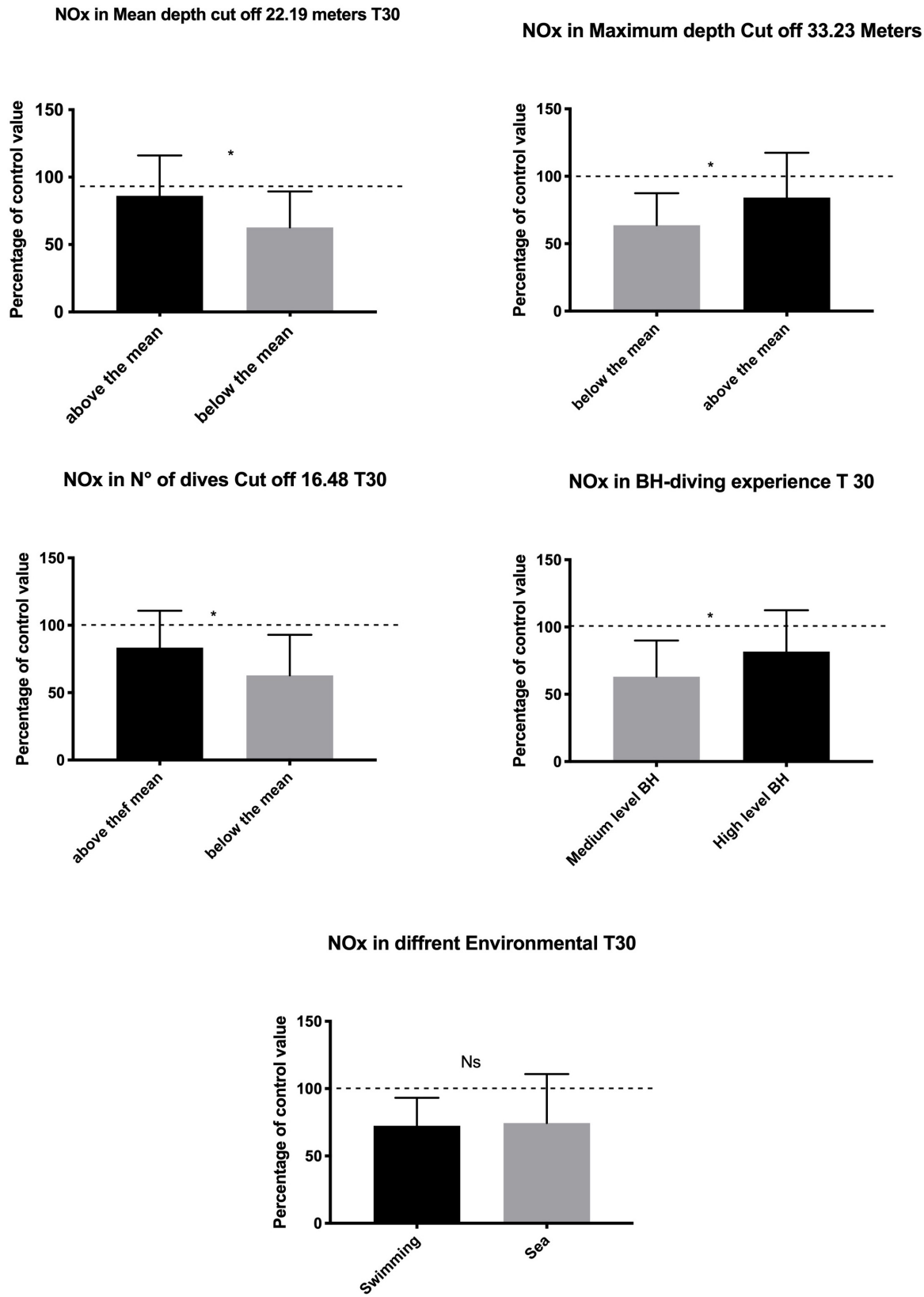

FIGURE 2 | Data at T30 show lower NO usage in highly exposed and experienced subjects indicating adaptation in the management of hyperbaric-related vascular changes. Swimming Pool vs. sea BH-diving data showed no significant differences. *$p < 0.05$.

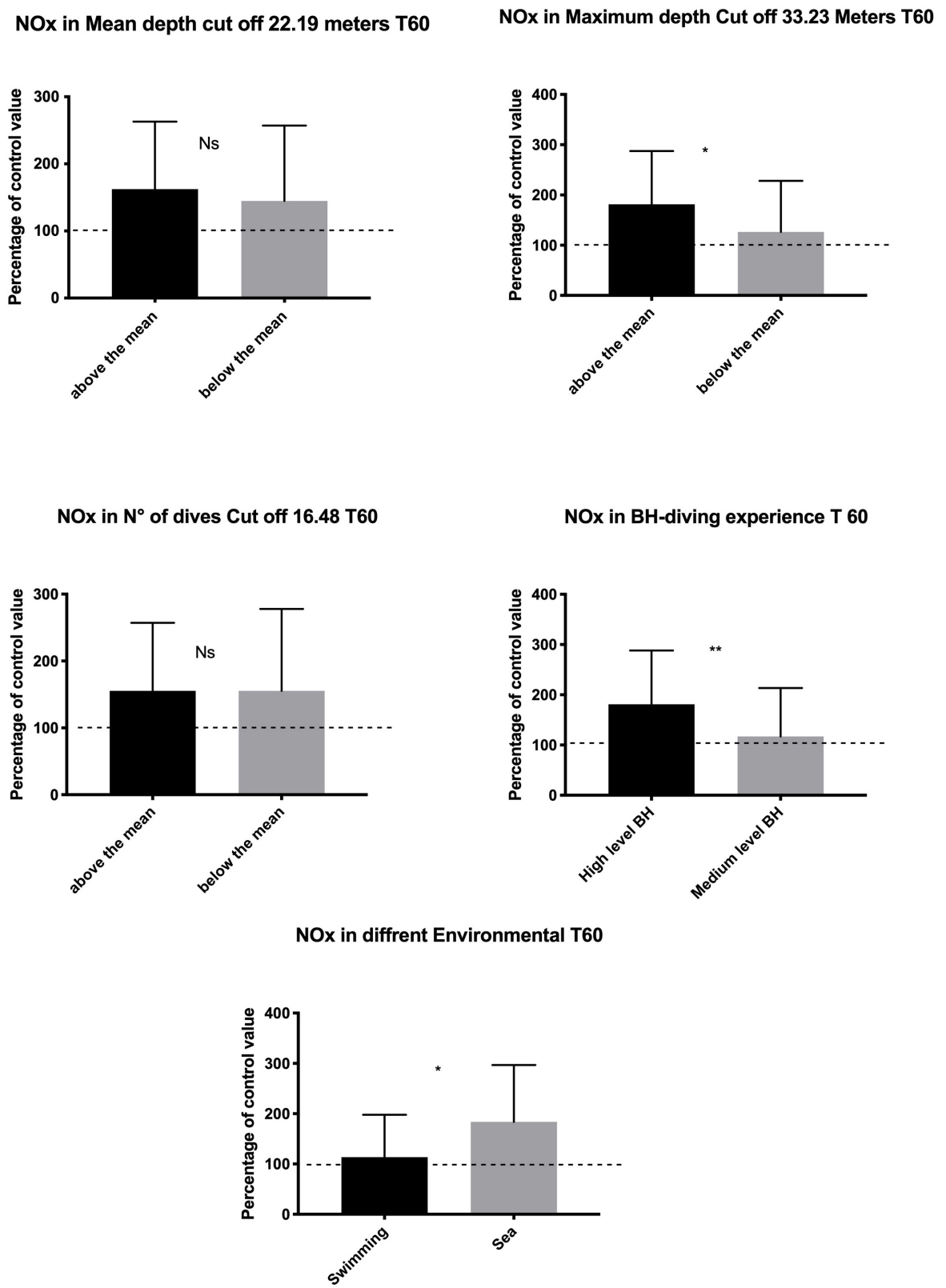

FIGURE 3 | Data at T60 show a rebound effect with an increase of NO available probably to compensate for the higher underwater consumption. This rebound effect was larger in the subject who shows a higher diving exposure and experience and in sea diving as compared with the swimming pool diving. $^{*}p < 0.05$, $^{**}p < 0.01$.

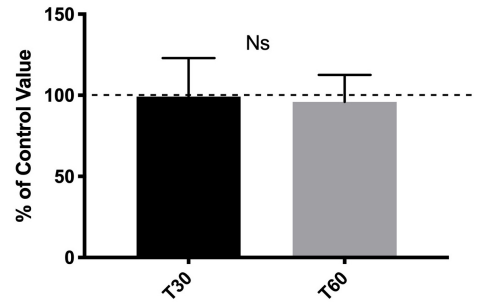

FIGURE 4 | No statistical difference in TAC levels were found between pre- and post-dive values at T30 and T60.

No statistical difference was detected in TAC levels between pre- and post-dive values (**Figure 4**).

Similarly, no differences were found at T30 in the groups above vs. below average for the four-diving risk levels (AD, MD, ND, and LD) and in the two different environmental conditions (sea vs. swimming pool). Only the follow-up at T60 demonstrated higher oxidative stress levels in divers who performed BH-diving above the mean of MD and in HE divers HE (p = 0.01 and p = 0.03, respectively).

No significant difference was found as well between the swimming pool and the sea BH-dives.

Finally, no significant relationship was found in the NOx and TAC levels in the different genetic variant (**Supplementary Table 2**).

DISCUSSION

The NOx (Tsukiyama et al., 2017) and the TAC changes (Marciniak et al., 2009) after a BH-diving training session were investigated in a group of 50 BH-divers divided into two groups with different hyperbaric exposure and different experiences.

In the appendix to this primary aim, we also observed the behavior of NOx and TAC in the same divers sorted by their genetic variant of 10 anti-inflammatory, vascular, and antioxidant related polymorphisms.

Nitric oxide (Koshland, 1992) plays an essential and complex role as a signaling molecule in several human physiological and pathological responses, including diving and hyperbaric-related adaptations (Theunissen et al., 2013a; Sureda et al., 2014). NO behavior is a complex molecule to be studied, for these multifaceted actions and the short half-life (Liu et al., 1998), especially, when adding the challenging test conditions of an extreme environment. A standard method to investigate plasma NO changes is by looking at the variations of its oxidation products, NOx, because the half-lives of NO_3 and NO_2 in blood circulation are 5–8 h and 20–40 min, respectively (Dejam et al., 2007; Lundberg et al., 2008). Physical exercise increases eNOS activity and resulting in a higher level of circulating NOx (Jungersten et al., 1997; Green et al., 2004).

Under particular conditions, such as hypoxia, NOx can be reconverted to NO through different pathways involving proteins (hemoglobin and myoglobin), enzymes (xanthine oxidase and xanthine oxidoreductase), and ascorbate to ensure NO production when O_2 supply is reduced. In blood vessels, NO_2 generates vasodilatory NO by reacting with deoxygenated hemoglobin (deoxy-Hb) and contributes to physiological hypoxic blood flow regulation. When hemoglobin O_2-saturation drops to 50%, the reduction of NO_2 to NO is enhanced. This effect results from two mechanisms: the availability of deoxyhemes (reaction substrate) to bind NO_2, which is maximal in deoxygenated hemoglobin, and the amount of oxygenated hemoglobin tetramer, which increases the intrinsic reactivity of the heme with NO_2 (Lundberg et al., 2008).

In a recent study, data obtained from an underwater blood draw (−42 m) carried out on 12 expert BH-divers clearly showed the NOx kinetics in BH-diving. These data indicate a significant underwater increase in plasma NOx concentration (+410.5% compared with pre-dive value) and an immediate return to baseline values after reaching the surface (Cialoni et al., 2021). These data confirmed a significant use of NO during BH-diving, compatible with the well-known BH-diving-related circulatory adaptations, but unexpectedly showed a swift return of circulating NOx to basal levels at the surface. This last aspect confirms that the NOx measured after diving reflects the availability of NO in real-time, without any diving-related "accumulation" effect in tissues. This observation helps to understand the results reported in this new protocol performed after a BH-diving training session, where we found a decrease of NOx 30 min after the training session, followed by an increase at T60. These data partially confirm a previous study where a similar increase was found, although without any initial decrease (Theunissen et al., 2013a). This difference could be easily explained by the different diving protocols of the previous test compared with that proposed in this research, especially concerning the ND and the descent technique.

Therefore, the T30 post-diving reduction of NO_x found in this experiment can be ascribed to the lower NO availability in the first few minutes after the dives caused by the higher use of this molecule during diving (Cialoni et al., 2021) to ensure the BH-diving related vascular adaptations. On the other hand, an increase at T60 could be a rebound of the efforts of the body to restore basal conditions after exceptional stress exposure (**Figure 1**).

Hyperbaric exposure-related oxidative stress is the second aspect taken into account due to the consequences potentially affecting BH-divers. Indeed, BH-diving results in higher ROS production and oxidative stress, as confirmed by several authors (Theunissen et al., 2013a; Mrakic-Sposta et al., 2019; Cialoni et al., 2021), along with the activation of endogenous antioxidant defenses (Bulmer et al., 2008; Sureda et al., 2015). As recently suggested (Cialoni et al., 2021), oxidative stress is transitory, increasing in the underwater phases but returning near pre-dive levels after reaching the surface.

Unlike the previous paper (decrease of TAC: −60% to pre-dive value) (Cialoni et al., 2021), TAC did not show any difference between pre- and post-diving in the present experiment. This fact

suggests the absence of an oxidative stimulus at the end of the training session, despite the hyperbaric exposure.

The data, in this study, indicate significant differences in NO consumption only when stratifying the divers into the two groups of high or low hyperbaric exposure or in the two groups of more expert vs. less expert subjects.

It is also intriguing to note that the lower NO consumption was observed in expert divers (HE) and those with higher hyperbaric exposure on the day of the experiment. We can hypothesize that a possible adaptation effect was undergone in these subjects who trained more intensely or had higher performances on the day of the investigation, with respect to the BH-divers that dived below the average. This aspect can also be explained by the "relax and comfort" training and diving techniques adopted by expert BH-divers, most likely decreasing the NO necessary to support the BH-diving induced hyperbaric-related physiological changes. However, this variable was not explicitly investigated and is worthy of further assessment in the future.

Another important observation concerns the changes in NOx and TAC when comparing swimming pool and sea exposures. Data at T30 were similar in the two different environments indicating a low influence of variables such as temperature (34 vs. 24°C) and salinity (freshwater vs. seawater). Therefore, NOx level changes are probably more influenced by the magnitude of hyperbaric exposure. An in-depth analysis of this aspect showed that the rebound effect noted at T60 is significantly higher in the sea subjects than in the swimming pool subjects (183.9 ± 112.9 vs. 113.5 ± 84.3). This could be related to the characteristics of sea BH-diving, requiring complex logistics for the training sessions, the use of a boat and a diving suit, more time inside the water (even if the ND is similar in the two groups), and more demanding environmental conditions (e.g., colder temperatures, waves, and currents).

However with all the limits that our genetic investigation shows, it is intriguing to note that the data did not show any differences in NOx and TAC values in the single nucleotide variant in all the 10 polymorphisms investigated. If this data will be confirmed by future studies, more focus on the genetic aspect could be indicated to confirm that the genetic predisposition is less critical as compared with hyperbaric exposure when concerning BH-diving related NOx and oxidative stress.

CONCLUSION

This study showed the importance of hyperbaric exposure and expertise regarding NO availability and oxidative stress in BH-divers. NO consumption seemed to be significantly lower in high-performance BH-divers and the expert group indicating a possible training-related adaptation process. On the other hand, expert BH-divers demonstrated higher oxidative stress due to higher hyperbaric exposure in the sessions. A preliminary genetic investigation seems to indicate a lack of specific influence of genetic predisposition as compared with the increase of diving exposure.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Committee of Università degli Studi di Milano, Italy (Aut. No. 37/17). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DC proposed the protocol and the search strategy, extracted and analyzed the data, and wrote the first draft. AB was involved in the conception and design of this work, reviewed the critical appraisal of selected articles, and assisted with the compilation of the systematic review. MP and NS extracted and analyzed the data and reviewed the manuscript. VL was involved in the test on the field and reviewed the manuscript. MS, AB, and AM supervised the entire process. All authors contributed to at least three of the four major components of a study and were involved in the conception and design of this work, contributed to the process of writing, and approval of the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.692204/full#supplementary-material

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Heart Rate and Muscle Oxygenation Kinetics During Dynamic Constant Load Intermittent Breath-Holds

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Introduction: Acute apnea evokes bradycardia and peripheral vasoconstriction in order to conserve oxygen, which is more pronounced with face immersion. This response is contrary to the tachycardia and increased blood flow to muscle tissue related to the higher oxygen consumption during exercise. The aim of this study was to investigate cardiovascular and metabolic responses of dynamic dry apnea (DRA) and face immersed apnea (FIA).

Methods: Ten female volunteers (17.1 ± 0.6 years old) naive to breath-hold-related sports, performed a series of seven dynamic 30 s breath-holds while cycling at 25% of their peak power output. This was performed in two separate conditions in a randomized order: FIA (15°C) and DRA. Heart rate and muscle tissue oxygenation through near-infrared spectroscopy were continuously measured to determine oxygenated (m[O_2Hb]) and deoxygenated hemoglobin concentration (m[HHb]) and tissue oxygenation index (mTOI). Capillary blood lactate was measured 1 min after the first, third, fifth, and seventh breath-hold.

Results: Average duration of the seven breath-holds did not differ between conditions (25.3 s ± 1.4 s, $p = 0.231$). The apnea-induced bradycardia was stronger with FIA (from 134 ± 4 to 85 ± 3 bpm) than DRA (from 134 ± 4 to 100 ± 5 bpm, $p < 0.001$). mTOI decreased significantly from 69.9 ± 0.9% to 63.0 ± 1.3% ($p < 0.001$) which is reflected in a steady decrease in m[O_2Hb] ($p < 0.001$) and concomitant increase in m[HHb] ($p = 0.001$). However, this was similar in both conditions ($0.121 < p < 0.542$). Lactate was lower after the first apnea with FIA compared to DRA ($p = 0.038$), while no differences were observed in the other breath-holds.

Conclusion: Our data show strong decreases in heart rate and muscle tissue oxygenation during dynamic apneas. A stronger bradycardia was observed in FIA, while muscle oxygenation was not different, suggesting that FIA did not influence muscle oxygenation. An order of mechanisms was observed in which, after an initial tachycardia, heart rate starts to decrease after muscle tissue deoxygenation occurs, suggesting a role of peripheral vasoconstriction in the apnea-induced bradycardia. The apnea-induced increase in lactate was lower in FIA during the first apnea, probably caused by the stronger bradycardia.

Keywords: diving response, face immersion, dynamic apnea, near-infrared spectroscopy, bradycardia, peripheral oxygenation

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INTRODUCTION

Acute apnea or breath-holding is known to induce a series of physiological responses which are called the "diving response." This response can be defined as a pattern of respiratory, cardiac, and vascular responses triggered by apnea (Gooden, 1994). The diving response is characterized by bradycardia, peripheral vasoconstriction, and an increase in blood pressure. It is believed to fulfill an oxygen conserving role (Schagatay et al., 1999; Foster and Sheel, 2005) prioritizing O_2 delivery to the vital organs, such as the heart and the brain. Indeed, acute breath-holding is believed to reduce overall O_2 demand (Gooden, 1994; Foster and Sheel, 2005; Costalat et al., 2017).

During exercise, however, the overall cardiovascular and metabolic demand increases, leading to an increased oxygen delivery to the working heart and skeletal muscles. During dynamic apnea, conflicting stimuli emerge simultaneously. At the cardiac level, the apnea-induced diving response elicits bradycardia through increased parasympathetic nerve stimulation, originating from the removal of the inspiratory-induced phasic tachycardia and reduced parasympathetic stimulation of lung stretch receptors (Bain et al., 2018). On the other hand, the exercise-induced sympathetic activity stimulates tachycardia to improve blood circulation to the working muscle. At the skeletal muscle level, increased muscle sympathetic nerve activity (MSNA) in response to apnea elicits peripheral vasoconstriction redistributing blood flow and prioritizing O_2 delivery to the vital organs (Heistad et al., 1968; Leuenberger et al., 2001), while exercise evokes local peripheral vasodilation which increases muscle blood flow. As face immersion is known to further enhance the diving response (Kawakami et al., 1967; Schuitema and Holm, 1988), dynamic apnea performed in an aquatic environment, such as practiced in synchronized swimming, underwater hockey, and dynamic apnea disciplines, can be expected to enforce these conflicting responses (Bergman et al., 1972; Hurwitz and Furedy, 1986).

The diving response has been shown to overrule the cardiovascular responses to exercise during dynamic apnea reaching heart rate values around and below resting heart rate (Scholander et al., 1962; Asmussen and Kristiansson, 1968; Bergman et al., 1972; Butler and Woakes, 1987; Joulia et al., 2009; Ichinose et al., 2018). This is especially true when the face is immersed in cold water (Bergman et al., 1972; Andersson and Evaggelidis, 2009) and during light exercise, while the response is less apparent in more vigorous exercise (Asmussen and Kristiansson, 1968). A similar manifestation of the diving response overruling the cardiovascular response to exercise was observed for peripheral vasoconstriction. Leg blood flow decreases during dynamic dry apnea (DRA) reaching resting baseline values and as such, overriding the exercise induced response (Nishiyasu et al., 2012; Ichinose et al., 2018). This illustrates that peripheral vasoconstriction occurs not only in resting but also in exercising muscle tissue. The peripheral blood flow response also appears to be related to the magnitude of bradycardia as a more pronounced decrease in leg blood flow was seen in the group of subjects with the strongest decrease in heart rate (Nishiyasu et al., 2012).

The effect of limited peripheral blood flow on muscle tissue oxygenation has occasionally been studied in dry static apnea through NIRS measurements (Palada et al., 2007; Eichhorn et al., 2015, 2017; Ratmanova et al., 2016; Bouten et al., 2020). These studies show a steady, continuous decrease in muscle tissue oxygenation starting within 10s after apnea onset and further developing throughout the breath-hold. This illustrates that the body is successful in maintaining cerebral oxygenation, only falling below resting value in the last part of the static breath-hold (Eichhorn et al., 2015; Bouten et al., 2020). However, only two studies reported muscle tissue oxygenation during either short DRAs at 60% VO_2max (Kume et al., 2013) and maximal dynamic face immersed apnea (FIA) at 30% of peak power output (Costalat et al., 2013). Although direct comparison is difficult due to different methodologies, dynamic FIA appears to evoke stronger muscle deoxygenation than dry static apnea. Data comparing muscle oxygenation for DRAs with FIAs are currently missing.

Lower O_2 supply to the working muscle due to a decrease in heart rate and peripheral vasoconstriction can also be expected to alter metabolic pathways during exercise from aerobic to more anaerobic energy delivery. Indeed, lactate has been observed to increase throughout series of apneas, with a stronger response in dynamic breath-holds (Elia et al., 2021).

The aim of this study was to investigate cardiovascular and metabolic responses and their kinetics during short dynamic apnea and to examine the role of face immersion with cold water (15°C) in enhancing these responses. We hypothesized that (1) face immersion augments the bradycardia which develops throughout the breath-hold and (2) face immersion augments muscle tissue deoxygenation due to peripheral vasoconstriction. We also expect that (3) hemodynamic changes shortly before and at onset of apnea occur in a specific order. Additionally, we hypothesized that (4) apnea-induced increases in lactate concentration can be observed which are enforced with face immersion due to a more pronounced muscle deoxygenation.

MATERIALS AND METHODS

Ethical Approval

The protocol was approved by the Ethics Committee of the Ghent University Hospital (EC UZG 2016/0809). After verbally clarifying test procedures and potential risks involved, written informed consent was signed by all subjects. For subjects who were still a minor at the onset of testing, a parent or guardian signed the informed consent as well. All participants underwent a medical screening by a skilled physician before engaging any tests.

Population

Ten female subjects (17.1 ± 1.8 years old) volunteered to take part in this study. These subjects were unexperienced in apnea-related sports, but were engaged in physical training for 6.7 ± 4.2 h per week. All subjects were non-smokers and were declared to be in good general health. Subject characteristics

TABLE 1 | Anthropometric and training characteristics, lung function parameters, and maximal incremental test parameters (mean ± SE).

General characteristics	
Age (years)	17.1 ± 0.6
Height (cm)	164.6 ± 1.7
Body mass (kg)	60.5 ± 1.6
Body fat (%)	19.5 ± 1.1
Max static apnea time (s)	118.2 ± 11.2
Exercise test results	
Ppeak (W)	265.9 ± 10.7
HRpeak (bpm)	192.4 ± 2.7
VEpeak (L.min^{-1})	86.0 ± 5.2
VO_2peak (mL.min^{-1}.kg^{-1})	40.2 ± 1.4
[La]peak (mmol.L^{-1})	11.6 ± 1.1
Lung function parameters	
FVC (L)	4.4 ± 0.2
FEV_1 (L)	3.7 ± 0.2

P, power; HR, heart rate; VE, ventilation; VO_2, oxygen uptake; La, lactate; FVC, forced vital capacity; and FEV1, forced expiratory volume in 1 s.

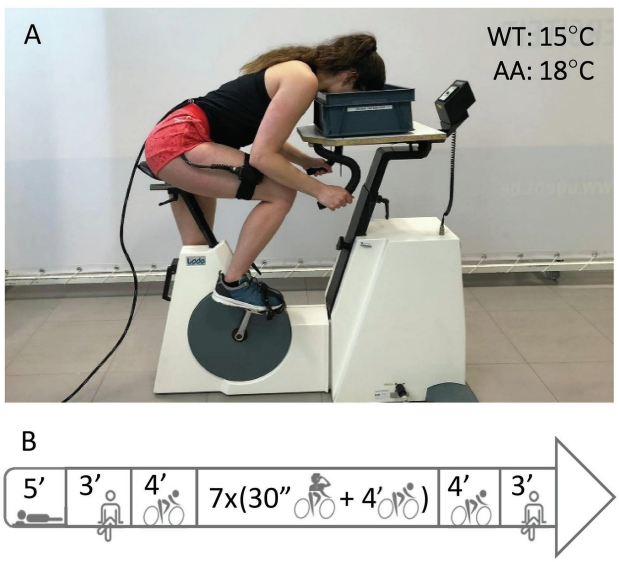

FIGURE 1 | Panel **(A)** shows an example of the experimental setup. Ambient air (AA) was set at 18°C, water temperature (WT) in the container for face immersed apneas (FIA) at 15°C. Panel **(B)** shows an overview of the protocol starting with 5 min of supine rest, 3 min of BL measurements while seated on the bike followed by 4 min dynamic BL at 25% PPO. Then, subjects performed seven bouts of 30 s-dynamic apnea (AP) interspersed with 4-min cycling intervals, followed by a 4-min dynamic and 3-min seated recovery.

can be seen in **Table 1**. All subjects were advised to avoid any vigorous activity 48 h prior to the test and instructed to eat and drink similarly and refrain from caffeinated and alcoholic beverages in the last 24 h before each test.

Procedures

The study consisted of three different testing days and took place in the Sport Science Laboratory Jacques Rogge (Ghent University, Belgium) at a constant ambient air temperature of 18°C and humidity of 45%. All tests were performed at least 1 week apart to avoid residual effects of the previous test day. During the first day, all participants were subjected to a medical screening with ECG analysis, anthropometric assessment, and pulmonary function testing. During this test, heart rate (HR), muscle tissue oxygenation, and blood lactate were measured. Additionally, subjects performed a series of five maximal dry static seated apneas with 2-min rest intervals. After 30 min of rest, subjects performed a maximal ramp incremental exercise test on a cycle ergometer. The incremental test started with 3 min of cycling at 30 W, followed by a continuously increasing power output (30 W. min^{-1}). During this test, maximal power output, pulmonary gas exchange, and lactate 30 s post-exercise were measured.

The following two test days, the subjects performed the two dynamic constant load intermittent apnea tests, one with face immersion (FIA) and one dry (DRA) in a randomized order. The protocol for the apnea tests is summarized in **Figure 1**. The test started with 5 min of supine rest in an isolated and silent room to measure the resting HR. After 3 min of seated rest in order to stabilize HR and NIRS data, subjects cycled 34.5 min at a cadence between 60 and 70 rpm and a power output of 25% of their peak power output obtained during the maximal incremental exercise test. During this period, subjects performed 7 bouts of 30-s dynamic apnea intervals, interspersed with 4-min bouts of regular cycling. The first bout was performed after 4 min of cycling. After the seventh (final) bout, subjects cycled for another 4 min followed by 3 min of seated rest on the ergometer to obtain recovery measurements. Capillary blood was collected for blood (lactate) determination 1 min after the first, third, fifth, and seventh apnea. Subjects were notified 30 s before each apnea and verbally guided with a 10-s countdown. Subjects were instructed to take a deep but not maximal breath. During apnea, subjects were not allowed to exhale and were verbally noticed if cadence was not kept constant. During the FIA test, a container with water (15°C) was placed on the steering wheel of the ergometer, positioned in a way that the subjects only had to bend the neck to submerge the face (**Figure 1A**). Subjects were instructed to submerge their face deeply, so the water contacted the skin in the regions surrounding the eyes, the forehead, the nose, and the mouth. After 30 s of apnea, the subjects lifted the head from the water and the face was dried with a towel. If the subjects were not able to sustain the 30-s apneas, breathing was resumed prematurely. During the 4-min breathing periods, the subjects' head was positioned just above the water surface. This allowed minimal postural adjustments when starting or ending an apneic period. During DRA tests, the same procedure was repeated, but with an empty container placed on the ergometer's steering wheel. All tests were performed at the same time of day for each participant to avoid bias by circadian rhythm.

Equipment and Measurements

Anthropometric Assessment

Weight and height were measured using a SECA scale and measure (model 708, Seca GmbH, Hamburg, Germany). Ten

skinfolds were taken to estimate the body fat percentage (cheek, chin, thorax 1, thorax 2, triceps, subscapular, abdomen, supra-iliac, thigh above the patella, and calf). All skinfolds were taken with a Harpenden skinfold caliper (Harpenden, West Sussex, United Kingdom) according to the ISAK (International Society for the Advancement of Kinanthropometry) guidelines. To avoid inter-observer variability, all skinfolds were taken by the same person (Fuller et al., 1991). Body fat percentage was calculated with the equation of Parizkova et al. (1961).

Incremental Exercise Test and Apnea Tests

All exercise tests were performed on an electrically braked cycling ergometer (Lode Excalibur Sport, Lode B.V., Groningen, Netherlands). A breath-by-breath gas analyzer (Jaeger Oxycon Pro, Viasys Healthcare GmbH, Höchberg, Germany) was used to perform lung function testing and measure gas exchange and ventilation during the incremental exercise test. Lactate was analyzed from a capillary blood sample collected from the right index finger (Radiometer ABL 90 Flex, Radiometer, Copenhagen, Denmark). Heart rate (HR) was recorded continuously (Polar HR monitor, Polar Electro, Kempele, Finland). After cleaning and shaving the skin, a near-infrared spectroscopy probe (NIRS; Oxiplex TS, ISS, Champaign, IL, United States) was positioned over the muscle belly of the M. Vastus Lateralis of the right quadriceps muscle and aligned with its vertical axis. The probe was securely attached with Velcro straps and tape. Muscle oxyhemoglobin (m[O_2Hb]), deoxyhemoglobin (m[HHb]), and tissue oxygenation index (mTOI) were continuously derived based on the absorption of infrared light emitted at different wavelengths (692 and 834 nm) and averaged into 1-s bins.

Data Analysis

Peak power output and peak HR were determined as the highest power output and HR reached during the incremental exercise test. The peak ventilation and peak oxygen uptake (VO_2 peak) were defined as the highest 30-s average. The best single apneic performance out of the five static maximal apneas was set as the maximal static apnea time.

All time series (HR, m[O_2Hb], m[HHb], and mTOI; 1 Hz) were synchronized. Baseline values for all four parameters were calculated for each apnea as the mean of the values between 90 and 30 s before the start of apnea. Extreme values represent the highest or lowest value obtained near or at the end of apnea, or in the first 30 s after apnea. The minimal value reached during or in the first 30 s after each apnea was taken for HR, mTOI, and m[O_2Hb]. For m[HHb], the extreme value was defined as the maximal value achieved in the same time interval. Overshoot values represent the opposite extreme while normalizing but before returning to the actual baseline and were calculated as the maximal value obtained in the first 30 s following each apnea for HR, mTOI, and m[O_2Hb]. For m[HHb], the minimal value was taken. Delta (Δ) values for HR, mTOI, m[O_2Hb], and m[HHb] were calculated as the difference between baseline and the extreme value for each apnea. For statistical analysis of the acute cardiovascular response, the average baseline values, values at −30, −25, −20, −15, −10, −5, 0, 5, 10, and 15 s after onset of apnea, extreme and overshoot values were calculated as the average of all seven apneas for each individual to obtain a general response for HR and NIRS values. Data at 20, 25, and 30 s are not presented because not all apneas were sustained for 20 s.

Statistical Analysis

Statistical analyses were performed with IBM SPSS 26. Statistical significance was set at $p < 0.05$. Shapiro-Wilks tests were performed to check normality of the data. Sphericity was verified by Mauchly's test of sphericity. When the assumption of sphericity was not met, the Greenhouse-Geisser correction was applied. Partial eta square (η_p^2) was used to indicate effect sizes for the RM MANOVA's, while Cohen's d was used to indicate effect sizes for pairwise comparisons. All data are presented as mean ± standard error.

Acute Cardiovascular Response

A 2 × 13 within-subjects RM MANOVA (Condition × Time Point) was used to analyze the acute response of the measures HR, mTOI, m[O_2Hb], and m[HHb] over 13 time points (baseline, −30 s, −25 s, −20 s, −15 s, −10 s, −5 s, 0 s, 5 s 10 s, 15 s, extreme value, and overshoot value) for two conditions (FIA and DRA). Pairwise comparisons (least square differences) were used as *post-hoc* analysis. When a significant interaction effect was found, time points were one by one excluded from the analysis to determine when this difference started to establish.

Longitudinal Analysis

Three 2 × 7 within-subjects RM MANOVAs (Condition × Apnea number) were used to analyze the pattern of HR, mTOI, m[O_2Hb], and m[HHb] for baseline, extreme, and Δ values throughout the seven apneas (apnea number 1 through 7) for two conditions (FIA and DRA). Pairwise comparisons (least square differences) were used as *post-hoc* analysis.

A 2 × 4 within-subjects RM ANOVA (Condition × Lactate measurement) was used to analyze the evolution of lactate throughout the apnea tests for the measurements after the first, third, fifth, and seventh apnea for both conditions (FIA vs. DRA).

RESULTS

Anthropometrics, Exercise, and Lung Function Tests

Subject characteristics are displayed in **Table 1**.

Apnea Tests

General

Two out of 10 subjects completed all 14 dynamic 30-s apnea bouts (both face immersed apneas, FIA, and dry apneas, DRA). Two of out 10 subjects failed to complete any 30-s apnea. Subjects completed 28 of 70 DRA bouts and 29 of 70 FIA

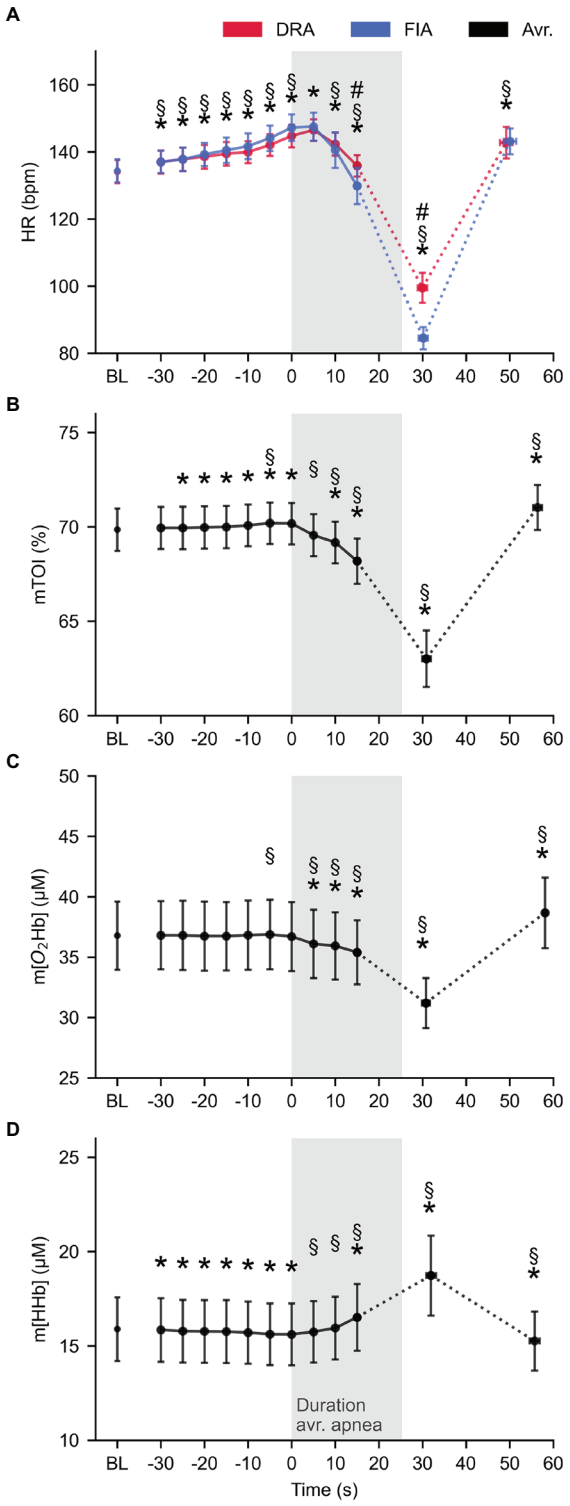

FIGURE 2 | The average pattern for heart rate (HR, panel **A**), tissue oxygenation index (mTOI, panel **B**), oxygenated (m[O2Hb], panel **C**), and deoxygenated hemoglobin concentration (m[HHb], panel **D**) during apnea (n = 10). Blue lines represent FIA, red lines represent dynamic dry apnea (DRA), and black lines represent the average of both conditions. * = statistically different from BL at $p < 0.05$; § = statistically different from the previous value at $p < 0.05$; and # = statistical time × condition interaction effect at $p < 0.05$.

bouts without premature resumption of breathing. Incomplete apneas were sustained for 22 ± 1.4 s in both FIA and DRA, while total average apnea time for all apneas, complete and incomplete, was 25 ± 1.4 s in both conditions (p = 0.231).

Acute Cardiovascular Response

The 2 × 13 RM MANOVA (Condition × Time point) only showed a significant interaction effect for condition × time (F = 8.205, $p < 0.001$, η_p^2 = 0.477) for HR. Significant main effects were found for mTOI (F = 77.041, $p < 0.001$, η_p^2 = 0.895), m[O_2Hb] (F = 36.421, $p < 0.001$, η_p^2 = 0.802), and m[HHb] (F = 34.409, $p < 0.001$, η_p^2 = 0.793) over time during acute apnea.

The interaction effect for HR revealed that the acute response in heart was more pronounced in FIA compared to DRA. This difference started to establish after 15 s (F = 10.104, p = 0.011, η_p^2 = 0.317) and was most obvious at the minimal HR (F = 15.391, p = 0.003, Cohen's d = 1.20). **Figure 2A** shows that HR increased similarly from baseline to onset of apnea in both conditions (F = 3.156, p = 0.057, η_p^2 = 0.260), from 134 ± 4 bpm to 147 ± 4 bpm in FIA ($p < 0.001$, Cohen's d = 1.09) and from 134 ± 4 bpm to 145 ± 3 bpm in DRA ($p < 0.001$, Cohen's d = 0.98). HR reached a maximum of 148 ± 4 bpm in FIA and 147 ± 3 bpm in DRA 5 s after onset of apnea. From then on, HR started decreasing and did not reach values significantly below baseline in the first 15 s after onset of apnea (FIA: 130 ± 5 bpm, p = 0.273, Cohen's d = 0.23; DRA: 136 ± 3 bpm, p = 0.495, Cohen's d = −0.17). Minimal HR fell significantly below baseline in both FIA (85 ± 3 bpm, $p < 0.001$, Cohen's d = 4.62) and DRA (100 ± 5 bpm, $p < 0.001$, Cohen's d = 2.7) and was reached after 30 ± 1 s in both conditions which is around 5 s after termination of apnea.

mTOI slightly increased from baseline to onset of apnea (69.9 ± 0.9% at baseline to 70.2 ± 0.9% at T0, p = 0.003, Cohen's d = −0.11) and immediately started decreasing after onset of apnea to 69.6 ± 0.9% at 5 s ($p < 0.001$, Cohen's d = 0.21). mTOI reached values significantly below baseline after 10 s (69.2 ± 0.9%, p = 0.017, Cohen's d = 0.20) and decreased even further till the minimum value (63.0 ± 1.3%, $p < 0.001$, Cohen's d = 1.83) which was reached 30.5 ± 1.6 s after onset of apnea (**Figure 2B**). m[O_2Hb] had already significantly decreased from 36.8 ± 2.7 μm at baseline to 36.1 ± 2.7 μm after 5 s ($p < 0.001$, Cohen's d = 0.21). **Figure 2C** illustrates that m[O_2Hb] steadily decreased (35.9 ± 2.7 μm after 10 s, $p < 0.001$, Cohen's d = 0.23 and 35.4 ± 2.5.2 after 15 s, p = 0.001, Cohen's d = 0.31) reaching an average minimum of 31.2 ± 1.9 μm ($p < 0.001$, Cohen's d = 0.92) after 29.1 ± 2.0 s. m[HHb] decreased from 15.9 ± 1.6 μm at baseline to 15.6 ± 1.6 μm at the start of apnea ($p < 0.001$, Cohen's d = 0.06) and immediately started increasing when the breath-hold started (p = 0.022, Cohen's d = 0.04). m[HHb] only reached values significantly above baseline after 15 s (16.5 ± 1.7 μm, $p < 0.001$, Cohen's d = 0.49). m[HHb] reached an average maximum of 18.7 ± 2.1 μm (p = 0.001, Cohen's d = 0.13) after 32.0 ± 1.4 s (**Figure 2D**).

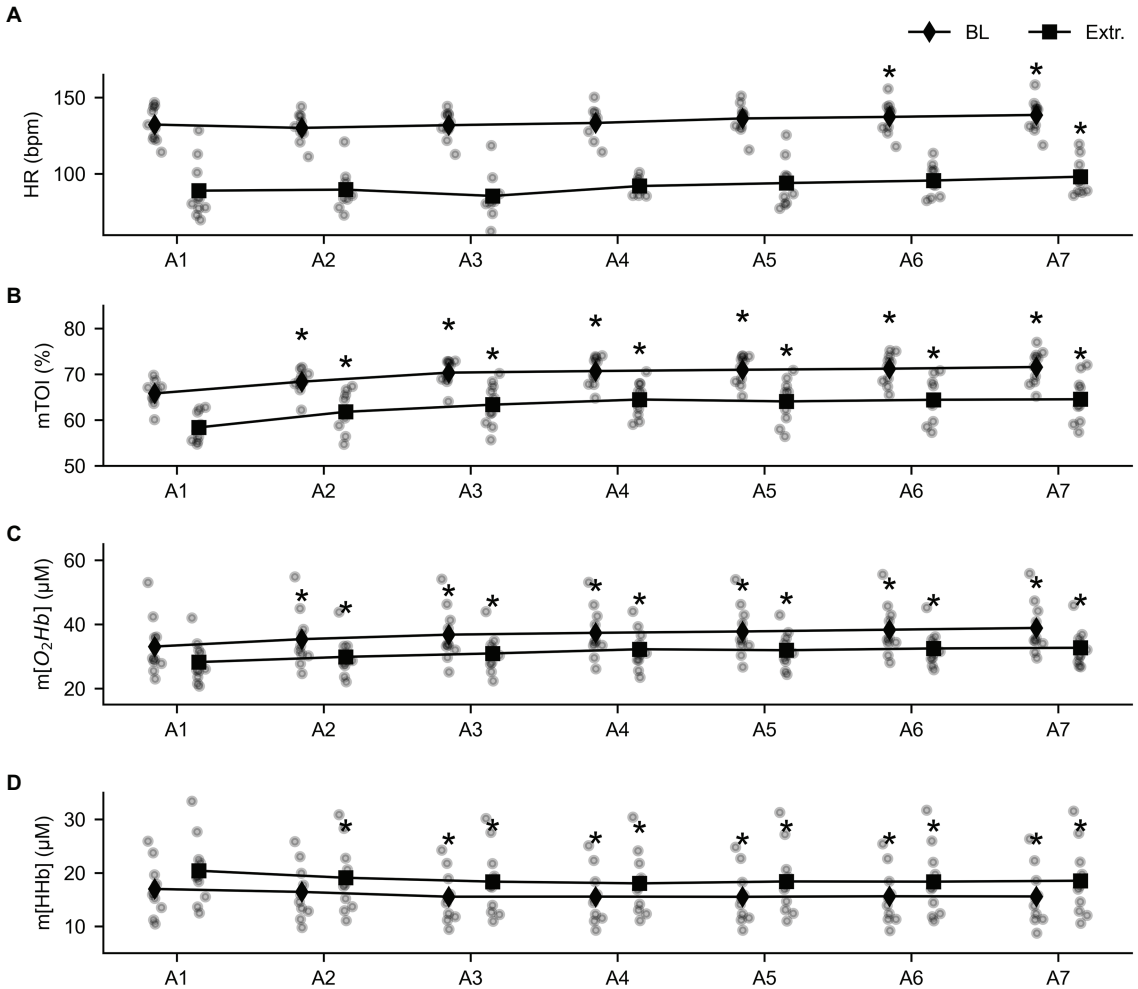

FIGURE 3 | Overview of the evolution of the baseline (diamond shapes) and extreme values (squares) throughout the series of seven apneas for heart rate (HR, panel **A**), tissue oxygenation index (mTOI, panel **B**), oxygenated (m[O_2Hb], panel **C**), and deoxygenated hemoglobin concentration (m[HHb], panel **D**). * = statistically different from A1 at $p < 0.05$.

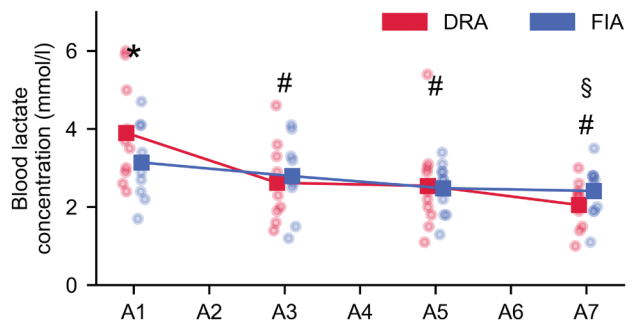

FIGURE 4 | Evolution of lactate during the series of seven apneas (A1–A7 = measurement after the first–seventh apnea). The blue graph represents the average for FIA, while the red graph represents the average for DRA. * = significant difference between FIA and DRA at $p < 0.05$; § = significantly different from BL for FIA; and # = significantly different from BL for DRA.

Longitudinal Analysis

The 2 × 7 RM MANOVA revealed that baseline values changed over time for all four parameters: Baseline HR ($F = 8.414$, $p < 0.001$, $\eta_p^2 = 0.483$), baseline mTOI ($F = 41.282$, $p < 0.001$, $\eta_p^2 = 0.821$), and baseline m[O_2Hb] ($F = 52.373$, $p < 0.001$, $\eta_p^2 = 0.853$) increased throughout the series of apneas, while m[HHb] decreased ($F = 17.594$, $p < 0.001$, $\eta_p^2 = 0.662$). Similarly, the 2 × 7 RM MANOVA main effects for time revealed differences for extreme values over time for mTOI, m[O_2Hb], and m[HHb]. Minimal HR following apnea tended to increase during the series ($F = 2.566$, $p = 0.073$, $\eta_p^2 = 0.22$). The lowest mTOI value ($F = 33.830$, $p < 0.001$, $\eta_p^2 = 0.790$) and m[O_2Hb] ($F = 24.068$, $p < 0.001$, $\eta_p^2 = 0.728$) increased from the first to last apnea, while maximal values for m[HHb] decreased ($F = 14.610$, $p < 0.001$, $\eta_p^2 = 0.619$).

Baseline and extreme values evolved similarly over the time course of the apneas **(Figure 3)**, as observed by the non-significant main effect for the delta values (ΔHR: $F = 0.489$, $p = 0.814$, $\eta_p^2 = 0.052$; ΔmTOI: $F = 1.371$, $p = 0.274$, $\eta_p^2 = 0.132$), Δ

m[O_2Hb] (F = 2.358, p = 0.098, η_p^2 = 0.208), and Δ m[HHb] (F = 2.292, p = 0.231, η_p^2 = 0.203).

Blood lactate values decreased over time from the value obtained 1 min after the first, to the value 1 min after the last apnea (F = 19.392, p < 0.001, η_p^2 = 0.683). This decrease was more pronounced for DRA than FIA (F = 4.386, p = 0.012, η_p^2 = 0.328; **Figure 4**); however, the blood lactate values at the respective time points only differed after the first breath-hold (p = 0.038, Cohen's d = 0.57).

DISCUSSION

This study was the first to compare both heart rate and muscle oxygenation kinetics during constant load exercise with intermittent dynamic apneas between FIA and DRA. The main findings were that these types of dynamic apneas in subjects naive to breath-holds induced (1) a stronger bradycardia in FIA compared to DRA and (2) a significant decrease in mTOI which was similar in both conditions. Additionally, (3) an order of events was observed during which all muscle oxygenation patterns immediately started to decrease, while bradycardia occurred later, possibly suggesting that peripheral vasoconstriction can facilitate bradycardia. Last, (4) a higher lactate concentration following apnea was seen in DRA compared to FIA, although this difference was only observed after the first apnea.

Despite the short duration of apneas (the target time was 30 s, while actual time was on average 25 s), both FIA and DRA were successful in decreasing heart rate (HR) and muscle oxygenation. After an initial increase before and at onset of apnea, heart rate started to decrease after 5 s and did not fall below baseline in the first 15 s. Muscle oxygenation parameters changed immediately upon the onset of apnea. This is consistent with a quick onset of peripheral vasoconstriction and in line with the immediate increase in MSNA (Heusser et al., 2009) limiting blood delivery to the working muscle. As the muscle needs to maintain the same workload, O_2 is still needed and muscle oxygenation therefore decreases.

Contrary to most studies comparing baseline values with end-apnea values and specific relative time points during breath-hold, we analyzed the data on an absolute time scale with short 5-s intervals. This allowed us to gain insight in the quick hemodynamic changes occurring before and at onset of breath-hold (Heusser et al., 2009) and especially in the order of events to understand the interaction of changes in heart rate and oxygenation. Looking deeper into the order of the response, it appears that the first response is an increase in heart rate, which already occurs before onset of apnea and lasts till 5 s after apnea. Aside from mental arousal and preparation, two mechanisms can be responsible for this increase in heart rate. First, the increase in lung stretch through deep inspiration stimulates the lung stretch receptors in the bronchi (Sroufe, 1971), which in their turn inhibit the cardiac vagal nerves and increase heart rate. Second, the deep inspiration at high lung volume is suggested to increase intrathoracic pressure which limits venous return and stroke volume and causes a drop in blood pressure and an increase in heart rate (Andersson and Schagatay, 1997; Schipke et al., 2019). This drop in blood pressure has been observed before in trained breath-hold divers (Schagatay et al., 1999; Sivieri et al., 2015; Ratmanova et al., 2016). Indeed, Heusser et al. (2009) showed that responses in blood pressure (MAP) and MSNA in the first 15–20 s of apnea closely resemble the responses of a Valsalva maneuver. This drop in blood pressure then increases peripheral resistance and leads to peripheral vasoconstriction, improving venous return. This quick vasoconstriction is consistent with our data showing an immediate decrease in m[O_2Hb] and mTOI, and allows the heart rate to decrease and blood pressure to normalize. The order of mechanisms suggested above is consistent with our data in naive subjects showing that the onset of bradycardia is observed after the changes in muscle oxygenation, both in dynamic apnea (DRA and FIA) but also in dry static breath-hold (Bouten et al., 2020). These initial hemodynamic changes are mechanical and neural in nature and are therefore able to elicit the rapid changes observed in the short period before and immediately after onset of apnea. These initial reflexes are supported by chemical responses to hypoxia later in the apnea. For example, a chemoreflex-induced MSNA increase leads to sustained and/or increased peripheral vasoconstriction (Leuenberger et al., 2001; Heusser et al., 2009; Badrov et al., 2017). The origin of increasing MSNA during apnea is still debated and might be more related to the lack of ventilation than the chemoreflex (Badrov et al., 2017). However, the duration of breath-holds in this study is too short for these responses to elicit.

In accordance with previous studies for static apneas (Bergman et al., 1972; Andersson et al., 2002, 2004), the bradycardic response was more pronounced with FIA compared to DRA. This difference, however, only tended to manifest after 10 s and did not reach statistical significance in the first 15 s. Heart rate dropped significantly from 134 ± 3 bpm during cycling at baseline to a minimal value of 100 ± 5 bpm in DRA and to 85 ± 3 bpm in FIA. Our data suggest that stimulation of the trigeminal nerve does not alter the initial reflexes eliciting an increase in heart rate, but influences the pattern only when these initial reflexes, increasing heart rate, are overruled. Additionally, due to the practical setup, subjects immersed their face after deep preparatory inspirations and onset of apnea, causing a latency period between onset of apnea and face immersion. The response to face immersion therefore occurs later than the immediate respiratory-induced reflexes. Contrary, muscle oxygenation was similar in both conditions. This suggests that face immersion did not impact peripheral vasoconstriction in our study. A likely explanation would be that, while face immersion triggers the trigeminal nerve which regulates vagal activity and thus improves bradycardia (Lemaitre and Schaller, 2015), peripheral vasoconstriction results from sympathetic activation (Leuenberger et al., 2001) and would therefore be independent from face immersion. This is however in contrast with the observations of stronger increases in MSNA in short FIA

compared to DRA (Fagius and Sundlöf, 1986) and greater increases in MAP during short dynamic FIA compared to DRA (Andersson et al., 2002), both supporting an enhanced sympathetic activation in response to face immersion (Heindl et al., 2004). This discrepancy in results might be related to differences in methodology. First, the short duration of the apneas in our study might be insufficient to develop a full dive response and differences might occur late in the apneas. However, increased MSNA (Fagius and Sundlöf, 1986) and increases in MAP and decreases in finger and forearm blood flow (Heistad et al., 1968) have been reported in apneas as short as 12 and 30s. Second, our subjects were inexperienced in breath-holding, while training is known to improve dive responses (Engan et al., 2013). A third consideration is that ambient air temperature (18°C) in our study was a lot lower than other studies and the difference between ambient air and water temperature (15°C) was only small. Indeed, Andersson et al. (2002) reported a water temperature of 10°C and ambient air temperature of 24°, while Fagius and Sundlöf (1986) showed the strongest increase in MSNA at the lowest water temperature (9°C) at an ambient temperature of 24°C. As cold air of 18°C already evokes noticeable increases in MSNA compared to 22°C (Fagius and Kay, 1991), it is possible that cold receptors stimulating MSNA were also partially stimulated without face immersion, making it more difficult to observe differences between FIA and DRA. A last consideration is that we only measured oxygenation in the working muscle. It is possible that perfusion to the working muscles is maintained to a certain level, while vasoconstriction appears to be more intense in inactive muscle tissues (Nishiyasu et al., 2012). Simultaneous oxygenation measurements of active and non-active muscles during dynamic apnea would be interesting to give more insight into this discussion.

When looking at the evolution of the baseline parameters during the prolonged constant load cycling at 25% of peak power output, we see that heart rate, mTOI, and m[O_2Hb] gradually increase during the test. We hypothesize that this response can be explained by the interaction of the breath-holds, exercise, and thermoregulatory mechanisms, termed cardiovascular drift (Coyle and González-Alonso, 2001). Extreme values changed similarly to baseline values, indicating that the amplitude of the response did not change throughout the series of breath-holds. Indeed, this is reflected by the observation that all delta values were similar during the series, indicating that the response did not improve from the first to last apnea. This is probably due to the short nature of the apneas in this study. As the maximum of the breath-holds was set at 30 s, breath-hold time did not increase significantly throughout the series.

Blood lactate decreases during the series of apnea in both conditions. As blood lactate concentration is the net result of lactate production, diffusion to the blood, and elimination, a decrease is caused by either a lower lactate production during apnea, a higher lactate elimination rate during both apnea and cycling intervals, or a combination of both. As the magnitude of the response for both heart rate and muscle oxygenation is similar throughout the entire protocol, we do not expect lactate production to change. Improved lactate elimination on the other hand is a logical explanation as the continued exercise leads to better muscle oxygenation observed throughout the protocol, facilitating a better lactate clearance. Lactate concentration was significantly higher after the first apnea in DRA than FIA. The observation that peripheral oxygenation was similar during apnea in both conditions suggests similar local oxygen supply, while heart rate decreases less in the dry condition which could indicate that the overall metabolic demand reduces less in DRA. This might lead to a greater reliance on the anaerobic system to meet the demand in the dry apneas, leading to a higher lactate production, which might be reflected by a higher lactate concentration. However, this was only seen in the first apnea. Later in the protocol, when lactate elimination is expected to be enhanced due to prolonged exercise in the moderate intensity domain, the duration of the apneas is most likely too short to observe differences between the conditions.

CONCLUSION

Our data show strong decreases in heart rate and muscle tissue oxygenation during short dynamic apneas in young female individuals naive to breath-holding. Contrary to previous research, only the response in heart rate was enforced through facial immersion, while muscle oxygenation was unaltered. This might indicate that the influence of face immersion on peripheral vasoconstriction is not a general response but only apparent in specific conditions and/or specific populations. Additionally, analyses of our data on a 5 s basis suggest an order of mechanisms through which heart rate increases due to inspiratory mechanical and neural mechanisms, followed by a quick onset of peripheral vasoconstriction as illustrated by rapid changes in muscle oxygen kinetics. Heart rate only starts to decrease after muscle tissue deoxygenation has started to establish, suggesting a role of peripheral vasoconstriction in the apnea induced bradycardia.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Ghent University Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/ next of kin.

AUTHOR CONTRIBUTIONS

All authors: conception, design, acquisition, analysis, and interpretation. JB and JGB: article drafting. All authors read, revised, and approved of the final version and agreed to be accountable for all aspects of the work.

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Association Between Arterial Oxygen Saturation and Lung Ultrasound B-Lines After Competitive Deep Breath-Hold Diving

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Breath-hold diving (freediving) is an underwater sport that is associated with elevated hydrostatic pressure, which has a compressive effect on the lungs that can lead to the development of pulmonary edema. Pulmonary edema reduces oxygen uptake and thereby the recovery from the hypoxia developed during freediving, and increases the risk of hypoxic syncope. We aimed to examine the efficacy of SpO_2, via pulse-oximetry, as a tool to detect pulmonary edema by comparing it to lung ultrasound B-line measurements after deep diving. SpO_2 and B-lines were collected in 40 freedivers participating in an international deep freediving competition. SpO_2 was measured within 17 ± 6 min and lung B-lines using ultrasound within 44 ± 15 min after surfacing. A specific symptoms questionnaire was used during SpO_2 measurements. We found a negative correlation between B-line score and minimum SpO_2 ($r_s = -0.491$; $p = 0.002$) and mean SpO_2 ($r_s = -0.335$; $p = 0.046$). B-line scores were positively correlated with depth ($r_s = 0.408$; $p = 0.013$), confirming that extra-vascular lung water is increased with deeper dives. Compared to dives that were asymptomatic, symptomatic dives had a 27% greater B-line score, and both a lower mean and minimum SpO_2 (all $p < 0.05$). Indeed, a minimum $SpO_2 \leq 95\%$ after a deep dive has a positive predictive value of 29% and a negative predictive value of 100% regarding symptoms. We concluded that elevated B-line scores are associated with reduced SpO_2 after dives, suggesting that SpO_2 via pulse oximetry could be a useful screening tool to detect increased extra-vascular lung water. The practical application is not to diagnose pulmonary edema based on SpO_2 – as pulse oximetry is inexact – rather, to utilize it as a tool to determine which divers require further evaluation before returning to deep freediving.

Keywords: hypoxia, apnea, hypoxic syncope, blackout, pulmonary edema, barotrauma, injury, extreme environment

INTRODUCTION

Breath-hold diving, also known as freediving, ranges from recreational and professional activities to a competitive sport with several disciplines for the maximal duration, distance, or depth performed on a single breath (Fitz-Clarke, 2018). Within each discipline, a specific combination of physiological factors determine performance (Schagatay, 2009, 2010, 2011; Bain et al., 2018).

Breath-hold diving is associated with an increased hydrostatic pressure that has a compressive effect on the body's air-filled cavities. As such, the rise in ambient pressure compresses the lungs in accordance with Boyle's law, thus reducing lung volume as the diver descends. The lungs are indeed compliant, but being delicate, exceeding the compliant threshold of the lung may lead to pulmonary edema (Lindholm et al., 2008), commonly referred to by divers as "lung squeeze." Pulmonary edema is associated with the accumulation of fluid in the alveoli, thus preventing oxygen uptake and causing hypoxemia (Lindholm and Lundgren, 2009). When affected, divers present with symptoms of productive cough, dyspnea, chest tightness (Cialoni et al., 2012), hemoptysis, and exhibit desaturation with a reduction in pulmonary function tests (Linér and Andersson, 2008). However, it would be just before, or upon surfacing where divers are most vulnerable because of the risk of hypoxic syncope, or "blackout" (Lindholm, 2007). The presence of fluid in the lungs would reduce the diffusion of oxygen from the alveolar space into the pulmonary circulation, thereby restraining the recovery from a low arterial oxygen saturation upon surfacing. Deep freediving additionally requires strenuous effort, and exercise stresses the pulmonary capillaries (West, 2004) increasing the risk of fluid leakage and increasing the diffusion barrier. One lethal case is known from a breath-hold diving competition in 2013, where a diver surfaced after a deep dive but experienced a "blackout" and was unable to recover due to severe lung squeeze (Vestin, 2015).

Ultrasound B-lines, also referred to as ultrasound lung comets, has been found to provide an accurate estimation of extravascular lung water (Jambrik et al., 2004; Agricola et al., 2005) and are considered to be a reliable tool to evaluate pulmonary edema (Lichtenstein, 2014; Chiumello et al., 2016; Wang et al., 2018; Soldati et al., 2019). B-lines have previously been measured in breath-hold divers, and depth does indeed appear to be a potent contributor to increase the B-line score (Frassi et al., 2008; Boussuges et al., 2011; Lambrechts et al., 2011) and pulmonary gas exchange impairment (Patrician et al., 2021). However, ultrasound imaging is not readily available outside of the research and clinical community. Therefore, it would be ideal to determine the efficacy of an easier and simpler tool for post-dive evaluation in freedivers, which provides insight into the potential of extravascular lung water. Preliminary data collected from 100 competitive dives have suggested that monitoring peripheral oxygen saturation (SpO_2) in the 20 min following the dive could be a tool to detect pulmonary edema/lung squeeze, as it was associated with reported and observed symptoms (Schagatay et al., 2015).

Our study sought to test the primary hypothesis that the impairment of SpO_2 recovery following deep breath-hold diving would be associated with elevated B-line score, an accepted and informative index of extravascular lung water. To examine this hypothesis, we studied breath-hold divers during a depth world championship.

MATERIALS AND METHODS

Participants

A total of 40 freedivers, 28 males and 12 females, with a mean ± SD age of 35 ± 7 years; 177 ± 7 cm height and 70 ± 8 kg weight and body mass index 22 ± 2 kg/m^2 volunteered to be in the study. All participants provided verbal informed consent, following written and oral information on the study and protocol. The study was reviewed by the local research ethics board and conformed to the Declaration of Helsinki. The study was also approved by and conducted in conjunction with the competition organizing committee.

While the measurements during this study likely capture maximal levels of physiological strain and provide valuable insight into the limits of the human body during freediving, the nature of a professional competition unavoidably led to variability in the timing of assessments. The competition dives were performed in three different dive disciplines: constant weight with fins (CWT), constant weight without fins (CNF), and free immersion (FIM) over 6 days of competition. A review of these disciplines has been covered elsewhere (Schagatay, 2011).

Procedures

Following each dive, SpO_2 and heart rate were recorded in 15-s intervals for 2 min via pulse oximetry (Nonin Onyx Vantage 9590) within 20 ± 11 min (range:10–45 min) of surfacing. Pulse oximetry data was collected in a finger, in the upright position, and only recorded when the oximetry signal was strong, as indicated by a bright green light signal. If the signal strength was sub-optimal (e.g., due to cold fingers), divers were given time to warm up before re-assessing. Ultrasound B-lines were collected in the supine position [within 45 ± 18 min (range: 18–85 min) of surfacing] and performed using 2-dimensional ultrasonic imaging, with a convex transducer 2–5 MHz (M-Turbo ultrasound system, FUJIFILM SonoSite Inc., Bothell, WA, United States). Bilateral imaging of the hemithorax (parasternal, mid-clavicular, anterior axillary line, and mid-axillary) from the second to fourth (fifth on the right side) intercostal spaces were performed, culminating in 28 zones. A B-line was defined as an echogenic, coherent, and wedge-shaped signal with a narrow origin in the near field of the image, spreading from the pleural line to the further border of the screen, which moves in concert with lung sliding (Lichtenstein, 2014). The same investigator (AL-S) performed all B-line measurements. The total number of B-lines in each zone was counted and summed to provide a total B-line score. For situations when the entire zone was full of B-lines (i.e., white-out) a maximum of 20 was assigned. B-lines are an index of extravascular lung fluid, which has been performed previously with high sensitivity and intra-patient reliability to fluid visible using radiographic imaging (Jambrik et al., 2004; Picano et al., 2006; Boussuges et al., 2011;

Lambrechts et al., 2011). As part of the evaluation, the participant was also asked for specific symptoms. Symptoms considered relevant were cough, mild-severe chest discomfort, tightness and/or irritation along the respiratory tract (i.e., throat to lungs) and hemoptysis. The participant was considered symptomatic if one or more of these symptoms was present. Post-dive evaluation and measurements were performed on the support boat for the competition.

In a subgroup of divers ($n = 6$), baseline B-line measurements were conducted before the competition, and with at least 12 h without diving activity.

Given the unavoidable range in measurement times (i.e., time from surfacing to measurement); a time-specific analysis was conducted – using pulse oximetry within 30 min as a cut-off. A total of 50 dives: 10 CNF, 21 CWT, and 19 FIM at a mean depth of 76 ± 21 m (range: 40–122 m) with pulse oximetry within 18 ± 6 min (range: 6–30 min) and B-lines within 41 ± 15 min (range: 17–82 min) were included. For correlational analysis (see section "Statistical analysis"), only the first dive for each diver were included (i.e., 36 dives), to ensure the independence of cases since each diver competed over several days.

As part of the competition, supplemental O_2 was available for divers upon surfacing, it was used in 36 dives (72%) and SpO_2 was measured 26 ± 11 min (range: 10–50 min) after exposure.

Statistical Analysis

Data was assessed for normality using Kolmogorov-Smirnov tests (IBM SPSS, Statistics, United States). Differences from baseline B-line score, the right vs. left lung and symptomatic vs. asymptomatic dives were evaluated using the Mann-Whitney test. Effect size (R) was calculated using the Mann-Whitney Z score, divided by the square root of the sample size (n). Correlations were evaluated using Spearman's (r_s) correlational analysis. All data is presented as mean ± SD and significance was assumed at $p < 0.05$.

RESULTS

B-Line Score and SpO_2

B-line score was negatively correlated with mean SpO_2 ($r_s = -0.335$, $p = 0.046$; **Figure 1A**) and minimum SpO_2 ($r_s = -0.491$, $p = 0.002$; **Figure 1B**). Across all zones, there were on average 35 ± 49 B-lines, with a mean and minimum SpO_2 of 96.4 ± 2.8 and 93.7 ± 4.3%, respectively.

Post-dive B-line score was positively correlated with depth ($r_s = 0.408$, $p = 0.013$; **Figure 2**). When intercostal regions II-IV were evaluated between sides (i.e., given intercostal region V is confounded by the heart on the left side), the right lung appeared to have a higher B-line score (19 ± 26 B-lines) following the dive compared to the left lung (9 ± 18 B-lines; $U = 784.5$, $p = 0.001$, $R = 0.322$).

Symptoms

When B-line and SpO_2 were grouped based on symptoms, symptomatic dives showed significantly lower SpO_2, higher B-line score and greater depth (**Figure 3** and **Table 1**). Mean SpO_2 also correlated positively with symptoms ($r_s = -0.421$, $p = 0.010$), and with depth ($r_s = -0.381$, $p = 0.022$). Minimum SpO_2 was negatively correlated with both symptoms ($r_s = -0.468$, $p = 0.004$) and depth ($r_s = -0.347$, $p = 0.038$).

Baseline and Post-Dive B-Lines

There was an increase in mean B-line scores from baseline at 5 ± 2 (range: 2–6; $n = 6$) to post-dive at 33 ± 46 (range: 1–183; $n = 50$; $U = 65.6$, $p = 0.012$, $R = 0.308$; **Figure 4**). Males (35 dives) demonstrated higher B-line scores (41 ± 50 B-lines) compared to females (15 dives; 9 ± 9 B-lines; $U = 340.5$, $p = 0.01$, $R = 0.362$). However, males had deeper dives (81 ± 20 m) than females (60 ± 13 m; $U = 131.5$, $p = 0.001$, $R = 0.460$).

DISCUSSION

Our main finding is that SpO_2 measured within 30 min of surfacing correlates inversely to the prevalence of B-lines. This shows that impairments in the recovery of SpO_2 during this period could reflect an excess of extra-pulmonary lung water. This relationship becomes further apparent as lower SpO_2 was associated with respiratory symptoms. Aligning with our hypothesis, the measurement of pulse oximetry shortly after a dive is a simple and valuable tool for breath-hold divers for identifying possible lung harm. Indeed, many athletes, coaches, and organizers have begun incorporating pulse oximetry monitoring; however, this is the first study to establish its correlation with extravascular lung water during an actual competition, along with a specific time for measurement.

The positive correlation between depth and B-lines score aligns with earlier studies (Frassi et al., 2008; Lambrechts et al., 2011), and those demonstrating an increase in the incidence of pulmonary edema, via reduced spirometric performance (Linér and Andersson, 2008). Depth is also associated with an increase in pulmonary edema-related symptoms (Lindholm et al., 2008; Cialoni et al., 2012). However, as also shown in our data, some susceptible individuals may present symptoms after relatively shallow dives (Cialoni et al., 2012).

Aligning with Linér and colleagues, our data shows a negative correlation between depth and SpO_2 (Linér and Andersson, 2008). While persistent post-dive hypoxemia may have multiple causes: (1) ventilation/perfusion mismatch due to atelectasis (Fahlman et al., 2009; Schipke et al., 2019). (2) increased right-to-left shunt; via increased strain on the right ventricle (Scherhag et al., 2005) and/or chronic pulmonary hypertension (Vestin, 2015), so if right atrial pressure becomes higher than left atrial pressure right-to-left shunting can occur (Layoun et al., 2017). A third cause could be diffusion limitation due to increased extravascular lung water (Picano et al., 2006; Boussuges et al., 2011; Picano and Pellikka, 2016). Finally, additional causes could be hypoventilation due to reduction in vital capacity, related to alveolar hemorrhage (Kiyan et al., 2001; Gouzi et al., 2007) or pulmonary barotrauma (Kiyan et al., 2001; Chung et al., 2010; Banham and Lippmann, 2019). Accordingly, persistent

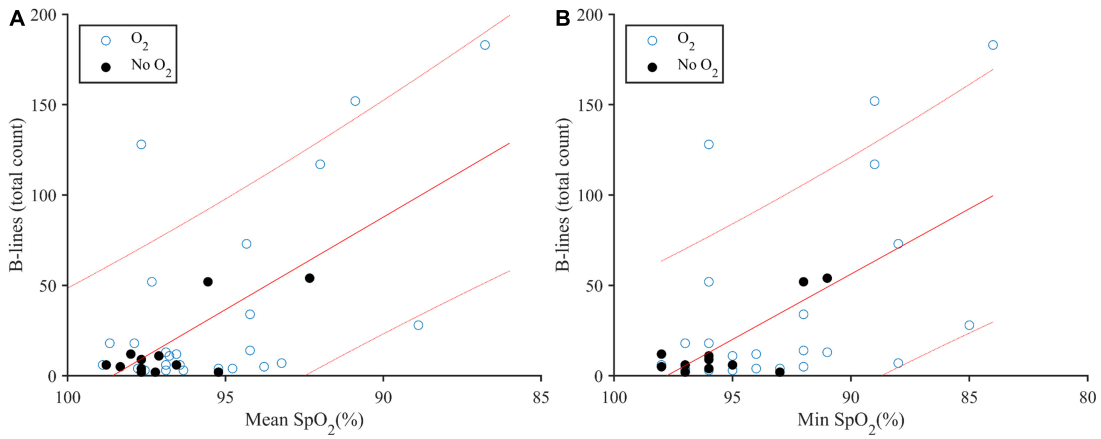

FIGURE 1 | (A) Correlation plot of B-lines and mean SpO_2 following deep breath-hold diving ($r_s = -0.335$, $p = 0.046$) for $n = 36$. Use of supplemental oxygen at the end of the dive is indicated by used (O_2; open circles) and not used (No O_2, closed circles). **(B)** B-lines and minimum (Min) SpO_2 following deep breath-hold diving ($r_s = -0.491$, $p = 0.002$) for $n = 36$. Linear regression (solid line) with 90% confidence intervals (dotted line).

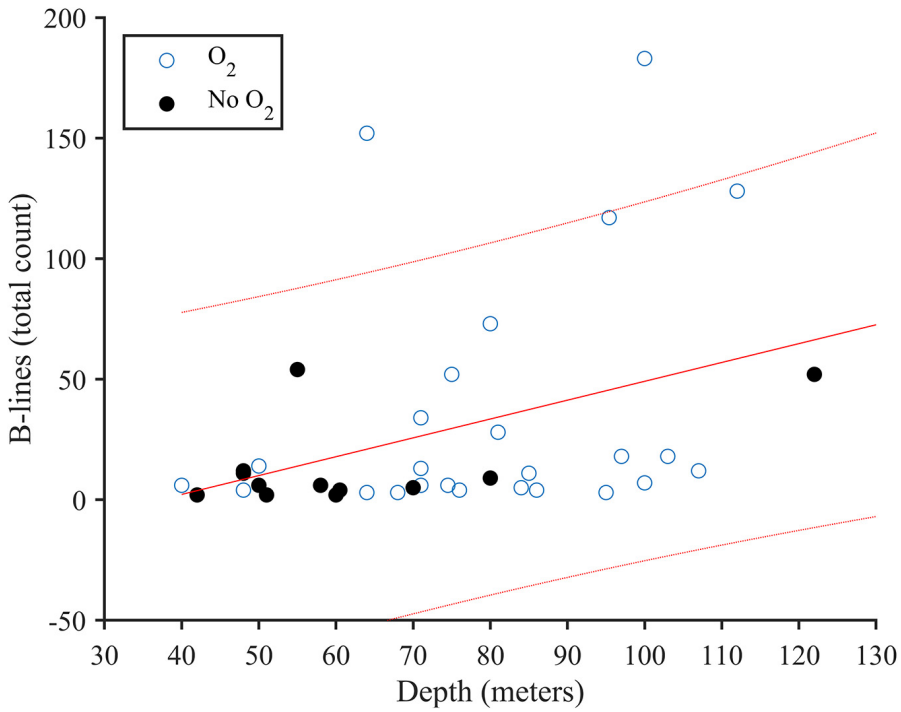

FIGURE 2 | Correlation plot of B-lines and diving depth ($r_s = 0.408$, $p = 0.013$) for $n = 36$. Use of supplemental oxygen at the end of the dive is indicated by used (O_2; open circles) and not used (No O_2, closed circles). Linear regression (solid line) with 90% confidence intervals (dotted line).

hypoxemia after diving, in a freediver, warrants a more in-depth analysis.

In addition, we found a clear difference in minimum SpO_2 between divers with or without pulmonary symptoms. Indeed, all symptomatic dives had SpO_2 below 95%, which further supports our main hypothesis that SpO_2 could be useful to detect cases. B-line prevalence has a high range of reporting, varying from 14 to 31% (Frassi et al., 2008; Linér and Andersson, 2008; Cialoni et al., 2012) that can be explained by different approach (prospective or retrospective) and in our group, it was 15%. If we use the value of minimum $SpO_2 \leq 95\%$ after a deep dive, the positive predictive value is 29% and the negative predictive value is 100%. In terms of safety, it is better to have false positives that can be discarded with further investigation, than to let divers with pulmonary edema resume diving too early.

The unique finding that the right lung demonstrated an increased B-line score compared to the left lung (when matched for zones), is difficult to discern. However, there are some reports

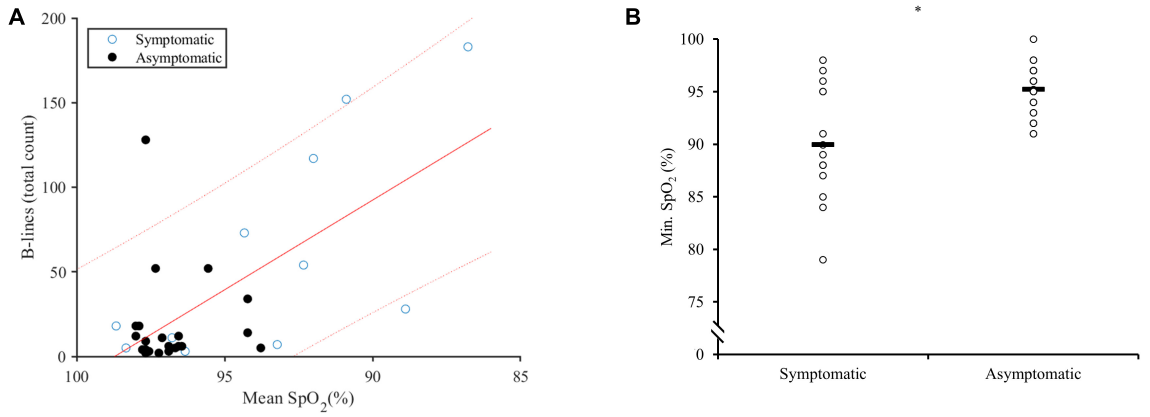

FIGURE 3 | (A) Correlation plot of respiratory symptoms and the relationship between B-lines and mean SpO_2. Symptomatic (open circles) and asymptomatic (closed circles). Linear regression (solid line) with 90% confidence intervals (dotted line). **(B)** Dot plot of minimum SpO_2 in symptomatic divers, versus asymptomatic divers. Horizontal line represents the means at 89.9 ± 5.4% for symptomatic and 95.2 ± 2.3% for asymptomatic divers. * Indicates $p < 0.05$ between groups.

of unilateral swimming-induced pulmonary edema (Mahon et al., 2002; Lund et al., 2003), which could be related to increased perfusion on dependent zones of the lung. In terms of the biomechanics of propulsion, important differences exist between each discipline (Schagatay, 2011), but we did not find differences among disciplines. When freedivers reach the bottom plate, they have to make a change of direction to start swimming upward, this turn requires some thoracic torsion. This movement produces a change in thoracic compliance which can lead to a heterogeneous distribution of transpulmonary pressure (Cortes-Puentes et al., 2015). While we did not record which arm the divers turned with, if our cohort was primarily right-handed, it is intriguing to postulate that this might account for the uneven distribution of B-lines between right and left lung.

Our study thus confirms previous studies documenting an increase in B-lines after breath-hold diving (Frassi et al., 2008; Lambrechts et al., 2011). A relevant discussion revolves around how many B-lines are meaningful in diagnosing pulmonary edema in freedivers. Usually, they are not pronounced in the normal lung (Dietrich et al., 2016) and can be seen in other conditions, such as atelectasis (Dietrich et al., 2016), pneumonitis, and fibrosis that are unrelated to pulmonary edema (Volpicelli et al., 2012; Soldati et al., 2016). However, an increase in B-lines does not always coincide with pulmonary edema-related symptoms (Linér and Andersson, 2008). But, the notion that B-lines increase after diving and usually disappear in 24 h (Frassi et al., 2008) reinforces that it is an indirect measure of extra-vascular lung water which has been confirmed on other causes of pulmonary edema (Dietrich et al., 2016; Picano and Pellikka, 2016; Assaad et al., 2018).

With males diving deeper than females in the current study, it was not possible to evaluate whether there is a difference between sexes in B-line prevalence. However, with sex-related structural and functional differences of the lungs (Dominelli et al., 2019), different stroke volume during apnea (Magnani et al., 2018), and the unique hormonal role of estrogen on the pulmonary vasculature (Lahm et al., 2008), this is a valuable question and warrants further investigation.

Interestingly, not all deep dives lead to a substantial increase in B-lines and there is considerable variation in B-line score between divers. For example, some dives over 100 m had B-line scores ≤20, which is comparable to most of the 60-m dives. Many inter-individual differences could influence the incidence of B-lines. On one hand, there could be possible modifiable factors as a pre-dive warm-up, discipline, ascent/descent speed, use of glossopharyngeal insufflation (Chung et al., 2010), depth training (Cialoni et al., 2012), involuntary breathing movements at depth (Kiyan et al., 2001; Koehle et al., 2005; Lambrechts et al., 2011) and rib cage compliance. On the other hand, unmodifiable factors could be psychological stress, cold water (Koehle et al., 2005), lung volumes (Lindholm et al., 2008), residual volume to total lung capacity ratio (Ferretti et al., 2012), previous acute lung injuries (Carter et al., 2014), comorbidities and medications (Koehle et al., 2005), and genetic predisposition (Cialoni et al., 2015).

Safety is crucial in freediving, and there are no guidelines for safe return-to-dive practices regarding pulmonary edema.

TABLE 1 | Symptomatology on post-dive measurements when pulse oximetry was collected within 30 min.

	Asymptomatic	Symptomatic	U	*p*-value	Effect size
Dives (count)	35	15			
B-lines (total count)	18 ± 27	67 ± 63*	118	0.002	0.433
TAS-B-lines (min)	43.2 ± 15.6	45.4 ± 14.7	239.5	0.624	0.069
Mean SpO_2	96.7 ± 1.7	93.8 ± 3.5*	133	0.006	0.388
Min. SpO_2	95.2 ± 2.3	89.9 ± 5.4*	108	0.001	0.467
HR (bpm)	92 ± 12	98 ± 12	193	0.141	0.208
TAS-P.Ox (min)	16.1 ± 6.1	20.0 ± 5.4*	169	0.047	0.281
Depth (m)	71 ± 20	88 ± 18*	135	0.007	0.382

Mean ± SD.
TAS, time after surfacing (i.e., the time between surfacing and measurement of B-lines and pulse oximetry [P.Ox], respectively).
*$p < 0.05$.

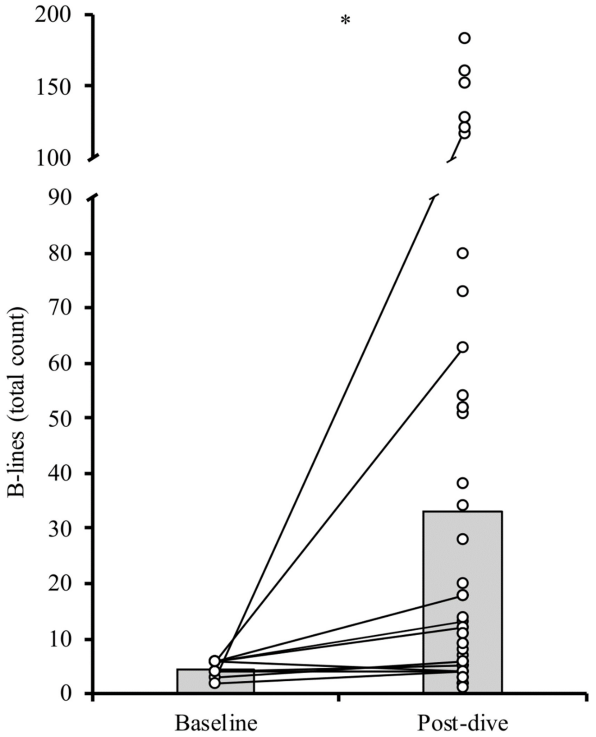

FIGURE 4 | Individual (circles) and mean (bar) B-line scores at baseline and following the competitive dives. Solid lines connect baseline with post-dive values in participants with a baseline score ($n = 6$). *$p < 0.05$.

The overarching goal of the current study was to evaluate if pulse oximetry can act as a reliable marker of potential damage, to provide divers with information to enable them to evaluate their symptoms, and competition safety team to detect possibly affected divers. A pulse oximeter is an easy to use low-cost option for divers to be able to self-evaluate their condition post-dive. Efficient recovery of SpO_2 is reliant upon efficient pulmonary gas exchange, which when impaired by fluid, prevents normal oxygenation, which is particularly dangerous for divers as they are severely hypoxic upon surfacing. Blood gas measurements of S_aO_2 and P_aO_2 in elite trained divers have been reported to drop well below 60% and 30 mmHg, respectively, during static apnea (Willie et al., 2015; Bain et al., 2017). Recently, the use of underwater near-infrared spectroscopy (NIRS) and pulse-oximetry reveals that even lower values develop after deep freediving (McKnight et al., 2021; Mulder and Schagatay, 2021). At such low levels of circulation oxygen, syncope can occur and the hypoxic blackout is not an uncommon occurrence (Pearn et al., 2015). Given the competitive progression of the sport of breath-hold diving, tools and guidelines aiming to improve diver safety are essential and important in keeping pace with the sport.

It is important to remark that there lacks a standardized definition of this type of pulmonary edema, induced by deep breath-hold diving. In the literature, there are a variety of terms in use, including squeeze (Lindholm and Lundgren, 2009; Schipke et al., 2019), pulmonary barotrauma, and immersion pulmonary edema (Moon et al., 2016; Kumar and Thompson, 2019). It has been suggested that a pathophysiological link exists between the pulmonary edema occurring during immersion and at high altitude. For example, high-resolution computed tomography imaging, albeit in a small cohort of patients ($n = 4$), suggests the heterogenetic blood flow response to hypoxia that occurs in high altitude pulmonary edema, may also be shared by those susceptible to immersion pulmonary edema (Lindholm et al., 2018). Furthermore, differences in lung structure and pulmonary lymphatics may also support a shared mechanism, especially in those susceptible to both high altitude pulmonary edema and immersion pulmonary edema (Carter et al., 2014). However, deep breath-hold diving imparts unique stress upon the pulmonary vasculature, since it is characterized by an increasing hydrostatic pressure that compresses both lung and thoracic cage (Fitz-Clarke, 2007). Due to differences in geometry and strain response (Plataki and Hubmayr, 2010; Andrikakou et al., 2016), the reduction in lung volume occurs at a quicker rate than the thoracic cage, and this interaction reduces intra-pleural pressure (Lai-Fook and Rodarte, 1991); thereby exaggerating the negative pleural pressure and increasing the hydrostatic pressure gradient. As the hydrostatic pressure gradient increases, any increase in the pulmonary artery pressure, due to the blood shift and hypoxic pulmonary vasoconstriction (Sylvester et al., 2012), could elevate pulmonary capillary pressure to the point of capillary stress failure (West et al., 1991). Together, this could lend support to distinct terminology of pulmonary edema because of breath-hold diving as a "depth-induced pulmonary edema."

Limitations

While our study is the first to correlate SpO_2 with the number of lung ultrasound B-lines, it is important to note some limitations.

The small cohort of baseline lung b-line scores in the majority of participants does not allow us to conclude with certainty that the elevation in the B-line score correlates directly with the dive. However, our data aligns with baseline B-line scores in other breath-hold diving cohorts (Frassi et al., 2008; Boussuges et al., 2011; Lambrechts et al., 2011; Patrician et al., 2021). Additionally, given the study was non-interventional and all measures were collected alongside the competition, variability in timing was unavoidable. However, competition diving provides valuable insight into the current limits of physiology, since divers are highly motivated and performing single dives of maximal (or even supra-maximal) effort. By excluding values too far from the intended timing, we believe the measurements were within acceptable time limits for these circumstances. Furthermore, the nature of lung B-lines (extravascular lung water), and how they affect SpO_2, would not disappear in one hour. Previous studies had shown that lung B-lines take around 24 h to disappear (Frassi et al., 2008; Cortellaro et al., 2017) and its reduction takes a few hours to occur (Picano et al., 2006). In that regard we will not expect substantial changes after 41 $\pm$ 15 min of surfacing, especially without any specific treatment (Dietrich et al., 2016), even oxygen inhalation does not seem to affect extra-vascular lung water (Pratali et al., 2012). Due to the lack of standardization of the dives (regarding warm-up, depth, and discipline), all

correlations have to be seen with caution and our findings require confirmation in a more controlled setting.

Regarding SpO_2, it is known that readings can be inaccurate when compared with SaO_2 from blood gases (Wilson et al., 2010; Nitzan et al., 2014). For example, the used device has reported inaccuracy of 3.29% (95% CI 2.39–4.20%) during moderate hypoxic conditions (Ross et al., 2013). Additionally, vasoconstriction can also affect the readings (Wilson et al., 2010), but its effects last only 60 s on simulated dives (Heistad et al., 1968); on the other hand, sympathetic nerve activity returns to normal values within seconds after resuming breathing and arterial blood pressure returns rapidly to normal values once respiration is resumed (Ferrigno et al., 1997). As we measured SpO_2 within 18 $\pm$ 6 min after surfacing and avoided recording from cold fingers/hands, we are confident in mediating the confounding influence of peripheral vasoconstriction. Therefore, we believe that persistent desaturation within 30 min will show real cases instead of false positives. However, as SpO_2 is a surrogate for SaO_2 and can be limited immediately post-dive, confirmation of these findings with arterial blood gas sampling is an important next step.

CONCLUSION

SpO_2 after dives was associated with ultrasound lung B-lines score, suggesting that SpO_2 via pulse oximetry could be a useful tool, when measured within 30 min after surfacing, taking into account the intrinsic inaccuracy of the device. Ultrasound lung B-lines correlates with diving depth, confirming that extra-vascular lung water increases with deeper dives. Our findings can be important to increase freediving safety, by identifying injured divers to prevent them from continuing without proper recovery. Our data support the use of a $SpO_2 < 95\%$ to identify possibly injured divers for further medical evaluation. Failure to identify these divers increases the risk of hypoxic syncope. This proof of concept study requires further research to evaluate its practical application.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors upon reasonable request.

ETHICS STATEMENT

The study was reviewed by the local research ethics board and conformed to the Declaration of Helsinki. The study was also approved by and conducted in conjunction with the competition organizing committee.

AUTHOR CONTRIBUTIONS

AL-S and ES contributed to the conception of the study. AP, AL-S and ES contributed to the data acquisition. AP, FP and ES contributed to data analysis and manuscript writing and review. All authors approved the submitted version.

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Underwater and Surface Swimming Parameters Reflect Performance Level in Elite Swimmers

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Although the role of underwater phases is well-known, no study has taken an interest in describing and quantifying the distance and time spent in apnea as a condition for optimal performance. This study aimed to investigate the impact of time and distance spent underwater and surface parameters on the swimming performance of elite swimmers. The performances of 79 swimmers in 100-m freestyle were analyzed (short-course). The underwater and spatiotemporal parameters of three groups have been recorded: finalists of the 2018 World Swimming Championships (WORLD), French swimmers who reached a 100 m performance time under 50 s at the 2018 French National Championships (UND50), and those who reached a 100 m performance time above 50 s (UP50). The WORLD group spent more distance underwater (37.50 ± 4.92 m) in comparison with UND50 (31.90 ± 4.88 m, $p < 0.05$) and UP50 (31.94 ± 4.93 m, $p < 0.01$) groups. The total percentage of non-swimming time was higher for WORLD (39.11 ± 4.73%) vs. UND50 (34.21 ± 4.55%, $p < 0.05$) and UP50 (33.94 ± 5.00%, $p < 0.01$). In addition, underwater speed was higher for WORLD (2.54 ± 0.05 m/s) compared with UND50 (2.46 ± 0.09 m/s, $p < 0.05$) and UP50 (2.38 ± 0.11 m/s, $p < 0.01$). Three parameters among the underwater phases (i.e. distance underwater, speed underwater, and total percentage of non-swimming time) determine the 100-m freestyle short course performance. These data suggest an appropriate focus on specific apnea training to improve underwater skills during short-course swimming performances.

Keywords: apnea training, breath-holding, immersion, freestyle swimming, elite swimmers, dolphin kick, apnea plan

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INTRODUCTION

Swimming competition analysis is highly documented, and numerous studies have investigated the features of spatiotemporal parameters during swimming competitions to determine their influence on the performance of swimmers (Craig et al., 1985; Huot-Marchand et al., 2005; Hellard et al., 2008).

A swimming race includes the swimming phases and the so-called non-swimming phases, namely, the start and the turns, such as underwater swimming segments. While, the work of

Tor et al. remains the link between start time and swimming performance for high-level swimmers (Tor et al., 2015), a recent paper has highlighted the correlation between the performance and the start and the turns, especially for the 100 m races (Morais et al., 2019a). Furthermore, the time after 15 m after each length depends on under or above water swimming (Arellano et al., 1994; Veiga et al., 2014a, 2016). During 200 m races, the 15 m time after the start is better for the swimmers to cover in apnea large underwater distances (Veiga and Roig, 2015), benefiting from the reduction of wave drag under the water (Vennell et al., 2006).

According to the literature, the duration of underwater swimming seems to be more related to the performance in 200 m than in 100 m events (Veiga and Roig, 2015). The modifications on the start or turn distances (especially in the last turn) could represent the overall time improvements of a practical importance for 200 m elite swimmers (Veiga et al., 2016). Conversely, in these 100 m events, the average velocity of these underwater sections seems to be a key for the race performances. Indeed, despite not spending longer underwater distance, the faster swimmers at the World Championships 100 m events traveled with faster velocities during the freestyle and breaststroke start than slower swimmers (Veiga et al., 2016). Changes in the start or turn velocities could represent moderate time improvements in most of the 100 m events (Veiga et al., 2016). In short-course events, the greater contribution of non-swimming phases could emphasize a more significant effect on the performance where underwater phases can represent up to 60% of the race distance (FINA rules).

The underwater phases require high skills when applying the dolphin kick technique, since the leg extension from above the ankle to the toes plays a huge part in producing a propulsive force (von Loebbecke et al., 2009). It depends on the importance of the trunk undulation (especially the chest bending) (Nakashima, 2009), and the ankle muscle strength and flexibility (Willems et al., 2014; Shimojo et al., 2019).

In addition, swimming such a distance underwater enhances the interest toward the physiological repercussions on the swimmer, during the non-swimming phases in an apnea situation. Apnea induces a typical cardiovascular response called a diving response, such as bradycardia (Foster and Sheel, 2005), which can compete with exercise tachycardia (Wein et al., 2007; Alboni et al., 2011) during the underwater apnea stages. If some swimming studies have focused on the physiological repercussions of apnea (vs. breathing) in a situation of surface swimming (Guimard et al., 2014, 2017, 2018), to our knowledge, one study has considered the underwater physiological aspects but without determining the influence of the non-swimming phases (Rozi et al., 2018).

The non-swimming phases seem essential during a 100 m short event, but these phases are most often used and worked on empirically by the swimmers and coaches. This study aimed to analyze the underwater and surface swimming parameters according to the level of performance of elite swimmers in a 100 m freestyle competition, and thus to highlight the strategies used in a competitive situation.

Abbreviations: UP50, French swimmers who performed the 100 m with a performance time upper to 50 s; UND50, French swimmers who performed the 100 m with a performance time under 50 s; SC, stroke count; SL, stroke length; SR, stroke rate; WORLD, world finalists.

MATERIALS AND METHODS

Participants

Data from the 100 m freestyle have been recorded in male swimmers for two events: (1) the 2018 Short Course World Championships (WORLD) ($n = 8$) in Hangzhou (China) and (2) the 2018 French National Championships ($n = 71$) in Montpellier (France). The two groups were divided among the French National Championships: the swimmers who reached a 100 m performance time under 50 s (UND50) ($n = 21$) and those who reached a time upper to 50 s (UP50) ($n = 50$). The swimming speed value of 2 m/s seems a threshold value to reach. The procedures have been conducted with adequate understanding and written consent of the participants and the study was carried out by the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Video Collection

Three cameras (Sony FDR AX700, Tokyo, Japan) have been positioned perpendicularly to the length axis of the pool, at 5, 12.5, and 20 m from the starting block. The film of each race has been analyzed with dedicated software to calculate the performance metrics of each swimmer.

Data Collection and Data Treatment

Race analysis software (Espadon, Actriss, Brest, France) was used for the calibration and the image was processed by manual digitalization, to obtain the time and distance of each stroke cycle as previously described (Hellard et al., 2008). The software converts the pixels into distance (meters, SI units), based on the calibration made using four poolside marks in the swimming pool (wall and lane). The video analyst made this calibration and then manually digitalized the head position at the beginning of each stroke cycle (right-hand entry) to obtain the time and distance of each stroke cycle, as already described by Hellard et al. (2008). The speed, stroke rate (SR), stroke length (SL), and stroke count (SC) were computed for each 50 m lap. In order to measure all the stroke variables, the time and spot of the first and last arm water entry for each lap was calculated, giving the beginning and the end of each "swim-time" period. SC is the total number of arm entries on the water surface. SL is calculated by dividing the free-swimming distance by SC. SR is obtained by dividing the free-swimming time by SC. SI (stroke index) is the product of the swimming speed (lap distance divided by lap time) and SL. The underwater distance is equal to the distance between the wall to the head of the swimmer at the moment of stroking resumption (Veiga and Roig, 2015). The underwater time (expressed in seconds) is equal to the time to cover that underwater distance. The underwater speed is equal to the official split time at the wall contact and the split time at the stroking resumption (Veiga and Roig, 2015). Finally, the non-swimming time (expressed in percentage) is equal to the total time minus

the free-swimming time, divided by the total time. In other words, the non-swimming time includes all the non-swimming segments of the race as start, underwater, and turns.

The assessment of performance metrics—used with the help of the software—was managed by a video analyst with a scientific background who is part of the French swimming staff.

Statistics

For all the variables, descriptive statistics (mean and standard deviation) were performed. Normal Gaussian distribution of the data was verified by Shapiro–Wilk's test and homogeneity of variance by a modified Levene's test. The differences between the groups (UP50 vs. UND50, UP50 vs. WORLD, and UND50 vs. WORLD) were compared using an unpaired Student's *t*-test. Null hypothesis was rejected at $p < 0.05$. The statistical analyses were undertaken using the software package STATISTICA (version 8.0, Statsoft, Tulsa, OK, USA).

RESULTS

The mean swimming performance was higher for the swimmers of WORLD with a total time matching 97.48% of the world-record, whereas the swimmers of UND50 and UP50, respectively, represented 91.27% and 88.27% ($p < 0.001$). All the swimmers of WORLD reached a 100 m performance time under 47 s.

Table 1 provides a comparison of the underwater and surface strategies for the 100-m freestyle swimming among the swimmers of WORLD, UND50, and UP50. **Figure 1** details the differences among the groups for each lap of the 100 m freestyle race.

The total underwater distance (m) was higher in the WORLD compared with UND50 ($p < 0.05$) and UP50 ($p < 0.01$). Similar results were observed for the total non-swimming time (%), which was higher in the WORLD compared with the UND50 ($p < 0.05$) and UP50 ($p < 0.01$). The mean underwater speed was higher in the WORLD compared with the UND50 ($p < 0.05$) and UP50 ($p < 0.05$), while the UND50 had a greater mean underwater speed than UP50 ($p < 0.01$).

Figure 1 shows that the WORLD had a higher underwater distance than the UP50 for each lap of the 100 m, and it was more significant for the last two laps of the 100 m race ($p < 0.01$). Furthermore, WORLD had a higher underwater distance than the UND50 group in the first lap ($p < 0.01$), the third lap ($p < 0.05$), and the final lap ($p < 0.01$), whereas no difference was observed during the second lap.

The underwater speed was higher for the WORLD than for the other groups in the first lap (corresponding to the underwater phase after the dive start) ($p < 0.01$). However, no difference in underwater speed was observed between the UND50 and WORLD in the following three laps. The non-swimming time (%) was superior for the WORLD than for the UP50 in each lap of the race, whereas only the first and the fourth laps were higher for the WORLD than for the UND50.

Besides, the main outcome observed was the higher SR for the WORLD than for the UND50 ($p < 0.01$) and the UP50 ($p < 0.05$). In addition, the total SC was lower for the WORLD than for the UND50 ($p < 0.01$) and UP50 ($p < 0.01$). Also, **Figure 1** highlights that there were no SL differences between the groups for each lap of the 100 m race. Finally, the WORLD had a higher SR than the UND50 and UP50 in the first lap ($p < 0.01$ and $p < 0.05$, respectively) and the third lap ($p < 0.01$).

DISCUSSION

The main outcome of this study investigation is that the difference in performance over 100 m is mainly in the non-swimming phases.

To our knowledge, this study was the first to examine the underwater and spatiotemporal parameters strategies depending on the three levels of swimming performance. Few differences occurred in the spatiotemporal parameters. The current study results show that the elite swimmers maintain a higher SR than the national level swimmers during the whole 100 m contest and both the groups obtain similar SL, while the UP50 has a smaller SL compared with the WORLD and UND50. This represents a new analysis of the swimming performance, since most of the studies have reported that the best swimmers have a greater SL than the other groups (Huot-Marchand et al., 2005). Such observation may challenge the idea of maximizing propulsion to improve swimming performance. Another study has already reported similar results to our work (Hellard et al., 2008), affirming that the best 200-m swimmers were much more distinguished by a higher SR, than a greater distance per cycle.

In our opinion, an SL "plateau" for the elite swimmers could exist, therefore to increase their swimming speed, swimmers would have to increase their SR. Otherwise, it is possible that the WORLD swimmers are able to sustain a higher SR because of their smaller SC, given by their larger underwater distance. As proposed by Alberty et al. (2008), the high metabolic demand required for an intense swim task is a restriction that could alter the stroke rate to maintain the required pace during the race. Thereby, the peripheral fatigue could be reduced during non-swimming phases by keeping the arms passive, to allow swimmers to keep up arm strength during the rest of the race. Ohkuwa and Itoh (1992) showed that the lactate in the blood predominantly originates from the arm muscle groups. Therefore, keeping a hydrodynamic position with a lengthened use of the leg muscles by the dolphin kicks technique could be a solution to have a positive impact on peripheral fatigue.

The results of this study highlight significant differences in the SC, particularly, during the first and fourth 25 m (as shown in **Figure 1**), not only with a smaller SC for the WORLD but also with a decrease in the total SC level. The differences noticed are linked to the larger underwater distance covered by the WORLD, allowing them to reduce the swimming distance. However, it should be noted that there were no significant results for the second and third turns. Besides, it is established that the last turn allows the speed of the swimmer to be maintained among the elite swimmers (Veiga and Roig, 2015). Mauger et al. (2012) have also shown that "fast-start" and "parabolic" (fast-start, speed decrease during the race, and a higher finish velocity) strategies are favored among the competitors in a 400-m swimming race. This statement confirms our results, with the same pattern as for the race management. Therefore, the two central laps do not

TABLE 1 | Comparison of mean ± *SD* for performance, underwater and spatio-temporal parameters among the world finalists (WORLD) vs. French swimmers under 50 s (UND50) vs. French swimmers upper to 50 s (UP50) during the 100 m freestyle.

Group		Performance		Underwater parameters			Spatio-temporal parameters		
		Total time (s)	Total underwater distance (m)	Mean underwater time (s)	Mean underwater speed (m/s)	Non-swimming time (%)	Mean stroke rate (c/min)	Mean stroke length (m/c)	Total stroke count
WORLD	Mean	46.10	37.50	14.71	2.54	39.11	53.32	2.27	53.38
	SD	0.40	4.92	2.10	0.05	4.73	2.54	0.13	3.62
UND50	Mean	49.24***	31.90*	13.02	2.46*	34.21*	50.46**	2.27	58.14**
	SD	0.59	4.88	2.29	0.09	4.55	2.39	0.11	4.23
UP50	Mean	50.91***###	31.94**	13.50	2.38**##	33.94**	50.70*	2.19#	60.52**
	SD	0.73	4.93	2.45	0.11	5.00	3.03	0.14	5.91

Significant difference between WORLD vs. UND50 and WORLD vs. UP50: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Significant difference between UND50 vs. UP50: $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$.

seem to impact the number of arms strokes. The skills developed at the start of the race and during the fourth turn are probably the most advantageous for elite swimmers. Hence, it is important to suggest possible physiological adaptations to apnea in these swimmers as observed in apnea-trained athletes, who are able to perform dynamic apneas with attenuate fatigue signs (Joulia et al., 2009). Then, elite swimmers maintain a high-intensity level during the last immersion at the turn in order to delay the effects of fatigue.

Major differences between the groups are observed on non-swimming phases during the 100 m race. The WORLD spent more time in apnea covering a greater distance underwater and also spent more total distance underwater than the UND50 and UP50. It agrees with previous studies, and it can explain why international swimmers are faster than the others (Veiga et al., 2014a; Veiga and Roig, 2015). Thus, a high level of underwater skills would allow the swimmer to cover a long underwater distance to benefit from a reduction of wave resistance below the surface and thus increase the speed (Vennell et al., 2006). In addition, the time after 15 m (highly influenced by the underwater phase) depends on the level of competence (Arellano et al., 1994; Veiga et al., 2014a, 2016). It appears predominant in short-course swimming, where the underwater parts could represent up to 60% of the distance covered by the best swimmers in the world (FINA rules). However, it should not be generalized, because a lower level of competence supports a reduction in the time spent underwater after the start (Nazeer et al., 2016). We could explain such results by a reduced general control of the non-swimming phases and, therefore, a voluntary choice to swim faster and waste less time underwater. Additionally, the underwater distance differences between the groups clearly increase in the second part of the race. This is a clear characteristic of the higher skilled performers who maintain greater stability on underwater parameters as fatigue appears (Veiga et al., 2014b; Morais et al., 2019b). It could partly explain the high-level competency of the elite swimmers, who are able to keep control of the non-swimming phases, which widen the gap with national level swimmers, as the race progresses (Huot-Marchand et al., 2005).

Moreover, although the underwater distance is greater for the WORLD, the underwater speed is only higher after the dive (not the case for the next three turns of the race). This is probably due to the better start skills of the elite swimmers (Tor et al., 2015). In addition, we showed that, despite the same underwater speed, international swimmers than national swimmers moved a greater distance, suggesting that they are more efficient during underwater parts. This efficiency would depend on both the biomechanics and physiological capacities to build a better underwater speed and performance. As suggested by Veiga and Roig (2015), the underwater phases would require great skills in terms of technical control of the dolphin kick (Veiga and Roig, 2015), but these non-swimming parts are fully covered in apnea.

To our knowledge, no study had yet quantified the total non-swimming time during a swim race. This study results show that the elite swimmers have a greater percentage of non-swimming time during the race, approaching 40% of the total race time. Therefore, the technical skills of the non-swimming phases do not seem sufficient to optimize the performance and the individual strategy. Indeed, swimming 40% of the time underwater enhances the interest in the physiological repercussions on the swimmer, during the non-swimming phases in an apnea situation. It is well-known that apnea induces a typical cardiovascular response called a diving response, such as bradycardia (Foster and Sheel, 2005) and vascular adjustments (Joulia et al., 2009) that are important defense mechanisms of the body against hypoxia (Alboni et al., 2011). The diving response and skeletal muscle activity exert opposite influences on the heart and peripheral circulation (Wein et al., 2007; Alboni et al., 2011). The best swimmers are thus the ones with the longest apnea phase. They are likely managing more efficiently, the conflictual requirements between physical activity (i.e., bringing oxygen through the blood supply to the skeletal muscles) and apnea (i.e., bringing oxygen through to the heart and brain). Therefore, during the non-swimming apnea stages, since the

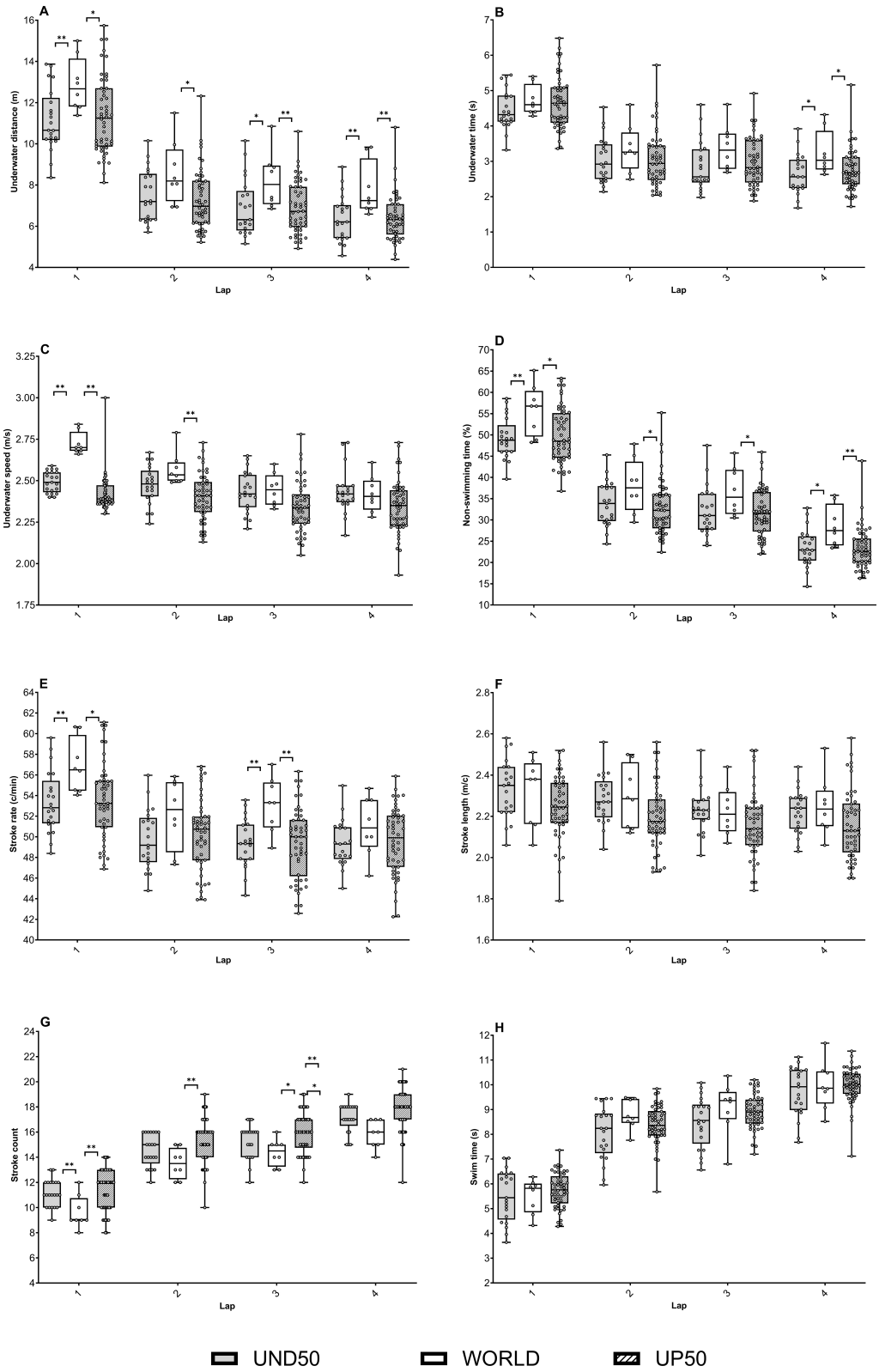

FIGURE 1 | Underwater and spatiotemporal parameters for the world finalists (WORLD), French swimmers under 50 s (UND50), and French swimmers upper to 50 s (UP50) over each lap of the 100-m freestyle race. **(A)** Underwater distance, **(B)** underwater time, **(C)** underwater speed, **(D)** non-swimming time, **(E)** stroke rate, **(F)** stroke length, **(G)** stroke count, and **(H)** swim time. Significant difference between the WORLD vs. UND50 and the WORLD vs. UP50: $^{*}p < 0.05$, $^{**}p < 0.01$.

conflict between the two inputs could appear, the physiological capacities involved might be more significant. Furthermore, apnea, by stopping the vital breathing function constitutes a psycho–physical stress coupled with repeated and prolonged apnea intervals and the intense dynamic exercises as suggested by Rodríguez-Zamora et al. (2014). This aspect needs to be considered when interpreting the differences noted regarding the non-swimming phases according to the performance levels.

The originality of this study is that it was performed during the competition conditions. On the other hand, the main limitation is the difficulty to measure the physiological data required in this ecological condition. Another limitation is the small differences observed between the UND50 and UP50, suggesting the necessity to introduce another lower-level swimming group such as regional level.

In conclusion, the current study presents new insights on the underwater parameters according to the level of swimming of the participants. Indeed, the international swimmers have covered more distance underwater, with a higher total non-swimming time, introducing new key data in the swimming performance. Coaches should, therefore, precisely monitor the race stages, with a focus on the underwater parts. Finally, various strategies of the underwater and surface parameters could be tested in order to reach optimal swimming performance. Thus, it is advisable to initiate further studies on the physiological dimension of apnea coupled with the biomechanical variables to investigate and understand the mechanisms involved during the non-swimming phases without neglecting the link with the swimming parts that precedes and follows the apnea but also to customize the training.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

RP and AG conceived and designed the project. RP performed the data collection. RP, AG, and GP performed the data analysis and the interpretation of the data. RP, AG, GP, FJ, and CS contributed to the preparation of the manuscript. All authors contributed to the article and approved the submitted version.

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A Baseline Model For Estimating the Risk of Gas Embolism in Sea Turtles During Routine Dives

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Sea turtles, like other air-breathing diving vertebrates, commonly experience significant gas embolism (GE) when incidentally caught at depth in fishing gear and brought to the surface. To better understand why sea turtles develop GE, we built a mathematical model to estimate partial pressures of N_2 (PN_2), O_2 (PO_2), and CO_2 (PCO_2) in the major body-compartments of diving loggerheads (*Caretta caretta*), leatherbacks (*Dermochelys coriacea*), and green turtles (*Chelonia mydas*). This model was adapted from a published model for estimating gas dynamics in marine mammals and penguins. To parameterize the sea turtle model, we used values gleaned from previously published literature and 22 necropsies. Next, we applied this model to data collected from free-roaming individuals of the three study species. Finally, we varied body-condition and cardiac output within the model to see how these factors affected the risk of GE. Our model suggests that cardiac output likely plays a significant role in the modulation of GE, especially in the deeper diving leatherback turtles. This baseline model also indicates that even during routine diving behavior, sea turtles are at high risk of GE. This likely means that turtles have additional behavioral, anatomical, and/or physiologic adaptions that serve to reduce the probability of GE but were not incorporated in this model. Identifying these adaptations and incorporating them into future iterations of this model will further reveal the factors driving GE in sea turtles.

Keywords: Physiology, ecological modeling, fisheries, decompression sickness, conservation, dive behavior

INTRODUCTION

Until the early 2000s, it was assumed that most air-breathing marine vertebrates were not susceptible to gas embolism (GE) or its associated diseases (Jepson et al., 2003). However, several studies have now provided direct evidence of GE in cetaceans, pinnipeds, and sea turtles (Van Bonn et al., 2011, 2013; Dennison et al., 2012a,b; Crespo-Picazo et al., 2020). Under natural conditions, most air-breathing marine vertebrates likely manage the risk of GE through a combination of behavioral, anatomical, and physiological adaptations (García-Párraga et al., 2018b; Fahlman et al., 2020, 2021). Yet there appears to be significant increase in the potential for GE if animals make abnormally rapid ascents after diving (Fahlman et al., 2021). This could be caused by animals being caught incidentally in fishing gear (Fahlman et al., 2017) or if they are disturbed by acoustic sources such as mid-frequency sonar (Tal et al., 2015). Severe cases of GE can even lead to immediate or delayed mortality after several days (Parga et al., 2020). Consequently, GE likely poses an underestimated threat to marine animals that are subject to high levels of fisheries bycatch such as sea turtles (Wallace et al., 2010, 2013).

The formation of gas emboli is driven by changes in gas dynamics associated with the variable hydrostatic pressure that an animal experiences while diving. During the descent, hydrostatic pressure increases in proportion to depth. For air-breathing vertebrates, the increased pulmonary gas tensions cause the gaseous N_2, O_2, and CO_2 in the lungs to dissolve into solution within the pulmonary circulatory system (Fahlman et al., 2006, 2021). The gases are then transported *via* the vascular system throughout the various tissues of the body (Fahlman et al., 2006, 2009). The amount of gas that dissolves into the tissues during a dive is, therefore, influenced by the level of gas exchange in the lungs, cardiac output (heart rate and stroke volume), blood flow distribution, and tissue perfusion (Fahlman et al., 2006, 2009, 2018). Initially, the rate at which gas diffuses across the lungs increases with depth as higher hydrostatic pressures increase the quantity of gas forced into solution in the blood (Scholander, 1940; Berkson, 1967). However, if hydrostatic pressures continue to increase, the lung alveoli (or ediculi and faveoli in reptiles) compress and eventually collapse (Bostrom et al., 2008; Fahlman et al., 2009; Mcdonald and Ponganis, 2012). When all the alveoli have collapsed, gas exchange in the lungs ceases. Many diving animals also further reduce the rate at which gases dissolve in the their tissues through changes in circulation and perfusion (Fahlman et al., 2006). Specifically, some animals reduce cardiac output and blood flow to the lungs while simultaneously redistributing blood flow to O_2-demanding tissues (Scholander, 1940).

On ascent, diminishing hydrostatic pressures decrease gas tensions and so the gases come out of solution. If this process occurs faster than the animal can transfer the excess blood gas to the lungs, intravascular gas bubbles will form (Fahlman, 2017; Fahlman et al., 2021). While minor GE can be tolerated, serious cases can damage neural and more vascularized tissues. In turn, this can lead to impaired motor skills, loss of consciousness, paralysis, and even death (van Hulst et al., 2003; Dennison et al., 2012b; García-Párraga et al., 2014).

Several recent studies have confirmed that GE in sea turtles is a wide-spread phenomenon associated with fisheries bycatch (García-Párraga et al., 2014; Crespo-Picazo et al., 2020; Parga et al., 2020) and can also occur in other interactions with submerged gear such as Hopper dredges (Harms et al., 2020). It has also been shown that the depth of capture is a key component in determining the likelihood of GE. Loggerhead turtles (*Caretta caretta*) caught by Mediterranean trawlers and gillnets at depths exceeding 65 m were 50% more likely to suffer from fatal GE than those caught at shallower depths (Fahlman et al., 2017). To understand why turtles are susceptible to fisheries-induced GE, we must first understand tissue gas dynamics during normal diving behavior. For example, it is possible that sea turtles live with elevated tissue N_2 levels, comparable to that of a saturation diver (Brubakk et al., 2011). If so, even minor changes to routine diving patterns or disruption to those anatomical or physiological adaptations governing cardiac output and blood flow could lead to GE.

Our goal in this study was to develop a baseline model to estimate the risk of GE formation in sea turtles. To build this model, we built upon a published model for estimating gas tissue N_2, O_2, and CO_2 tensions of marine mammals and penguins (Fahlman et al., 2006, 2007, 2009, 2018, 2021) and adapted it for use with loggerhead turtles, green turtles (*Chelonia mydas*), and leatherback turtles (*Dermochelys coriacea*). While there are many anatomical and physiological differences between marine mammals/penguins and sea turtles, building off these earlier models provided us with an initial framework for modeling gas dynamics that had been previously validated for use with air-breathing vertebrates. We parameterized the model based on estimates of key tissue compartment volumes in sea turtles derived from necropsies and computed tomography. We also gleaned other parameters on sea turtle metabolic rate and cardiac output data from the published literature. Next, we used this model to estimate partial pressures of N_2 (PN_2), O_2 (PO_2), and CO_2 (PCO_2) in various body compartments during routine dive patterns for each turtle species. Finally, we used this model to investigate whether (1) the percentage of body-fat (as a proxy for body-condition) and (2) cardiac output influenced the risk of GE. We chose to investigate body-fat as it changes the dynamics of the body N_2 stores and has been considered as a potential risk variable in GE in humans (Lam and Yau, 1989; Schellart et al., 2012) and cetaceans (Fahlman et al., 2021). In addition, the volume of body fat is strongly influenced by reproduction

Abbreviations: ATA, Atmospheres Absolute; DVfA, Faveolar Volume; GE, Gas Embolism; Mb, Myoglobin; Pamb, Ambient Partial Pressure; PN_2, Partial Pressure of N_2; PO_2, Partial Pressure of O_2; PCO_2, Partial Pressure of CO_2; $\dot{Q}_{tot}$, Cardiac Output; TDR, Time-Depth Recorder; TLC, Total Lung Capacity; VD, Volume of Dead Space in the Trachea and Bronchi; VfAmax, Maximum Faveolar Volume; s $\dot{Q}_{tot}$, Mass-Specific Cardiac Output.

in sea turtles (Davenport et al., 2011). We chose to investigate cardiac output as modeling efforts for marine mammals have shown this to be a key component influencing the risk of GE (Fahlman et al., 2006, 2009, 2021; Hooker et al., 2009).

MATERIALS AND METHODS

Data Sets

We analyzed time-depth recorder (TDR) datasets from three loggerhead, six leatherback, and four green turtles (**Table 1**). The TDRs deployed on loggerhead turtles were Satellite Relay Data Loggers (SRDL–Sea Mammal Research Unit, United Kingdom), while MK10 tags (Wildlife Computers, United States) were used for both leatherback and green turtles. The loggerhead TDRs were attached to the top of the carapace using epoxy (for details, see Patel et al., 2018). For leatherback and green turtles, the TDRs were tethered to the posterior position of the carapace (for details, see Robinson et al., 2016). The loggerhead turtles were sub-adults sampled at foraging areas in the Mid-Atlantic Bight, while the leatherback and green turtles were inter-nesting females sampled on the east coast of South Africa and the northwest coast of Costa Rica, respectively (**Table 1**). The TDRs recorded depth to within 1 m accuracy at time-intervals of either 4 or 10 s. We standardized the sampling interval for all TDRs by interpolating the data to 1 s intervals.

TDR datasets ranged in duration 187–476 days for loggerhead turtles, 10–34 days for leatherback turtles, and 11–54 days for green turtles. As loggerheads are known to modify their diving behavior in response to seasonal variation in water temperatures (Iverson et al., 2019) and conduct extended "hibernation" dives during winter (Hochscheid et al., 2005), we only analyzed loggerhead data from the first 46 to 139 days (**Table 1**). This ensured that all data were collected during the summer months (May to November) when tag-derived sea surface temperature values were between 19 and 26°C. In contrast, tracking duration was short enough for leatherback and green turtles to assume that there were no significant changes in diving behavior due to seasonal factors within the available datasets. Moreover, all the leatherback and green turtles' data were collected from tropical or sub-tropical habitats, where the sea surface temperature values only varied between 24 and 29°C. The loggerhead TDR data have been previously published in Patel et al. (2018). The leatherback and green turtle data have been published in Blanco et al. (2013), Robinson et al. (2017), and Clyde-Brockway et al. (2019).

Model

To estimate gas dynamics in diving sea turtles, we adapted a model that was developed for estimating blood and gas tissue N_2, O_2, and CO_2 tensions in marine mammals and penguins (Fahlman et al., 2006, 2007, 2009, 2018, 2021). In the model, gas exchange first occurs between the respiratory system and arterial blood, and then between the arterial blood and four compartments: (1) the brain; (2) fat and bone; (3) the central circulatory system, which included the heart, kidney, liver, and digestive tract but not blood; and (4) muscle, which included muscle, skin, connective tissue, and all other tissues and organs that were not included in the other compartments. Gas exchange next occurs between the four compartments and the mixed venous blood, and finally between the mixed venous blood and the respiratory system.

Gas exchange was driven by partial pressure/tension gradients as detailed in Fahlman et al. (2006), and a physiologic pulmonary shunt was allowed at increasing pressures due to alveolar compression (Bostrom et al., 2008; Fahlman et al., 2009). While

TABLE 1 | Time-depth data used in gas dynamics model. Data were analyzed from three loggerhead, six leatherback, and four green turtles.

Species	*ID*	*Body mass* (kg)	Life-stage/Sex	Sampling frequency(s)	Recording duration (days)	Location
Leatherback	Dc1	334[1]	Adult female (gravid)	10	21	SA
Leatherback	Dc2	356[1]	Adult female (gravid)	10	11	SA
Leatherback	Dc3	392[1]	Adult female (gravid)	10	10	SA
Leatherback	Dc4	217[1]	Adult female (gravid)	10	34	SA
Leatherback	Dc5	371[1]	Adult female (gravid)	10	21	SA
Leatherback	Dc6	360[1]	Adult female (gravid)	10	10	SA
Loggerhead	Cc1	44	Subadult, Sex Unknown	4	476(*139)	MAB
Loggerhead	Cc2	89	Subadult, Sex Unknown	4	187(*46)	MAB
Loggerhead	Cc3	60[2]	Subadult, Sex Unknown	4	401(*116)	MAB
Green	Cm1	71	Adult female (gravid)	10	54	CR
Green	Cm2	81[3]	Adult female (gravid)	10	41	CR
Green	Cm3	65[3]	Adult female (gravid)	10	22	CR
Green	Cm4	70[3]	Adult female (gravid)	10	11	CR

*For each individual, we note the species, ID, body-mass, life-stage/sex, time-depth sampling frequency, recording duration, and the sampling location (SA- iSimangaliso Wetland Park, South Africa; MAB- Mid-Atlantic bight; CR- Playa Cabuyal, Costa Rica). To estimate body-mass for leatherback turtles, we first converted Curved Carapace Length to Straight Carapace Length following an equation in (Blanco et al., 2013) and then from Straight Carapace Length to body-mass following an equation in Georges and Fosette (2006). Subscript numbers refer to reference for the equations used to convert Curved Carapace Length to body-mass equations: [1](Jean-Yves and Sabrina, 2006). [2]In-situ weights collected. [3](Bjorndal and Bolten, 1988). *number of days used for modeling.*

reptilian lungs have faveoli or ediculi instead of the alveoli in mammals, the similarities in their structures mean that they likely respond similarly under pressure differentials. For ease of use, we hereafter refer to these differing features under the singular term faveoli. It should also be noted that sea turtles have the capacity to perform a right-to-left intracardiac shunt. As the role of this cardiac shunt in regulating N_2 is unknown in aquatic reptiles (Burggren et al., 2020), we therefore adjusted the parameters for the modeled pulmonary shunt, so that it mirrored the arterial PN_2 levels that previously reported for a sea turtles during forced submergence (Berkson, 1967).

The model assumed that breathing began the instant the animal reached the surface (i.e., a depth of 0 m). However, due to a combination of measurement error, the use of tethered tags on both leatherback and green turtles, and the original sampling frequency of the TDRs, not all breathing events were associated with a depth measurement of 0. While many studies address this issue by defining a dive as the period when a turtle descends beyond a pre-determined depth (e.g., 2 m), we chose to not use this dive definition as previous models have shown the risk of GE increases rapidly as a diver approaches the surface (Fahlman et al., 2018). Instead, we subtracted 2 m from all depth values. While this ensured that all breathing events were associated with a measurement of 0 depth, we acknowledge that this also slightly reduced the maximum depth of the dives.

When breathing, the arterial blood gas tensions of N_2, O_2, and CO_2 were set to 0.741 atmospheres absolute (ATA), 0.164 ATA, and 0.033 ATA, respectively, as have been previously reported for loggerhead turtles (Lutcavage and Lutz, 1991) with water vapor being 0.062 ATA (Lutcavage and Lutz, 1991; Fahlman et al., 2008). We also assumed that arterial blood gas tensions while breathing were equal to the partial pressures in the faveoli, and that all CO_2 that exchanged for O_2 remained in gas phase in the lungs and did not dissolve into the lung parenchyma (Fahlman et al., 2006).

Lung Compression and Pulmonary Shunt

We used the model published by Bostrom et al. (2008) to estimate faveolar volume at depth and compared the estimated faveolar collapse depth from this model with empirical data previously published in green turtles forcibly submerged inside hyperbaric chambers (Berkson, 1967). This model assumed that the total lung capacity (TLC) includes both the maximum faveolar volume (VfA_{max}) and the volume of dead space in the trachea and bronchi (VD; Fahlman et al., 2009, 2018). We obtained measurements of TLC for each species from the relevant literature (**Table 2**; Berkson, 1967; **Table 3**; Lapennas and Lutz, 1982; Lutz and Bentley, 1985; Lutcavage et al., 1990; Lutcavage and Lutz, 1991; Hochscheid et al., 2007). However, as we were only able to find measurements of VD for green turtles (Berkson, 1967; Gatz et al., 1987), we assumed that VD was equivalent (7% of TLC) in both loggerhead and leatherback turtles. We assumed that gas exchange only occurred in the faveoli, and that gas exchange stopped immediately upon faveolar collapse (i.e., when the faveolar volume was zero, VfA = 0).

To account for the relationship between pulmonary shunt and faveolar collapse, we used Eq. 4 in Fahlman et al. (2009). We also estimated the faveolar volume (Eq. 4 in Bostrom et al., 2008), using the parameters as previously defined for human alveoli (a = 1.04, b = 0.20, and c = 1.21). Finally, we estimated dead space in the upper airways (Eq. 5 in Bostrom et al., 2008) and applied the parameters as previously defined for a compliant human trachea (Kp = −6.44, n = 0.74). We used the same parameters for all three turtle species.

Compartment Size

We generated estimates of the relative size of the four compartments in the model using computed tomography of five loggerhead turtles ranging in body mass from 3.2 to 48.7 kg (**Appendix 1**). To support these results, we also performed mass dissections of two loggerheads, one leatherback, and nine green turtles ranging in body mass from 1 to 355.9 kg and encompassing both sexes. Dissections were conducted by the Fundación Oceanogràfic de la Comunidad Valenciana which is registered as a research unit under the official ID number: ES460250001024 under the collaboration agreement with the Conselleria d'Agricultura, Medi Ambient, Canvi Climàtic i Desenvolupament Rural of the Valencian Regional Goverment, the National Oceanic and Atmospheric Administration (Florida Fish and Wildlife Conservation Commission Marine Turtle Permit 20–081), and North Carolina State University (North Carolina Endangered Species Permit 20ST42). Organs were removed intact and as much blood as possible drained from the tissues prior to weighing. To weigh the heart, the great vessels were severed at the pericardium. Due to the difficulties separating fat and muscle, we did not attempt to estimate the fat/bone and muscle compartments during dissections.

To estimate blood volumes, we used a value midway between those measured in leatherback and green turtles (Thorson, 1968; Lutcavage et al., 1992; **Table 3**). Blood was separated into arterial and venous, respectively, 33 and 67% of the total blood volume (Fahlman et al., 2006). After the arterial blood gases were exchanged with the tissue, the venous side from all compartments joined immediately to form mixed venous blood, as previously detailed (Fahlman et al., 2006, 2009).

As we observed higher variability between individuals than between species and proportions in compartment sizes among species were similar, we combined the results from all the species to characterize compartment sizes for a "generic" sea turtle (**Table 3**). This turtle was composed of 7.00% blood, 0.06% brain, 30.00% fat/bone, 9.00% central circulation, and 53.94% muscle.

Cardiac Output and Blood Flow Distribution

We defined cardiac output ($\dot{Q}_{tot}$) at the surface (i.e., depth = 0) based on measurements from green turtles ($body\ mass$ = 1.2 kg; West et al., 1992). To account for allometric differences

TABLE 2 | Estimated model variables for mass-specific total lung capacity (TLC, ml • kg^{-1}), O_2 stores (ml • kg^{-1}) of the lung (l-O_2), blood (b-O_2), and muscle (m-O_2), hemoglobin concentration ([Hb], g • dl^{-1}), packed cell volume (PCV, %), myoglobin concentration ([Mb], g Mb • kg^{-1} muscle).

Species	TLC	l-O_2	b-O_2	m-O_2	[Hb]	PCV	[Mb]	P50
Loggerhead	(113.6 *body mass*$^{0.923}$) • *body mass*$^{-1}$	16.4	7.84	3.93	0.088	30	2.9*	25
Leatherback	64	9.25	13.8	4.50	0.156	39	4.9	40
Green	115	16.6	8.72	3.93	0.098	29	2.9	47

*The hemoglobin P50 (mmHg) was selected from measurements in each species, and the Hill coefficient for the O2 dissociation curve was assumed to be 2.8 from measurements in the loggerhead sea turtle (Wood et al., 1984). (Berkson, 1967; Lapennas and Lutz, 1982; Lutz and Bentley, 1985; Lutcavage et al., 1990; Lutcavage and Lutz, 1991; Hochscheid et al., 2007).*No data so assumed equal to another species.*

between species in body mass, we used the following equation:

$$s\dot{Q}_{tot} = \dot{Q} \bullet \left(bodymass^{-0.25} \bullet 1.2^{-0.25} \right) \quad (1)$$

where 8.4 was the $\dot{Q}_{tot}$ (l • min^{-1}) and s$\dot{Q}_{tot}$ is the mass-specific $\dot{Q}_{tot}$ (Davis and Kanatous, 1999). At the surface, the model transferred 60% of $\dot{Q}_{tot}$ to the central circulation compartment, 34% to the muscle compartment, 4% to the brain compartment, and 2% to the fat and bone compartment. As no empirical data on this exist for sea turtles, we chose these values based on comparable data from marine mammal studies (Fahlman et al., 2018). While diving, the distribution of blood flow was tested iteratively to optimize O_2 utilization and thus maximize the aerobic dive duration (Fahlman et al., 2006, 2009). This resulted in 92.8, 5.0, 2.0, and 0.2% of s$\dot{Q}_{tot}$ being directed to the central circulation, muscle, brain, and fat and bone, respectively, while diving.

When at the surface, we used a metabolic rate of 4.23 ml O_2 • min^{-1} • kg^{-1} for each compartment. This is comparable to those reported in the literature for exercising leatherback, loggerhead, and green turtles on land under air temperatures between 26 and 30°C (Jackson and Prange, 1979; Butler et al., 1984; Lutcavage et al., 1987, 1990, 1992; West et al., 1992). We used values for exercising MR instead of resting MR, as cardiac output is often higher at the surface to recover from previous dives (Southwood et al., 1999). When diving, we assumed that the metabolic rate was 5% of that at the surface, i.e., 0.21 ml O_2 • min^{-1} • kg^{-1} based on data from a leatherback turtle conducting a 34-min dive (Southwood et al., 1999). This value also lies within the range of metabolic rates reported by Jackson and Prange (1979), Butler et al. (1984), Lutcavage et al. (1987, 1990, 1992), and West et al. (1992), and it also ensured that the modeled turtles did not run out of O_2 during long dives.

Hemoglobin and Myoglobin Characteristics

We used published data on blood packed cell volume (PCV, %), hemoglobin ([Hb]), and muscle myoglobin ([Mb], **Table 2**) concentrations for each species (Lapennas and Lutz, 1982; Lutz and Bentley, 1985; Lutcavage et al., 1990). A P_{50} of the O_2 dissociation curve for each species was gleaned from published literature (**Table 3**). We used a Hill coefficient of 2.7 for all species (Berkson, 1967; Wood et al., 1984; Lutcavage et al., 1990).

Gas Dynamics Computations

We used O_2, CO_2, and N_2 gas balance equations from Fahlman et al. (2006) to estimate gas transport in the lungs and tissues over short-time intervals and small blood volumes (package volume). For these equations, the partial pressure/tension of each gas was used as the driving force for gas exchange, and each compartment was modeled as a single homogenous tissue with a one-to-one perfusion rate and tissue gas solubility (Fahlman et al., 2006).

Experiments

We ran several model variations on each of the turtle datasets to test the effects of varying: (1) body-condition and (2a) the surface s$\dot{Q}_{tot}$ and (2b) diving s$\dot{Q}_{tot}$ on venous PN_2

(1) To assess how changes in body-fat affect venous PN_2, we varied the relative volume of the fat/bone compartment in the control model (30%) to either 20 or 40% to represent extremes of nutritional condition. When changing the relative size of the fat/bone compartment, the contribution of the other compartments was adjusted accordingly to ensure that they maintained the same overall mass (**Table 3**)

(2a and b) To assess the effect of varying the s$\dot{Q}_{tot}$, we adjusted the surface s$\dot{Q}_{tot}$ from the control model (s$\dot{Q}_{tot}$ of 7 ml • min^{-1} • kg^{-1}) to either 2.5 ml • min^{-1} • kg^{-1} (Surface-Low) or 10 ml • min^{-1} • kg^{-1} (Surface-High). We also examined the effect of varying the s$\dot{Q}_{tot}$ during diving from the control model (5.0% of the control s$\dot{Q}_{tot}$) to either 10.0% (dive-low) or 3.3% (dive-high).

Finally, to evaluate how changes in body-fat or cardiac output affected the risk of GE, we calculated the end dive PN_2 saturation of the mixed venous blood during the last 5 s of each dive. We then measured the percentage change between the mean (over all dives) and the maximum end dive PN_2 for the different models using the formula:

$$\text{Saturation}(\%) = (\text{control model} - \text{model variation}) \bullet \text{control model}^{-1} \bullet 100 \quad (2)$$

Model Validation and Analysis

While there was no published data available on arterial PN_2 levels in free-diving sea turtles, Berkson (1967) measured

TABLE 3 | Body compartment size as a percentage of body mass in loggerhead, green, and leatherback turtles combined.

Model	Blood	Brain	Fat/Bone	Central Circulation	Muscle
Control	7.0*	0.06	30	9.0	53.94
Obese	6	0.054	40	8	45.946
Emaciated	8	0.066	20	10	61.394

Blood volume assumed between the values measured in the leatherback and green turtles (Lutcavage et al., 1968, 1992).

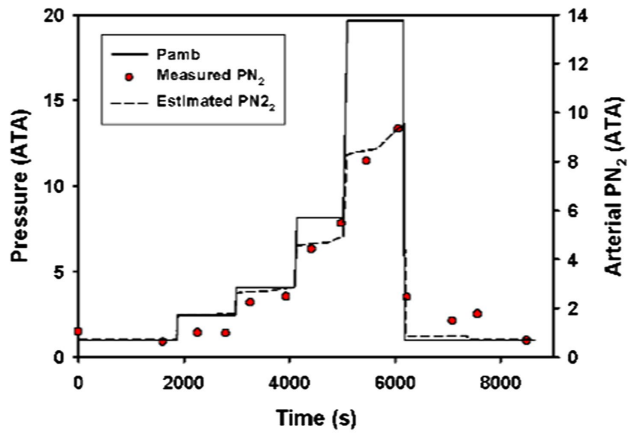

FIGURE 1 | Measured (red circles) and estimated (broken black line) arterial N_2 tension (PN_2) in a Pacific green turtle (*Chelonia mydas agassizii*) during a step-wise forced dive to 19.4 ATA (Berkson, 1967). The model used a surface sof 3.42 ml • min^{-1} • kg^{-1} and a diving s0.17 ml • min^{-1} • kg^{-1} and a diving lung volume 50% of total lung capacity.

arterial PN_2 in green turtles during forced submergence. Thus, to assess the accuracy of our model for estimated arterial PN_2, we compared the modeled results to those observed in Berkson (1967) during comparable dives.

As these are modeling based on the physiological assumptions from a limited number of individuals, we opted to only perform limited statistical analyses on the results. As such, these results should be considered largely descriptive and are simply to provide a framework for developing testable hypotheses in future studies.

RESULTS

Dive summaries for each turtle are reported in **Table 4**. We divided these summaries between shallow (0–30 m), medium (30–90 m), and deep (>90 m) dives. We selected these thresholds according to the values in Berkson (1967) as there should be limited impact of pressure on lung diffusion at 30 m or less and there should be almost no lung diffusion deeper than 90 m. The table includes the average (±SD) dive duration, the maximum depth reached during each dive, and the average depth for each dive (**Table 4**).

Model Validation

When comparing data on arterial PN_2 during forced submergence from Berkson (1967) to the model output, the modeled data were never more than 1.5 ATA from the measured data (**Figure 1**). Furthermore, the modeled data strongly reflected the observed changes in arterial PN_2 at different pressures.

Control Model

The model output showed that the accumulation of N_2 into the central circulation occurred faster than it did in the fat (**Figure 2**). Because of this, PN_2 within the central circulation compartment tended to reflect the ambient pressure, while fat PN_2 was more influenced by the dive profiles over a series of hours to days (**Figure 3**). As such, central circulation only briefly contributes to GE risk during the ascent as N_2 was rapidly removed and the supersaturation decreased. The fat compartment, on the other hand, slowly varied with time and depended on the previous diving pattern. Furthermore, unlike the central circulation, supersaturation occured close to the surface and during the entire surface interval. Nevertheless, the overall fat PN_2 saturation seldom exceeded 1 ATA and therefore does not contribute appreciably to GE risk.

Using the control model and combining the results from all three species, mean end-dive PN_2 increased with depth for central circulation (χ^2 = 32, 2 df, $p < 0.01$), muscle (χ^2 = 22, 2 df, p < 0.01), brain (χ^2 = 20, 2 df, $p < 0.01$), and mixed venous (χ^2 = 34, 2 df, $p < 0.01$) but not for the fat compartment (χ^2 = 2.4, 2 df, $p > 0.2$). When comparing dives within the shallowest dive bin (<30 m) to the deepest dive bin (>90 m), mean end-dive PN_2 increased by 245% for central circulation, 11% for muscle, 189% for brain, and 221% for the mixed venous compartment. When the three species were considered separately, leatherback turtles had the lowest mean end-dive PN_2, with green turtles having intermediate values, and loggerhead turtles having the highest (χ^2 = 8.49, 2 df, p = 0.014; End-dive PN_2 = 2.73 (±0.31)−0.56 (±0.28) × leatherback + 0.54 (±0.41) × loggerhead−0.63 (±0.30) medium−1.71 (±0.29) shallow).

With data from all three species combined, maximum end-dive PN_2 increased with depth for central circulation (χ^2 = 11.5, 2 df, $p < 0.01$), brain (χ^2 = 8.8, 2 df, p = 0.012), and mixed venous blood (χ^2 = 12.1, 2 df, $p < 0.01$), but decreased for fat (χ^2 = 7.9, 2 df, p = 0.019), and did not change for muscle (χ^2 = 5.1, 2 df, p = 0.079). Comparing dives in the shallow depth range to those in the deep depth bin, maximum end-dive PN_2 increased by 80% for central circulation, by 68% for brain, and by 81% for mixed venous blood. For brain, both depth range and species helped to explain the variation (χ^2 = 7.39, 2 df, p = 0.025). Overall, mean end-dive PN_2 for brain increased with depth and leatherback turtles had the lowest maximum end-dive PN_2, while green turtles had intermediate values, and loggerhead turtles had the highest (change in end-dive brain PN_2 (%) = 4.23 (±0.65) − 0.50 (±0.66) × leatherback + 1.95 (±0.92) × loggerhead – 1.03 (±0.54) medium −1.86 (±0.53)). The maximum end-dive PN_2 for mixed venous blood was 9.40

TABLE 4 | Dive duration summaries from six leatherback, three loggerhead, and four green turtles.

		Shallow (< 30 m)			Medium (30–90 m)			Deep (> 90 m)		
Species	**ID**	**Dive duration(s)**	**Mean max. Depth(m)**	**Proportion of dives (%)**	**Dive duration(s)**	**Mean max. Depth(m)**	**Proportion of dives(%)**	**Dive duration (s)**	**Mean max. Depth(m)**	**Proportion of dives(%)**
Leatherback	Dc1	209 ± 251	6.3 ± 7.0	87.2	827 ± 157	49.1 ± 14.4	11.6	839 ± 97	108.7 ± 15.3	1.2
Leatherback	Dc2	177 ± 222	4.1 ± 5.3	90.3	1,155 ± 248	55.5 ± 18.6	6.5	1,345 ± 141	136.4 ± 88.3	3.2
Leatherback	Dc3	74 ± 138	9.5 ± 7.0	84.0	244 ± 349	51.3 ± 15.8	14.2	367 ± 473	134.1 ± 67.8	1.9
Leatherback	Dc4	175 ± 162	7.8 ± 8.0	75.0	581 ± 161	48.2 ± 13.8	23.2	812 ± 133	120.7 ± 36.9	1.8
Leatherback	Dc5	230 ± 231	9.3 ± 9.4	71.7	775 ± 234	49.5 ± 15.2	25.0	1,166 ± 211	114.3 ± 20.0	3.2
Leatherback	Dc6	156 ± 174	4.3 ± 5.5	92.8	911 ± 199	54.9 ± 15.9	6.2	1,063 ± 98	115.9 ± 38.7	0.9
Loggerhead	Cc1	150 ± 499	0.7 ± 1.9	97.3	1,396 ± 604	44.6 ± 6.5	2.7	------	------	------
Loggerhead	Cc2	181 ± 396	1.1 ± 2.3	94.2	1,685 ± 840	53.3 ± 7.5	5.8	------	------	------
Loggerhead	Cc3	256 ± 544	1.0 ± 2.1	93.9	2,117 ± 439	66.3 ± 8.9	6.1	------	------	------
Green	Cm1	183 ± 438	2.3 ± 4.4	88.0	2,705 ± 1,215	58.1 ± 17.7	10.7	3,106 ± 891	96.9 ± 6.5	1.2
Green	Cm2	502 ± 542	5.0 ± 5.1	96.1	1,314 ± 479	48.4 ± 9.7	3.9	------	------	------
Green	Cm3	324 ± 382	4.6 ± 5.0	97.6	892 ± 390	44.2 ± 11.1	2.4	944	90.5	<0.01
Green	Cm4	431 ± 500	2.4 ± 1.8	100	------	------	------	------	------	------

Dives were separated by depth into shallow dives (F 30 m), medium dives (30–90 m), and deep dives (> 90 m). Within each depth category, we calculated the mean dive duration (± SD), mean maximum dive depth (m), and the proportion of dives within each dive bin (%).

ATA, 6.17 ATA, and 5.03 ATA for leatherback, green, and loggerhead turtles, respectively, representing supersaturation ratios [(mixed venous PN_2 – ambient pressure) • ambient pressure^{-1}, or M-ratios] of 12.7, 8.3, and 6.8.

Model Variation – Body-Fat

When body fat was increased from 30 to 40% of body mass or decreased from 30 to 20% of body mass, there was no effect observed on mean or maximum end-dive mixed venous PN_2 for either shallow or intermediate depths when all three species were combined (**Figure 5**). However, for the deepest depth bin, increasing or decreasing body-fat reduced the mean end-dive mixed venous PN_2 (χ^2 > 10.0, 2 df, p < 0.01; **Figures 4A,B**) and the maximum end-dive mixed venous PN_2 (**Figures 5A,B**). Thus, a model that included depth, species, and body-fat was warranted; % change in maximal end-dive mixed venous PN_2 = −13.3 (±1.2) − 12.4 (±2.6) leatherback – 5.2 (±3.8) logger + 12.5 (±3.0) medium depth + 11.0 (±2.9) shallow depth + 8.6 (±2.3) emaciated (χ^2 > 14.4, 1 df, p < 0.01).

Model Variation – Cardiac Output at Surface

When s$\dot{Q}_{tot}$ was either increased or decreased, there were no consistent differences in mean mixed venous end-dive PN_2 among species at the shallow depth bin (p > 0.3 for all, **Figures 4C,D**). For both medium and deep depths, when s$\dot{Q}_{tot}$ was increased to 10 ml • min^{-1} • kg^{-1} (Surface-High) this increased mean end-dive PN_2 by 10.6% (**Figure 4D**), while a decrease in s$\dot{Q}_{tot}$ to 2.5 ml • min^{-1} • kg^{-1} (Surface-Low; **Figure 4C**) caused a 4.5% decrease in mean end-dive PN_2 [χ^2 > 26.5, 1 df, p < 0.01, change in end-dive PN_2 (%) = 10.6 (±1.9) – 15.1 (±2.7) × (Surface-Low)].

For maximum end-dive mixed venous PN_2, the changes were similar, with 43% increase in surface s$\dot{Q}_{tot}$ (Surface-High) causing an increase in maximum end-dive PN_2 by 13.4% (**Figure 5D**), and 64% decrease (Surface-Low) in s$\dot{Q}_{tot}$ causing a 5.4% decrease [**Figure 5C**, χ^2 > 31.0, 1 df, p < 0.01, change in end-dive PN_2 (%) = 13.4(±2.1) – 18.7 (±3.0) × (Surface-Low)].

Model Variation – Cardiac Output at Depth

When altering diving s$\dot{Q}_{tot}$, there was no statistically significant effect of dive depth on mean end-dive mixed venous PN_2 when all three species were combined (p > 0.5 for all, **Figures 4E,F**). Considering all species and depths together, an increase in diving s$\dot{Q}_{tot}$ from 5 to 10% lead to 20.6% increase in mean end-dive mixed venous PN_2 (**Figure 5E**). A decrease in diving s$\dot{Q}_{tot}$ from 5 to 3.3% (**Figure 4F**) lead to a decrease of 13.2% in the mean end-dive mixed venous PN_2 [χ^2 > 52.8, 1 df, p < 0.01, change in end-dive PN_2 (%) = 20.6(±2.7) – 33.9 (±3.8) × (1/30)].

When varying diving s$\dot{Q}_{tot}$, there was uniform effect on the maximal end-dive PN_2, however, there were differences between species. Specifically, the maximum end-dive mixed venous PN_2 was higher for leatherback turtles than either

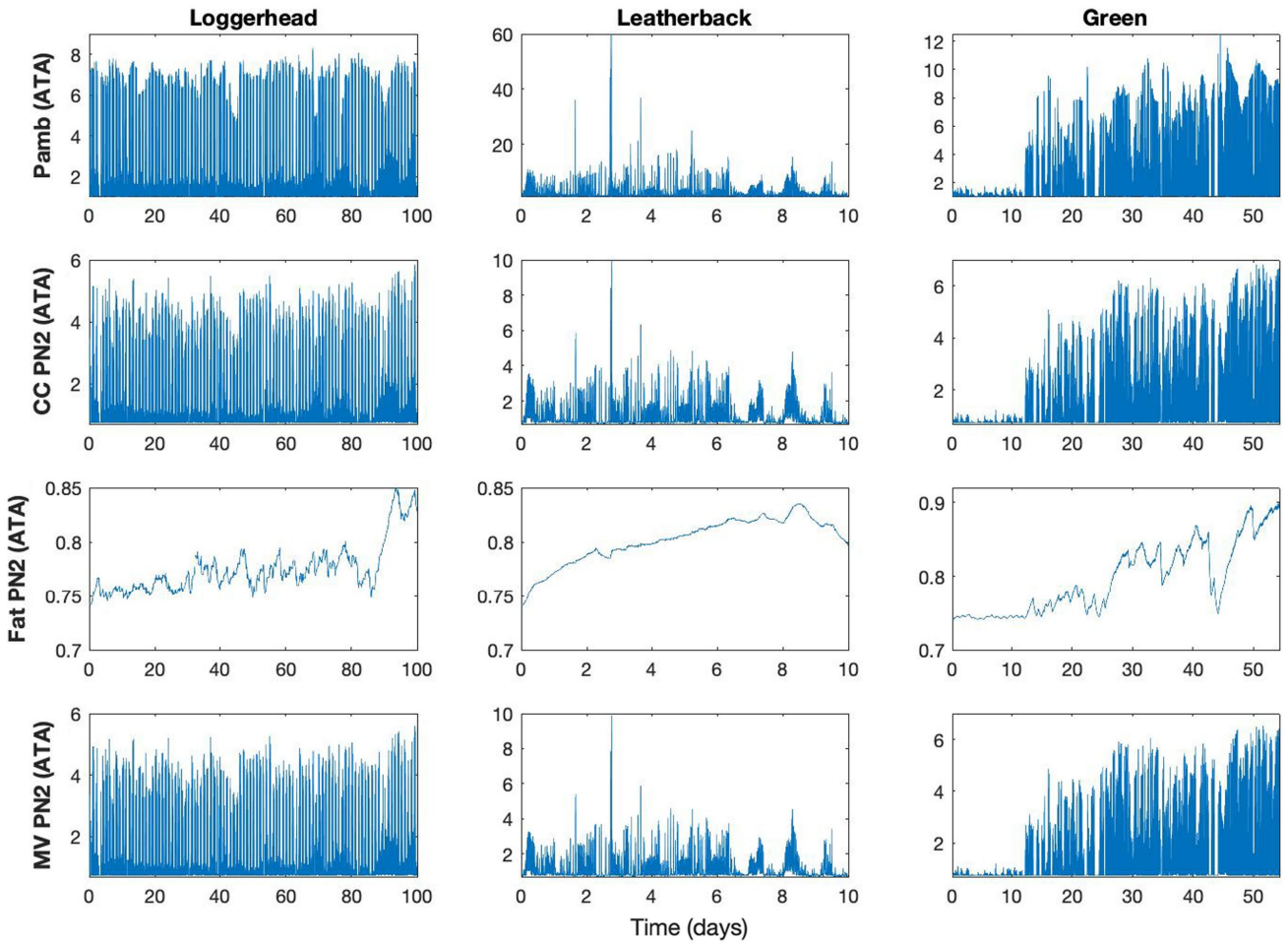

FIGURE 2 | Model result for control model, showing ambient pressure (Pamb, ATA), and tissue gas tension (PN_2) for central circulation (CC), fat, and mixed venous blood (MV) in a single loggerhead (Turtle ID 14293), leatherback (Turtle ID 10A1059a), and green sea turtles (Turtle ID 37800, **Table 2**). Data are shown for 100, 10, and 55 days for loggerhead, leatherback, and green turtles, respectively. The Pamb was used to generate the model output for the various tissues and the difference in tissue PN_2 between CC (fast tissue) and fat (slow tissue), with the PN_2 in MV is the composite of all tissues and their relative blood flow.

loggerhead or green turtles at all depths. In addition, the lower diving $s\dot{Q}_{tot}$ significantly reduced the maximal end-dive PN_2 (**Figures 5E,F**); [$\chi^2 > 6.21$, 1 df, $p = 0.013$, change in maximal end-dive PN_2 (%) = 32.2 (±3.1) – 44.9 (±3.8) × (1/30) – 9.6 (±3.8) loggerhead/green].

DISCUSSION

GE associated with fisheries bycatch and other submerged gear interactions could be a significant threat to sea turtles worldwide (García-Párraga et al., 2014; Fahlman et al., 2017; Crespo-Picazo et al., 2020; Harms et al., 2020; Parga et al., 2020). To better understand how sea turtles manage the risk of GE during natural diving behavior, we adapted a theoretical model to estimate blood and tissue N_2 tensions in diving marine mammals to fit loggerhead, leatherback, and green sea turtles. We also applied the new model to assess how body-fat and cardiac output influence the potential for GE during normal diving behavior in sea turtles. Demonstrating the accuracy of the model, it provided a reasonable estimate for blood and tissue PN_2 from previously conducted forced diving experiments (Berkson, 1967). By varying the model, we observed that higher fat reserves can reduce end-dive PN_2 by up to 25%. We also observed that an increase in surface $s\dot{Q}_{tot}$, as likely occurs as the turtle reaches the surface to help to increase O_2 uptake and CO_2 removal (Okuyama et al., 2020), led to an increase in end-dive PN_2. Finally, the model showed that leatherback turtles have lower mean end-dive blood and tissue PN_2, but higher maximum end-dive values, than loggerhead or green turtles during routine diving behavior. While higher maximum end-dives values may be expected considering that leatherback turtles dive considerably deeper than the other two species (Robinson and Paladino, 2015), the fact that they had lower mean end-dive blood and tissue PN_2 also suggests that their increased body-size helps to reduce the risk of GE.

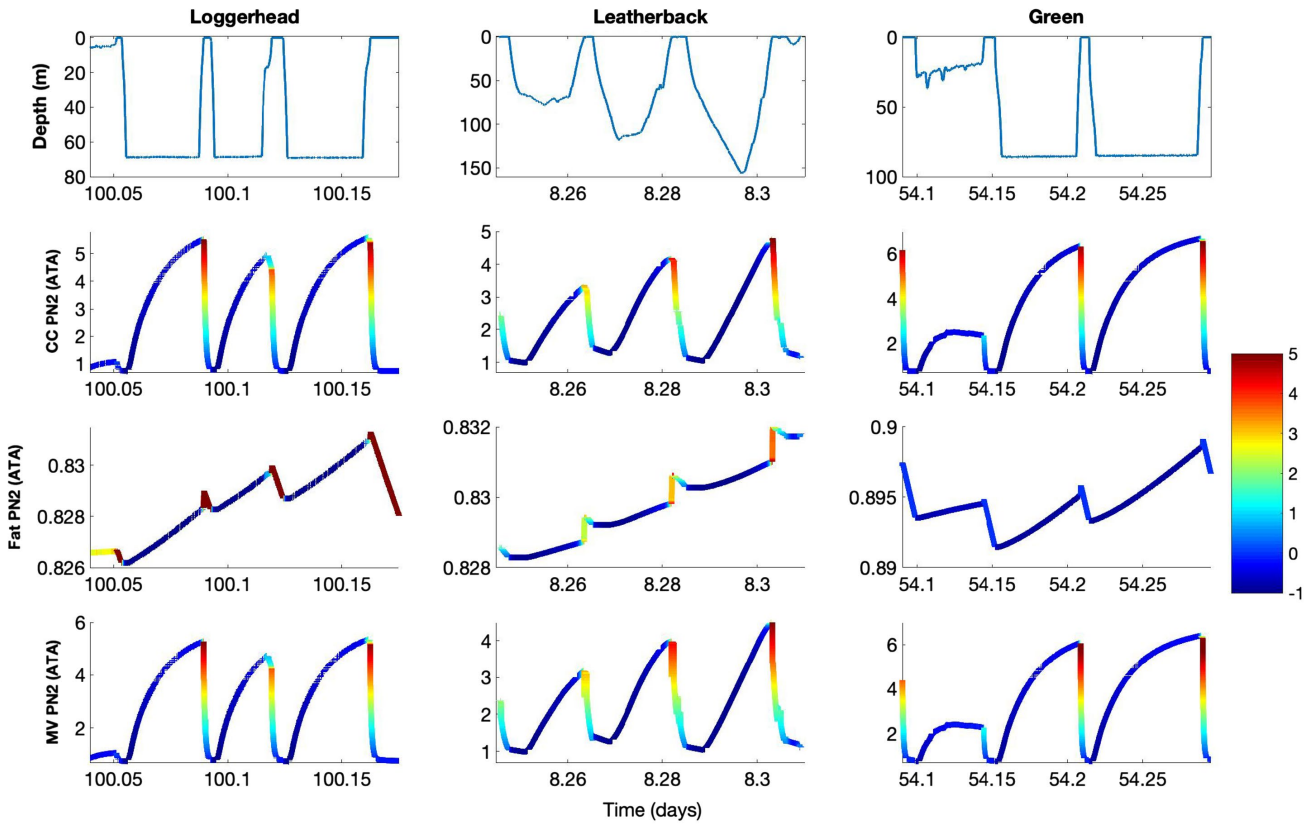

FIGURE 3 | Model result for control model, showing depth (m), and tissue gas tension (PN_2) in central circulation (CC), fat, and mixed venous blood (MV) in loggerhead (Turtle ID 14293), leatherback (Turtle ID 10A1059a), and green sea turtles (Turtle ID 37800, **Table 2**). The data presented are a subset of those in **Figure 2** to show specific dive patterns and blood and tissue PN_2 levels. The theoretical supersaturation [(mixed venous PN_2 – ambient pressure) • ambient pressure^{-1}, in ATA] for each of the tissues and the mixed venous blood are shown in color. The color bar shows the level of supersaturation, where negative values (blue) indicate N_2 uptake and positive values indicates the whole body, or mixed venous PN_2, exceeds the ambient pressure, i.e., risk of Gas Emboli formation (GE) and pathology (GEP). Thus, increasing value, i.e., yellow to red, indicates increasing risk of GEP. The results show increasing risk of GEP during ascent and how ascent rate or how sudden stops during ascent reduces risk, e.g., ascent from second dive in loggerhead or leatherback turtles.

Model Assumptions and Limitations

Our goal was to construct the initial framework for a model to understand gas dynamics in diving sea turtles. As no such models previously existed, a logical first step was to adapt a previously constructed and validated model for another air-breathing vertebrate. While we chose to adapt a model originally designed for marine mammals (Fahlman et al., 2006, 2009, 2021), there are several key anatomical and physiological differences between marine mammals and sea turtles that have not yet been incorporated into the new model. Firstly, and of considerable importance, is that sea turtles, unlike marine mammals, are at least partially ectotherms. Specifically, even though several studies have shown that sea turtles can maintain body-temperature elevated above ambient conditions (Paladino et al., 1990; Sato et al., 1995), their body temperatures still fluctuate over wider ranges than marine mammals. While we attempted to control for this by only analyzing diving data for sea turtles within a restricted range of sea surface temperatures (19–29°C), the effect of temperature on both the metabolic rate and the gas tensions/solubilities cannot be ignored (Lutz et al., 1989).

Another important difference between marine mammals and sea turtles is that sea turtles have muscular sphincters within their pulmonary arteries and a partially compartmentalized ventricle that allow for central intracardiac shunting (García-Párraga et al., 2018a; Burggren et al., 2020). These features could allow a complete shunt and cessation of gas exchange as the animal begins the dive. In fact, this could be the mechanism by which sea turtles avoid GE (García-Párraga et al., 2018b; Fahlman et al., 2021). In this model, we assumed that the pulmonary shunt develops due to the passive compression of the terminal air spaces and the structural properties of the conducting airways and faveoli (Bostrom et al., 2008; Fahlman et al., 2009). While this is not necessarily the case in reptiles, the exact factors influencing the cardiac shunt in sea turtles are not well-established (Burggren et al., 2020). Without having a solid understanding of the functioning of the cardiac shunt, we adjusted the model so that the arterial PN_2 reflected those of forced-diving sea turtles in Berkson (1967). This meant that the physiological shunt and the potential anatomical shunt were expressed together as a pulmonary physiological shunt. In addition, we acknowledge that forced-diving experiments

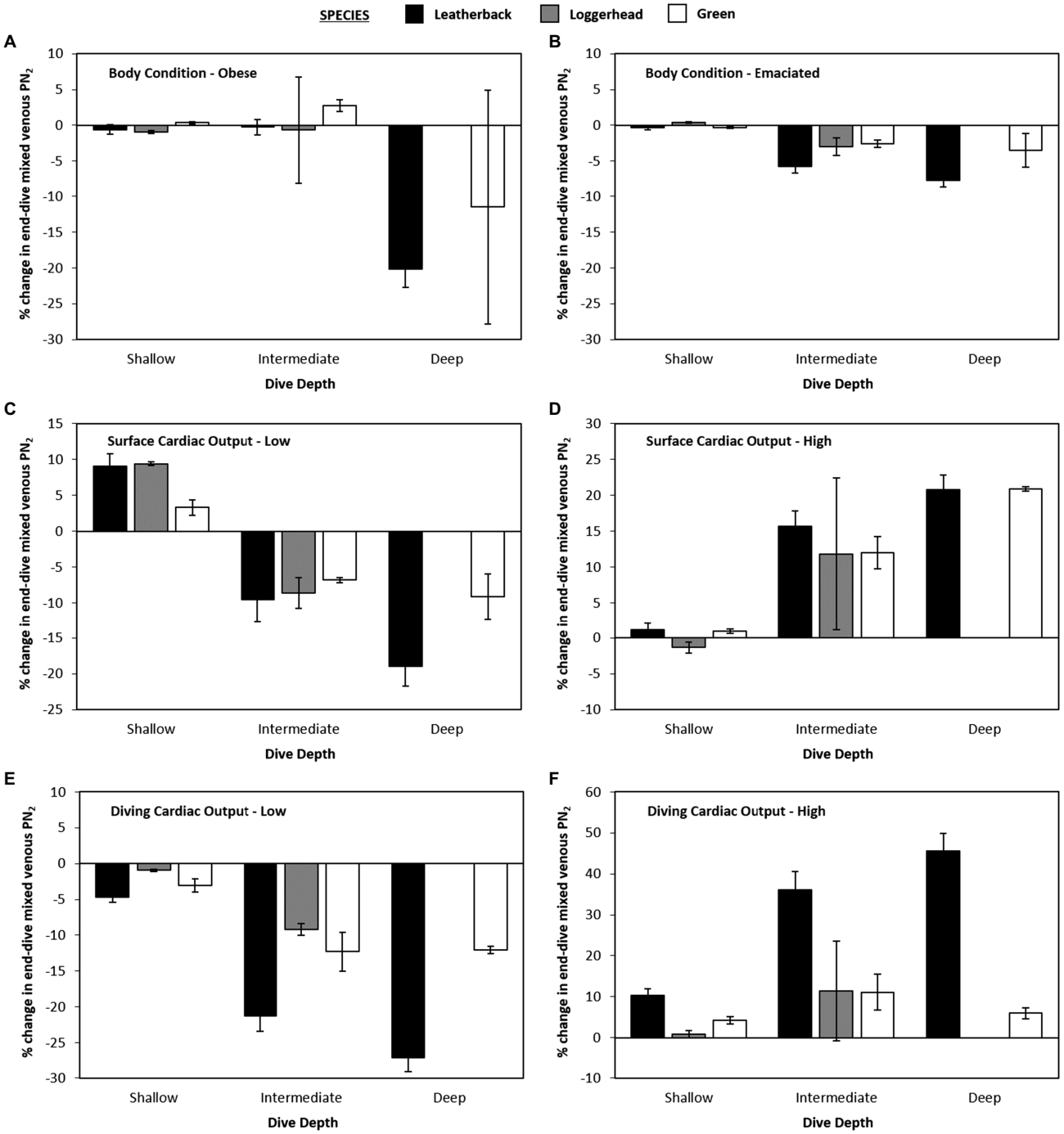

FIGURE 4 | Data showing percent changes in mean end-dive PN_2 for variation in gas dynamics model as compared with the control model [(new model – control model) • control model^{-1} • 100].

in Berkson (1967) would likely elevate blood and tissue N_2 levels beyond normal values, and our model likely thus provides a conservative estimate of blood and tissue N_2 levels. However, if we consider exercise as one of the main driving forces in the cardiac output adjustment (Okuyama et al., 2020), physically restrained animals could minimize N_2 uptake reflecting lower N_2 values compared to free swimming or entrapped individuals with restricted movement.

Other significant assumptions with the model resulted from differences between the datasets available for each species. For example, we utilized diving data from subadult loggerhead in foraging habitats, while the data for leatherback and green

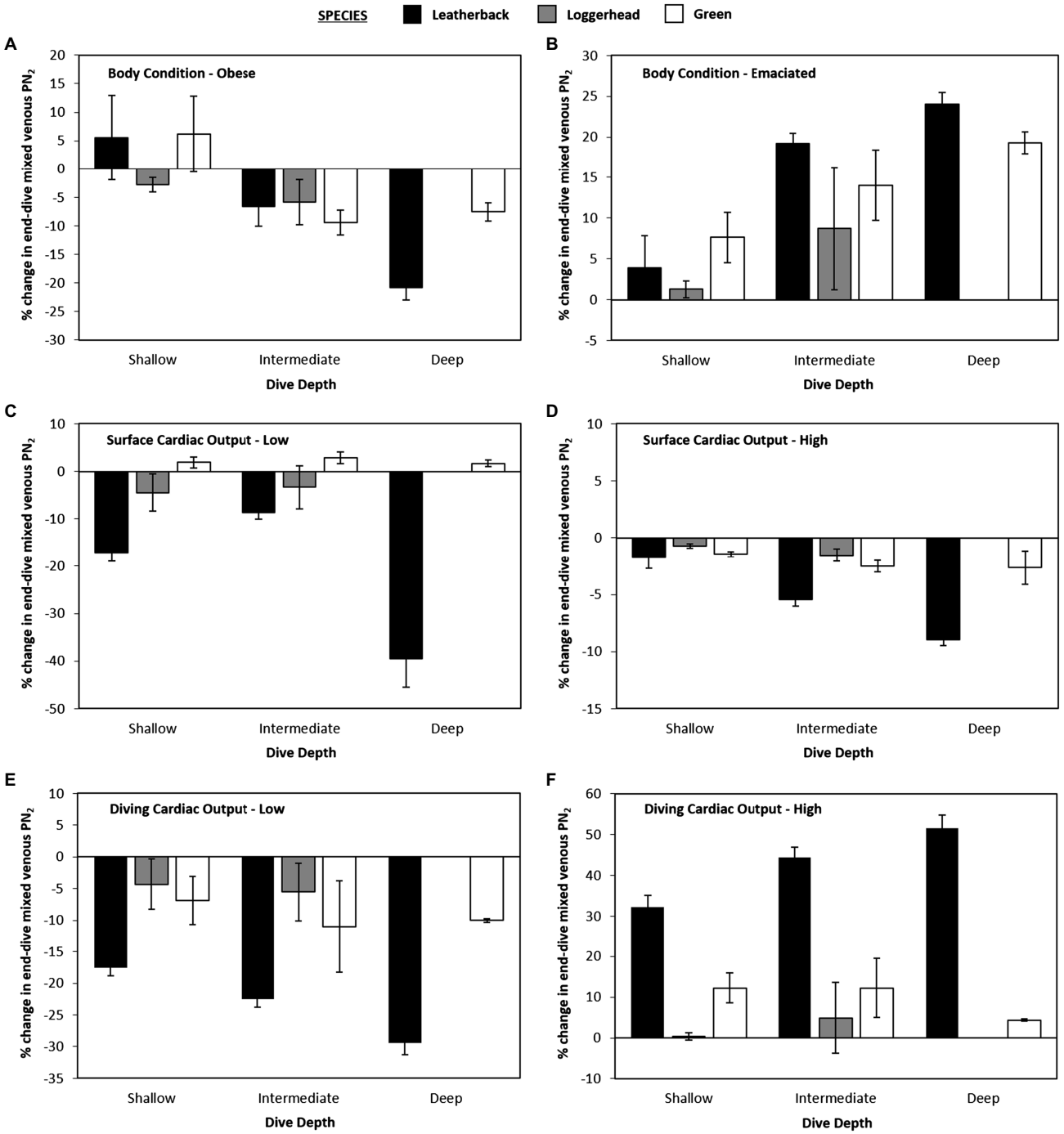

FIGURE 5 | Data showing percent changes in maximal end-dive PN_2 for variation in gas dynamics model as compared with the control model [(new model – control model) • control model^{-1} • 100].

turtles were from adult females in inter-nesting habitats. Indeed, it is known that foraging and inter-nesting turtles exhibit differing dive behaviors (Robinson and Paladino, 2015). Other factors including life-stage and temperature are also known to influence diving behavior but are once again not standardized between our turtle diving datasets.

As is the case for all theoretical physiological models, the complexity of the entire system cannot be incorporated holistically into a mathematical model. While such arguments are often used to disparage the value of such exercises, we stress that we aimed to build the foundation upon which more detailed assessments of how factors, such as behavior, life-stage, or

life-stage, can influence the risk of GE in sea turtles. As such, the goal of this exercise was to try and to identify which factors and assumptions need to be prioritized in future models to gain a more accurate understanding of gas dynamics in sea turtles.

General Patterns in PN_2 and Inter-Specific Variation

In the basic model, the mean end-dive PN_2 differed between species and maximum depth of the previous dive. For shallow and deep dives, the end-dive PN_2 values were, respectively, between 38–111% and 193–342% higher as compared with the surface equilibrium PN_2. Interestingly, maximum PN_2 increased with dive depth in all turtle species. This suggests that the risk of GE increases with depth as observed in previous studies (Fahlman et al., 2017).

Effect of Body-Fat on Gas Emboli Formation

While we observed no effect of body-fat on end-dive mixed venous PN_2 at depths less than 90 m, significant differences were observed beyond 90 m. Interestingly, end-dive mixed venous PN_2 was highest at intermediate body-fat levels, and it dropped if body-fat was either increased to 40% or dropped to 20%. Moreover, these differences were up to 25% lower in leatherback turtles relative to loggerhead or green turtles. These findings suggest that the higher levels of fat in leatherback turtles could perhaps reduce the effects of GE and play a role in the ability of leatherbacks to dive several times deeper than any other sea turtle species (Robinson and Paladino, 2015). That said it is unclear why lower body-fat values also result in reduced end dive PN_2.

Effect of Cardiac Output on Gas Emboli Formation

Past studies have shown that variation in blood flow, through changes in cardiac output, and the level of gas exchange are the physiological variables that have the greatest effect on N_2 uptake and removal (Fahlman et al., 2006, 2009; Hooker et al., 2009). Our model provided evidence that this may also be the case for sea turtles as a reduction of the diving $s\dot{Q}_{tot}$ to 3.3% caused a mean 13.2% decrease in end-dive PN_2, while increasing the diving $s\dot{Q}_{tot}$ to 10% caused a mean 20.6% increase in end-dive PN_2. Similar patterns were also observed for maximum end-dive PN_2. Nevertheless, these patterns differed by species and leatherback turtles exhibited lower mean end-dive PN_2 at both higher and lower diving $s\dot{Q}_{tot}$.

Implications for Gas Embolism Formation

Our model suggests that during "natural" diving behavior sea turtles experience blood and tissue N_2 levels that would cause decompression sickness in land mammals of similar size. Indeed, the maximal end-dive PN_2 values between species ranged from 5.03 ATA to 9.40 ATA, which is considerably higher than the level of end-dive PN_2 that would result in severe DCS in 50% of similarly sized humans (Fahlman et al., 2020). As it is highly unlikely that sea turtles are perpetually suffering from GE during routine diving behavior, we propose that turtles, much like marine mammals, must have additional behavioral, anatomical, and/or physiological mechanisms to reduce N_2 uptake that are not currently considered in this model (García-Párraga et al., 2018a; Burggren et al., 2020; Fahlman et al., 2021).

One mechanism that sea turtles may employ to lower the risk of GE is the *Selective Gas Exchange* hypothesis (García-Párraga et al., 2018b; Fahlman et al., 2021). This hypothesis, which is supported by theoretical, anatomical, and physiological studies (Fahlman et al., 2009, 2018, 2020; Olson et al., 2010; Hodanbosi et al., 2016; García-Párraga et al., 2018a), suggests that if ventilation and perfusion in the lung can be managed, it would allow exchange of O_2 and CO_2, with little or no exchange of N_2 (García-Párraga et al., 2018b; Fahlman et al., 2021). In support of this, it has been shown that the pulmonary arterial sphincters contract and relax when exposed to parasympathetic and sympathetic neurotransmitters, respectively (García-Párraga et al., 2018a). If sea turtles exhibit *Selective Gas Exchange*, this would help to significantly reduce our modeled blood and tissue N_2 levels and help to explain how turtles avoid GE during natural dives. It would also explain how the sympathetic stress response could result in elevated pulmonary blood flow to the still ventilated lung, yielding excessive N_2 uptake and GE (Fahlman et al., 2021).

It is also possible that turtles couple physiological and behavioral mechanisms to further reduce the risk of GE. Indeed, sea turtles increase their heart rate, and likely cardiac output, during the ascent phase (Okuyama et al., 2020). In addition, several breath-hold diving vertebrates, including sea turtles, the beluga (*Delphinapterus leucas*; Martin and Smith, 1992), beaked whales (*Hyperoodon ampullatus*; Hooker and Baird, 1999), macaroni penguin (*Eudyptes chrysolophus*; Sato et al., 2004), and king penguins (*Aptenodytes patagonicus*; Sato et al., 2004) have been shown to reduce the ascent rate while approaching the surface. It has been suggested that this reduction may be an adaptation to reduce end-dive PN_2 before reaching the surface (Fahlman et al., 2006; Fossette et al., 2010). When the reduction in ascent rate is coupled with increases in heart rate during the ascent, it may reduce end-dive PN_2 by as much as 45% in marine mammals (Fahlman et al., 2006). A similar reduction in the supersaturation was seen in all the turtles in this study (**Figure 4**). It also appears that the tendency of sea turtles to conduct a short "pause" in their accent around 10–20 m, which we observed in all three species, may also help to reduce the supersaturation as the turtle returned to the surface (Fossette et al., 2010).

These adaptations to reduce PN_2 during normal dives could also explain why sea turtles are predisposed to GE when caught by fisheries. For example, rapid accent to the surface in a fishing net coupled with disruption of the normal dive profile and physiological response to underwater entrapment may alter cardiac output and shunting, leading to rapid GE. It also suggests that slower retrieval of the fishing nets or trawls may help to significantly reduce blood and tissue N_2 levels in bycatch sea turtles. If operationally possible, this may provide a simple and cost-effective strategy to minimize GE in turtle by-catch and could have the added benefit of reducing fish by-catch

as well (Grothues et al., 2017). However, a significant concern with longer ascent times is increased risk of drowning, which may negate any benefits.

Future Directions

Our model provides a framework for future studies on the analysis of GE in sea turtles. While we adapted the model to test the effects of body-fat and cardiac output on GE, this model could be adapted in other beneficial ways. Indeed, incorporating a simulated capture event, whereby the speed of the ascent to the surface or the total time spent at depth is varied. The model could also be adapted to assess how variation in the cardiac shunt plays a role in GE during both routine dives and during incidental capture. The strong effect of cardiac output on GE also highlights that more information is needed on cardiac dynamics in diving sea turtles.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Purdue Animal Care and Use Committee, NOAA Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

NR and AF designed the experiment with input from BS. NR and AF wrote the manuscript with input from all authors. AF built and ran the model. NR, GB, CC-B, HH, and SP provided the data from the data loggers. BS, DG-P, and AC supervised necropsies and computed tomography. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: AF was employed without salary by the company Global Diving Research Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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APPENDIX 1

Relative compartment size (blood, brain, fat, central circulation, and muscle) from seven loggerhead, eight green, and one leatherback turtles. Compartment size in five of the seven loggerhead turtles was assessed using computed tomography, while it was measured in-situ *via* necropsy in the remaining animals. *Blood was not measured directly and was estimated from values in the published literature.

				Compartment size (% of total body mass)				
Species	**Body mass (kg)**	**Sex**	**Method for estimating compartment mass**	**Blood***	**Brain**	**Fat/Bone**	**Central circulation**	**Muscle**
Cc	3.2	Unknown	Computed tomography	6.7	<0.1	15.9	23.2	58.4
Cc	4.7	Unknown	Computed tomography	6.7	<0.1	20.2	29.7	48.0
Cc	20.0	Unknown	Computed tomography	6.7	<0.1	28.9	18.7	53.8
Cc	20.7	Unknown	Computed tomography	6.7	<0.1	32.2	17.2	48.4
Cc	48.7	Unknown	Computed tomography	6.7	<0.1	28.7	26.2	39.0
Cc	95.9	Female	Necropsy	6.7	<0.1	Not measured	12.2	Not measured
Cm	1.9	Female	Necropsy	6.7	0.1	Not measured	11.0	Not measured
Cm	4.0	Female	Necropsy	6.7	0.1	Not measured	10.0	Not measured
Cm	2.7	Female	Necropsy	6.7	0.1	Not measured	8.9	Not measured
Cm	2.6	Male	Necropsy	6.7	0.1	Not measured	8.9	Not measured
Cm	117.2	Female	Necropsy	6.7	<0.1	Not measured	8.3	Not measured
Cm	139.6	Female	Necropsy	6.7	<0.1	Not measured	9.8	Not measured
Cm	150.5	Female	Necropsy	6.7	<0.1	Not measured	9.2	Not measured
Cm	2.0	Unknown	Necropsy	6.7	0.1	Not measured	10.2	Not measured
Cm	2.2	Unknown	Necropsy	6.7	0.1	Not measured	13.2	Not measured
Dc	355.9	Female	Necropsy	6.7	<0.1	Not measured	5.0	Not measured

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REVIEW
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Decompression Illness in Repetitive Breath-Hold Diving: Why Ischemic Lesions Involve the Brain?

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Nitrogen (N_2) accumulation in the blood and tissues can occur due to breath-hold (BH) diving. Post-dive venous gas emboli have been documented in commercial BH divers (Ama) after repetitive dives with short surface intervals. Hence, BH diving can theoretically cause decompression illness (DCI). "Taravana," the diving syndrome described in Polynesian pearl divers by Cross in the 1960s, is likely DCI. It manifests mainly with cerebral involvements, especially stroke-like brain attacks with the spinal cord spared. Neuroradiological studies on Ama divers showed symptomatic and asymptomatic ischemic lesions in the cerebral cortex, subcortex, basal ganglia, brainstem, and cerebellum. These lesions localized in the external watershed areas and deep perforating arteries are compatible with cerebral arterial gas embolism. The underlying mechanisms remain to be elucidated. We consider that the most plausible mechanisms are arterialized venous gas bubbles passing through the lungs, bubbles mixed with thrombi occlude cerebral arteries and then expand from N_2 influx from the occluded arteries and the brain. The first aid normobaric oxygen appears beneficial. DCI prevention strategy includes avoiding long-lasting repetitive dives for more than several hours, prolonging the surface intervals. This article provides an overview of clinical manifestations of DCI following repetitive BH dives and discusses possible mechanisms based on clinical and neuroimaging studies.

Keywords: bubbles, AMA, stroke, cerebral infarct, mechanism

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INTRODUCTION

Underwater breath-hold (BH) diving is practiced casually by millions of beachgoers and snorkelers. Risks of casual BH diving involve ear and sinus barotrauma, shallow water black-out, and drowning but not decompression sickness (DCS) because of limited depth and time involved. However, the extreme BH diving exposure as seen in professional harvester divers, competitive spearfishermen, and freedivers are sometimes associated with acute brain injuries, and in some cases, with chronic dysfunction. There are also some reports about asymptomatic brain lesions in BH divers without a history of acute neurological symptoms. The causes and

mechanisms of neurological post-dive conditions are not clear yet, and the term BH diving neurologic deficit (BHDND) was suggested in a symposium (**Table 1**; Wong, 2006). The stroke-like manifestations and time relation to BH diving in acute cases are suggestive of DCS, cerebral arterial gas embolism (CAGE), and hypoxic brain injury as the most likely causes. Imaging of brains in BHDND reveals lesions similar to stroke and micro-stroke caused by embolism of various origins. While this does not exclude hypoxic injury due to hypoxemia or hemodynamic hypoperfusion, it shifts the focus of discussion toward various forms of decompression illness (DCI), including paradoxical venous gas emboli (VGE), CAGE, and intraarterial growth of bubbles from nanobubble buds on hydrophobic vascular surfaces.

After 25 years of studying dive-related brain injuries in Ama divers, we find that the evidence, although not yet unequivocal, points toward the DCI as a primary cause. This paper aims to review the epidemiology, clinical manifestation, imaging, functional studies, and possible brain injury mechanisms in BH divers, especially Japanese Ama divers. Available data pertain to several distinctive groups, including Polynesian indigenous harvesters, spear-fisher, and competitive freedivers.

DIVING PRACTICES OF AMA

Polynesian harvester divers may have the longest history of BH diving, extending into the 20th century. They have used googles and no other equipment or protective suit, but some could dive deeper than 30 m and stay underwater longer than 5 min. They dived repeatedly for several hours a day with a variable surface time between descents. At the end of the day, divers sometimes manifested neurological symptoms usually of short duration, which became known as "taravana"(Cross, 1965).

Commercial or professional BH divers of Japan and Korea, known in the scientific literature as "Ama" (men and women of the sea) have been in existence for more than 2,000 years (Teruoka, 1932). One theory contends that this diving tradition originated from Polynesian pearl divers (Hong and Rahn, 1967). In Japan, Ama divers start their profession at the age of 15–16 years and continue working for more than 20 years. Divers older than 60 years are not rare (Hong and Rahn, 1967; Shiraki et al., 1985).

Ama divers use one of two primary methods: Cachido unassisted diving and Funado assisted diving. Ama divers usually begin their carrier as Cachido divers and dive without any aids to depths of 3–10 m. With the experience, they may graduate to Funado and, using weight for faster descent, dive deeper – occasionally over 30 m (**Figure 1**). Funado divers hyperventilate and emit a pursed-lip whistle before descending to the bottom, spending 15–45 s for harvesting (**Figure 2**). They usually stay at the surface 30–60 s between the two dives. They work 2–6 h a day, usually in two shifts, with a short break for lunch (Tamaki et al., 2010a; Lemaître et al., 2014). In Japan, local unions regulate harvesting season, daily shifts, and diving patterns and do not allow the use of wetsuits to protect their natural resources from over-harvesting in some areas.

Sport spearfishers in the Mediterranean regularly use submarine scooters to achieve quick descents and ascents. They may do 15–20 dives per hour for 3–8 h, in a depth range of 20–60 m lasting for 2 min or more each, with surface intervals in between sometimes as short as 2 min or less (Batle, 1999). Freedivers can dive to depths greater than 100 m and stay up to 5 min and ascend fast to the surface. They practice glossopharyngeal insufflation and exsufflation to increase their diving capacities, which exposes them to additional risks (Lemaître et al., 2009; Schipke et al., 2019).

TABLE 1 | Clinical manifestations of decompression illness (DCI) in compressed-air and breath-hold (BH) divers.

	Breath-hold divers (Wong, 2006)	Compressed-air divers (Vann et al., 2011)
Non-neurological	✓ Dizziness/vertigo	✓ Pain
	✓ Constitutional*	✓ Constitutional
	✓ Pain	✓ Dizziness/vertigo
	✓ Cardiovascular	✓ Cutaneous
		✓ Muscle discomfort
		✓ Pulmonary
		✓ Auditory
		✓ Lymphatic
		✓ Cardiovascular
Neurological	✓ Motor weakness	✓ Numbness/paresthesia
	✓ Numbness/paresthesia	✓ Motor weakness
	✓ Consciousness	✓ Mental status
	✓ Mental status	✓ Coordination
	✓ Altered speech	✓ Consciousness
	✓ Visual disturbance	✓ Bladder/bowel
	✓ Coordination	
	✓ Convulsions	
	✓ Bladder/bowel	

Constitutional symptoms include headache, fatigue, agitation, nausea, and vomiting.

CLINICAL MANIFESTATIONS OF BH DIVING NEUROLOGICAL SYNDROME

Post-dive Neurological Manifestation in Ama Divers

Commercial breath-hold divers Ama of Japan and Korea have been studied extensively. A survey among Ama conducted in one diving village in Japan showed that nine out of 16 Funado divers had histories of neurological accidents during or immediately after repetitive BH diving (Kohshi et al., 2001). This prompted a much broader survey which showed that 12 of 173 Ama divers (6.9%) had experienced post-dive stroke-like neurological events (**Table 2**). The incidence was much higher in Funado (11 out of 29) than in Cachido divers (one out of 144). All affected divers were males (Tamaki et al., 2010a). The most common symptoms were sensory numbness in eight cases, and hemiparesis in six cases. Other symptoms were dizziness, vertigo, nausea,

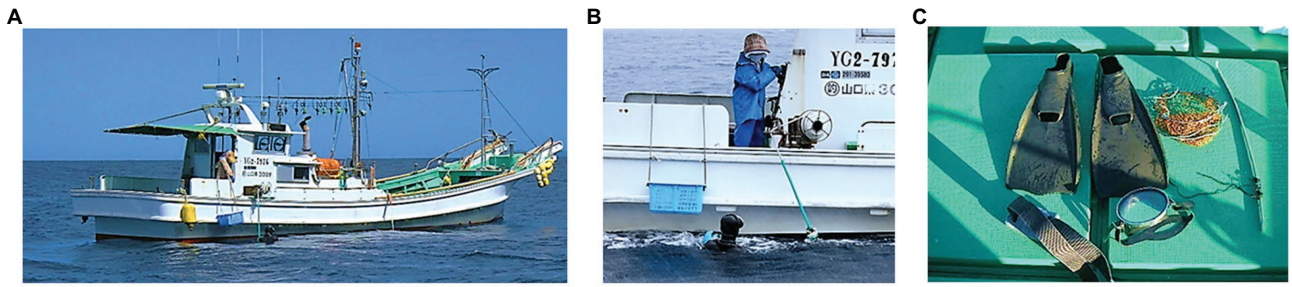

FIGURE 1 | An assisted male Ama diver, called Funado needs his partner (generally, wife) to operate fishing boat, which has a rolling machine to pull up his weight and a basket for seafoods **(A)**. His wife on the boat pulls up an iron-weight and gets closer to Ama after each his dive **(B)**. Ama's diving equipment includes weight belt, fins, goagle, and hook and net for sea foods **(C)**.

FIGURE 2 | Photos of an assisted male Ama diver during breath-hold diving. He uses an iron weight of 20-kg to descend passively to a depth of 10–30 m **(A)**, and he searches and gathers sea foods on the bottom **(B)**. After 1 min dive, he swims to the surface without assistance **(C)**.

and limb pain. Dizziness was particularly common after continuous long-lasting dives in Funado divers. However, although explicitly asked, Ama divers never reported symptoms suggesting pulmonary barotrauma, like chest pain, hemoptysis, or dyspnea. Two of 12 divers with neurological events also had severe knee and limb pain, but none had a skin rash nor swelling. In 10 of these divers neurological disorders wholly resolved without any treatments, one diver had a residual partial visual deficit, and another had a sensory numbness of the hand. All instances of neurological conditions appeared to be involving the brain. There was no apparent spinal cord involvement, frequently seen in compressed-air diving injuries.

Symptoms in Ama divers were similar to those in Polynesian divers. The stroke-like brain insults appeared during or few minutes after ending several hour-day shifts of repetitive diving to a depth exceeding 20 m. Dizziness or blurred vision sometimes preceded the onset of other neurological symptoms (Wong, 2006). The symptoms usually start as mild and may resolve in half an hour or deteriorate within a few hours (Kohshi et al., 1998, 2000, 2020; Tamaki et al., 2010b). One of the clinical characteristics of DCI in Ama divers is that severe neurological symptoms, unlike in CAGE following pulmonary barotrauma, rarely occur suddenly.

Post-dive Neurological Manifestation in Other Repetitive BH Divers

Batle (1999) reported 25 cases of spearfishers with neurological symptoms appearing immediately on surfacing (**Table 2**). All divers received recompression therapy and their symptoms entirely resolved. Wong (2006) reported eight cases of Australian BH spearfisher with similar symptoms, short duration, and no sequelae in most cases.

Post-dive Neurological Manifestation in Single Deep BH Dives

Single deep BH dives following a few shallow dives can cause DCI-like insults, though the cases are less frequently reported. Tetzlaff et al. (2017) summarized reports of stroke-like symptoms following single deep BH dives, including one case they treated and three reported by others. In all four cases, diving depths exceeded 100 m (**Table 2**). The symptoms included motor weakness, sensory numbness, unconsciousness, and speaking difficulty. The case they treated was a 31-year-old man who did three dives to a depth of 100 m with 15 min of surface intervals and developed speaking difficulty and right-sided motor weakness immediately after the last dive. His laboratory studies and chest CT findings were normal.

TABLE 2 | Diving events in different types of BH divers.

	Spanish spearfishermen (25 cases; Batle, 1999)	Japanese Ama divers (12 cases; Tamaki et al., 2010a)	Competitive athletes (four cases; Tetzlaff et al., 2017)
Manifestation	✓ Constitutional (23)*	✓ Dizziness/vertigo (8)	✓ Numbness/paresthesia (3)
	✓ Numbness/paresthesia (17)	✓ Numbness/paresthesia (8)	✓ Motor weakness (2)
	✓ Consciousness (13)	✓ Motor weakness (6)	✓ Altered speech (2)
	✓ Motor weakness (11)	✓ Altered speech (3)	✓ Dizziness/vertigo (1)
	✓ Visual disturbance (10)	✓ Constitutional (2)	✓ Unconsciousness (1)
	✓ Altered speech (7)	✓ Pain (2)	
	✓ Dizziness/vertigo (5)	✓ Visual disturbance (1)	
	✓ Coordination (5)		
	✓ Memory loss (4)		
	✓ Convulsions (1)		
	✓ Sphincter relaxation (1)		
	✓ Auditory disturbance (1)		
	✓ Cardiorespiratory arrest (1)		
	✓ Pain (1)		

Parenthesis means total number of cases.

Mental Disorders in BH Diving

Male Ama divers have reported no psychiatric disorders following diving work, although, they may occasionally complain of anxiety during deep and long-lasting dives. In contrast, female Ama divers have suffered specific psychiatric disorders called "Chiyamai" related to their dives on an island (Tochimoto et al., 1998). A survey of 44 female, Ama divers noted that nine of them had mental disturbances related to anxiety attacks. On this island, their diving depths and durations were deeper and longer in other areas (Hong et al., 1991; Mohri et al., 1995). Their diving patterns were similar to those of male Ama divers with diving accidents (Tamaki et al., 2010a). Although, the clinical features of psychiatric disease closely resemble those of some types of panic disorders, female divers did not have depersonalization or de-realization. The clinical symptoms included palpitation, dizziness or unsteady feelings, dyspnea, nausea, and hot flushes; palpitation was the most frequent (Tochimoto et al., 1998). Several Ama divers who had experienced the disorder could not dive and had to stop their diving work. While female Ama divers may have recovered from the disorder, they could not dive at great depths and always had to take anti-anxiety medicine prior to diving. No diving-related psychiatric disturbances have been reported among female or male Ama who dive shallower, in contrast, Polynesian pearl divers frequently felt mental anguish as a form of "taravana" syndrome. A few of them were mentally affected with such symptoms as restlessness, irritability, and poor understanding (Cross, 1965). Mental disorders associated with deep and long-lasting repetitive BH dives are rare, and there is no evidence that their causes are organic.

Neuropsychological Examinations in BH Diving

Results of neuropsychological studies in BH divers vary. We will mention here only two illustrative studies. Ridgway and McFarland (2006) evaluated neuropsychological investigation in 21 elite freedivers and found no significant differences between the different diving careers. Another study comparing trained BH divers with matched controlled subjects found that divers had slower responses on a Stroop test and more errors on interference card tests (Billaut et al., 2018). These findings were correlated with the maximal BH abilities ($r = 0.73$, $p < 0.05$) and years of training ($r = 0.79$, $p < 0.001$), collectively suggesting that apnea training can cause persistent episodic memory impairments.

NEUROIMAGING STUDIES

We first reported cerebral infarction in Ama divers occurring after repetitive dives to the depths of 15–25 m (Kohshi et al., 1998). Since then, we documented by MRI many more cases (Kohshi et al., 2000, 2020; Tamaki et al., 2010b). In Ama divers with post-breath-hold stroke-like symptoms, brain MRIs showed single or multiple cerebral infarcts in areas corresponding to the symptoms and elicited signs. The brain lesions are localized in the cerebral cortex, subcortex, basal ganglia, brainstem, and cerebellum (**Figure 3**). The neuroradiological findings are consistent with the vascular pathogenesis of the lesions; the presence of cerebral infarcts suggests a shower of various sized emboli migrating into the watershed areas and distal vascular territories of deep perforating arteries (Wodarz, 1980; Mull et al., 1997).

Watershed infarcts are classified into two broad categories as external (cortical) and internal (subcortical) infarcts (Momjian-Mayor and Baron, 2005; Mangla et al., 2011). The formers involve the junction of the distal fields of cortical arteries and are usually wedge-shaped or ovoid, and they may be embolic rather than hemodynamic in nature. The latter are located at the junctions of the cortical arterial territories with deep

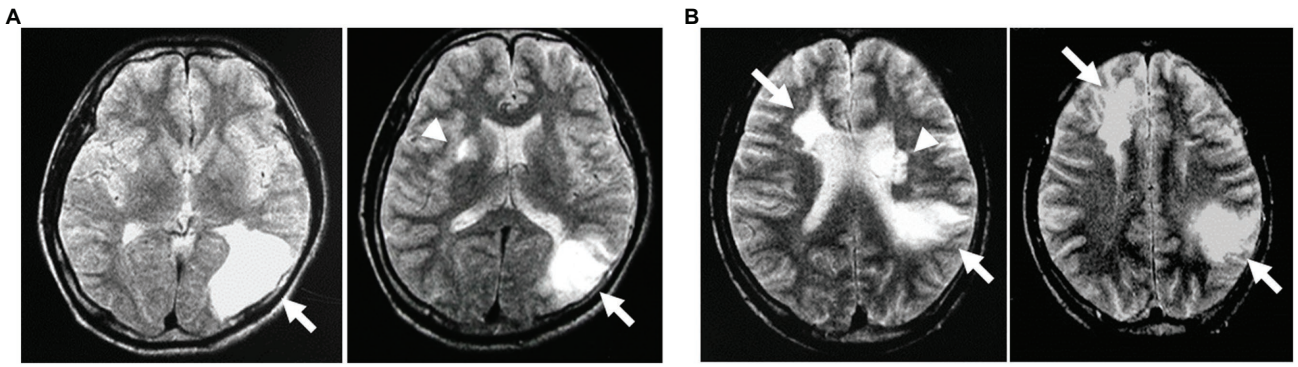

FIGURE 3 | MRI of the brain in a 33-year-old assisted Ama diver with right homonymous hemianopsia. T2-weighted MRI obtained on the 4th day after the diving event shows two increased signal intensities in the left occipital lobe (arrow) and the right basal ganglia (arrow head; **A**). A 39-year-old assisted male Ama diver with right-sided hemiparesis and hemisensory numbness. He suffered from transient left hemiparesis at the age of 17, 25, and 27 years, and his T2-weighted MRI on the 3rd day after the event shows three increased signal intensities in the right frontal lobe and left parietal lobe (arrows) and the left basal ganglia (arrow head; **B**; taken from Kohshi et al., 2000 with permission).

perforating arteries, showing the rosary-like pattern in the centrum semiovale; they are mainly affected by hypoperfusion due to arterial stenosis or hemodynamic impairment. The most common cerebral lesions revealed by MRI in Ama divers with BHDND appear external watershed infarcts suggesting arterial embolism.

The second group of common lesions seems like lacunar infarcts in the territories of the deep perforating arteries in the basal ganglia and brainstem. The occlusions causing lacunar infarcts are considered to be due to atheromatous changes, secondary hypertension, or diabetes mellitus. Still, one-third may include emboli from cardiac or carotid sources (Horowitz et al., 1992). Lacunar lesions were also reported in compressed gas divers with or without a known history of DCI (Palmer et al., 1992).

The third group of lesions in Ama divers involves the cerebellum (Tamaki et al., 2010b), located in the watershed area between the penetrating cerebellar arterial branches (Savoiardo et al., 1987). Generally, cerebellar infarcts are uncommon in strokes, and more than half of the cases originate from cardiac embolic sources (Kase et al., 1993). In summary, neuroimaging findings in Ama divers with BHDND support the possible role of DCI emboli affecting the external watershed areas (arrows, **Figure 3**) and the territories of deep perforating arteries of the brain (arrowheads, **Figure 3**).

In a radiological study of Ama divers without neurological deficits, we found brain changes in 11 out of 12 divers. The age of divers was 44 to 61 (median: 56), and four of them had a history of BHDND (Kohshi et al., 2014). Brain changes in divers without a history of BHDND may be due to the cumulative effects of repeated transient ischemic injury. Even in the absence of MRI signs, single-photon emission tomography (SPECT) in five elite BH divers found diffuse abnormal perfusion of the brain (Potkin and Uszler, 2006). Recently, Moir et al. (2019) have suggested a possible impairment of cerebral autoregulation in elite competitive BH divers. Long-term repetitive BH diving probably affects perfusion and causes local or diffuse cerebral ischemia, and it deserves further studies.

POSSIBLE MECHANISMS OF DCI IN BH DIVING

While the signs and symptoms of acute BHDND appear consistent with DCI there are still unresolved questions of underlying mechanisms (Schipke and Tetzlaff, 2016). In BHDND, neuroradiological imaging shows large cerebral infarcts like those in CAGE following pulmonary barotrauma or iatrogenic embolism (Wityk et al., 2001; Gao et al., 2009), but the clinical course is different. The post-dive neurological symptoms in commercial BH divers appear mild at onset and may gradually deteriorate within several hours (Kohshi et al., 1998, 2000; Tamaki et al., 2010b; Cortegiani et al., 2013), while in the CAGE symptoms occur all at once immediately after surfacing (Neuman, 2003). Prompt symptoms resolution with hyperbaric oxygen (HBO_2) treatment (Paulev, 1965; Tetzlaff et al., 2017) or oxygen (O_2) inhalation alone (Gempp and Blatteau, 2006) may support the hypothesis that brain insults in BH divers are caused by inert gas bubbles in the brain. However, Ama divers with acute BHDND have neither symptoms nor signs of pulmonary barotrauma, and CAGE appears less likely. It may instead be DCS, but intravascular bubbles in BH divers are rarely detected. Thus, the question of what could cause the cerebral insults in BH divers remains an unresolved issue.

Nitrogen Accumulation

Direct measurement of nitrogen (N_2) in peripheral venous blood of unassisted Korean Ama divers after a series of diving to 3–6 m has shown a significant but insufficient accumulation to cause DCS (Radermacher et al., 1992). However, N_2 accumulation could amount to levels necessary to cause DCI in multiple deeper and longer-lasting BH dives with short intervals.

Calculations of saturation and desaturation using compressed gas diving models show that conditions for DCS could occur with deep, repetitive BH dives and short surface intervals (Paulev, 1965; Olszowka and Rahn, 1987). Based on these

calculations, it appeared that repeated BH diving to less than 20 m would be safe if the surface time is equal to or longer than the dive time (Lanphier, 1965). However, our interview survey found that some Ama divers experienced neurological events after a series of dives to a depth of around 15 m and a surface time to a dive time ratio of 0.4 (Tamaki et al., 2010a). Gempp and Blatteau (2006) reported a case of a 21-year-old man who had transient neurological disorders 2 h after 10–12 times of BH dives to 10–18 m over 60–90 min; the diving time was 1–2 min each with surface intervals of 5–6 min. BHDND after dives predicted safe by models may still be the DCS, but the compressed gas models do not fit BH diving. Neurological problems were also reported in shallow (6–8 m) BH diving for more than 4 h (Wong, 2000, personal communication), which seems too shallow for DCS.

Single BH dive does not provide enough N_2 to cause DCS (Paulev, 1965; Olszowka and Rahn, 1987; Radermacher et al., 1992), unless extremely deep and preceded with few shallower preparatory dives. Tetzlaff et al. (2017) presented four cases of competitive freediving athletes experiencing stroke-like insults after BH dives to maximum depths of 100–249.5 m. Such dives have been mathematically shown to create supersaturation and generate gas bubbles.

Detectable Venous Gas Emboli

After repetitive BH dives, N_2 bubbles are probably formed in the small veins of tissues and flow into the right atrium. Spencer and Okino (1972) used continuous-wave Doppler. They described the signals for 1 hour, suggesting air bubbles in a Japanese Ama diver after a 51-min period of 30 dives to 15 m in depth. Boussuges et al. (1997), however, using wave Doppler and two-dimensional echocardiography, did not find any circulating bubbles in 10 BH divers for a mean duration of 4 h and 3 min (2–6 h) and at a mean maximum depth of 35 m (24–40 m). Lemaître et al. (2014) recorded the "lowest" intravascular bubbles (Spencer's grade 1; occasional bubbles) by precordial Doppler monitoring in only one of 12 Ama divers (mean age: 55.6, 48–66) after 186 min of 99 repetitive dives; mean maximal depth and dive duration were 15.8 m and 68 s, respectively. The bubbles were identified after 2 min of detection and lasted only for 10 s. While out of these 12 Ama divers, four had previously experienced neurological diving events, ischemic cerebral lesions were found by MRI in 11 of them (Kohshi et al., 2014). Huggins and Stepanek (2006) followed a competitive freediver during 4 consecutive days of training dives with consistent dive profiles up to 50 m depth. Only on one occasion, at the end of the day, when the diver overused his right hand, they detected Grade 1 bubbles in his right subclavian vein. Remarkably, this diver did his preparatory dives on functional residual capacity, meaning that the availability of N_2 in alveoli was minimal. The only impressively high and lasting bubble grade (Eftedal-Brubakk's grade 4; continuous bubbling) after BH diving was reported in a spearfisher performing 15 training dives in a deep pool with a water temperature of 33°C. The median depth of dive was 40.2 m (6.2–41.7 m), and the mean duration 140.9 s. Interestingly, the computer calculated gradient factor at the end of the dive session was 0.33, usually not associated with such a high bubble grade in scuba diving (Cialoni et al., 2016). The diver's BMI was 24, which is similar to Ama divers' (Lemaître et al., 2014). In another recent echocardiography study, Barak et al. (2020) recorded the "lowest" venous bubbles in six out of 11 BH divers for 15 min after dives at rest or after provocation. Interestingly, the results were similar after eight dives to 35 m for 1 h and after 6 h of competitive dives to depths between 15 and 40 m.

So far, detectable VGEs in BH divers are rare, and it appears that there are more divers with cerebral symptoms than with VGE. However, the measurement results of such bubble detection may simply mean that we have no data of the best measurable condition under which VGEs are the most detectable in BH divers.

On the other hand, in compressed-air diving, despite the abundance of VGE often detected (Nishi et al., 2003), DCS is rare, and spinal involvement is more common than cerebral (Vann et al., 2011). The risk of VGE causing DCS, in general, is low because the pulmonary circulation effectively filters VGE. Why lesions in BH diving mainly involve the brain is an unresolved dilemma. A possibility that BH may generate undetectable microbubbles cannot be excluded (Nishi et al., 2003; Eftedal et al., 2007), despite recent improvement in the resolution of VGE detection tools (Le et al., 2021). VGE in BH diving deserves more systematic studies. Gas embolization alone may not be sufficient to cause DCI. Repeated bubble insults and involvement of other processes may be necessary (Thalmann, 2003; Balestra et al., 2019; Barak et al., 2020).

Microbubbles and Nanobubbles

Mammalian lungs usually constitute a complete filter for bubbles larger than 21 μ in diameter (Butler and Hills, 1979; Butler and Katz, 1988). Smaller bubbles would not usually cause detectable brain lesions since they can pass through the capillaries of the brain. In an experimental study, Hills and James (1991) showed that such bubbles could transiently impair the blood-brain barrier. One could assume that a prolonged and repeated release of microbubbles might cause permanent damage to the brain. Moreover, gas bubbles serve as an interface for aggregating blood components such as platelets and leukocytes acting as emboli (Francis and Mitchell, 2003).

An interesting theory of decompression bubbles developing from gas micronuclei called nanobubbles has been published recently. The nanobubbles have been shown to occur on active hydrophobic spots. They may also appear on endothelial surfaces in the lungs, pass through intrapulmonary shunts or the patent foramen ovale (PFO), and lodge in remote tissues where they continue to grow. Nanobubbles may also appear as autochthonous bubbles in distal small arteries or capillaries of the brain. With further growth, they can cause cerebral blood flow disorder (Goldman and Solano-Altamirano, 2015; Arieli and Marmur, 2017; Arieli, 2019a,b). While this hypothesis is supported by *ex vivo* experiments (Le et al., 2021), there is no experimental evidence that it happens *in vivo*, and there are also some concerns regarding its' basic assumptions (Doolette, 2019).

The nanobubbles hypothesis implies multiple small infarcts in the internal watershed areas most vulnerable to hemodynamic impairment (Moody et al., 1990; Momjian-Mayor and Baron, 2005). However, the cerebral lesions in Ama divers with neurological DCI are not situated in these areas (Kohshi et al., 1998, 2000, 2020; Tamaki et al., 2010b), but instead in the external watershed areas (Matsuo et al., 2012).

Intracardiac and Intrapulmonary Shunts

Gas bubbles formed in the venous blood after long-lasting repetitive BH dives can cross from the venous side to the arterial side (arterialization) through intracardiac or intrapulmonary right-to-left shunt (RLS). The intracardiac RLS, including the PFO and atrial septal deficit, are present in 10–30% of healthy adults (Hagen et al., 1984; Lynch et al., 1984). Bubbles that pass through these shunts can cause DCI as a paradoxical embolization of the brain. In compressed-air divers, the proportion of cerebral ischemic lesions was closely related to intracardiac RLS presence (Knauth et al., 1997; Gempp et al., 2010). The RLS has been documented in at least one BH divers with post-dive DCS-like neurological symptoms (Gempp and Blatteau, 2006), but it has not been detected in Ama divers with the BHDND (Kohshi et al., 2000; Matsuo et al., 2012). These reported cases suggest that neurological DCI in BH divers cannot be explained only by intracardiac RLS and that several possible mechanisms may be involved in cerebral DCI (Fitz-Clarke, 2018). Another pathway allowing blood flow to bypass the lung capillaries are intrapulmonary arteriovenous anastomoses (IPAVA), which provide a route for right-to-left transmission of embolus of 25–50 μ in diameter. IPAVA can open during submaximal exercise (Eldridge et al., 2004; Ljubkovic et al., 2012), or even at rest in hypoxic conditions (Laurie et al., 2010). Schipke and Tetzlaff (2016) suggest that hypoxia enhanced IPAVA opening plays a key role in brain damage in BH divers. Barak et al. (2015) have shown in compressed-air divers that exercise-induced IPAVA enables arterialization of VGE but only a few arterial emboli reach cerebral circulation. Regardless, while the risk may be small, possible role of IPAVA in cerebral DCI after BH diving may not be dismissed.

Pulmonary Barotrauma

Cerebral arterial gas embolism following pulmonary barotrauma remains a possible cause of DCI in BH divers, at least in some cases. It has been reported in a shallow BH undersurface swimming (Harmsen et al., 2015), in fishermen and freedivers. Lungpacking by glossopharyngeal insufflation can cause lung barotrauma (Jacobson et al., 2006), and AGE before descent (Liner and Andersson, 2010). Deep diving, not necessarily to the depth of total lung collapse, may cause lung injury and AGE. Alaimo et al. (2010) reported a case of a 41-year-old diver who, after 5 h of repetitive BH diving to 20–24 m of depth, developed neurological DCI like symptoms. A brain CT revealed gas bubbles in carotid and left ophthalmic arteries consistent with AGE. Cortegiani et al. (2013) presented a stroke-like case in a 57-year-old BH fishing champion. The dive pattern and the MRI findings of a subcortical hypointense area in the temporal region were suggestive of DCS. Still, the chest CT findings of a ground-glass pattern indicated the lung squeeze, which could be the source of AGE in this case.

However, acute hemoptysis due to pulmonary barotrauma during deep descents in healthy freedivers is not uncommon (Boussuges et al., 1999; Kiyan et al., 2001; Cialoni et al., 2012; Schipke et al., 2019). Ama divers with BHDND had never reported symptoms of pulmonary barotrauma despite a systematic interrogation (Kohshi et al., 2001; Tamaki et al., 2010a), and their chest radiograms in acute cases demonstrated no abnormal shadows (Kohshi et al., 2000, 2020; Tamaki et al., 2010b; Matsuo et al., 2012). Thus, we believe that pulmonary barotrauma and CAGE is less likely cause of stroke-like neurological disorders with large ischemic cerebral lesions in Ama divers.

Other Factors

The pathophysiology of DCI in BH divers is not clear at all and is probably multifactorial. On the comments for the viewpoint, Foster et al. (2016) discussed some pathogenetic factors including increase in cardiac output or cerebral blood flow, hypercapnia, and hypoxia. In compressed-air diving, other factors like vascular dysfunction, microparticles, and neutrophil activation have been identified as potential contributors (Thom et al., 2015). Recently, Barak et al. (2020) have also described in BH diving that microparticles play an essential role in endothelial dysfunction of the brain. Although, the circulating microparticles could induce cerebral small vessel disease, the microparticle theory may not completely explain the differences in clinical characteristics of DCI between compressed-air and BH diving, nor the number and location of the large cerebral lesions.

A rapid increase in blood pressure, endothelial dysfunction, a transitory cerebral vascular autoregulation dysfunction, and blood-brain barrier breakdown in BH diving (Barak et al., 2015; Balestra et al., 2019; Patrician et al., 2021) may play a similar role as in a reversible cerebral vasoconstriction syndrome (RCVS) and posterior reversible encephalopathy syndrome (PRES) resulting from a cerebral arterial constriction in a context of major inflammatory diseases or injuries (Ducros, 2012; Pilato et al., 2020). However, clinical presentation and imaging in BHDND is different. Endothelial dysfunction has been observed after BH diving (Nossum et al., 2002; Theunissen et al., 2013; Barak et al., 2020), which may stimulate aggregation of blood components and form thrombi in vessels (Francis and Mitchell, 2003). However, acute neuroradiological findings in BHDND indicate the absence of multiple small cortical infarcts (Kohshi et al., 1998, 2000, 2020; Tamaki et al., 2010b; Matsuo et al., 2012), which suggest a typical shower of thrombotic emboli (Wityk et al., 2001).

The formation of cerebral ischemic lesions in BH divers probably requires gas bubble emboli in the main arteries of the brain. In addition, DCS appears a systemic disease that is related to gas bubbles, but its manifestations depend

on multiple factors and individual response of the diver (Balestra et al., 2019).

NEW HYPOTHESIZED MECHANISM

The neuroimaging studies show considerably large ischemic lesions in the external watershed areas and the territories of perforating arteries, a pattern seen in embolic brain injuries. However, VGEs after BH diving are detected rarely and in small quantities (Spencer and Okino, 1972; Lemaître et al., 2014; Barak et al., 2020). Thus, we believe that VGE need not be detectable to initiate processes resulting in cerebral ischemic lesions.

They may cause occlusion of cerebral arteries perfusing the external watershed areas and the perforating arteries. Major surface arteries with higher blood flow, branch, and taper into capillaries in the external watershed areas (Mangla et al., 2011); hence, small bubbles may be propelled by hydrostatic forces to reach this region (Dutka et al., 1988) and migrate into small cerebral arteries (average diameter, 30–-60 μ; Dutka, 1985).

These bubble seeds are the first step of neurological DCI in BH divers. Small amounts of intravascular bubble cause endothelial dysfunction (Nossum et al., 2002; Theunissen et al., 2013; Barak et al., 2020), may form thrombi and affect arterial occlusion of the brain. Or microparticles may induce bubble nucleation and contribute to vascular injuries (Thom et al., 2015; Barak et al., 2020). The progressive evolution of neurological disorders in most Ama divers seems compatible with gradual bubble growth. The expansion of bubbles is possible due to N_2 influx from the end of occluded arteries and surrounding brain tissue (Arieli, 2019a,b). At present, this hypothesis (**Figure 4**) may explain why cerebral DCI in BH dives, its clinical course, and neuroimaging findings.

TREATMENT AND PREVENTION

Experience with the treatment of DCI in BH diving is limited. However, HBO_2 is the treatment of choice for bubble-related disease. It appears effective for iatrogenic arterial gas embolism in the hyperacute phase (Blanc et al., 2002; Tekle et al., 2013), but not for acute ischemic stroke (Bennett et al., 2014). HBO is very effective in cerebral DCI of compressed gas divers when administered within 6 h of symptom onset (Blatteau et al., 2011). Similarly, when administered with a short delay, the HBO is effective in BH diving DCI (Batle, 1999; Wong, 2006; Cortegiani et al., 2013; Tetzlaff et al., 2017).

Breathing normobaric O_2 immediately upon symptom onset has been beneficial in compressed gas diving DCI (Longphre et al., 2007), and has become a standard first aid in recreational scuba diving.

Treatment of DCI in commercial and competitive BH divers should follow the practices adopted in compressed gas diving. Normobaric O_2 should be administered immediately upon symptom onset, followed by HBO_2 therapy as soon as possible. Early treatment may prevent permanent brain injury.

Prevention of DCI is critical for BH divers; the best risk mitigation strategy is to reduce exposure and increase surface times between consecutive dives. While diving depth and duration and bottom time are well known as risk factors for DCI in BH diving, the short surface interval and long diving shifts are probably major causes in BH dives to 10–20 m.

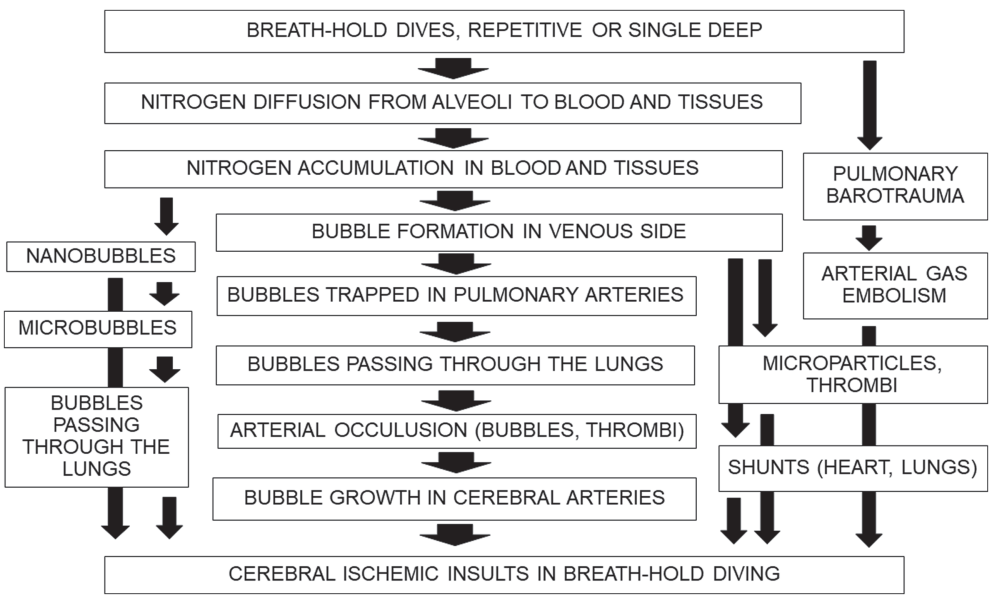

FIGURE 4 | Overview on pathophysiologic mechanisms of DCI in repetitive breath-hold diving. Large arrows show the new hypothesized mechanism and small arrows suggest other possible factors.

Breathing normobaric O_2 after long-lasting repetitive BH dives appears protective in BH and compressed gas diving (James, 2007; Blatteau and Pontier, 2009; Castagna et al., 2009). However, diving fishermen around the world may have no access to use O_2 for their diving work. Thus, the best strategy for mitigating the DCI risk in repetitive BH diving remains taking longer surface intervals and limiting the diving shift to less than 2 h.

CONCLUSION

The BH diving neurological deficit, both acute stroke-like manifestations and asymptomatic lesions, in our opinion, are decompression disorders initiated by gas embolism. Underlying injuries occur in the external watershed areas and the territories of perforating arteries of the brain, an area vulnerable to arterialized venous gas bubbles, which in the conditions of repetitive BH diving can grow inducing processes that lead to DCI. The mechanisms of brain injury following repetitive BH diving are not clear and seem to be multifactorial, but more research is necessary to establish complete understanding.

AUTHOR CONTRIBUTIONS

FL, KK, and TI contributed to concept and design of the study. HT, KK, TI, and YM obtained divers' data (images and clinical history), and FL took all photos. KK wrote the first draft of the manuscript. PD critically reviewed the paper. All authors contributed to the article and approved the submitted version.

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Effect of Apnea-Induced Hypoxia on Cardiovascular Adaptation and Circulating Biomarkers of Oxidative Stress in Elite Breath-Hold Divers

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Given the previous evidence that breath-hold diving is a cause of physiological stress, this study aimed to determine whether a combination static and dynamic apnea would affect total oxidant status, nitric oxide, heat shock proteins and cardiovascular parameters in elite freedivers. Thirteen finalists of the World and European championships in swimming pool breath-hold diving participated in the study. Whole-body plethysmography and electrocardiography was performed to determine the cardiorespiratory variables at baseline and during the simulation static apnea. An assessment of the heart rate, blood oxygen saturation and biochemical variables was performed before and in response to a combination of a static followed by a dynamic apnea. Static and dynamic breath-holding had a significant effect on oxidative stress, as evidenced by an increase in the total oxidant status/capacity ($p < 0.001$). The post apnea concentrations of heat shock proteins 27 (HSP27) were significantly elevated ($p < 0.03$, but total antioxidant status (TAS), HSP90, HSP70, and nitric oxide (NO) changes were not significant. levels under the influence of the static and dynamic breath-hold protocol. A significant positive correlation between HSPs and TAS ($r = 0.63$; $p < 0.05$) as well as NO levels was associated with beneficial cardiovascular adaptation. An increase in serum HSP27 levels mediated in nitric oxide levels could explain its important role in improving cardiovascular functions in elite freedivers. Further studies are necessary to explain the exact mechanisms of breath holds training of cardiovascular adaptation responsible for maintaining adequate oxygen supply in elite divers.

Keywords: freediving, cardiac function analysis, oxidative stress, hypoxia, heat shock (stress) proteins

INTRODUCTION

Individual and team world freediving championships include disciplines, such as Static Apnea (STA) in which the diver holding his breath for as long as possible with his nose and mouth immersed, or Dynamic Apnea in which the diver travels underwater attempting to cover the greatest possible distance with or without fins (Elia et al., 2021). The trends in improving world

records in this sport indicate specific training protocol including endurance training and breath-hold training with hypoxia exposition (Heusser et al., 2009; Cross et al., 2013). The diving reflex is a protective, multifaceted physiologic response whose aim is to preserve oxygen stores during times of water immersion. It is characterized by bradycardia, apnea, and increased peripheral vascular resistance which redistribute blood to the brain and hart while limiting oxygen consumption by non-essential muscle groups (Eichhorn et al., 2017; Vega, 2017). The reflex mechanism and physiological adaptation depend on various diving conditions (i.e., depth of diving, static or dynamic apnea, and water temperature). In well-trained freedivers, the ability to reduce oxygen saturation in the blood relatively slowly (Schagatay, 2011; Engan et al., 2013), the centralization of blood circulation, and bradycardia by stimulation of the trigeminal nerve (Buchholz et al., 2017) are important mechanisms to increase hypoxic tolerance. However, muscle contraction and higher energy demand during dynamic apnea decrease apnea tolerance.

The precise molecular changes responsible for cardiovascular adaptation in response to the independent and combined contribution of two factors, i.e., exercise and hypoxic exposure during static and dynamic apnea are still not well understood (Costalat et al., 2017; Bain et al., 2018; Elia et al., 2019; Cialoni et al., 2021b). It has been suggested that hypoxia and exercise alter molecular compounds of tissues, such as nitric oxide (NO) and endothelial relaxation factor (EDRF) levels. NO is known to play a crucial role in cytoprotection through its vasodilatation effect and its ability to modulate mitochondrial function. Exposure to hypoxia can stimulate the release of NO and increase inducible NO synthase (iNOS) gene expression, which has been suggested as the beneficial endothelial-dependent vasodilatation mechanisms in response to lowering oxygen availability (Lundberg et al., 2015). It has been evidenced that NO might play an important role in optimizing oxygen transport and restoration of arterial oxygen saturation after repeated breath-hold diving at a depth (Theunissen et al., 2013a; Cialoni et al., 2019; Mrakic-Sposta et al., 2019).

Some cardiovascular effects have been explained by the result of binding NO to the superoxide anion (O_2^{2+}) and reducing oxidative stress. Holding the breath during diving can cause adverse changes resulting from too long exposure to hypoxia and hypercapnia. These changes include the imbalance between the antioxidant capacity and the generation of reactive oxygen species (ROS; Cialoni et al., 2021a). Moreover, exposure to oxidative stress increases during the first breath when divers re-surface and the oxygen-deprived cells are flooded with oxygen. Tissue damage from ischemia and reperfusion, where there is an increased ROS production, might be partially inhibited by the higher release of NO and increase expression of heat shock proteins (HSPs; Inaguma et al., 1995; Djurhuus et al., 2010).

The induction of proteins from the HSP 27, HSP 70, and HSP 90 family in cardiomyocytes, endothelial cells and in the coronary vessels has a cytoprotective effect which is important in reducing the rate of stress factors-induced apoptosis (Snoeckx et al., 2001). Different functions of HSPs have been described to explain their physiological functions in response to hypoxia, including their role in the regulation in protein folding, oligomerization, translocation as well as anti-apoptotic properties (Lin et al., 2001; Arya et al., 2007).

Despite much-published data concerning the role of the above-mentioned cytoprotection in response to exercise training (Krüger et al., 2019), there is little information available on the effects of breath-hold training on oxidative stress, the antioxidant capacity of the blood, and the concentration of HSPs in breath-hold divers.

This study aimed to assess the relationship between the total antioxidant status (TAS), the NO levels, the concentration of HSPs and cardiovascular adaptation in response to a combination of a static followed by a dynamic apnea in elite freedivers.

MATERIALS AND METHODS

Subjects

Thirteen elite freedivers (three women and ten men) mean age: 35.8 ± 5.7 years, body height 180.5 ± 8.8 cm, body mass 78.3 ± 17.0, BMI 23.8 ± 3.6) participated in the study. They were the members of the National Team and finalists of the World and European championships in swimming pool breath-hold diving (AIDA, 2021). The sample size reflect the target population of total members of Polish Freediving Association who have regularly competed if freediving competitions prior to the study. The sample size (n = 13) were calculated with confidence level 95% and the confidence interval 20% from the total population of 40 freedivers.

The training status of the subjects was 7.2 ± 2.0 years and the experience of freediving coincided with the career length in competition was 7.1 ± 2.0 years. The training protocol of freedivers comprised three components: (1) aerobic endurance training (stationary cycling, treadmill, and swimming training with intensity of individual 70–80%, (2) STA and different disciplines of dynamic apnea training, (3) strength training, and (4) hypoxic training with increase the time of hypoxia exposition from 20% in preparatory training period to 70% of maximal BHT freediving training in specific and pre- competitive phase of training.

They were the members of the National Team and finalists of the World and European championships in swimming pool breath-hold diving (AIDA, 2021). The mean best results in the three freediving competitions were: STA 6.25 ± 1.29 min (minimum 5.28 min; maximum, 9.35 min.), distance without fins (DNF) 148.92 ± 45.09 m (minimum, 88.0 m; maximum 244.0 m), and the distance with fins 190.92 ± 60.56 m (minimum, 106 m; maximum, 300 m). All participants had valid medical certificates qualifying them to practice freediving. Two weeks prior to the study, subjects were asked to consume the recommended mixed diet. The daily intakes for energy were 30–35 kcal/kg/day with the proportion of protein, lipids, and carbohydrates 20, 20, and 60%, respectively. The diet were formulated with food items commonly available. Three weeks before the study and during the study protocol none of the respondents consumed supplements that would additionally modify an endogenous antioxidant protection.

All subjects were instructed to abstain from exercise within 24 h before the biochemical measurements.

The participants' age, height, body mass, body mass index (BMI), body composition, and training status are presented in **Table 1**. The body composition of all participants was evaluated using a model In Body220 analyzer (Biospace Inc., Seoul, Korea). At the baseline before the study protocol, the graded treadmill exercise test (HP/Cosmos-Pulsar, Germany) was performed to measure individual maximal oxygen uptake (VO_{2max}) (Matalyzer 3B, Cortex, Germany).

Before entering the study, lung function and cardiovascular variables were assessed. Whole-body plethysmography was used to measure lung volumes and diffusing capacity for carbon monoxide (Elite Platinum, Med. Graphics 2010, United States) according to ATS/ERS guidelines (Miller et al., 2005; **Table 2**). Electrocardiography (System ECG RScribe5, MDS Cardio, United States) was performed to evaluate the selected variables of cardiac function at baseline and during the simulation STA test (**Table 3**). At baseline, arterial oxygen saturation (SaO_2) (Konica Minolta PULSOX-300i, Japan) and systolic and diastolic blood pressure (SBP/DBP) (HEM-907 XL, Omron Corporation, Kyoto, Japan) were measured in all subjects.

All participants were informed about the aim of the research, the possibility of refusal of the participation and provided written informed consent. The study was approved by the Local Bioethical Committee (Decision No 3/2018) and conducted in accordance with the Declaration of Helsinki of the World Medical Association.

Study Protocol

All measurements were made in a pool environment with a water temperature of 27°C wearing a 5 mm-thick for STA and 1 mm-thick for dynamic apnea diving suits. Each diver performed two tests organized according to the (AIDA, 2021) competition protocol. The first immersion consisted in a STA, while the second consisted in a dynamic apnea without fins (DNF). The time between the two immersion protocols was 10 min. The BHT was recorded from the moment the face was immersed and finished when a diver emerged from the water. During the study protocol, the participants were controlled with a safety diver.

Before the STA test, all subjects performed a warm-up in which they repeated diving with 30, 50, and 60% of their maximum breath-hold-time obtained in the last 6 months. Then, the subjects started the STA test by performing hyperventilation of individual duration, inhaled to the maximum and dived into the swimming pool in a horizontal position (**Figure 1**) for a total immersion time of 70% of the individual maximum breath-hold time (BHT). After the STA test, the freedivers changed their diving suit and proceeded to the DNF test, in which the combination of apnea with exercise (swimming distance) was additionally assessed.

Measurements and Biochemical Analyses

An assessment of the heart rate (HR) and blood oxygen saturation (SpO_2) was continuously monitored during STA and before and immediately after dynamic apnea trials. To compare the results of STA, the values measured at 30, 50, and 70% of the subject's BHT were included.

At the beginning of the study (rest) and the end of the breath-hold test (post DNF test), all subjects had venous blood drawn for the determination of HSPs (HSP 70, HSP 27, and HSP 90), NO, total oxidant status and total oxidant capacity (TOS/TOC), and TAS.

Total oxidant status and total oxidant capacity showing the total lipid peroxide concentration directly related to the level of oxygen radicals could be a good representative of the level of oxidative stress in biological fluids (Sadowska-Krępa et al., 2021). The reference ranges for TOS/TOC <200, 200–350, and >350 μmol/l correspond to low, moderate, and high oxidative stress, respectively.

Blood samples from the antecubital vein were collected to separator tubes and, after 30 min, centrifuged for 20 min at 1,000× *g*. Obtained serum was kept frozen at −80°C until analyzed.

The serum HSPs levels were measured by enzyme-linked immunosorbent assay ELISA Kit (ELISA Cloud-Clone Corp, Germany). The intra-assay and inter-assay coefficients of variation values were <12.0% and the test sensitivity was

TABLE 1 | Characteristics of subjects.

Variables		Age (year)	Height (cm)	Weight (kg)	BMI (kg/m^2)	FAT (%)	FFM (kg)	TBW (kg)	BSA (m^2)	VO_{2max} (ml/kg/min.)	Training status (year)
n = 13	*X*	35.8	180.5	78.3	23.8	13.9	67.8	49.5	2.0	41.8	7.2
	SD	5.7	8.8	17.0	3.6	6.0	13.4	9.7	0.3	4.7	2.0

BMI, body max index; FAT, fat mass; FFM, free fat mass; TBW, total body water; BSA, body surface area; VO_{2max}, maximal oxygen uptake.

TABLE 2 | Lung function assessment.

Variables		TLC (l)	TLC Pred (%)	RV (l)	RV Pred (%)	IC (l)	IC Pred (%)	ERV (l)	ERV Pred (%)	DLCO (ml/min/mmHg)	DLCO Pred (%)
n = 13	*X*	8.3	118.7	1.8	96.8	4.6	134.3	1.9	128.4	39.6	115.0
	SD	1.5	1.5	0.4	22.9	1.2	19.1	0.7	50.2	10.5	20.3

TLC, total lung capacity; RV, residua volume; IC, inspiratory capacity; ERV, residual expiratory volume; DLCO, diffusing capacity for carbon monoxide; Pred, predicted values.

TABLE 3 | Electrocardiographic cardiac function at baseline (REST) and in response to simulation static apnea test (STA).

Variables	REST		STA	
	x	SD	*x*	SD
HR (b/min)	66.0	12.0	62.0	22.0
PR (ms)	164.0	18.0	179.0	70.0
QRS (ms)	103.0	16.0	106.0	20.0
QT/QTC	1.1	0.1	1.0	0.1
Mean RR (ms)	924.0	167.0	1059.0	335.0
QTcB (ms)	415.0	31.0	407.0	60.0

HR, heart rate; PR, interval represents the time between the onset of atrial depolarization and the onset of ventricular depolarization; QRS, ventricular depolarization; QTc, duration of ventricular repolarization; RR, intervals; QTcB, QT correction to HR.

FIGURE 1 | Static breath-hold test in water immersion (STA).

1.33 ng/mL for serum HSP70 concentrations. Intra- and inter-assay coefficients of variation (CV) for HSP27 were <12.0% and the test sensitivity was 0.31 ng/mL, and for serum levels of HSP90, the CV values were <12% and the test sensitivity was 1.22 ng/mL.

The total NO and nitrate/nitrite (xNO) parameters were measured by Assay Kit (R&D System, BIOTechne Brand, North America). Intra-assay precision was 2.5%, and inter-assay precision was 4.6%. Total NO, nitrite, and nitrate levels in various samples were measured with a sensitivity of 0.78 μmol/L, and the assay range was 3.1–200 μmol/L.

Total antioxidant status was measured by enzyme immunoassay (Randox UK, NX 2332), and total oxidant status (TOS/TOC) was determined using the PerOx diagnostic kit and enzyme immunoassay (TOS/TOC) ELISA Kit (Germany, REF 5100) with a sensitivity of 7 μmol/L and CV <6.63%. Plasma samples for TAS and TOS were stored at −20°C until biochemical analyses were performed.

Biochemical analyses were performed in our certified laboratory, meeting the requirements of PN EN-ISO 9001:2009 (certificate No. 129/2015).

Statistical Analyses

Descriptive statistics were calculated, and the results were presented as means and standard deviations (mean ± SD). All analyses were performed using the Statistica v. 10 statistical software package (StatSoft, Tulsa, OK, United States). The data were analyzed by one-way ANOVA followed by the Bonferroni test and the *U* Mann-Whitney test when appropriate. The statistical analysis includes a one-way ANOVA (rest vs. post-test), and Spearman correlation coefficients were analyzed to determine the inter-variable relationships. Statistical significance was set at $p < 0.05$.

RESULTS

The physical performance of the subjects, expressed as maximal oxygen consumption (VO_{2max}), was 41.8 ± 4.7 ml/kg/min. There were no significant differences between the plethysmography variables (TLC, RV, IC, and DLCO) and ECG variables of the studied group compared to references norms for this age group. A tendency to higher inspiratory capacity, expiratory reserve volume, and DLCO compared to the predicted values was observed (**Table 2**). A comparison between baseline and simulation STA test showed a trend toward slower heart rate rhythm (HR) and a longer time for ventricular depolarization (QRS) compared to rest. At the basis of ECG analyses no cardiac function abnormalities in the participants were found.

A significant effect of breath-hold and immersion on SpO_2 and HR was demonstrated in the STA test (**Figure 2**). Significant differences in the SpO_2 levels were observed between the values measured at 50 and 70% BHT compared to the bassline levels ($p < 0.001$ and $p < 0.001$, respectively). In the STA test, significant differences in the HR were demonstrated throughout the whole apnea time ($p < 0.001$).

Comparison of breath-holding time between the STA and DNF tests clearly indicated the effect of exercise on decrease apnea tolerance ($F = 85.2$; $p < 0.001$). There was a significant effect of apnea and exercise (DNF) on post-test HR ($F = 35.6$; $p < 0.001$). Higher BHT during the STA test corresponds to a significant decrease of HR but not SpO_2 levels.

A negative correlation was observed between 70% of BHT and SpO_2 ($r = -0.74$; $p < 0.05$). Significant correlation was observed between SpO_2 measured during 70%BHT and TLC $r = -0.59$; $p < 0.05$) as well as SpO_2 and DLCO ($r = -0.61$; $p < 0.05$).

The effects of breath-hold diving on serum HSPs and NO concentrations in freedivers were compared to baseline levels (**Table 4**). Analysis of variance revealed a significant effect of breath-hold diving protocol on serum HSP27 concentration (6.3; $p = 0.03$). ANOVA showed a non-significant effect of the intervention (rest vs. post-test) on serum total NO level ($p > 0.05$). ANOVA revealed a significant effect of the intervention on ($F = 18.2$; $p < 0.001$) on serum PerOx levels. A significantly higher post-test PerOx concentration was observed compared to the rest values ($p < 0.001$). There were no significant changes in the HSP70, HSP90 (**Figure 3**), and TAS under the influence of the static and dynamic breath-hold protocol (**Table 4**).

A significant positive correlation between post-test HSP70 and TAS ($r = 0.63$; $p < 0.05$) was observed. An inverse correlation between post-test TAS and SpO_2 ($r = -0.70$; $p < 0.01$) was also revealed. Significant positive correlations were detected between post-test NO and HSP70 ($r = 0.88$; $p < 0.001$); NO post-test

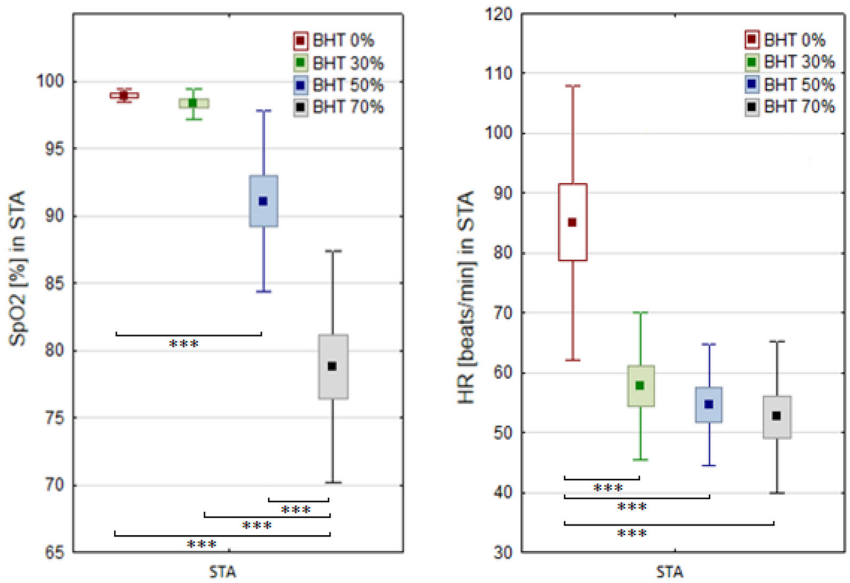

FIGURE 2 | Blood oxygen saturation (SpO_2) and heart rate (HR) changes before (BHT 0%) and at 30, 50, and 70% of breath holding time (BHT) during static apnea (STA). Significant differences ***$p < 0.001$.

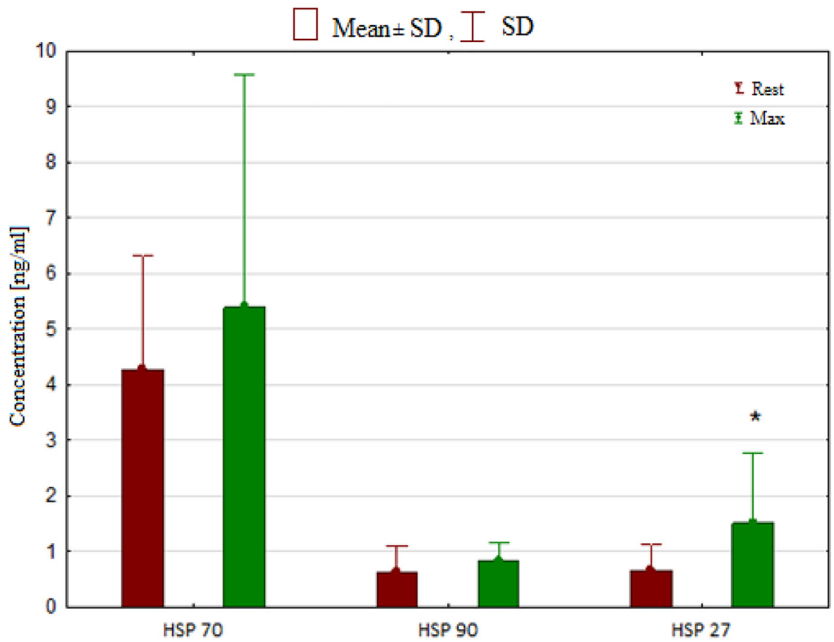

FIGURE 3 | Concentration of heat shock proteins (HSP) in rest and after breath hold diving. Significant differences *$p < 0.05$.

and HSP90 ($r = 0.59$; $p < 0.05$); and NO post-test and HSP 27 ($r = 0.66$; $p < 0.05$).

DISCUSSION

The present study investigated whether breath-holding will induce adaptive cardiovascular mechanisms during the combined contribution of two factors hypoxic exposure STA and hypoxia and exercise training during dynamic apnea in elite freedivers. It also aimed to determine if this effect, if present, is accompanied by changes in serum levels of NO, HSPs (HSP27, HSP70, and HSP90), and blood antioxidant status.

Our results demonstrate an increase RR, the interval time (PR) and ventricular repolarization time (QT/QTc) and a decrease sinus rhythm the after breath-hold maneuver during STA

test compared to the baseline values in freedivers. Significant changes in the QT interval depend on the frequency of heart depolarizations. This study may indicate a significant relationship between bradycardia and QT prolongation in response to STA. Breath-hold diving did not adversely affect the heart function of elite divers who participated in this study, and the analyzed variables fell within the reference values (Charbit et al., 2006; Mason et al., 2007). Freedivers had normal electrocardiographic parameters both at rest and after the STA test. The results of this study confirm the latest reports in the study of Kafes et al. (2020), who observe the lower risk of cardiac dysfunction by monitoring the ECG and hemodynamic parameters after maximum breath-hold of 24 athletes participating in the freediving competition. Similarly with our study, in divers with an average BHT of 113 s and low SpO_2 value (88%), normal heart function was demonstrated (Laurino et al., 2012).

It has been evidenced that maximal apnea time in divers was accompanied by marked oxygen desaturation (Stewart et al., 2005; Willie et al., 2015). At the end of apnea, divers showed a >5-fold greater muscle sympathetic nerve activity increase with increased vascular resistance. The rise in muscle sympathetic nerve activity correlated with oxygen desaturation and with the increase in mean arterial pressure (Lemaitre et al., 2005; Heusser et al., 2009). Contrary to these results, cardiac arrhythmias by monitoring the electrocardiogram (ECG) were also found in 12 out of 16 recreational divers during voluntary immersed breath holds. It has been suggested that the occurrence of cardiac arrhythmias was significantly associated with BHT and was associated with individual factors, such as the tolerable SpO_2 decrease (Hansel et al., 2009).

In our study, the average duration of breath-hold in elite athletes was 269 ± 62 s in the STA test and was significantly longer compared to results presented in previous studies (Hansel et al., 2009; Laurino et al., 2012). There was a significant reduction in HR and SpO_2 depending on BHT, and significantly lower values were observed during the STA test compared to the dynamic test (DNF). On this basis, we can conclude that the training used in the studied group of freedivers increased apnea tolerance. However, a limitation of our study is the lack of the possibility of referring to the values of cardiovascular indices in the respondents to the values before their training period. Importantly, apnea time was negatively correlated with SpO_2 at the level of 70% of maximum apnea, and lower SpO_2 was associated with higher lung capacity (TLC) and diffusing capacity for carbon monoxide (DLCO). These results might indicate an increase in lung volume and function as an important factor in adapting the mechanism to freediving (Overgaard et al., 2006). Several other adaptive mechanisms cannot be ruled out that protect against hypercapnia and hypoxemia developing during apnea (Lindholm and Lundgren, 2008; Bain et al., 2018; Taboni et al., 2019; Elia et al., 2021).

From the literature data, it may be suggested that the slowing of the HR was the result of the activation of cardiac vagal fibers following the stimulation of peripheral chemoreceptors by hypoxemia (Lemaitre et al., 2005, 2008; Wierzba and Ropiak, 2011; Willie et al., 2015). Moreover, stimulation of parasympathetic fibers leads to increased bradycardia resulting from immersing the face in the water (Lemaitre et al., 2015). The phasic HR responses throughout a dry, static breath-holding in elite divers (Perini et al., 2008) and three distinct phases (i.e., an initial reduction-phase I, plateau-phase II, and further reduction-phase III) have been observed. The results of the presented study do not clearly confirm the phase HR changes. It should be emphasized that the bradycardia observed was 30% of BHT might be explained by an increase in the sensitivity of diving reflex receptors due to long-term apnea training.

Molecular mechanisms protecting against tissues and myocardium ischemia and hypoxia, although well known in clinical studies, are relatively rarely assessed in research data of athletes who practice diving (Marongiu et al., 2015; Zelenkova and Chomahidze, 2016). The protective mechanisms that have been confirmed in previous studies include: expunction of brain vessels due to the increase in carbon dioxide levels, increased dissociation of oxygen from hemoglobin, and the use of oxygen reserves (Tocco et al., 2012; Cross et al., 2013).

Previously, a significant role for NO and the HSPs in adapting the vascular system (Andreadou et al., 2015) and important cardioprotective factors after repeated breath-holds has also been considered (Joulia et al., 2009; Marlinge et al., 2019; Cialoni et al., 2021b). The most important results of this study include the increased release of HSPs (HSP 27) and the tendency of a higher concentration of HSP 70 and HSP 90 after the end of breath-hold trials compared to the resting values. Post static and dynamic apnea TOS was significantly greater in response to breath-holding maneuvers. We also found significant correlations between the post-test NO and HSP70, NO and HSP 90, and NO and HSP 27 levels. Serum HSP 70 concentration increased in response to apnea correlated with TAS. The observed increase of TAS was evident at the lowest SpO_2. In our study, the TOS/TOC measured in response to static and dynamic apnea pointed to moderate oxidative stress. The results indicate that changes in oxygen availability (SpO_2) during the breath-hold dive were associated with a significant increase in the total oxidant status (TOS/TOC) in the blood. Interestingly, changes in NO serum concentrations after performing breath-hold maneuvers may have no significant impact on the activation of protective

TABLE 4 | Results of selected biochemical indices before and after breath hold diving.

Variables	*n* = 13				*p*	*F*	Post-hoc
	Rest		Post test				
	X	SD	*X*	SD			
HSP 70 (ng/ml)	4.26	1.97	5.39	4.03	0.20	1.88	0.20
HSP 90 (ng/ml)	0.62	0.45	0.82	0.34	0.18	2.17	0.18
HSP 27 (ng/ml)	0.66	0.45	1.50	1.20	0.03	6.26	0.03
NO (μmol/L)	45.3	21.5	41.2	17.9	0.22	1.68	0.14
PerOx (μmol/L)	226.79	118.12	308.99	168.73	0.002	16.14	0.00
TAS (mmol/L)	1.47	0.11	1.50	0.14	0.15	2.07	0.15

HSP, heat shock protein; NO, total nitric oxide; PerOx, lipid peroxidation products; TAS, total antioxidant status.

vasoconstrictive mechanisms. However, the beneficial effects may be confirmed by the positive correlation between NO and HSPs levels.

Nitric oxide synthase (iNOS) and cyclooxygenase (COX-2) have been previously reported as important factors responsible for exercise-induced cardioprotection mechanisms (Bolli, 2007; Powers et al., 2014). Theunissen et al. (2013a,b) reported that during breath-hold diving, an increase in iNOS activity and superoxide anions levels had been observed as a result of transient hyperoxia followed by hypoxia and CO_2 accumulation. Therefore, NO has been suggested as an important element in initiating cardioprotective signals. However, its excessive accumulation during ischemia might contribute to the formation of nitrites and, consequently, reperfusion damage by nitrogen stress (Andreadou et al., 2015). The induction of ROS production and nitrate concentration in response to changes in molecular oxygen pressures might affect endothelial function (Mrakic-Sposta et al., 2019). Similar to the presented study, there was also an increase in the TOS levels but significantly lower antioxidant capacity after diving was observed. Joulia et al. (2003) suggested that the presence of oxidative stress after breath-hold sessions, as evidenced by an increase in the concentration of reactive substances in thiobarbituric acid. Contrary, no changes in the level of secondary lipid peroxidation products and the decrease of superoxide dismutase activity have also been observed (Mila-Kierzenkowski et al., 2015). These authors suggested that inhibition of free-radical processes occurs and/or the products of lipid peroxidation are quickly removed due to adaptation, which protects the elite divers against damages on the cellular level.

Our findings support the hypothesis that physiological and molecular processes are responsible for increasing tolerance in hypoxic conditions in breath-hold divers during a combination of static and dynamic apnea. It has been suggested that exercise training increases the concentrations of basal levels of HSPs (Lin et al., 2001; Peake et al., 2015). The protective effect of HSPs has been documented in experimental animal studies in which exercise-induced HSPs reduced the risk of cardiovascular diseases (Demirel et al., 2003; Senf et al., 2008). An increase in HSPs is believed to protect the heart from ischemia-reperfusion damage by increasing its antioxidant capacity. The significant role of HSPs for mitochondria by protecting cells against apoptosis has also been suggested (Dimauro et al., 2016). In the present study, an increase in HSP 27 concentration after a few minutes of breath-hold during static and dynamic apnea indicates a contribution from this protein to a protective mechanism against hypoxia (Arya et al., 2007). Importantly, the obtained positive relationship between HSP 70 and TAS might indicate an important role of this protein in protecting cells against oxidative stress (Dimauro et al., 2016). It has been documented that exercise training facilitates the expression of HSPs in the heart (Demirel et al., 2003), increasing their cardio protective effects (Powers et al., 2014). In these studies, there was a trend toward higher concentrations of HSP 70 and HSP 90 depending on antioxidant defense (TAS) and a significant effect of the TOS in the blood, was also observed. Lipid peroxidation, the oxidation process of polyunsaturated fatty acids leading to the formation of peroxides of these compounds, is the best-known oxidation process initiated by free radicals. The levels of lipoperoxidation products referred to as the oxidative potential indicate the activity of ROS in the examined tissues (Finaud et al., 2006; Sureda et al., 2015).

We detected an increase in lipid peroxidation products after breath-hold and exercise, confirming the higher activity of the free radical process under the influence of the repeated apnea maneuvers. Surprisingly, we did not observe a significant increase in the antioxidant potential based on the TAS after breath-hold compared to resting values. It can be presumed that the state of antioxidant defense is not responsible for protecting cells against ROS in the studied group of divers. However, it cannot be ruled out that the determination of pro-oxidative-anti oxidative status in the minutes following the long-term recovery would indicate that such a defense was induced.

CONCLUSION

Static and dynamic breath-holding had a significant effect on oxidative stress, as evidenced by an increase in the total oxidant status and total oxidant capacity. An increase in serum heat shock protein levels in response to higher nitric oxide levels could explain its important role in improving cardiovascular adaptation to hypoxia in elite freedivers.

Further studies are necessary to explain the exact mechanisms of breath holds training of cardiovascular adaptation responsible for maintaining adequate oxygen supply in elite divers.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Bioethical Committee of Academy of Physical Education (Decision No 3/2018). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MS-T, AŻ, DJ, and PS contributed to conception and design of the study. MS-T, SK-N, RM, and DZ organized the database. RM performed the statistical analysis. MS-T and AŻ wrote the first draft of the manuscript. AŻ and PS wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Large Lung Volumes Delay the Onset of the Physiological Breaking Point During Simulated Diving

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During breath holding after face immersion there develops an urge to breathe. The point that would initiate the termination of the breath hold, the "physiological breaking point," is thought to be primarily due to changes in blood gases. However, we theorized that other factors, such as lung volume, also contributes significantly to terminating breath holds during face immersion. Accordingly, nine naïve subjects (controls) and seven underwater hockey players (divers) voluntarily initiated face immersions in room temperature water at Total Lung Capacity (TLC) and Functional Residual Capacity (FRC) after pre-breathing air, 100% O_2, 15% O_2 / 85% N_2, or 5% CO_2 / 95% O_2. Heart rate (HR), arterial blood pressure (BP), end-tidal CO_2 ($etCO_2$), and breath hold durations (BHD) were monitored during all face immersions. The decrease in HR and increase in BP were not significantly different at the two lung volumes, although the increase in BP was usually greater at FRC. BHD was significantly longer at TLC (54 ± 2 s) than at FRC (30 ± 2 s). Also, with each pre-breathed gas BHD was always longer at TLC. We found no consistent $etCO_2$ at which the breath holding terminated. BDHs were significantly longer in divers than in controls. We suggest that during breath holding with face immersion high lung volume acts directly within the brainstem to actively delay the attainment of the physiological breaking point, rather than acting indirectly as a sink to produce a slower build-up of PCO_2.

Keywords: face immersion, human diving response, lung volume, physiological breaking point, chemoreceptor stimulation

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INTRODUCTION

The diving response, which is present in all mammals, including humans, includes a parasympathetically-mediated bradycardia that decreases cardiac output, a sympathetically-mediated increase in arterial blood pressure through selective restriction of blood flow to non-vital tissues, and apnea (for reviews see Gooden, 1994; Foster and Sheel, 2005; Lindholm and Lundgren, 2009; Castellini, 2012; Fitz-Clarke, 2018). Although voluntary breath holding in air can produce cardiovascular responses similar to that of the diving response, stimulation of trigeminal receptors on the face is necessary for full development of the diving response (Manley, 1990; Gooden, 1994; Andersson et al., 2000). The initiation of the diving response with immersion of the face into water, especially cold water, is well documented and is consistent with research in other animals and in humans with full body immersion (Kawakami et al., 1967; Moore et al., 1972; Fitz-Clarke, 2018).

The duration of a breath hold in either naïve subjects or experienced divers can be quite variable. Breath hold durations (BHDs) typically vary from 20 to 270 s (Lin, 1982), although

voluntary breath holds during submergence reaching 11 min have been reported (Fitz-Clarke, 2018). After a voluntary inhibition of breathing and immersion of the face into water, a person can easily hold their breath, called the "easy-going" phase (see Lin, 1982). Throughout this time a physiological urge to breathe develops, primarily through changes in blood gases (i.e., increases in $PaCO_2$ and/or decreases in PaO_2). The point where these physiological parameters have built up to levels that initiate the termination of the breath hold is called the "physiological breaking point." At the physiological breaking point there is an onset of involuntary ventilatory activity, such as an increase in diaphragmatic activity. Some subjects can continue to hold their breath past the physiological breaking point by consciously suppressing their urge to breathe, and overcoming the discomfort of hypoxia, hypercapnia, and acidosis, etc. (Fitz-Clarke, 2018). This phase is called the "struggle phase," and may involve psychological and/or motivational factors that inhibit the termination of the breath hold. Strong involuntary breathing movements during the "struggle phase" may also increase brain blood flow (Dujic et al., 2009) and splenic contraction (Palada et al., 2008). The end of the struggle phase is the "conventional breaking point," where the breath holding can no longer be continued, and the subject voluntarily terminates their breath hold, reemerges from the face submersion, and resumes normal ventilatory activity.

The physiological breaking point is sharply defined by chemical status (Lin, 1987, 1988). However, in addition to changes in blood gases, lung volume may also play a role in determining the duration of the easy-going phase. Both the easy going phase and total BHDs are longer when breath holding at total lung capacity (TLC) than at functional residual capacity (FRC) (Andersson and Schagatay, 1998). This may be because, with a larger lung volume, it could take longer for $PaCO_2$ and PaO_2 to reach levels that signal the physiological breaking point (Andersson and Schagatay, 1998), an effect perhaps aided through a potentiation of diving bradycardia (Manley, 1990). Alternatively, the level of lung inflation may provide afferent signals, through pulmonary stretch receptor activity, that could directly constitute a physiological signal that affects the onset of the physiological breaking point. Accordingly, we designed the present study to determine whether lung volume *per se* can affect the duration of the easy-going phase in either untrained or experienced divers (members of an underwater hockey team). Also, since chemical status can affect BHD, we had subjects pre-breath different gas mixtures to enhance or diminish chemoreceptor activity during the breath holds. We hypothesized that activation of pulmonary stretch receptors at large lung volumes act to prolong the attainment of the physiological breaking point.

MATERIALS AND METHODS

Subjects

This study was approved by the Midwestern University Institutional Review Board (IRB) prior to subject enrollment. All subjects gave informed consent and understood the procedures, risks, and voluntary nature of the study.

Subjects (ages 21–42) were recruited from Midwestern University in Downers Grove, IL ("controls": N = 9; 4M, 5F) and a local underwater hockey club ("divers" N = 7; 4M, 3F). All subjects were healthy, non-smokers without apparent cardiovascular or respiratory disease and not taking any medication that would interfere with study parameters. In addition, subjects abstained from caffeine use on the day of testing. No adverse experiences were reported.

Study Procedures

A total of eight trials were performed at two lung volumes—four at TLC and four at FRC. Before each breath hold trial, subjects were asked to breathe one of the following four gas mixtures to alter chemoreceptor activity. Standard Air (21% O_2) was the control gas; 100% O_2 was chosen to decrease peripheral chemoreceptor activity; 15% O_2 / 85% N_2 was chosen to increase peripheral chemoreceptor activity; and 5% CO_2 / 95% O_2 was chosen to increase central chemoreceptor activity and decrease peripheral chemoreceptor activity. The assignment of each gas was random, predetermined, and conducted in a single-blind fashion—subjects were blinded to the gas mixture administered before each trial.

Two practice trials were performed prior to recorded testing to acclimate the subjects to the testing format. Four trials with each of the four gas mixtures were conducted at TLC followed by four additional trials at FRC.

The subjects were seated and breathed the appropriate gas mixture for 5 min according to a randomization list, *via* a disposable facemask. After 3 min. of breathing the gas, an expired CO_2 measurement was obtained by having the subject take a full breath, remove the face mask without releasing any air and blow into an end-tidal CO_2 ($etCO_2$) analyzer (see below). The subject then replaced the facemask without taking a breath and continued to breathe the gas mixture for an additional 1 min. At the end of the last minute the subject was given a verbal cue to halt their breathing at either TLC or FRC, remove the facemask, and immediately immerse their face in a basin of room temperature water. To ensure full stimulation of the diving response the forehead and eye areas were required to be submerged.

Subjects were instructed to hold their breath as long as comfortably possible, but to end their breath hold after they felt the urge to breathe. They were also instructed to refrain from hyperventilation before the breath hold, to refrain from movements of the chest or other skeletal muscles, including swallowing movements, during the breath hold, and not to release any air into the water. At the end of the breath hold, the subject removed their face from the water and immediately provided a second $etCO_2$ measurement before taking a breath of room air. They were allowed to breathe normally once this measurement was obtained. Five-minute rest periods separated each trial.

Heart Rate [HR, in beats per minute (BPM)] and arterial blood oxygen saturation (SaO_2, in percent saturation) were recorded throughout each trial using a cardiosync pulse oximeter (Noni^{TM}n Medical, Model 8700) with toe clip sensor and contact thermal printer printing continuously in real time. The oximeter

had a stated accuracy of ± 2% of full scale in the range of 70–100% SaO_2. Systolic and diastolic blood pressure were measured using an automatic digital blood pressure monitor with printer (Omron®, Model HEM-703CP), and were used to calculate mean arterial blood pressure (BP, in mm Hg). $EtCO_2$ (in percent) was measured using a CO_2 analyzer (BIOPAC $CO_2$100C). BHD (in s) was recorded using the touch-print feature of the oximeter. Lastly, an elastic chest band recorded chest excursion to evaluate lung volume and extraneous chest movements during each trial (BIOPAC TSD101B).

Blood pressure was obtained once approximately 30 s prior to the breath hold and once during the breath hold. However, BP readings may not have been obtained during the trials that lasted less than 30 s. $EtCO_2$ was measured 1 min prior to the breath hold and again immediately after the breath hold was terminated. SaO_2 and HR were measured throughout the 3 min prior to the breath hold until completion of the second CO_2 measurement. Data points were collected at 10-s intervals as well as at the timepoints of breath hold initiation and termination. To minimize recording errors, the subjects were instructed to keep their foot still while the toe clip was in place. Water and air temperatures were recorded prior to the first trial. Finally, all subjects were questioned about adverse experiences.

Statistical Analysis

All data is presented as mean ± standard error (SE). Statistical comparisons were made with a computer statistical package (SigmaStat v14) and $p < 0.05$ was used as the level of significance. For both the controls and divers, data for males and females were not significantly different, and so were grouped together. For all data, three-way ANOVAs compared controls vs. divers, TLC vs. FRC, and the four gases pre-breathed before the face immersions. Two-way ANOVAs compared TLC vs. FRC and the four gases pre-breathed before the face immersions for HR and SaO_2 at specific time points during the face immersions. One-way repeated measures ANOVAs were used to compare HR and SaO_2 during the face immersion to the control value before the face immersion. Even if the BHD lasted longer than 35 s, statistical analysis on HR and SaO_2 was only done on the first 35 s of the face immersion to facilitate statistical comparisons between groups. If statistical significance was reached during comparisons, it was then followed by *post hoc* testing (Tukey's multiple comparison procedure or Dunnett's multiple comparison vs. control) to determine which values were different from the others. *T*-tests were used to compare BP and $etCO_2$ before and after the face immersions.

RESULTS

Initial analysis combines data from control and divers to determine the effects of lung volume and pre-existing chemoreceptor stimulation on HR, BP, SaO_2, $etCO_2$ and BHD responses to face immersion. We then subsequently analyze data between the control and divers to determine whether breath hold diving experience affects any of these parameters.

Face immersion into room temperature water produced a significant decrease in HR when breath holding at both TLC ($F = 80.109$, $p < 0.001$) and FRC ($F = 69.145$, $p < 0.001$) (**Figure 1**). HRs were significantly higher when breath holding at TLC vs. FRC at pre-immersion ($F = 5.230$, $p = 0.024$), 5 s ($F = 9.734$, $p = 0.002$), and 15 s ($F = 17.522$, $p < 0.001$) (**Figure 1**). However, the magnitude of the bradycardias was not significantly different when breath holding at either TLC or FRC. Chemoreceptor stimulation by breathing different gases before face immersion had little effect on the decrease in HR (**Table 1**). There were no significant differences in the HR responses between the four TLC face immersions, nor between the four FRC face immersions. Additionally, for each of the four gases, there were no significant differences in the HR responses between TLC and FRC face immersions.

Face immersion produced a significant increase in BP when breath holding at both TLC ($p < 0.001$) and FRC ($p < 0.001$; **Figure 2**). BP increased by 6 ± 1 mm Hg when breath holding at TLC, and by a significantly greater 10 ± 2 mm Hg when breath holding at FRC ($p = 0.028$). Chemoreceptor stimulation by breathing different gases before face immersion had little effect on increase in BP (**Table 2**). There were no significant differences between the BPs for the four TLC face immersions, nor between the BPs for the four FRC face immersions.

During face immersion in room temperature water, SaO_2 had a slight downward trend, but remained greater than 98% in both TLC and FRC face immersions. There were no differences in SaO_2 between TLC and FRC during face immersion. When pre-breathing 100% O_2 or 5% CO_2 / 95% O_2, SaO_2 was statistically greater than when pre-breathing air or 15% O_2 ($F = 14.628$, $p < 0.001$). However, pre-breathing different gases had little effect on SaO_2 during face immersion, and even when pre-breathing 15% O_2 before face immersion, SaO_2 did not fall below 98% during face immersion.

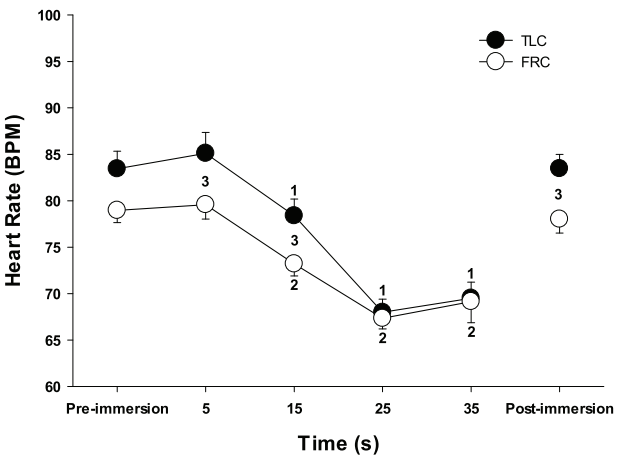

FIGURE 1 | Face immersion into room temperature water produced a significant decrease in HR when breath holding at both TLC and FRC. 1, HR is significantly less than TLC pre-immersion HR; 2, HR is significantly less than FRC pre-immersion HR; 3, TLC HR is significantly greater than FRC HR.

TABLE 1 | Chemoreceptor stimulation by breathing different gases before face immersion did not affect the HR response (presented as HR ± SE) to face immersion.

Time	AIR		15% O_2		100% O_2		5% CO_2/95% O_2	
	TLC	FRC	TLC	FRC	TLC	FRC	TLC	FRC
Pre-immersion	86 ± 4	81 ± 3	85 ± 4	78 ± 2	81 ± 3	79 ± 3	82 ± 4	78 ± 3
5 s	87 ± 4	82 ± 4	88 ± 6	79 ± 3	81 ± 4	80 ± 3	84 ± 4	78 ± 3
15 s	77 ± 2[1]	75 ± 3	76 ± 3	71 ± 3[2]	78 ± 3	73 ± 3	82 ± 6	74 ± 2
25 s	69 ± 3[1]	69 ± 3[2]	67 ± 2[1]	66 ± 22	68 ± 4[1]	66 ± 2[2]	67 ± 3[1]	67 ± 2[2]
35 s	70 ± 4[1]	70 ± 6[2]	69 ± 3[1]	69 ± 6[2]	69 ± 3[1]	70 ± 3[2]	70 ± 4[1]	69 ± 4[2]
Post-immersion	82 ± 3	76 ± 3	83 ± 3	76 ± 4	85 ± 3	81 ± 2	84 ± 3	78 ± 3

There were no significant differences between the four TLC face immersions, or between the four FRC face immersions. Additionally, for each of the four gases, there were no significant differences between the TLC and FRC face immersions. 1, HR significantly less than TLC pre-immersion HR; 2, HR significantly different than FRC pre-immersion HR.

During face immersion in room temperature water, $etCO_2$ increased significantly from 6.13% ± 0.08 pre-immersion to 6.98% ± 0.09 post-immersion with TLC ($p < 0.001$), and from 5.92% ± 0.08 pre-immersion to 6.81% ± 0.09 post-immersion with FRC ($p < 0.001$) (**Figure 3**). Pre-breathing different gases did not change the pre-immersion $etCO_2$, or the fact that there was a significant increase in $etCO_2$ during the immersion ($F = 10.52$, $p < 0.001$). However, there was no consistent end-immersion $etCO_2$ at which the breath holds terminated (**Figure 4**). After pre-breathing 5% CO_2 / 95% O_2 post-immersion $etCO_2$ (7.24% ± 0.12) was significantly greater than for air (6.66% ± 0.12; $F = 7.42$, $p = 0.003$) or 15% O_2 (6.59% ± 0.10; $F = 7.42$, $p < 0.001$). Also, after pre-breathing 100% O_2, the post-immersion $etCO_2$ (7.09% ± 0.13) was significantly greater than for air ($F = 7.42$, $p = 0.028$) or 15% O_2, ($F = 7.42$, $p = 0.013$). In addition, there was no consistent increase in $etCO_2$ after which the breath holds terminated. After breath holding at TLC, $etCO_2$ increased by 1.21% ± 0.11 after pre-breathing 100% O_2, which was a significantly greater increase in $etCO_2$ ($F = 7.39$, $p < 0.001$) than that seen after pre-breathing air (0.66% ± 0.001; $p < 0.001$) or 15% O_2 (0.64% ± 0.08; $p < 0.001$).

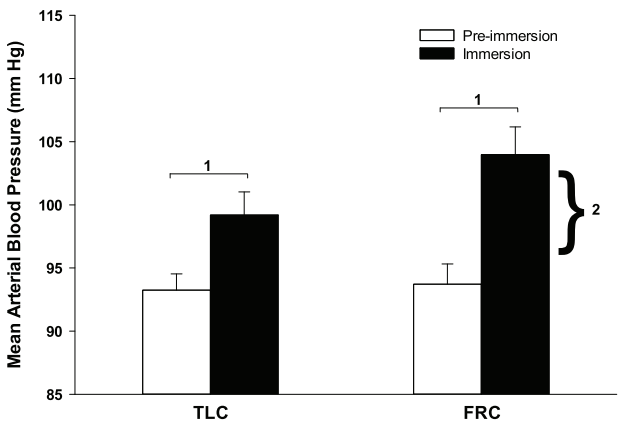

FIGURE 2 | Face immersion into room temperature water produced a significant increase in BP when breath holding at both TLC and FRC. The increase in BP was significantly greater during FRC face immersions than TLC face immersions. 1, immersion BP is significantly greater than pre-immersion BP; 2, the increase in BP at FRC is significantly greater than the increase in BP at TLC.

BHD after face immersion was significantly longer ($F = 55.18$, $p < 0.001$) at TLC (59 ± 3 s) than at FRC (32 ± 2 s), although it is notable that the post-immersion $etCO_2$ at TLC and FRC was not significantly different from each other ($F = 2.248$, $p = 0.137$). Additionally, for each gas breathed before the breath hold, BHD when breath holding at TLC was always significantly longer than BHD when breath holding at FRC (air, $p < 0.001$; 15% O_2, $p < 0.001$; 100% O2, $p = 0.003$; 5% CO_2 / 95% O_2, $p = 0.003$) (**Figure 4**).

Divers had a qualitatively similar cardiovascular diving response to the control subjects, and the differences between TLC and FRC face immersions that were seen in controls were also seen in divers. The pre-immersion HR of divers was significantly lower than the pre-immersion HR of controls (66 ± 1 for divers vs. 81 ± 1 for controls; $F = 67.702$, $p < 0.001$). Consequently, the face immersions HRs were significantly lower in divers than in controls (5 s, $F = 54.868$, $p < 0.001$; 15 s, $F = 66.178$, $p < 0.001$; 25 s, $F = 100.817$, $p < 0.001$; 35 s, $F = 88.106$, $p < 0.001$). However, the magnitude and time course of the bradycardias were similar between divers and controls. During face immersion divers showed a significant increase in BP ($p < 0.001$) that was similar in magnitude to that of controls (8 ± 2 mm Hg for controls vs. 7 ± 1 mm Hg for divers). As in controls, SaO_2 had a slight downward trend during the breath holds in divers but did not decrease below 98% for any of the gases that were pre-breathed. The pre-immersion $etCO_2$ of divers was significantly lower than that of controls (5.81% ± 0.10 for divers vs. 6.02% ± 0.06 for controls; $F = 12.65$, $p < 0.001$), although the end-immersion $etCO_2$'s were not significantly different. Consequently, the increase in $etCO_2$ during the face immersions was significantly greater for divers than for controls (0.97% ± 0.05 for divers vs. 0.79% ± 0.06 for controls; $F = 6.38$, $p < 0.013$). BHD in divers (50 ± 4 s) was significantly longer than BHD in controls (42 ± 2 s) ($F = 4.34$; $p = 0.039$), but not when specifically comparing at either TLC or FRC. In addition, for divers BHD at TLC (64 ± 6 s) was significantly longer ($p < 0.001$) than at FRC (35 ± 4 s). For controls BHD at TLC (54 ± 2 s) was significantly longer ($p < 0.001$) than at FRC (30 ± 2 s) (**Figure 5**).

TABLE 2 | Chemoreceptor stimulation by breathing different gases before face immersion did not affect BP response (presented as BP ± SE) to face immersion.

Time	AIR		15% O_2		100% O_2		5% CO_2/95% O_2	
	TLC	FRC	TLC	FRC	TLC	FRC	TLC	FRC
Pre-immersion	89 ± 2	97 ± 5	93 ± 2	93 ± 2	96 ± 4	92 ± 2	94 ± 2	93 ± 2
Immersion	100 ± 3[1]	105 ± 5	98 ± 3	103 ± 4[1]	99 ± 4	102 ± 3[1]	100 ± 4	108 ± 7[1]

The increase in BP during face immersion was usually greater at FRC than at TLC.
There were no significant differences between the BPs for the four TLC face immersions, or between the BPs for the four FRC face immersions.
1, Immersion BP significantly greater than pre-immersion BP.

DISCUSSION

The most important finding from this study is that lung volume, and therefore presumably pulmonary stretch receptor activity, is an important physiological factor that contributes to the onset of the physiological breaking point during voluntary breath holding in room temperature water. BHD at TLC was always significantly longer than BHDs at FRC, even though the cardiovascular responses to face immersion at the two lung volumes were similar. Additionally, BHDs at TLC were always longer regardless of which gas was breathed before the breath hold or whether the subject had previous breath holding experience. Although PCO_2 is the most important factor that contributes to the onset of the physiological breaking point (Lin et al., 1974; Fitz-Clarke, 2018), we found that there was no consistent $etCO_2$ at which breath holds terminated.

Human Diving Response

Like previous studies investigating the human diving response, we found that face immersion in room temperature water produced bradycardia and an increase in blood pressure (Gooden, 1994; Foster and Sheel, 2005; Lindholm and Lundgren, 2009; Fitz-Clarke, 2018). Changing chemoreceptor stimulation by pre-breathing different gases before face immersion had little effect on the development of the bradycardia or the increase in BP. The HR response to breath holding after face immersion was similar at either TLC or FRC. Similar results were found by Andersson and Schagatay (1998) who reported that lung volume [60, 85, or 100% of Vital Capacity (VC)] does not appreciably change HR, skin capillary blood flow or blood pressure responses to breath holding. However, unlike Andersson and Schagatay (1998), we found that there was a greater increase in BP when breath holding at lower lung volumes (FRC) than at higher lung volumes (TLC). The intensification of peripheral vasoconstriction when breath holding at FRC would presumably lead to greater oxygen conservation during the breath hold (Andersson et al., 2000). Instead, however, BHD at FRC was significantly shorter than BHD at TLC.

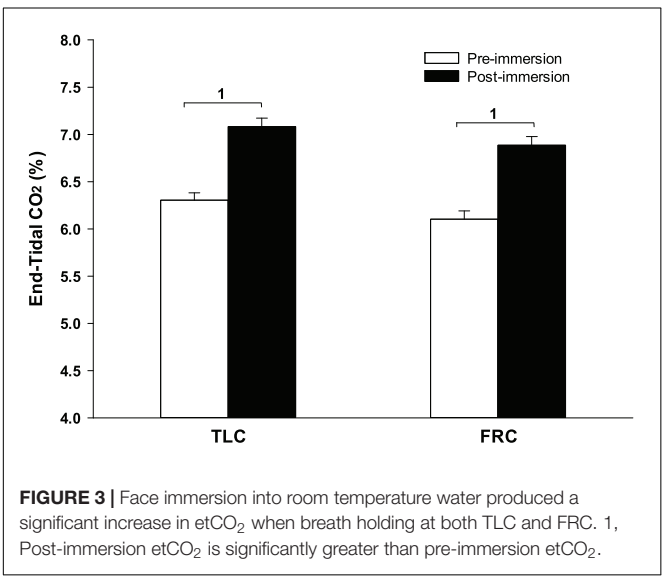

FIGURE 3 | Face immersion into room temperature water produced a significant increase in $etCO_2$ when breath holding at both TLC and FRC. 1, Post-immersion $etCO_2$ is significantly greater than pre-immersion $etCO_2$.

Chemoreceptor Stimulation and the Physiological Breaking Point

The physiological breaking point signals the end of the easy-going phase of a voluntary breath hold. When physiological variables build up to the point where a signal to terminate the breath hold has been reached, the breath hold can only be continued through voluntary suppression of the drive to terminate the breath hold (Lin et al., 1974; Hentsch and Ulmer, 1984). The primary physiological variable that is thought to determine the physiological breaking point is an increase in chemoreceptor activity through an increase in PCO_2 or a decrease in PO_2 (Lin et al., 1974; Parkes, 2006). When breath holding with oxygen without face submersion, the physiological breaking point is reached when alveolar PCO_2 reaches 48–54 mm Hg (Lin et al., 1974; Parkes, 2006), although the PCO_2 will be lower if there is simultaneous hypoxia (Lin, 1987).

$EtCO_2$ increased significantly during face immersions, and we found that breath holds at both TLC and FRC ended at an $etCO_2$ of about 7%. Andersson and Schagatay (1998) found that breath holds at 60%, 85% and 100% of VC ended at an $etCO_2$ of approximately 7.4%. When subjects pre-breathed different gases before breath holding, we found no post-immersion $etCO_2$ at which all face immersions ended. Rather, pre-breathing 100% O_2 or 5% CO_2 / 95% O_2 at FRC, or 5% CO_2 / 95% O_2 at TLC, significantly increased the post-immersion $etCO_2$. Also, the difference between the pre-immersion $etCO_2$ and post-immersion $etCO_2$ was increased after pre-breathing 100% O_2, at least at TLC. These data suggest that there is a synergistic effect between CO_2 central chemoreceptor stimulation and O_2 peripheral chemoreceptor stimulation (Fowler, 1954; Lin et al., 1974). Breathing a high O_2 gas permits breath holds to terminate

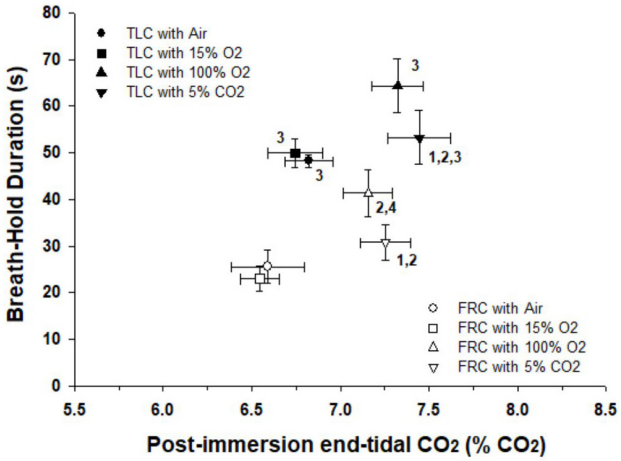

FIGURE 4 | Breath holds after face immersion into room temperature water did not end at a consistent $etCO_2$, and BHD was always longer at TLC than at FRC. There were significant differences between the post-immersion $etCO_2$'s after pre-breathing different gases before breath holds. Thus there was no consistent $etCO_2$ that initiated the termination of the breath holds. For each gas breathed before the breath hold, BHD at TLC was always longer than BHD at FRC. 1, Post-immersion $etCO_2$ is significantly greater than post-immersion $etCO_2$ after pre-breathing air at that lung volume. 2, Post-immersion $etCO_2$ is significantly greater than post-immersion $etCO_2$ after pre-breathing 15% O_2 at that lung volume. 3, BDH at TLC is significantly longer than BHD at FRC after pre-breathing that specific gas. 4, BHD after pre-breathing 100% O_2 at FRC is significantly longer than BHD after pre-breathing 15% O_2 at FRC.

at higher $etCO_2$'s. However, there is no absolute $etCO_2$ that, when reached, terminates a breath hold.

In the present study, BHDs were so short that SaO_2 decreased very little during face immersion. Hemoglobin remained above 98% saturated with oxygen, even after pre-breathing hypoxic gas. Consequently it was unlikely that arterial chemoreceptors were greatly stimulated through increasing hypoxemia during any of these breath holds. Therefore the peripheral chemoreflex, by itself, probably did not contribute to the cardiovascular (i.e., HR and BP) changes observed during the breath holds, or to the attainment of the physiological breaking point. However, even though oxygen saturations did not decrease substantially during the breath holds, PO_2 had a synergistic effect with PCO_2, as evidenced by significant differences in post-immersion $etCO_2$ after pre-breathing different gases (**Figure 4**).

Lung Volume and the Physiological Breaking Point

Although beginning a breath hold at either TLC or FRC did not appreciably change the cardiovascular responses to breath holding, breath holding at either TLC or FRC did significantly change BHD. BHDs were always longer at TLC than at FRC, even when subjects pre-breathed different gases. Andersson and Schagatay (1998) found that larger lung volumes increased the duration of both the easy-going phase and total breath holding duration. It is thought that a larger lung volume provides a sink that allows a build-up of CO_2 and/or depletion of O_2, thus allowing for a longer BHD (Sterba and Lundgren, 1985, 1988). However, even though there were significant differences in $etCO_2$'s after pre-breathing different gases, there were no significant differences in BHD for the four face immersions at each of the two lung volumes (**Figure 4**). This suggests that lung volume, in addition to $etCO_2$, is an important factor in determining physiological breaking point and breath holding capability.

The effect of lung volume on attainment of the physiological breaking point could stem from slowly adapting pulmonary stretch receptor (SAR) afferent activity. With greater expansion of the lungs with greater lung volumes, SAR activity will increase (Sant'Ambrogio, 1982; Schelegle and Green, 2001). When lung inflation is maintained, SARs characteristically show a long-lasting and sustained discharge (Sant'Ambrogio, 1982; Schelegle and Green, 2001). When breath holding at a large lung volume without face immersion, a Breuer–Hering like reflex may act

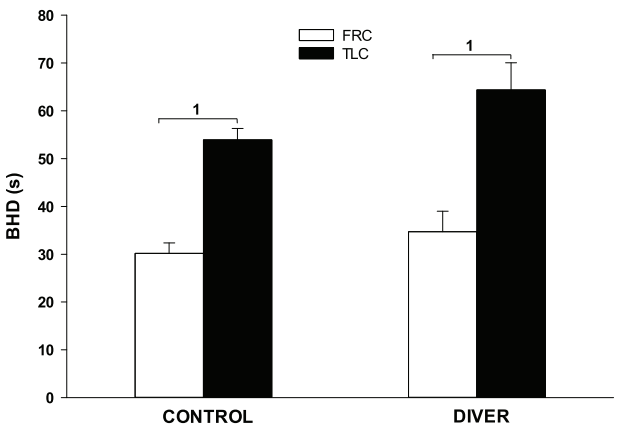

FIGURE 5 | Face immersion into room temperature water at TLC significantly increases BHD in both controls and divers. 1, BHD is significantly longer at TLC vs. FRC.

to prolong maximal breath hold duration (Muxworthy, 1951; Chapin, 1955). We suggest that SAR activity during voluntary breath holding with face immersion at large lung volumes acts within the brainstem central rhythm generator to actively prolong phase II expiration and/or to inhibit generation of inspiration (D'Angelo and Agostoni, 1975; St. John and Zhou, 1990; Sant'Ambrogio and Sant'Ambrogio, 1991). The result of the SAR activity would be to extend the duration of the easy-going phase of a breath hold by suppressing attainment of the physiological breaking point that primarily results from an increase in PCO_2. Thus at large lung volumes, inhibition of breath hold termination would occur due to neuronal interactions within the brainstem central rhythm generator, rather than due to a slower build-up of PCO_2 within the lungs. Additionally, strong involuntary breathing movements are less frequent at high lung volumes, possibly explaining why higher lung volumes allows for longer BHDs (Fitz-Clarke, 2018).

Other explanations of the direct effect lung volume plays on BHD could be that the prolonged absence of SAR activity when breath holding at low lung volumes is, in itself, a respiratory stimulus that leads to the termination of the breath hold and reestablishment of eupnea (Fowler, 1954; Godfrey and Campbell, 1968, 1969). At low lung volumes there would be neither rhythmic nor static SAR afferent activity, because with eupnic breathing, and thus at lung volumes near to FRC, there is normally no SAR activity in humans (Sant'Ambrogio and Sant'Ambrogio, 1991). Additionally, the absence of any SAR input due to the voluntarily initiated apnea may act to terminate a breath hold (Mithoefer, 1959; Delapille et al., 2001). A technique that experienced breath hold divers sometimes use to prolong BHD is to engage in fictive breathing movements by pumping the thoracic cavity while keeping the glottis closed [personal observations; (Lin, 1982)]. This would have the effect of changing lung volume, and thus provide SAR input to the brainstem, even if no fresh air reaches the lungs. Rebreathing during a breath hold extends BHD, even though arterial blood gases do not improve (Fowler, 1954; Godfrey and Campbell, 1968, 1969). Another technique used to prolong breath holds is to open the epiglottis and swallow. Although this would not change lung volume or SAR activity, it would provide a rhythmic input into brainstem respiratory centers. However, swallowing or taking a "false breath" while breath holding reduces the magnitude of diving bradycardia (Gandevia et al., 1978), and involuntary breathing movements during breath holding are thought to be too small to influence the diving response (Andersson and Schagatay, 1998).

Effect of Previous Breath Holding Experience

The experienced divers in our study were members of a local underwater hockey club. Qualitatively, the diving response of divers was similar to that of controls. However, the BHD of divers was significantly longer than that of controls, although not as long as that seen in experienced underwater hockey or rugby players (Davis et al., 1987; Schagatay and Andersson, 1998), or synchronized swimmers (Oldridge et al., 1978; Bjurström and Schoene, 1987). However, our divers were recreational underwater hockey players, rather than national caliber players as in the study by Davis et al. (1987). Additionally, through our instructions to them, divers ended their face immersions at or near their physiological breaking points, rather than near to their conventional breaking points. During the struggle phase of breath hold diving, the physiological drive to terminate the breath hold is consciously suppressed. Breath holding experience or apnea training can prolong total BHD by delaying the conventional breaking point (Davis et al., 1987; Schagatay et al., 1999, 2000). Thus, with our instructions to our subjects to end their breath holds when they felt the urge to breath and not to use fictive breathing techniques, we negated the training effects that could have given the trained divers a breath holding "advantage".

Conclusion

We conclude many factors bring about the physiological breaking point during breath holding. These include increased $PaCO_2$ (as measured by $etCO_2$), although there is no absolute $etCO_2$ or change in $etCO_2$ that, when reached, will initiate the termination of the breath hold. We also conclude that high lung volume, or more specifically the increased pulmonary stretch receptor activity at high lung volume, is an important factor that contributes to the physiological breaking point. We suggest that high lung volume acts centrally to inhibit the termination of breath holds, and thus prolong the duration of the easy-going phase of voluntary breath holding. We also conclude that increased arterial chemoreceptor activity due to the development of hypoxia during the breath hold plays a limited, if any, role in terminating most short duration breath holds. Finally, we conclude that previous breath holding experiences increases breath hold durations without substantially changing cardiorespiratory (HR, BP SaO_2, and $etCO_2$) responses to face immersion.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Midwestern University Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

PM and BG conceptualized and planned the study, formulated research protocols, and obtained IRB approval. BG and JS collected data. PM performed statistical analysis, created figures, and wrote the manuscript. All authors contributed to the article and approved the submitted version (except JS).

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Blood Adenosine Increase During Apnea in Spearfishermen Reinforces the Efficiency of the Cardiovascular Component of the Diving Reflex

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The physiopathology consequences of hypoxia during breath-hold diving are a matter of debate. Adenosine (AD), an ATP derivative, is suspected to be implicated in the adaptive cardiovascular response to apnea, because of its vasodilating and bradycardic properties, two clinical manifestations observed during voluntary apnea. The aim of this study was to evaluate the adenosine response to apnea-induced hypoxia in trained spearfishermen (SFM) who are used to perform repetitive dives for 4–5 h. Twelve SFM (11 men and 1 woman, mean age 41 ± 3 years, apnea experience: 18 ± 9 years) and 10 control (CTL) subjects (age 44 ± 7 years) were enrolled in the study. Subjects were asked to main a dry static apnea and stopped it when they began the struggle phase (average duration: SFM 120 ± 78 s, CTL 78 ± 12 s). Capillary blood samples were collected at baseline and immediately after the apnea and analyzed for standard parameters and adenosine blood concentration ([AD]b). Heart rate (HR), systolic (SBP), and diastolic (DBP) blood pressures were also recorded continuously during the apnea. During the apnea, an increase in SBP and DBP and a decrease in HR were observed in both SFM and CTL. At baseline, [AD]b was higher in SFM compared with CTL (1.05 ± 0.2 vs. 0.73 ± 0.11 μM, $p < 0.01$). [AD]b increased significantly at the end of the apnea in both groups, but the increase was significantly greater in SFM than in controls (+90.4 vs. +12%, $p < 0.01$). Importantly, in SFM, we also observed significant correlations between [AD]b and HR ($R = -0.8$, $p = 0.02$), SpO_2 ($R = -0.69$, $p = 0.01$), SBP ($R = -0.89$, $p = 0.02$), and DBP ($R = -0.68$, $p = 0.03$). Such associations were absent in CTL. The adenosine release during apnea was associated with blood O_2 saturation and cardiovascular parameters in trained divers but not in controls. These data therefore suggest that adenosine may play a major role in the adaptive cardiovascular response to apnea and could reflect the level of training.

Keywords: adenosine, breath-hold, diving reflex, free-diving, hypoxia, training, spearfishing

INTRODUCTION

The cardiovascular adaptive response to breath-hold diving, also known as the diving response, has long been a matter of debate, especially since the popularization of recreational apnea and spearfishing. These aquatic activities commonly involve hours of immersion with repeated voluntary dynamic apneic phases causing severe hypoxia streak (Marlinge et al., 2021). The main cardiovascular responses occurring during breath-hold diving are bradycardia, peripheral vasoconstriction, and an increase in arterial blood pressure (Lindholm and Lundgren, 2009; Bain et al., 2018), while depending on the apnea duration, severe hypoxemia, and hypercapnia can also develop (Joulia et al., 2009). Several neurohumoral factors have also been suggested to be implicated in the cardiovascular response to hypoxemia, including cortisol, copeptin (Marlinge et al., 2019), catecholamines (Chmura et al., 2014; Eichhorn et al., 2017), and growth hormone (Djarova et al., 1986).

Adenosine (AD), an ATP derivative, is also implicated in the response to hypoxemia during breath hold (Joulia et al., 2013, 2014; Marlinge et al., 2019). Adenosine is released by endothelial cells and myocytes during hypoxia or ischemia. Indeed, with a drop in PaO_2, the rephosphorylation of AD into ATP is limited because of the inhibiting action of the hypoxia-inducible factor (HIF) on adenosine kinases (Morote-Garcia et al., 2008). Adenosine thus accumulates in the extracellular spaces and strongly impacts the cardiovascular system through its four G-coupled membrane receptors named A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R receptors. The activation of A_1R can lead to bradycardia, sinus arrest, or atrioventricular block (ATVB), while the activation of A_2 subtypes leads to strong vasodilation and hypotension. Moreover, A_3R are implicated in ischemia–reperfusion protection (Guieu et al., 2020; Paganelli et al., 2021).

Interestingly, there are major differences between breath-hold divers (BHDs) and spearfishermen (SFM) in their average total apnea durations in 1 day of training or competition (BHD 39 ± 12 min vs. SFM 86 ± 39 min). Consequently, spearfishing training could be viewed as highly repetitive hypoxia exposure whereas shorter breath-hold training could be compared to acute hypoxia exposure, and there is a paucity of data on the responses of this population to hypoxia. Thus, the aim of this study was to evaluate the amplitude of the AD release and its role in the cardiovascular response to hypoxia. In SFM who are exposed to hypoxemia for several hours per week, we expected an accentuation of the adenosine release as well as an emphasis of the diving response compared to control (CTL).

MATERIALS AND METHODS

Twelve SFM (2 women and 10 men) with competitive experience at national and/or international levels and 10 CTL participants (1 woman and 9 men) volunteered to participate in this study (**Table 1**). All participants were non-smokers, without medical treatment and free from inflammatory or cardiovascular disease.

TABLE 1 | Spearfishermen (SFM, *n* = 12) and control (CTL, *n* = 10) characteristics.

	SFM	CTL
Age (years)	41 ± 3 (24–58)	44 ± 7 (35–55)
Men/women	10/2	8/2
Body mass (kg)	78 ± 11 (60–93)	78 ± 9 (57–90)
Height (cm)	177 ± 13 (162–190)	174 ± 12 (165–185)
Experience in spearfishing (years)	18 ± 9 (4–27)	0
Mean apnea duration per training session	86 ± 39 min	0
Training days per week	3 ± 2	0
Dry apnea duration (s)	120 ± 37 (90–220)	78 ± 9 (63–91)

(Data are given as means, standard deviations, and range).

The protocol was approved by our institutional Ethics Committee (CPP Sud Marseille N° 13/41) and the study was carried out by the Code of Ethics of the World Medical Association (in agreement with the Declaration of Helsinki). The procedures have been conducted with the adequate understanding and written consent of the participants.

Protocol

The dry static apnea protocol has been described in detail elsewhere (Joulia et al., 2013, 2014). Briefly, all participants rested in supine position breathing normally in a controlled temperature room (25 ± 2°C) for 10 min. Apnea can be separated in two phases (Hentsch and Ulmer, 1984). The first one, or "*easy-going phase*" depends on physiological training adaptations whereas the second one known as the "*struggle phase*" is related to psychological capacity to fight against unpleasant feelings. To avoid the influence of training skills and allow better comparisons of physiological responses between SFM and CTL, participants were asked to perform a dry static apnea and to stop the breath hold when they began the struggle phase. The breath hold was also started without glossopharyngeal insufflation. The entire protocol was performed between 9 and 11 am.

Peripheral blood oxygen saturation (SpO_2) and heart rate (HR) were measured continuously on the left index finger (NPB 40; Nellcor Puritan Bennett, Pleasanton, CA, United States). Systolic (SBP) and diastolic (DBP) blood pressures were measured before and at the end of the apnea session. Blood samples were collected on the right index at baseline and immediately after the apnea cessation after finger puncture for capillary analysis of pH, PCO_2 and lactate concentration [La] (Epoc®, Blood Analysis System, Siemens). A separate drop of capillary blood was deposited on a blotting paper (Whatman®) for adenosine blood concentration ([AD]b) measurement as previously described (Marlinge et al., 2021).

Statistical Analysis

Data's distribution was tested using a Kolmogorov–Smirnov test. Since it did not reflect a normal distribution, the *U* Mann–Whitney test was used for inter-group comparisons (CTL vs.

SFM) and the Wilcoxon matched-pair signed-Rank test for intra-group comparisons (before vs. after apnea). *P*-values < 0.05 were considered as significant. Analyses were performed with Statistica software 6.0.

RESULTS

There were no significant differences between SFM and CTL regarding their anthropometric characteristics (**Table 1**) and the resting values of HR, DBP, SBP, pH, SpO_2, PCO_2, and [La] (**Table 2**). As expected, the apnea durations were longer in SFM compared to CTL (**Table 1**).

Individual apnea-induced changes in [AD]b are displayed in **Figure 1**. In basal conditions, [AD]b was higher in SFM compared with CTL (1.05 ± 0.2 vs. 0.61 ± 0.11 μM, $P < 0.01$). While [AD]b increased significantly at the end of apnea in both groups, the increase was greater in SFM compared to CTL (+1.05 vs. 0.16 μM, $P < 0.01$).

Apnea induced a bradycardia and increased in systolic (SBP) and diastolic (DBP) blood pressures in both SFM and CTL. The bradycardia was higher in SFM compared to CTL (−22 vs. −12%, $P < 0.01$) (**Table 2**). There was no significant difference between groups regarding DBP whereas the SBP increase was higher in SFM compared to CTL (17.1 vs. 6.2 mmHg, $P < 0.01$). Only in SFM, linear correlations were found between [AD]b measured at the end of the apnea and the cardiovascular parameters: HR ($R = -0.83$, $P < 0.01$, **Figure 2**), SBP ($R = 0.83$, $P < 0.01$, **Figure 3**), and DBP ($R = 0.82$, $P < 0.01$, **Figure 4**). Apnea induced a similar increase in $PaCO_2$ in both SFM (+12.8 mmHg, $P < 0.01$) and CTL (+9 mmHg, $P < 0.01$). Apnea also induced a low but significant decrease of pH in both groups with no significant difference between groups (**Table 2**). Apnea generated a [La] increase in SFM only (+23%, $P < 0.01$). Although a decline in SpO_2 was observed at the end of apnea in both groups, this decrease was significantly larger in SFM compared to CTL (−24 vs. −8.4%, $P < 0.01$). Finally, linear correlations were found between SpO_2 and [AD]b in both SFM ($R = -0.85$, $P < 0.01$) and CTL ($R = -0.71$, $P < 0.01$, **Figure 5**).

DISCUSSION

This study investigated the response of blood AD to sub-maximal apnea in trained spearfishermen vs. novice control participants, and its association with key cardiovascular parameters. While the [AD]b increased in both groups during the apnea, SFM displayed a significantly greater increase. Importantly, this increase in [AD]b was correlated with the decline in HR as well as the increase in blood pressure in SFM only.

Such cardiovascular changes are well known to be part of the diving response (Ferrigno and Lundgren, 2010), which constitutes an integrative protective mechanism against hypoxia to decrease the work load on the heart and increase perfusion during the diastolic phase (Alboni et al., 2011). The diving reflex intensity is known to accentuate with the reduction in O_2 saturation (Andersson and Schagatay, 1998; Andersson et al., 2002), the face immersion (Craig and Medd, 1968; Andersson et al., 2004), the depth (Ferrigno et al., 1991), and the level of expertise (Lemaître et al., 2010). Since participants only performed dry static apnea in our study, it could explain the significant but low bradycardia observed in SFM despite the apnea durations.

Vasoconstriction occurs during apnea in elite free-divers in order to limit skeletal muscle oxygen uptake and to facilitate blood redistribution toward the brain and the heart (Joulia et al., 2009). Vasoconstriction and the associated increase in systolic blood pressure are caused, at least in part, by the increase in epinephrine and norepinephrine concentrations during the apnea, and bradycardia is inversely correlated with the increase in norepinephrine (Eichhorn et al., 2017). Despite the bradycardia, the vasoconstriction is known to increase peripheric arterial pressure in well trained free-divers (Joulia et al., 2009). An [AD]b increase was previously described during apnea and higher in

TABLE 2 | Biological and physical markers of spearfishermen (SFM) and control (CTL) recorded before and immediately at the end of the static apnea.

	SFM		CTL		
Biological markers	**Baseline**	**Apnea**	**Baseline**	**Apnea**	«**b**» *P*-values
Hb (g l^{-1})	15.4 ± 1.2	15.1 ± 0.8	15.4 ± 1.1	15 ± 1.3	NS
Hematocrit (%)	45 ± 1.7	46.4 ± 1.8	45.2 ± 1.4	44 ± 2	NS
Lactates (mM l^{-1})	1.10 ± 0.15	$1.38 \pm 0.14^{a,b}$	1.15 ± 0.3	1.16 ± 0.4	$P < 0.01$
SpO_2 (mmHg)	110.5 ± 7^{b}	87.2 ± 4.4^{a}	96 ± 4	90 ± 4^{a}	$P < 0.01$
Saturation (%)	99 ± 0.8	$84 \pm 7^{a,b}$	98 ± 0.4	90 ± 2^{a}	$P < 0.001$
PCO_2 (mmHg)	27.9 ± 2.5^{b}	41 ± 2.9^{a}	28 ± 1.8	37 ± 2^{a}	$P < 0.001$
pH	7.41 ± 0.01	7.37 ± 0.01^{a}	7.40 ± 0.02	7.37 ± 0.03^{a}	$P = 0.01$
[AD]b (μM)	1.05 ± 0.2	$2.1 \pm 0.95^{a,b}$	0.59 ± 0.1	0.75 ± 0.1^{a}	$P = 0.01$
Cardiovascular parameters					
SBP (mmHg)	116 ± 11.8	133 ± 14.4^{a}	125 ± 6	131 ± 5^{a}	$P < 0.01$
DBP (mmHg)	64 ± 8.9	71 ± 10.7^{a}	70 ± 11	77 ± 6^{a}	$P < 0.01$
HR (beats per min)	76 ± 3.9^{b}	$59 \pm 6.2^{a,b}$	76 ± 5.2	67 ± 6.5	$P < 0.01$

Data are given as mean ± SD. The symbol "a" was used when there is a significant difference between baseline and apnea values and "b" when there is a significant difference between SFM and CTL values.

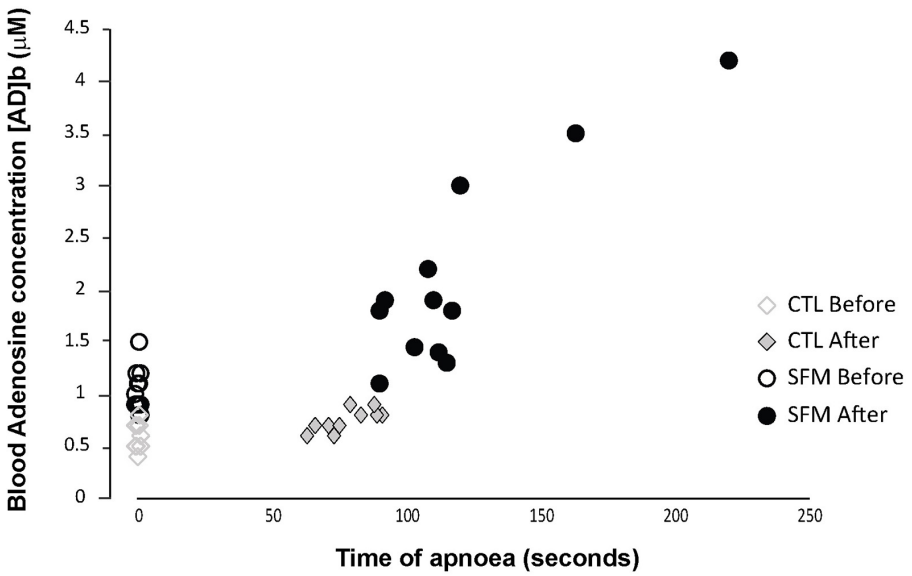

FIGURE 1 | Individual values of blood adenosine concentration measured in spearfishermen (SFM) and control (CTL) subjects before and immediately at the end of the static apnea.

elite free-divers (Joulia et al., 2013), however, it is the first time that changes in [AD]b are correlated with the "intensity" of cardiovascular components of the diving reflex.

The high intensity of spearfishing training vs. free-diving training (Joulia et al., 2009) could induce a double protection against hypoxia during apneas. In fact, an accentuated diving reflex associated with a high [AD]b could limit the blood circulation in peripheral territories and subsequently increase heart and brain perfusion despite the cardiac output decrease and a skeletal muscles activation. Chronic hypoxia exposure increases circulating AD in the blood which acts on A_1R situated on proximal and terminal skeletal muscle arterioles (Marshall, 2001), thereby limiting the blood pressure increase. The maintenance of the peripheral vasoconstrictor reflex during apnea in SFM, despite the high increase in [AD]b, is favoring the increase in brain perfusion previously describe in free-divers (Joulia et al., 2009; Vestergaard and Larsson, 2019). The [AD]b increase on A_2R, the predominant receptor subtype responsible for coronary blood flow regulation, induces a coronary arteries dilatation favoring the myocardial perfusion whereas its effect on the A_1R in supraventricular tissues (atrial myocytes, sinoatrial node, and atrioventricular node) exerts a negative chronotropic effect by suppressing the automaticity of cardiac pacemakers, and a negative dromotropic effect through inhibition of AV-nodal conduction (Mustafa et al., 2009). Finally, since the arterial blood pressure increase and the bradycardia were moderate in CTL and not correlated with the slight increase in [AD]b, we can hypothesize that the brain and heart in this population would be less protected against apnea-induced hypoxia even during short apnea durations.

The [AD]b increase was found to be secondary to the decrease in the erythrocyte nucleoside transporter 1 (ENT1) that regulates the extracellular concentration of AD, and which is down-regulated in free-divers thereby preventing AD uptake by erythrocytes and leading to an increase in [AD]b (Marlinge et al., 2021). It is likely that [AD]b is not simply correlated with HR and blood pressure, but actively participates in the control of the cardiovascular system since it is well established that its endogenous increase, *via* the activation of A_1R, decreases heart rhythm and causes vasodilation through A_2A or A_2B receptor activation (Saadjian et al., 2009; Guieu et al., 2020; Paganelli et al., 2021). Furthermore, exogenous administration of AD leads to bradycardia, sinus arrest, and sometimes ATVB (Brignole et al., 2003; Guieu et al., 2015; Deharo et al., 2018). Interestingly, the increase in [AD]b in control participants does not seem sufficient to induce vasodilation. Indeed, the K_D for the activation of $A_{2A}R$, the main receptors implicated in vasodilation, is in the range of 1.8 μM (Shryock et al., 1998). Such a concentration is usually reached only during prolonged apnea (Marlinge et al., 2021) which is only the case for the SFM group in our study.

Conversely, the [AD]b necessary to activate A_1R is in the range of 0.8 μM (Cohen et al., 1996). This may explain the bradycardia observed both in SFM and CTL at the end of the apnea in the current study. In basal condition, however, the relatively high [AD]b measured in divers was not correlated with HR and there was no difference between basal HR and arterial blood pressure recorded in SFM and CTL. This is suggesting that high basal [AD]b could be associated with a down regulation of AD receptors as previously reported in participants suffering from vasovagal manifestations and chronically exposed to high basal [AD]b (Brignole et al., 2017) and/or a compensating increase in sympathetic activity and catecholamine release to counterbalance the bradycardic and peripheric vasodilator effects of [AD]b (Dibner-Dunlap et al., 1993). Finally, since it was recently shown that the succession of oxygen partial pressure variations as hyperoxia–normoxia succession could modify the

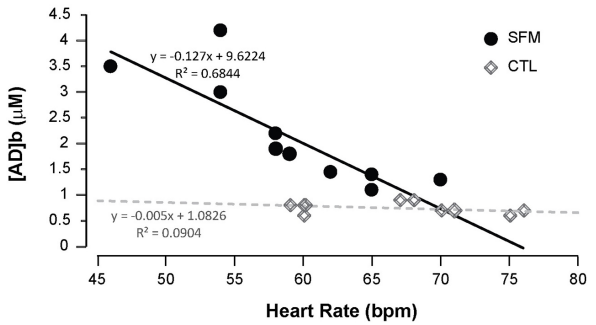

FIGURE 2 | Correlations between the minimal heart rate recorded during the apnea and the blood adenosine concentration in spearfishermen (SFM) and control (CTL) subjects.

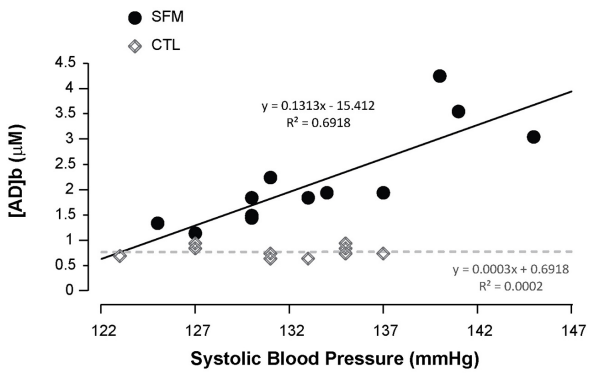

FIGURE 3 | Correlations between the systolic blood pressure recorded during the apnea and the blood adenosine concentration in spearfishermen (SFM) and control (CTL) subjects.

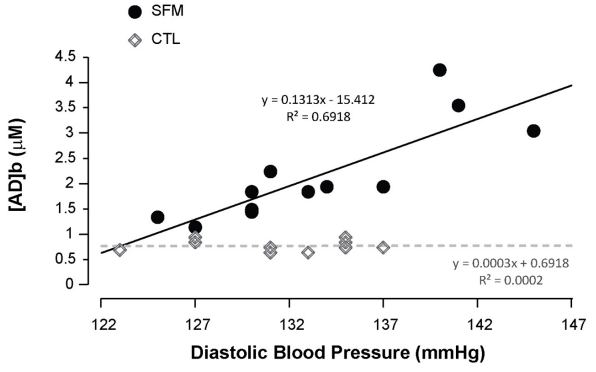

FIGURE 4 | Correlations between the diastolic blood pressure recorded during the apnea and the blood adenosine concentration in spearfishermen (SFM) and control (CTL) subjects.

regulation of the HIF transcription factor activity (Fratantonio et al., 2021) we can suppose that the succession of apneas during spearfishing could modify the HIF transcription activity known to be linked to the rephosphorylation of AD into ATP (Morote-Garcia et al., 2008).

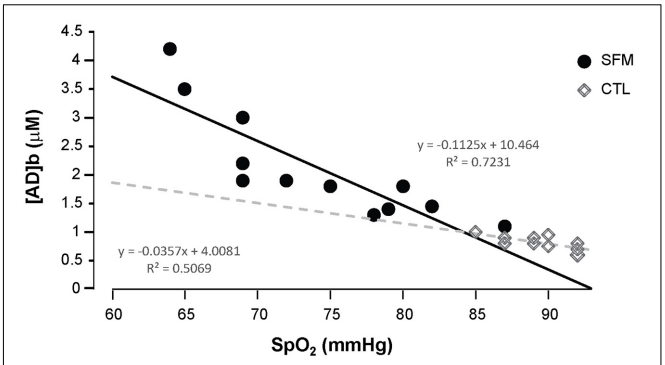

FIGURE 5 | Correlations between the SpO_2 values recording during the apnea and the blood adenosine concentration in spearfishermen (SFM) and control (CTL) subjects.

In conclusion, the AD release during apnea seems to play a major role in the adaptive cardiovascular response to hypoxia. The release of AD in the bloodstream, triggered by hypoxia, limits the increase in blood pressure and maintains sufficient flow to key organs through its vasodilation properties, and further protects the myocardium *via* its HR slowing effects. Finally, the AD release observed in SFM was higher compared to the one observed in free-divers suggesting that spearfishing training is more efficient to increase the diving response than apnea training.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the CPP Sud Marseille N° 13/41. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FB, RG, FJ, and MM conceived and designed the study. MCh and MZ performed the molecular biology and recruited the participants. FB, MCo, RG, FJ, and J-CR critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Cardiovascular Responses to Simultaneous Diving and Muscle Metaboreflex Activation

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Background: The aim of study was to assess hemodynamic changes during the simultaneous activation of muscle metaboreflex (MM) and diving reflex (DR) in a laboratory setting. We hypothesized that as long as the exercise intensity is mild DR can overwhelm the MM.

Methods: Ten trained divers underwent all four phases (randomly assigned) of the following protocol. (A) Postexercise muscle ischemia session (PEMI): 3 min of resting followed by 3 min of handgrip at 30% of maximum force, followed immediately by 3 min of PEMI on the same arm induced by inflating a sphygmomanometer. Three minutes of recovery was further allowed after the cuff was deflated for a total of 6 min of recovery. (B) Control exercise recovery session: the same rest-exercise protocol used for A followed by 6 min of recovery without inflation. (C) DR session: the same rest-exercise protocol used for A followed by 1 min of breath-hold (BH) with face immersion in cold water. (D) PEMI-DR session: the same protocol used for A with 60 s of BH with face immersion in cold water during the first minute of PEMI. Stroke volume (SV), heart rate (HR), and cardiac output (CO) were collected by means of an impedance method.

Results: At the end of apnea, HR was decreased in condition C and D with respect to A (−40.8 and −40.3%, respectively vs. −9.1%; $p < 0.05$). Since SV increase was less pronounced at the same time point (C = +32.4 and D = +21.7% vs. A = +6.0; $p < 0.05$), CO significantly decreased during C and D with respect to A (−23 and −29.0 vs. −1.4%, respectively; $p < 0.05$).

Conclusion: Results addressed the hypothesis that DR overcame the MM in our setting.

Keywords: diving reflex, metaboreflex, heart rate, stroke volume, face immersion

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INTRODUCTION

The human diving response (DR) is characterized by a hemodynamic remodeling where sympathetic and parasympathetic components of the nervous system simultaneously work to evoke bradycardia, reduced cardiac output, vasoconstriction of selected vascular beds, and increased arterial pressure. The outcome resulting is an oxygen sparing effect (Andersson et al., 2004). Conversely exercise is known to increase arterial pressure, heart rate, myocardial contractility, and ventilation. The type of exercise performed has important effects on how these autonomic effects are expressed (Kaufman and Hayes, 2002). A previous research, which investigated hemodynamic changes during simulated dynamic apnea, found a particular cardiovascular response in the second phase of dynamic apnea when a delayed increase in myocardial performance and stroke volume (SV) occurred and obscured the cardiovascular effects of diving reflex (Tocco et al., 2012). The

TABLE 1 | Anthropometric parameters of divers involved in the study.

Age (years)	BM (kg)	Height (cm)
43.6 ± 10.1	68.1 ± 9.4	172.0 ± 7.5

BM, body mass.

authors of the aforementioned article hypothesized that muscle metaboreflex (MM) may act in opposition to the DR to maintain cardiovascular homeostasis. In fact, even though a subject is specifically trained, during prolonged dynamic apnea the peripheral accumulation of metabolic end-products could cause a stimulation of group III and IV nerve-endings, thereby recruiting the MM, which is known to be able to improve myocardial performance (Crisafulli et al., 2003, 2006). However, in the study by Tocco et al. (2012), the MM interference on diving reflex was only theoretical by authors since the experimental condition did not allow to isolate and to measure its hemodynamic effect. Thus, we conceived the idea to use a protocol able to evoke MM by trapping muscle metabolites in the exercising limb and maintaining stimulation of the metaboreceptors, previously tested by Crisafulli et al. (2003), along with an apnea with face immersion that evoked the diving reflex. We hypothesized that as long as the exercise intensity is mild DR can overwhelm the MM. The aim of the present study was to assess, for the first time, hemodynamic changes during isolated and simultaneous activation of MM and DR in a laboratory setting.

MATERIALS AND METHODS

Participants

The study was conducted on 10 trained instructors of diving (six men and four women). At the time of tests, none of the female divers were in their menses phase. Information on the study procedures was provided to all participants and the study was approved by the Cagliari University Ethics Committee and conducted according to the Declaration of Helsinki. Written informed consent was obtained before subjects entered the study.

Experimental Design

Hemodynamic, respiratory, and metabolic changes were assessed in a temperature-controlled, air-conditioned room (22°C, relative humidity 50%) at the same time in the morning. Upon arrival of subjects in the room, basic anthropometric parameters were collected for each diver (**Table 1**). After instrumentation, the divers adopted the sitting posture. Five minutes were allowed to achieve steady-state conditions for cardio-respiratory and metabolic parameters, and then the subjects underwent all the four phases of the following protocol randomly assigned to eliminate any order effect (**Figure 1**).

Postexercise Muscle Ischemia Session (PEMI)

Divers observed 3 min of resting, followed by 3 min of mild exercise, consisting of handgrip at 30% of maximum force, followed by 3 min of PEMI on the left arm induced by rapidly inflating a tourniquet to 50 mmHg above systolic blood pressure. Three minutes of recovery was further allowed after the cuff was deflated, for a total of 6 min of exercise recovery. As stated before, this protocol has been used in several previous investigations and it has been demonstrated to be capable of trapping the muscle metabolites in the exercising limb and of maintaining stimulation of the metaboreceptors (Crisafulli et al., 2003, 2006).

Control Exercise Recovery Session (CER)

The same rest–exercise protocol used for PEMI followed by a control exercise recovery of 6 min without tourniquet inflation.

Diving Reflex Session (DR)

After the same rest–exercise protocol each diver performed 60 s of breath-hold with face immersion in cold water (water temperature 17°C). Previous investigations have demonstrated that apnea with face-immersion is capable of evoking the typical diving response (Andersson et al., 2004; Tocco et al., 2012).

PEMI+Diving Reflex Session (PEMI-DR)

During the first minute of PEMI divers performed 60 s of breath-hold with face immersion in cold water. Sessions were spaced by a 30-min interval during which the subject rested in order to recover completely.

Measurements

During all the protocol phases, cardiodynamic variables were measured by means of impedance cardiography (IC) (NCCOM 3, BoMed Inc., Irvine CA). The impedance method provides noninvasive reliable data of thoracic fluid index (TFI), left ventricular ejection time (VET), SV, heart rate (HR), and cardiac output (CO). IC has been commonly employed in resting and exercising subjects (Concu and Marcello, 1993; Crisafulli et al., 2003). Impedance and ECG recorded traces were analyzed with a digital chart recorder (PowerLab 8sp, ADInstruments, Castle Hill, Australia). The SV-to-VET ratio was also assessed and considered as an index of myocardial performance (Tanaka et al., 1986; Concu and Marcello, 1993). Systemic vascular resistance (SVR) was obtained by dividing mean blood pressure (MBP, calculated as diastolic blood pressure + 1/3 systolic blood pressure – diastolic blood pressure) by CO. Systolic and diastolic blood pressure measurements were performed every 30 s. Measurements were always taken in the morning at least 2 h after light breakfast.

Data Analysis

Since divers showed a wide dispersion in hemodynamic values at the end of exercise phase, we chose to report data as mean ± SD percent changes from end exercise level. Data were averaged for 1 min, except for the breath-hold phases, where variables were averaged for 20 s. Thus, during each trial we recorded nine time points: one for end exercise (EXE 3, which was taken as the last minute of exercise), three for the breath-hold phase (20, 40, and 60 s), and five for the recovery phase (REC 2 to REC 6, averaged for 1 min). Differences between conditions were studied by means of the two-way analysis of variance (ANOVA) for repeated measures (factors: condition and time). Tukey's *post hoc* was performed when appropriate. The level of significance was set at $P < 0.05$ in all cases.

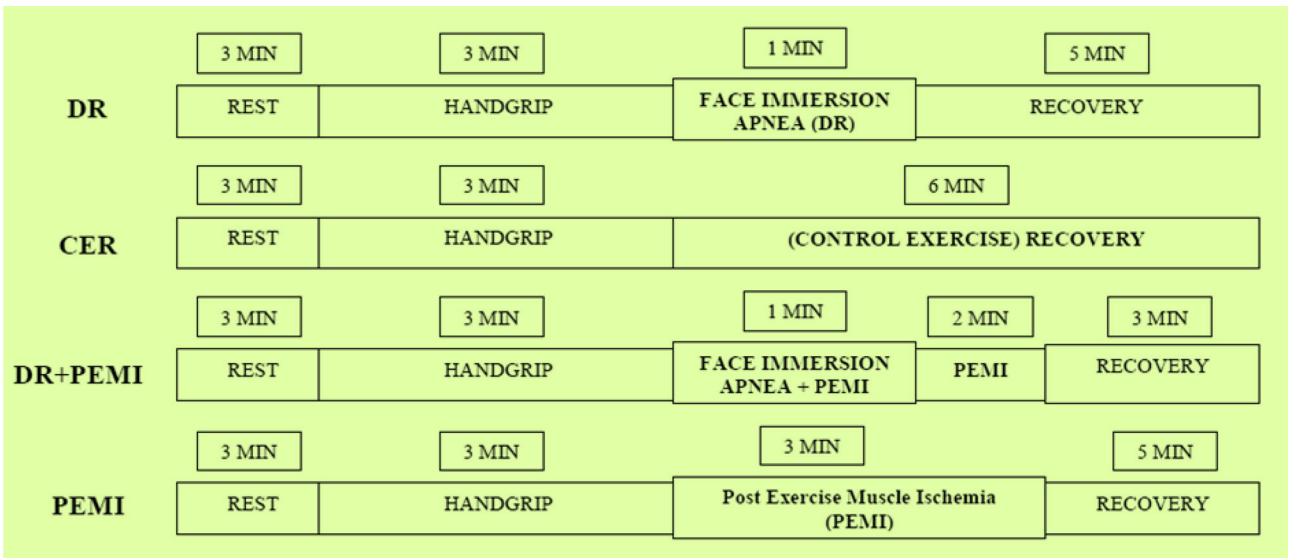

FIGURE 1 | Schematic representation of the four phases of the experimental design. See text for details.

TABLE 2 | Absolute hemodynamic and metabolic data in divers at rest preceding the apnoeas.

Parameter (Units)	HR (bpm)	SV (ml)	CO (L·min^{-1})	SV/VET (ml·sec^{-1})	SVR (dyne·s/cm^5)	MBP (mmHg)	TFI (Ohm)
Value	72.8 ± 0.6.1	56.1 ± 14.4	3.9 ± 0.8	245.3 ± 31.2	1773.7 ± 268.8	81.6 ± 8.2	33.9 ± 1.5

HR, heart rate; SV, stroke volume; CO, cardiac output; SV/VET, stroke volume/ventricular ejection time; SVR, systemic vascular resistance; MBP, mean blood pressure; TFI, trans-thoracic fluid index. Values are mean ± SD.

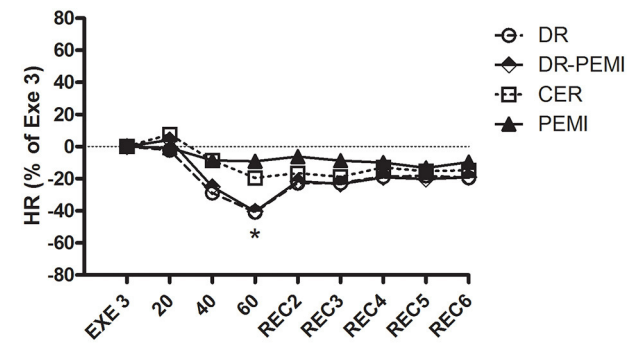

FIGURE 2 | HR time courses during all four tests. Values are means ± SD percentages of the end of handgrip (EXE3). * = $P < 0.05$ vs. EXE3.

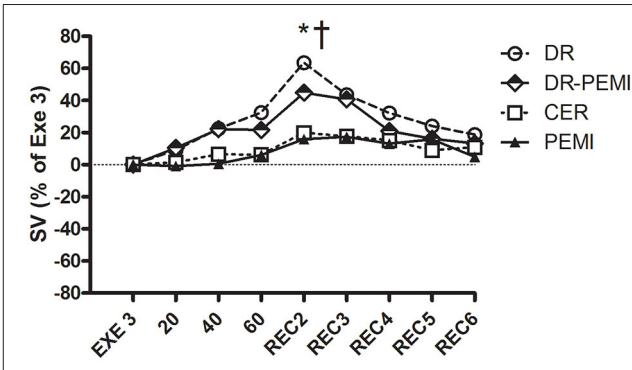

FIGURE 3 | SV time courses during all four tests. Values are means ± SD percentages of the end of handgrip (EXE3). * = $P < 0.05$ vs. EXE3. † = $P <$ 0.05 vs. PEMI.

RESULTS

The protocol was completed by all divers, and no symptoms were reported. The anthropometric values of the divers are shown in **Table 1**. Baseline absolute values of hemodynamic parameters are shown in **Table 2**. **Figures 2–8** show the time courses of each cardiovascular parameter measured in divers during trials.

Figure 2 shows that during DR and DR-PEMI trials, HR significantly decreased with respect to Exe 3 at time point 60. **Figure 3** demonstrates that SV tended to increase in response to both DR and DR-PEMI maneuvers with respect to Exe 3. Moreover, at time point REC2 of test DR SV significantly increased with respect to PEMI. As a consequence of HR and SV behaviors, CO decreased at time point 60 with respect to Exe3 during tests DR and DR-PEMI. It increased at REC2 especially during DR trial, and then returned to reference values in the following periods (**Figure 4**). The SV/VET ratio (index of

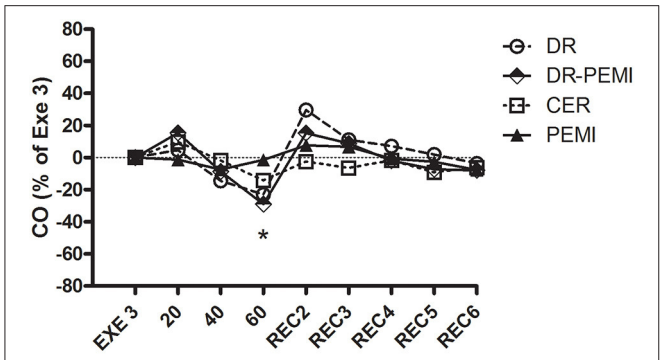

FIGURE 4 | CO time courses during all four tests. Values are means ± SD percentages at the end of handgrip (EXE3). * = $P < 0.05$ vs. EXE3.

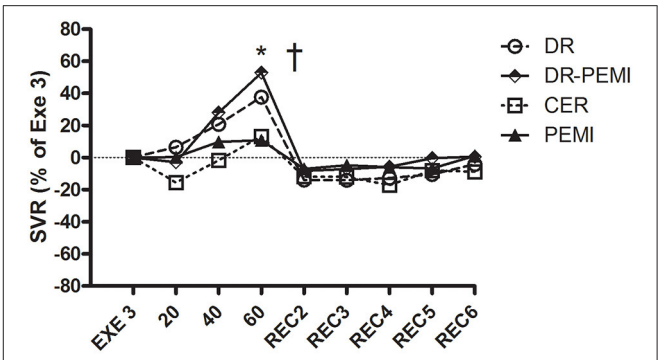

FIGURE 7 | SVR time courses during all four tests. Values are means ± SD percentages of the end of handgrip (EXE3). * = $P < 0.05$ vs. EXE3. † = $P < 0.05$ vs. PEMI.

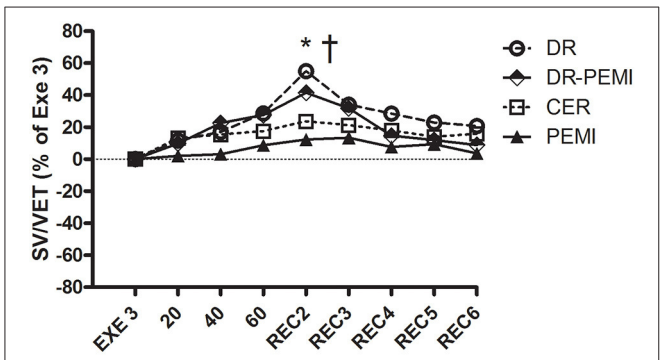

FIGURE 5 | SV/VET time courses during all four tests. Values are means ± SD percentages of the end of handgrip (EXE3). * = $P < 0.05$ vs. EXE3. † = $P < 0.05$ vs. PEMI.

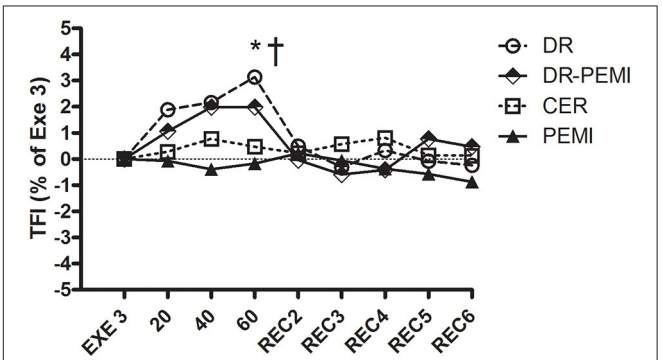

FIGURE 8 | TFI Values are means ± SD percentages of the end of handgrip (EXE3). * = $P < 0.05$ vs. EXE3. † = $P < 0.05$ vs. PEMI.

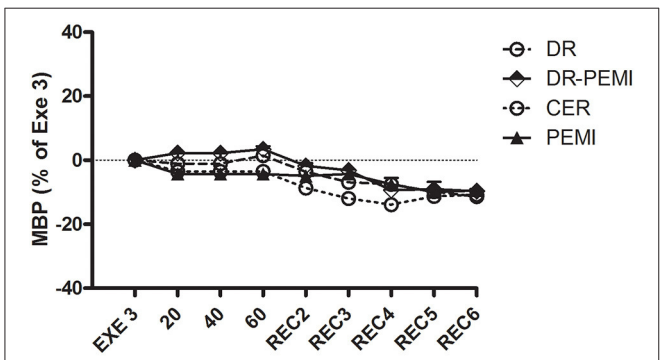

FIGURE 6 | MBP time courses during all four tests. Values are means ± SD percentages of the end of handgrip (EXE3).

myocardial performance, **Figure 5**) gradually increased during the apnea and REC2 periods of both test DR and DR-PEMI compared with Exe3, and it was significantly higher in DR than in PEMI trial at time point REC2 ($P = 0.007$). This fact meant that myocardial performance was improved by the apnea maneuvers during recovery. Then, in both trials DR and DR-PEMI, SV/VET gradually returned to the Exe3 level.

Despite aforementioned hemodynamic changes during all conditions, there was no detectable divergence of MBP values (**Figure 6**). In DR and DR-PEMI trials, apnea induced a rise in SVR compared with EXE 3, and these differences became significant at time point 60 versus PEMI trial (**Figure 7**). Finally, TFI increased (i.e. thoracic fluids decreased) during the apnea phases of tests DR and DR-PEMI, and this gain was significantly higher in DR compared with PEMI at time point 60 (**Figure 8**). In summary, the main differences between trials detected in our study were that: 1. apnea led to bradycardia in both DR and DR-PEMI trials; 2. there was a SV delayed increment in response to apnea in both DR and DR-PEMI trials; 3. in the same trials the SV increment did not compensate for the bradycardia during apnea and, as a consequence, CO was reduced; 4. SVR augmented during apnea in DR and DR-PEMI, thus MPB was maintained.

DISCUSSION

Our goal it was to investigate the hemodynamic changes evoked by a simultaneous condition of apnea and mild exercise in a laboratory simulation. It is plausible that during various diving disciplines (constant weight diving, variable weight, dynamic apnea, etc.) both mechanisms (apnea and exercise reflexes) are

activated. Indeed, Ichinose et al. (2018) recently demonstrated that voluntary apnea during dynamic exercise activates the MM in humans. Thus our hypothesis was that during a light intensity exercise (as happens in some diving disciplines) the diving reflex, despite metaboreflexes, could lead to cardiac output decrement and therefore an oxygen sparing effect. From the overall analysis of our results, it would seem that this is indeed the case. DR prevailed over MM in our simulation. This outcome is quite different from findings of Tocco et al. (2012) which found a particular cardiovascular response in the second phase of simulated dynamic apnoeas when a delayed increase in myocardial performance and SV occurred and obscured the cardiovascular effects of the DR. This opposite outcome could be due to the different protocol used. In fact, in the aforementioned research (Tocco et al., 2012) the divers performed a continuous dynamic exercise pedaling on a cycle-ergometer against a workload of 0.5 W kg^{-1} of body mass. At that exercise intensity level, an increase in myocardial contractility was already evident during the last part of apnea, with a consequent increase in SV. In the current research divers carried out 3 min of mild exercise, consisting of handgrip at 30% of maximum force, and there was instead an increase in contractility (SV/VET) and consequently in SV and CO immediately after apnea was stopped (REC2).

The prevalence of the apnea reflex over the exercise response is a debated topic among researchers. Delahoche et al. (2005) highlighted great importance of adequate apnea training in enhancing oxygen-saving capacity. During underwater dynamic apnea conditions, Joulia et al. (2009) found that elite divers presented a potentiating of the apnea response, whereas Tocco et al. (2013) concluded that sympathetic activation induced by exercise partially obscured the effects of the diving response. During a real free-diving in the sea, Lemaître et al. (2013) reported that the diving response was strong enough to override the stimulus of muscular exercise in elite divers that showed bradycardia performing deep dives. Different outcome was shown by Marongiu et al. (2015), which found that exercise performed during free-diving counteracted the cardiovascular effects of the diving response to ensure adequate CO toward exercising muscles.

Nutrition and dietary supplementation also appear to influence the response to diving. Engan et al. (2012) suggested that acute dietary NO_3^- supplementation improved static apnea performance by reducing metabolic costs. Patrician and Schagatay (2017) found an oxygen conserving effect of dietary nitrate supplementation during dynamic maximal apnea performance. Ghiani et al. (2016), during real diving in the sea, reported that divers with an adequate balance between metabolic and splancnic status (i.e. during a fasting by 12 hours) improved their diving response.

The present research emphasizes the importance of exercise intensity as more or less influencing the DR. This fact has important practical implications for divers. Maximizing movements required for dynamic apnea or constant weight immersion, keeping the exercise of light intensity, would allow the DR to prevail over the response to exercise better. In our opinion, an opinion supported by the results of the present and previous researches, this is an aspect that can tip the balance between two reflexes toward the DR. Divers should focus the most of their training on the diving propulsive and sliding technique, optimizing it as much as possible. This would also improve their safety when diving.

LIMITATIONS ON THE STUDY

The experimental design allowed us to study the diving reflex and exercise pressor response and their interactions simultaneously, but the results must be considered limited to our experimental context. In fact, for practical needs, it lacked some components of immersion such as especially the blood shift, linked to the increase in atmospheric pressure. Previous studies conducted under real diving conditions have shown how this component can modify hemodynamics compared with a laboratory situation (Joulia et al., 2009; Lemaître et al., 2013; Marongiu et al., 2015). Another possible limitation of the present investigation was the low number of subjects enrolled. However, we chose the use of percent changes instead of absolute values to describe time courses of variables, and this procedure allowed to curtail quantitative difference among subjects.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Cagliari State University Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AD, GG, FrT, and FiT contributed to the data analysis and interpretation of the data, drafting, and revising the manuscript, and approved the final version of the manuscript. The original study design was made by FiT and discussed with the other authors. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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The Effect of Breathing Patterns Common to Competitive Swimming on Gas Exchange and Muscle Deoxygenation During Heavy-Intensity Fartlek Exercise

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During competitive freestyle swimming, the change of direction requires a turn followed by ∼15 m of underwater kicking at various intensities that require a ∼5 s breath-hold (BH). Upon surfacing, breathing must be regulated, as head rotation is necessary to facilitate the breath while completing the length of the pool (∼25 s). This study compared the respiratory and muscle deoxygenation responses of regulated breathing vs. free breathing, during these 25–5 s cycles. It was hypothesized that with the addition of a BH and sprint during heavy-intensity (HVY) exercise, oxygen uptake (VO_2) and oxygen saturation ($S_{at}O_2$) would decrease, and muscle deoxygenation ([HHb]) and total hemoglobin ([Hb_{tot}]) would increase. Ten healthy male participants (24 ± 3 years) performed 4–6 min trials of HVY cycling in the following conditions: (1) continuous free breathing (CONLD); (2) continuous with 5 s BH every 25 s (CONLD-BH); (3) Fartlek (FLK), a 5 s sprint followed by 25 s of HVY; and (4) a combined Fartlek and BH (FLK-BH). Continuous collection of VO_2 and $S_{at}O_2$, [Hb_{tot}], and [HHb] *via* breath-by-breath gas analysis and near-infrared spectroscopy (normalized to baseline) was performed. Breathing frequency and tidal volumes were matched between CONLD and CONLD-BH and between FLK and FLK-BH. As a result, VO_2 was unchanged between CONLD (2.12 ± 0.35 L/min) and CONLD-BH (2.15 ± 0.42 L/min; $p = 0.116$) and between FLK (2.24 ± 0.40 L/min) and FLK-BH (2.20 ± 0.45 L/min; $p = 0.861$). $S_{at}O_2$ was higher in CONLD (63 ± 1.9%) than CONLD-BH (59 ± 3.3%; $p < 0.001$), but was unchanged between FLK (61 ± 2.2%) and FLK-BH (62 ± 3.1%; $p = 0.462$). Δ[Hb_{tot}] is higher in CONLD (3.3 ± 1.6 μM) than CONLD-BH (-2.5 ± 1.2 μM; Δ177%; $p < 0.001$), but was unchanged between FLK (2.0 ± 1.6 μM) and FLK-BH (0.82 ± 1.4 μM; $p = 0.979$). Δ[HHb] was higher in CONLD (7.3 ± 1.8μM) than CONLD-BH (7.0 ± 2.0μM; Δ4%; $p = 0.011$) and lower in FLK (6.7 ± 1.8μM) compared to FLK-BH (8.7 ± 2.4 μM; $p < 0.001$). It is suggested that the unchanged VO_2 between

CONLD and CONLD-BH was supported by increased deoxygenation as reflected by decreased $\Delta[Hb_{tot}]$ and blunted Δ[HHb], *via* apneic-driven redistribution of blood flow away from working muscles, which was reflected by the decreased $S_{at}O_2$. However, the preserved VO_2 during FLK-BH vs. FLK has been underpinned by an increase in [HHb].

Keywords: apnea, regulated breathing, gas exchange, muscle deoxygenation, swimming, front crawl

INTRODUCTION

Competitive swimming requires the performance of high-intensity work while performing regular periods of apnea. For example, the swimming "flip-turn" and push-off technique, facilitating a change in direction at the end of the pool, is a maneuver that requires high-power output (PO) of the lower extremities (kicking) combined with apnea. International swimming rules stipulate that the swimmers must surface after covering a maximum of 15 m from the underwater kicking phase. This underwater kicking phase endures for ∼5 s. While the backstroke affords swimmers to breathe freely during swimming as their face is not underwater, the breaths during the front crawl, performed in the prone position, is confined to the rhythmical rotation of the body along the sagittal plane in the position to when the face is exposed to the air once every stroke cycle. This imposes a regulated breathing paradigm dictated by the specific characteristics of this swimming stroke.

Previous work by Lim et al. (2018) simulated the cardiorespiratory response of the lower extremities to multiple laps of backstroke swimming by repeating cycles of 25 s of free-breathing, the approximate duration of swimming one length of a 50 m pool, with 5 s of breath-holding (BH) as are experienced during the aforementioned flip-turn and push-off phases. However, since these trials were performed on a cycle ergometer on land, as opposed to in water with facial submersion, their observations provided only a cursory understanding of the physiological effects of the ventilatory techniques that may be common to swimmers. Some swimmers, with better underwater hydrodynamics, may choose to perform a 5 s sprint kick, in combination with the BH, with the hope that the added swimming speed will offset any negative physiological effects of the sprint in the latter stages of the race. These apneic exercise interventions were separated into four 6 min bouts on a cycle ergometer performed at an intensity corresponding to a PO of 50% of the difference between the lactate threshold (LT) and maximal oxygen consumption (VO_{2max}) of an individual (Δ50%): (1) constant load (CONLD), (2) CONLD with 5 s BHs every 25 s (CONLD-BH), (3) Fartlek (25 s at Δ50% and a 5 s sprint) (FLK), and (4) FLK with 5 s BHs every 25 s (FLK-BH). The addition of a BH, which reduced breathing opportunities within CONLD (CONLD-BH), elicited an increase in minute ventilation (V_E) during the 25 s free-breathing periods, along with the elevated deoxyhemoglobin-to-VO_2 ratio ([HHb]/VO_2) during the 5 s BHs. This reflected greater local muscle deoxygenation that supported O_2 utilization and mitigated the repeated hypoxia of the 5 s BH. They suggested that the stimulus for increased ventilation was underpinned by the transient increases in the end-tidal partial pressure of CO_2 ($P_{ET}CO_2$) and decreases in O_2 ($P_{ET}O_2$) during the 5 s BHs, as these factors have been indicated to modulate ventilatory responses (Whipp and Davis, 1979). Similar to previous apnea studies, the observed decrease in mean concentration of total hemoglobin ($[Hb_{tot}]$) and mean concentration of deoxyhemoglobin ([HHb]) during the 5 s apnea period of CONLD-BH compared to CONLD suggests an overall reduction in blood flow and greater reliance on O_2 extraction to support O_2 utilization, reflecting the previously observed apnea-driven O_2 conservation response at the level of the muscle (Andersson et al., 2004). The BH did not impose a great enough physiological stress to cause a reduction in PO to either the CONLD-BH or FLK-BH condition. However, the added stress of the sprint resulted in decreased VO_2, despite increases in $[Hb_{tot}]$ and [HHb]. The redistribution of blood flow and reduced cardiac output (Andersson et al., 2004) have been replicated earlier under apneic conditions (Lindholm and Linnarsson, 2002; Hoffmann et al., 2005) and are similarly experienced during deep diving (Ferrigno et al., 1997). The supply of Hb to the blood has also been observed during these dives as a function of splenic contractions (Hurford et al., 1990), which elicits greater O_2 and CO_2 carrying capacities in blood. This deep diving response does not appear to extend to high-intensity knee extensions (Kennedy et al., 2008) or cycling exercise (Chacaroun et al., 2019) under hypoxic conditions.

Prone swimming strokes impose a regulated breathing paradigm that should abolish the transient increases in V_E during free-breathing. However, to what extent this is true remains unknown and could be evaluated by imposing a paradigm where regulation of frequency of breathing (f_B) and inspiratory tidal volume (VTI) of each individual are controlled for each minute of exercise.

Therefore, the purpose of this study was to investigate respiration and muscle deoxygenation under regulated breathing vs. free-breathing conditions between the aforementioned protocols. We hypothesized that with the reduced breathing opportunities from the regulated ventilation during the 25 s breathing periods, (1) mean VO_2 would decrease, and mean VCO_2 would decrease in CONLD-BH compared to CONLD and in FLK-BH compared to FLK. Moreover, (2) in comparison to the conflicting results between CONLD-BH and FLK-BH as suggested by Lim et al. (2018), we expected to observe increases in $[Hb_{tot}]$, [HHb], and $[La^-]$ to account for the increased physiological stress of regulated breathing during both the conditions.

MATERIALS AND METHODS

Ten males (mean 23.7 ± 2.5 years) participated in this study after their written informed consent was given. Inclusion criteria were that the participants were healthy and active (i.e., exercising one to three times per week). Smokers and individuals who take medication for the cardiopulmonary system were excluded. None of the participants were involved in sports. However, they were participating in 30–60 min of moderate- to heavy-intensity (HVY) resistance training of the upper and lower extremities two to three times per week. All procedures were approved by the Western University Research Ethics Board for Health Sciences Research Involving Human Participants and were in accordance with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Baseline characteristics for the participants are summarized in **Table 1**.

Test Conditions

The activity levels of participants were maintained throughout the duration of this study. Participants were asked to avoid caffeine for 6 h before each test. Five tests were performed on an electronically braked cycle ergometer, each on a separate day with a minimum interval of 48 h between the tests. Participants wore a nose clip to prevent nose breathing and a mouthpiece to facilitate gas exchange analysis and ventilatory measurements.

Ramp Incremental Test (Day 1)

Participants were instructed to complete a ramp test to exhaustion on a cycle ergometer. The PO was increased by 25 W/min, while a cadence of 70 rpm was maintained. Verbal encouragement was given to participants to maximize their performance. When participants were unable to cycle above 60 rpm for more than five consecutive seconds, the protocol was stopped. This incremental test was used to determine the maximal aerobic capacity (VO_{2max}), peak power output (PPO), and the estimated LT. Above the LT, excess [H^+] derived from an increase in lactate production is buffered by the carbonic anhydrase reaction, which yields greater CO_2 output in relation to O_2 utilization than that observed when exercising below the LT (Beaver et al., 1986). This increased CO_2 results in an increase in V_E, which is reflective of the LT. Therefore, this estimated LT was used as a proxy of the actual LT as the exercise intensity at which VCO_2/VO_2 began to increase disproportionately to increases in PO (also referred to as the gas exchange threshold).

TABLE 1 | Baseline participant characteristics and results from the incremental ramp test (*n*=10).

Variable	Mean ± SD
Age	23.7 ± 2.5
Height (cm)	175 ± 6
Weight (kg)	78.5 ± 9.0
VO_{2max} (L/min)	3.23 ± 0.63
VO_2 at LT (L/min)	1.72 ± 0.16
PO at LT (W)	155 ± 23
PO at Δ20% (W)	189 ± 23
PPO (W)	303 ± 36

LT, lactate threshold; SD, standard deviation.

Constant Load Exercise (CONLD)

A constant load step procedure (CONLD) was performed at the PO at 20% of the difference between LT and VO_{2max} (Δ20%) of an individual. A 4 min baseline period at 20 W was followed by constant load cycling at 70 rpm for 6 min while free-breathing in order to stabilize the gas exchange response. Five f_B measurements were recorded at 1, 2, 3, 4, and 5 min after the onset of the PO.

Constant Load With BHs (CONLD-BH)

Another square-wave test similar to CONLD was performed by participants at Δ20% at 70 rpm. Every 25 s from the beginning of the warm-up, participants performed a 5 s BH. A 5 s countdown was given before each BH period. During the remaining 25 s of the 30 s cycles, breathing was regulated to CONLD: participants matched their f_B and VTI for each minute of exercise to that achieved during the CONLD condition, with the guidance of a metronome and feedback from the attending researcher, respectively.

Fartlek

After the initial 4 min warm-up, participants commenced HVY work at Δ20% for 6 min with a cadence of 70 rpm. Every 25 s, a 5 s interval at the PPO of an individual was performed. Subjects could breathe freely. Like in CONLD, f_B measurements were recorded for each of the first 5 min after the onset of exercise.

Fartlek With BHs (FLK-BH)

Finally, a similar protocol to FLK was performed by participants. Notably, 5 s BHs were incorporated every 25 s, so the sprints were performed under apnea. f_B and Vt were matched to the minute-by-minute measurements taken in FLK.

The ramp protocol was performed always on day 1. Each participant was required to complete CONLD and FLK before CONLD-BH and FLK-BH, respectively, to establish the ventilatory thresholds for the BH protocols. Otherwise, each participant was randomly prescribed one of the six possible orders to complete the submaximal exercise conditions (e.g., CONLD, FLK, FLK-BH, and CONLD-BH) by an online random sequence generator. Comparisons of the physiological outcomes were made within the performance of each participant between apneic and non-apneic conditions, such that participants acted as their own controls.

Experimental Considerations

During pilot testing, we noted that no individuals were able to complete the 6 min work at the prescribed Δ50% PO with the periodic BHs and/or sprints. The PO was reduced until all participants were able to complete these 6 min trials. This PO corresponded with a prescribed PO of Δ20%.

Measurements

Breath-by-breath gas exchanges and ventilatory rates at the mouth were assessed using a mass spectrometer (Innovision, AMIS 2000, Lindvedvej, Denmark) and are described in detail

elsewhere (Babcock et al., 1994). Briefly, flow rates during inspiration and expiration were determined with a low dead space bidirectional turbine (Alpha Technologies, Laguna Beach, CA, United States, VMM 110) and pneumotach (Hans Rudolph, Shawnee, Kansas, United States, Model 4813) calibrated with a 3 L syringe. Gas samples at the mouth were analyzed for O_2, CO_2, and N_2 concentrations. Changes in concentrations of gases were matched to the corresponding increase or decrease in volumes of the gases. There was a 20 ms interval between collection samples that were sent electronically to a computer to analyze individual breaths. Each breath started with inspiration and ended with expiration. Therefore, each 5 s BH was recorded as a single breath.

The procedures for near-infrared spectrometry (NIRS) data collection were similar to those previously described (Inglis et al., 2017). Continuous measurement of the quadriceps was performed with a NIRS device (Oxiplex TS, model 95,205, ISS, Champaign, IL, United States). Laser diodes pulsed quickly (110 MHz) at two different wavelengths near the infrared region (690 and 828 nm). These were connected to a plastic probe that was placed midway between the lateral epicondyle and the greater trochanter of the femur on the belly of the vastus lateralis muscle head. An elastic strap secured the device in place. An optically dense, black vinyl sheet was placed over the device to prevent the exposure to extraneous light. A tensor bandage was wrapped gently around the leg of the participant to secure the NIRS device to the site of interest and to further prevent the intrusion of external light into the site of NIRS measurement. Care was taken to ensure that no blood flow occlusion occurred to the leg. Deoxygenated hemoglobin concentration ([HHb]) and oxygenated hemoglobin ([HbO]) were measured, whereas [Hb_{tot}] and tissue oxygen saturation ($S_{at}O_2$) were derived with this apparatus. [Hb_{tot}] was calculated as the sum of [HHb] and [HbO], and $S_{at}O_2$ was estimated as the percentage of [HHb] to [Hb_{tot}]. To account for individual differences in absolute tissue absorption, [HHb] and [Hb_{tot}] were adjusted to baseline values (Δ[HHb] and Δ[Hb_{tot}], respectively).

Rubbing alcohol was applied to the left index finger of each participant before each test. Blood lactate concentrations ([La^-]) were taken 3 min pre- and post-exercise for each test. A lancet (ACCU-CHEK Safe-T-Pro Plus) exposed blood on the finger, which was examined by the SensLab GmbH Lactate SCOUT arterialized-capillary lactate analyzer.

Analysis

Gas exchange and NIRS data were cleaned by the removal of aberrant data points that were at least 3 SDs from the local mean. The data were interpolated linearly to convert from breath-by-breath to 1 s intervals. Then, data points were averaged into 5 s bins. This analysis technique has been described previously (Keir et al., 2014). [HHb] and [Hb_{tot}] were adjusted to zero with baseline as described earlier. These baselines represented the average of 60 s before the square-wave change in PO. Δ[HHb]/VO_2 was determined as the ratio of the normalized [HHb] to VO_2. Between-group comparisons on gas exchange and NIRS variables were performed from the start of the square-wave change in PO (the exercise on-transient) to the end of the bout (0–360 s). Within-group comparisons were performed between the first 25 s and the last 5 s of each 30 s cycle for VO_2 and VCO_2. Lamarra et al. (1987) described this mono-exponential function that models the on-transient VO_2 curve during a step transition:

$$y(t) = y_{BSL} + A_p(1 - e^{-(t-TD)/\tau}) \quad (1)$$

where $y(t)$ represents VO_2 as a function of time during the transition to the steady state of new PO. y_{BSL} is VO_2 before the transition, A_p is the amplitude (increase above y_{BSL}), t is the dependent time variable, TD is the time delay, and τ is the time constant (time that elapses for 63% of the response to occur). The curve was fit to the data by applying the Levenberg–Marquardt algorithm for non-linear least squares analysis (Origin 9.7; OriginLab, Northampton, MA, United States).

Statistics

Ten participants were recruited based on a sample size power calculation of the measured VO_{2max}. It was found that 10 participants were sufficient (80% power) to calculate pre vs. post VO_{2max} (α = 0.05) to within an SD of $\pm$ 30 mL with the consideration of a 20% dropout rate. Mean analyses from 0 s (start of square-wave change in PO) to 360 s (end of exercise) (n = 10) of VO_2, VCO_2, $P_{ET}O_2$, $P_{ET}CO_2$, V_E, f_B, VTI, Δ[HHb], Δ[Hb_{tot}], $S_{at}O_2$, and Δ[HHb]/VO_2 between conditions (CONLD, CONLD-BH, FLK, and FLK-BH) were performed by a one-way repeated measures (1-RM) analysis of variance (ANOVA). [La^-] was compared between conditions and time (PRE vs. POST) by a two-way RM (2-RM) ANOVA. The first 25 s and last 5 s of each apneic cycle were analyzed for VO_2 and VCO_2 *via* a 2-RM ANOVA. The Shapiro–Wilk and Brown–Forsythe tests were performed to assess the normality and heteroscedasticity of the data, respectively. The 1-RM ANOVA comparisons for VCO_2, $P_{ET}O_2$, $P_{ET}CO_2$, V_E, f_B, VTI, Δ[HHb], Δ[Hb_{tot}], $S_{at}O_2$, and Δ[HHb]/VO_2 were performed by a Friedman RM ANOVA on ranks due to a rejection of either or both tests, whereas mean VO_2 was evaluated with a parametric 1-RM ANOVA. Data are reported as mean $\pm$ SD. The statistical significance threshold was $p < 0.05$.

RESULTS

Participants successfully matched V_E between CONLD and CONLD-BH (p = 0.889) and between FLK and FLK-BH

TABLE 2 | Breathing frequency (f_B), inspiratory tidal volume (VTI), and minute ventilation (V_E) under each condition.

Variable	CONLD	CONLD-BH	FLK	FLK-BH
f_B (breaths/min)	22.0 ± 4.7	21.2 ± 4.5	25.2 ± 5.2[a,b]	24.2 ± 6.0[a,b]
VTI (L)	2.93 ± 0.21	2.91 ± 0.27	2.86 ± 0.30[a,b]	2.94 ± 0.24[a,c]
V_E (L/min)	64.8 ± 16.9	62.4 ± 17.5	72.7 ± 20.0[a,b]	73.1 ± 22.1[a,b]

SD, standard deviation. CONLD-BH was matched to CONLD and FLK-BH to FLK. Values are mean ± SD.
[a] *Significantly different than CONLD.*
[b] *Significantly different than CONLD-BH.*
[c] *Significantly different than FLK.*

(p = 0.889) with regard to the onset of the square-wave increase in PO and exercise cessation (**Table 2** and **Figure 1A**). This was supported by the sustained mean f_B between CONLD and CONLD-BH (p = 0.72) and between FLK and FLK-BH (p = 0.99) (**Figure 1B**) and mean VTI between CONLD and CONLD-BH (p = 0.953) (**Figure 1C**). VTI was statistically greater in FLK-BH compared to FLK ($p < 0.001$) by ∼80 mL/min.

Gas Exchange Variables

Mean VO_2 from time 0 to 360 s was similar between CONLD and CONLD-BH (p = 0.406) and between FLK and FLK-BH

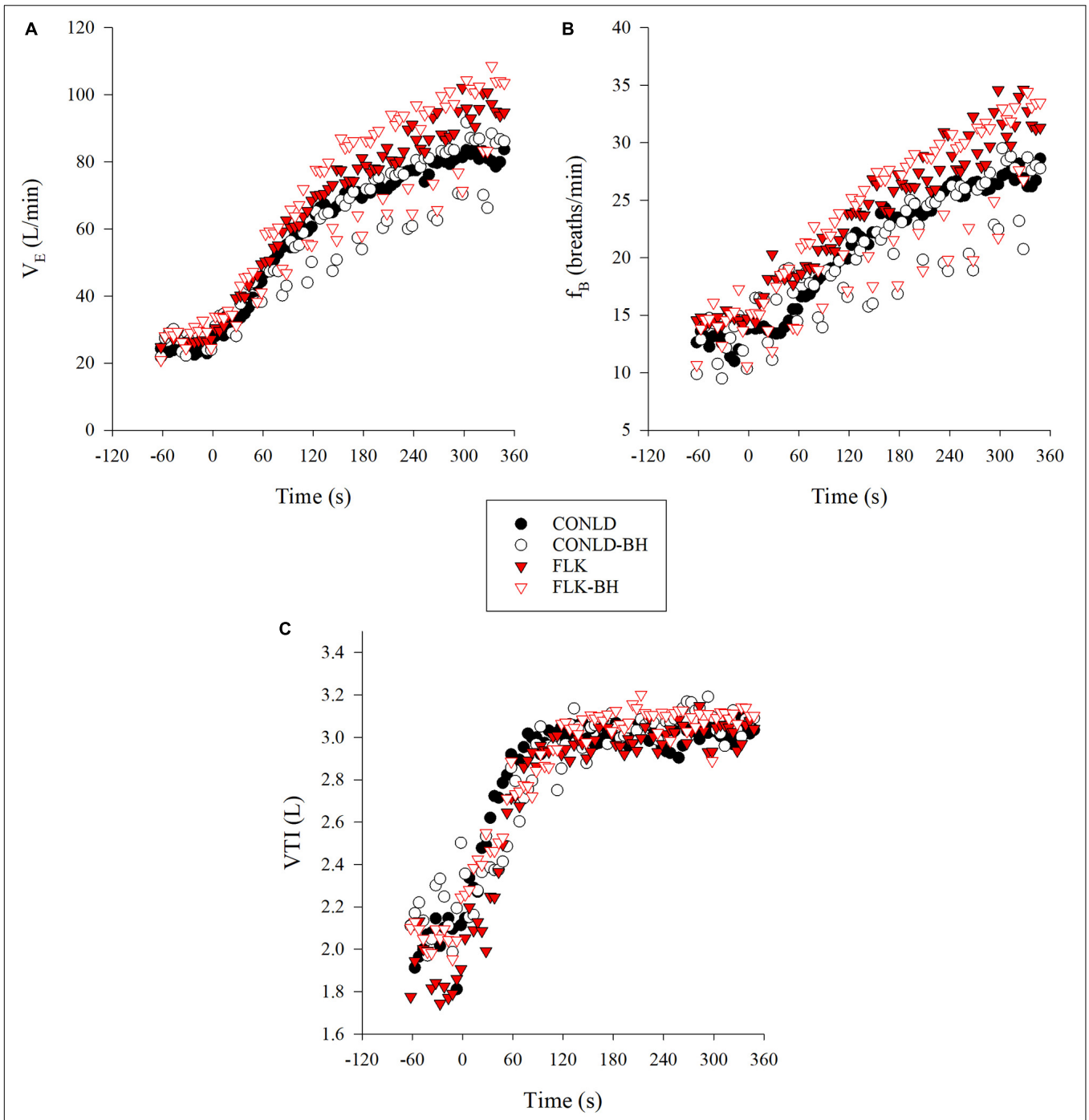

FIGURE 1 | Breathing data during CONLD (filled circles), CONLD-BH (open circles), FLK (filled red triangles), and FLK-BH (open red triangles). **(A)** Minute ventilation (V_E), **(B)** frequency of breathing (f^B), and **(C)** inspiratory tidal volume (VTI). Time 0 marks the start of the exercise transition. Data were interpolated from breath-by-breath to second-by-second and 5 s averaged for graphical representation.

(p = 0.165) (**Table 3** and **Figure 2A**). VO_2 was greater in FLK and FLK-BH than in CONLD ($p < 0.001$ for both comparisons) and greater in FLK than CONLD-BH ($p < 0.001$) over the same time period. Mean VO_2 for the last 5 s of each 30 s cycle was greater in CONLD than CONLD-BH (p = 0.001) and greater in FLK than in FLK-BH ($p < 0.001$) and CONLD-BH ($p < 0.001$) (**Table 4**). Mean $P_{ET}O_2$ was greater in CONLD than CONLD-BH ($p < 0.001$) but was unchanged in FLK compared to FLK-BH (p = 0.682) (**Table 3** and **Figure 2C**). $P_{ET}O_2$ was lower in CONLD and CONLD-BH than in FLK and FLK-BH ($p < 0.001$ for all comparisons) (**Table 3** and **Figure 2C**). $P_{ET}CO_2$ was lower in CONLD than CONLD-BH ($p < 0.001$) but was greater in FLK than FLK-BH ($p < 0.001$) (**Table 3** and **Figure 2D**). VCO_2 from 0 s to the end of exercise was similar between CONLD and CONLD-BH (p = 0.914) and between FLK and FLK-BH (p = 0.641), but was greater in FLK and FLK-BH than in CONLD and CONLD-BH ($p < 0.001$ for all comparisons) (**Table 3** and **Figure 2B**).

$\Delta[Hb_{tot}]$, Δ[HHb], $S_{at}O_2$, and $\Delta[HHb]/VO_2$

NIRS-derived normalized total hemoglobin content ($\Delta[Hb_{tot}]$) was greater in CONLD compared to CONLD-BH ($p < 0.001$), FLK ($p < 0.001$), and FLK-BH ($p < 0.001$), and also in FLK compared to FLK-BH ($p < 0.001$) (**Table 3** and **Figure 3C**). Deoxygenated hemoglobin normalized to baseline (Δ[HHb]) was greater in CONLD than in CONLD-BH (p = 0.011) and FLK ($p < 0.001$), and also in FLK-BH than in other three conditions ($p < 0.001$ for all comparisons) from the onset to the cessation of exercise (**Table 3** and **Figure 3A**). $S_{at}O_2$ was greater in CONLD compared to CONLD-BH ($p < 0.001$), FLK ($p < 0.001$), and FLK-BH ($p < 0.001$), and it was not significantly different in FLK compared to FLK-BH (p = 0.434) (**Table 3** and **Figure 3D**). The ratio of $\Delta[HHb]/VO_2$ was similar between CONLD and CONLD-BH (p = 0.086), but lower in FLK than FLK-BH ($p < 0.001$) (**Table 3** and **Figure 3B**).

TABLE 3 | Mean outcome measures for each of the four conditions, namely, CONLD, CONLD-BH, FLK, and FLK-BH from 0 to 360 s.

Variable	CONLD	CONLD-BH	FLK	FLK-BH
VO_2 (L/min)	2.12 ± 0.35	2.15 ± 0.42	2.24 ± 0.40[a,b]	2.20 ± 0.45[a]
VCO_2 (L/min)	2.30 ± 0.55	2.29 ± 0.64	2.55 ± 0.65[a,b]	2.43 ± 0.70[a,b]
$P_{ET}O_2$ (mmHg)	99.4 ± 5.3	96.9 ± 4.9[a]	101.3 ± 5.0[a,b]	101.5 ± 5.4[a,b]
$P_{ET}CO_2$ (mmHg)	45.0 ± 2.2	46.3 ± 3.0[a]	45.1 ± 2.6[b]	44.0 ± 2.6[a,b,c]
$\Delta[Hb_{tot}]$ (μM)	3.3 ± 1.6	-2.5 ± 1.2[a]	2.0 ± 1.6[a,b]	0.82 ± 1.4[a,b,c]
Δ[HHb] (μM)	7.3 ± 1.8	7.0 ± 2.0[a]	6.7 ± 1.8[a,b]	8.7 ± 2.4[a,b,c]
$S_{at}O_2$ (%)	62.6 ± 1.9	59.4 ± 3.3[a]	61.2 ± 2.2[a,b]	61.7 ± 3.1[a,b]
$\Delta[HHb]/VO_2$	3.38 ± 0.52	3.25 ± 0.83	2.96 ± 0.64[a,b]	3.88 ± 0.98[a,b,c]
POST [La^-] (mM)	9.4 ± 2.4	10.0 ± 2.6	11.5 ± 2.5[a,b]	11.3 ± 2.8[a,b]

SD, standard deviation. Data are reported as mean ± SD.
[a] Significantly different than CONLD.
[b] Significantly different than CONLD-BH.
[c] Significantly different than FLK. POST is 3 min post-exercise.

Lactate ([La^-])

Post-exercise arterialized capillary lactate concentration ([La^-]) was unchanged in CONLD compared to CONLD-BH (p = 1.000), and both were lower compared to FLK and FLK-BH ($p < 0.001$ and p = 0.007, respectively) (**Table 3** and **Figure 4**). Post-exercise [La^-] was similar between FLK and FLK-BH (p = 1.000) (**Table 3** and **Figure 4**).

DISCUSSION

The goal of this study was to monitor the physiological adjustments to repeated cycles of 5 s BHs followed by 25 s of regulated breathing during CONLD and FLK exercise that lasted 6 min. Main findings included (i) unchanged VO_2 between CONLD and CONLD-BH, and in FLK and FLK-BH, despite the imposed regulatory breathing paradigm; (ii) $\Delta[Hb_{tot}]$ and $S_{at}O_2$ were lower, and a marginal decrease in Δ[HHb] was observed in CONLD-BH compared to CONLD, whereas Δ[HHb] and $S_{at}O_2$ were greater in FLK-BH compared to FLK.

CONLD and CONLD-BH

The changes in PO in CONLD and CONLD-BH, relative to the previous related research (Δ50%-Δ20%) (Lim et al., 2018), reflect the additions of the regulated breathing to this 25 s breathing/5 s apnea protocol. The purpose of this modification was to simulate the difference in breathing patterns between swimming backstroke (25 s free-breathing) and front crawl (25 s regulated breathing). By introducing these restrictions, participants were forced to perform a much lower intensity (189 W, Δ20%) compared to that suggested by Lim et al. (2018) (218 W, Δ50%, p = 0.027), despite having similar aerobic fitness (VO_{2max}: 3.23 and 3.17 L/min, p = 0.82, respectively), LTs (VO_2 at LT: 1.72 and 1.77 L/min, p = 0.57, respectively; PO at LT: 155 and 156 W, respectively), and PPO (303 and 314 W, p = 0.58, respectively) to perform the 6 min trials. Moreover, the reduced PO of this study elicited lower mean V_E (68 L/min) compared to that suggested by Lim et al. (99 L/min) (Lim et al., 2018). Within the context of this PO adjustment of this study, VO_2 was unchanged with the addition of the 5 s apneic periods and matching of f_B and VTI during the 25 s breathing periods (**Table 2**) of CONLD-BH, demonstrating that any potential increase in O_2 cost derived from the periodic apneas had been met. The data in this study show that the aerobic metabolic demand (i.e., VO_2) was supported by the decreased $S_{at}O_2$ (**Table 3** and **Figure 3C**) and increased muscle deoxygenation under the reduced perfusion conditions, as reflected by the much greater decrease in mean $\Delta[Hb_{tot}]$ (**Table 3** and **Figure 3D**) and modest decrease in Δ[HHb] (**Table 3** and **Figure 3A**). A similar reduction in $S_{at}O_2$ and an increase in muscle deoxygenation were observed by Kume et al. (2016) at a PO corresponding to 65% of VO_{2max} interspersed with 4 s exhalations followed by a maximal inhalation, simulating hypoventilation. Furthermore, previous work by Hoffmann et al. (2005) under apneic and rebreathing exercise conditions during higher intensity exercise, which generated similar hypoxia (decreased $P_{ET}O_2$ in rebreathing), demonstrated greater mean arterial pressure and lower heart

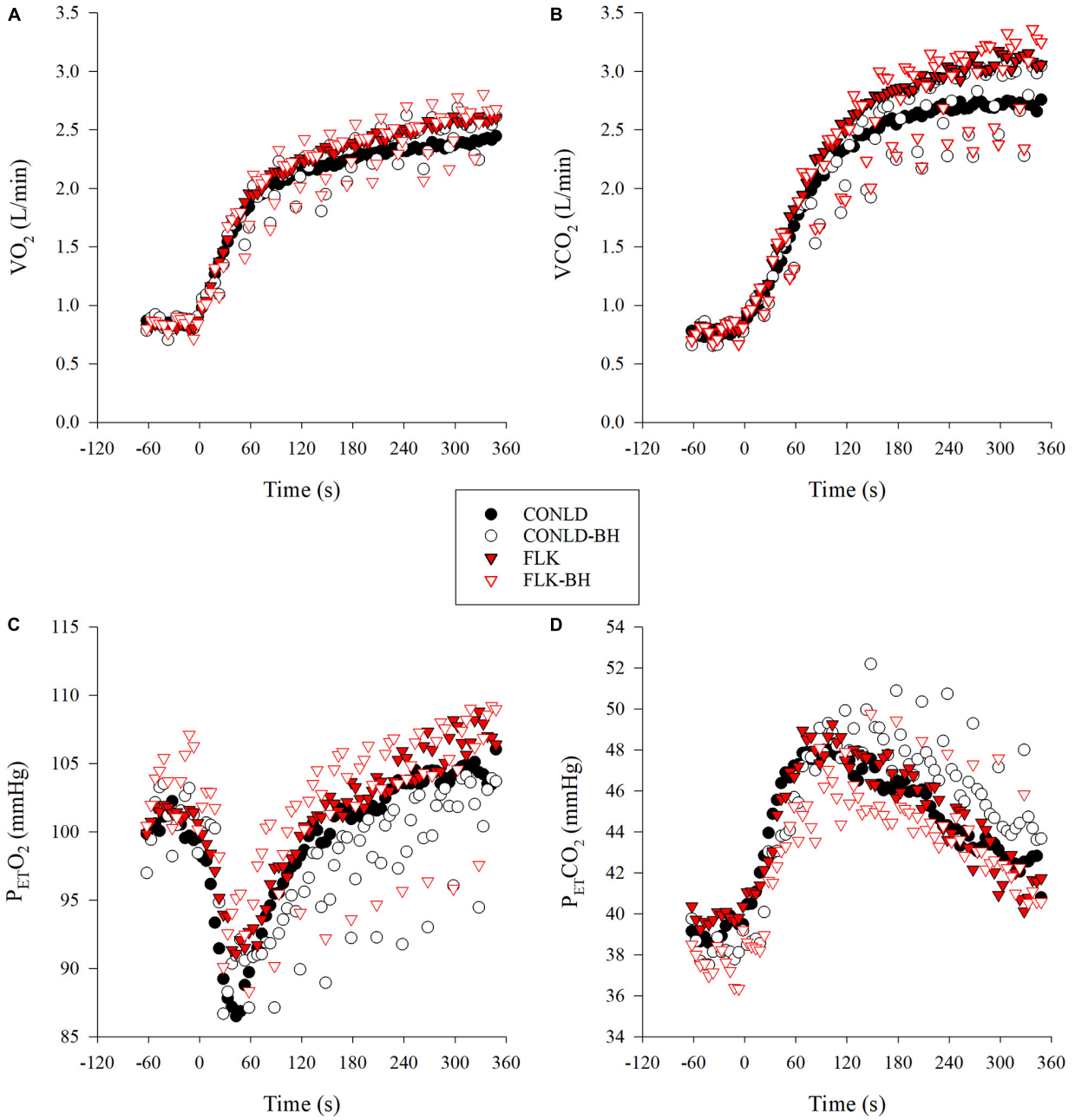

FIGURE 2 | Mean participant respiratory variables during constant A20% work (CONLD, filled circles), constant A20% work with 5 s BHs every 30 s (CONLD-BH, open circles), ?20% work with 5 s sprints at peak power output (PPO) every 30 s (FLK, filled red triangles) and A20% work with 5 s BHs, and sprints at PPO every 30 s (FLK-BH, open red triangles) for 6 min. **(A)** Oxygen consumption (VO_2), **(B)** carbon dioxide elimination (VCO_2), **(C)** end-tidal partial pressure of oxygen ($PETO_2$), and **(D)** end-tidal partial pressure of carbon dioxide ($PETCO_2$). Time 0 marks the start of the exercise transition. Data were interpolated from breath-by-breath to second-by-second and 5 s averaged for graphical representation.

rate in the apneic compared to the rebreathing condition, demonstrating that only the apneic state elicited the observed O_2 conservation or diving response that is associated with the decreased intramuscular blood flow. They concluded that the mechanical cessation of breathing was the key stimulus to this drop in perfusion. This has been corroborated by similar protocols comparing rebreathing with apnea (Lindholm et al., 1999). The data of this study confirm a similar response under the combination of the regulated breathing and BH paradigm. Moreover, the observed decrease in $\Delta[Hb_{tot}]$ shown in this study was not observed during hypoxic exercise (~12% fraction of oxygen in inspired air [F_IO_2]) performed at low-moderate (single-leg knee extensions) (DeLorey et al., 2004) and supra-LT intensities (Ainslie et al., 2007), suggesting again that the reduced

TABLE 4 | Mean outcome measures for both BH conditions (CONLD-BH and FLK-BH) for each 30 s apneic cycle (25 s regulated breathing, 5 s apnea) from 0 to 360 s.

Variable	CONLD-BH		FLK-BH	
	25 s	5 s	25 s	5 s
VO_2 (L/min)	2.19 ± 0.24	1.93 ± 0.27*	2.25 ± 0.38	1.89 ± 0.27*
VCO_2 (L/min)	2.33 ± 0.23	2.04 ± 0.27*	2.49 ± 0.34	2.11 ± 0.22*
$P_{ET}O_2$ (mmHg)	98.0 ± 4.4	90.9 ± 3.7*	103.0 ± 4.9	93.5 ± 4.4*
$P_{ET}CO_2$ (mmHg)	45.8 ± 4.1	48.8 ± 4.1*	43.4 ± 4.6	47.2 ± 4.3*
Δ[HHb] (μM)	7.0 ± 6.2	7.3 ± 6.5*	8.6 ± 9.6	8.9 ± 9.8*

SD, standard deviation. Data are reported as mean ± SD.
**25 s significantly different than 5 s.*

blood flow in this study was apnea-related. Furthermore, earlier research by Lim et al. (2018), within a comparable protocol as this study, and Kume et al. (2013), performing intermittent apneas during continuous exercise at a comparable intensity, identified similar temporal reductions in $\Delta[Hb_{tot}]$ resolved by similar increases in muscle deoxygenation. This decrease was observed despite previous suggestions that $\Delta[Hb_{tot}]$ is increased during deep diving *via* splenic contractions (Hurford et al., 1990).

The drop in $P_{ET}O_2$ (**Table 4** and **Figure 2C**) and increased VO_2 (**Table 4** and **Figure 2A**) observed immediately post-apnea in this study reflect continued alveolar to capillary O_2 diffusion facilitating the unchanged VO_2. This response has also been observed under 20 s apneas (Lindholm and Linnarsson, 2002) and also reflected the actual arterial O_2 (P_aO_2) under these transient apneic conditions (Suskind et al., 1950).

The maintenance of mean VO_2 between CONLD and CONLD-BH after the exercise on-transient response was supported not only by the microvasculature hemodynamic changes outlined earlier, but also by the increased VO_2 during the 25 s of regulated breathing (**Table 4** and **Figure 2A**) and the reduced $P_{ET}O_2$ immediately post-BH (**Table 4** and **Figure 2C**) suggested earlier (Yamamoto et al., 1987). Specifically, Woorons et al. (2007) found that intermittent apneas performed at a high pulmonary volume (i.e., near total lung capacity) during higher intensity exercise (∼70% VO_{2max}), similar to this study, maintained pulmonary arterial $S_{at}O_2$.

The unchanged mean VCO_2, between CONLD and CONLD-BH, suggests that the H^+ buffering, associated with the lactate production of anaerobic glycolysis, has been reflected in the observed increase in $P_{ET}CO_2$ and that the BH did not impose a greater anaerobic glycolytic contribution. The unchanged post-exercise [La^-] between CONLD and CONLD-BH trials is in contrast to other studies that have reported increased [La^-] accumulation during incremental (Seo et al., 2020) (50 W + 12.5 W/min to exhaustion) and intermittent (Lim et al., 2018) exercise under hypoxic or apneic condition, respectively. However, these studies were performed at much higher supra-LT PO, which would elicit much greater blood [La^-].

From a swimming front crawl perspective, at this Δ20% PO intensity, the addition of the regulated breathing paradigm and the 5 s BH, while maintaining a similar intensity of kicking, may be performed without negative physiological consequences. Notably, this is within the context of the legs only paradigm of this experiment as opposed to the simultaneous arm and leg action that is performed after the underwater portion of the swim.

FLK and FLK-BH

The FLK condition mimics the strategies of those swimmers who believe that sprint kicking during the underwater portion after the turn, along with their own superior hydrodynamics compared to their opponents, will give them a competitive advantage. In contrast to the study by Lim et al. (2018), utilizing a similar protocol, albeit at much higher PO, our results showed that implementing the intermittent 5 s periods of apnea to FLK (FLK-BH) did not affect mean VO_2. Similar to CONLD-BH and CONLD, f_B and VTI were also regulated in FLK-BH to FLK; however, although VTI was statistically greater in FLK-BH (**Table 2**), the unchanged V_E suggested that this ∼80 mL/min change in VTI (Δ1.6% over the 6 min) had no physiological effects. It was notable that VTI was lower in FLK compared to CONLD (2.86 ± 0.30 L and 2.93 ± 0.21 L, respectively). However, coupled with the increased f_B (25.2 ± 5.2 breaths/min and 22.0 ± 4.7 breaths/min), a higher V_E, in conjunction with the higher VCO_2 in FLK vs. CONLD, was observed (**Table 2**). The lowered $P_{ET}O_2$ immediately post-apnea (**Table 4** and **Figure 2A**) suggests that the observed pulmonary alveoli to capillary diffusion continued during the 5 s facilitating O_2 transport. Moreover, the increased Δ[HHb] and Δ[HHb]/VO_2 in FLK-BH compared to FLK, which reflected a greater reliance on muscle deoxygenation, at the active muscle was responsible for this result (duManoir et al., 2010; Murias et al., 2011).

In elite swimmers, similar maintenance of VO_2 has been demonstrated during 4 min of submaximal intensity swimming with apnea induced by regulated breathing conditions of every two arm strokes up to a maximum of five-arm strokes, notwithstanding the reduced V_E in the latter condition (Dicker et al., 1980). The consequences of the imposed regulated breathing and comparable intensity in the current study were apparently resolved by the greater alveolar to pulmonary capillary O_2 diffusion. The muscle deoxygenation response was not recorded in the earlier work by Dicker et al. (1980). However, a similar increase in Δ[HHb] was observed by Billaut and Buchheit (2013) during 10 repeated 10 s maximal sprints followed by 30 s rest, under hypoxic conditions (13% F_IO_2) compared to normoxia. Conversely, others have studied the effects of repeated 3 s loadless cycling recovery periods, as opposed to the 5 s of high PO as in this study, and observed an improved microvascular O_2 delivery reflected by decreased Δ[HHb]/VO_2 (Belfry et al., 2012). It is suggested that the contrasting results of this study were attributed to the apneic O_2 conservation effect (Kume et al., 2013), as reflected by the decreased $\Delta[Hb_{tot}]$ in FLK-BH compared to FLK. This result is similar to the CONLD-BH compared to CONLD, of this study and others, reflecting a similar redistribution of blood flow away from working muscles during apnea (Kume et al., 2013; Lim et al., 2018). Furthermore, it is suggested that the unchanged mean VCO_2 and [La^-] during FLK-BH compared to FLK despite the apneic periods was a consequence of the continued buffering and pulmonary capillary to alveolar diffusion and buffering

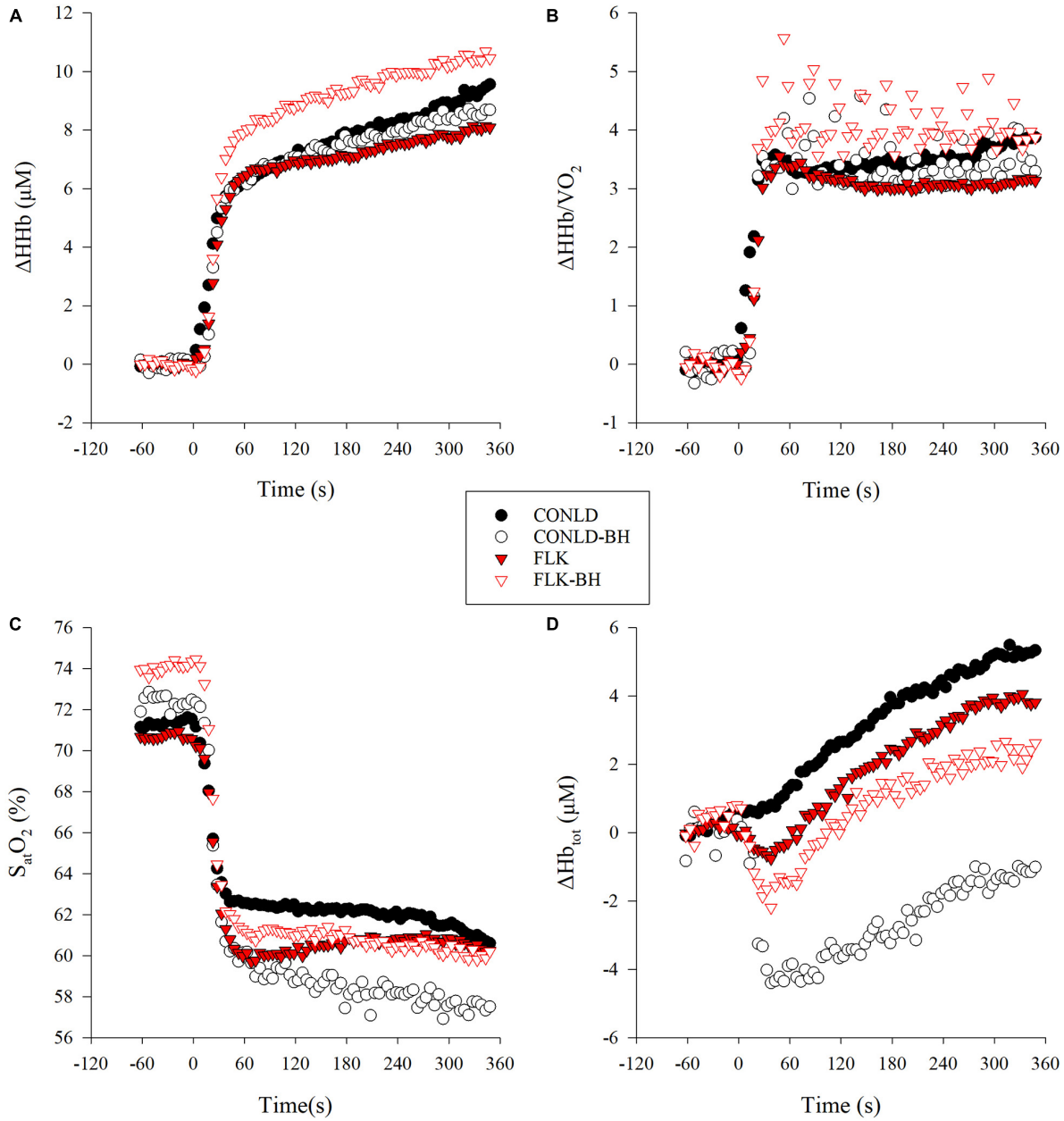

FIGURE 3 | Local muscle deoxygenation variables from near infrared spectrometry (NIRS) data in CONLD (filled circles), CONLD-BH (open circles), FLK (filled red triangles), and FLK-BH (open red triangles). **(A)** Deoxyhemoglobin concentration normalized to baseline (Δ[HHb]), **(B)** ratio of normalized deoxyhemoglobin to VO_2 (Δ[HHb]/VO_2), **(C)** oxygen saturation of hemoglobin $S_{at}O_2$, and **(D)** total hemoglobin concentration normalized to baseline (Δ[Hb_{tot}]). Time 0 marks the start of the exercise transition. Data were interpolated from breath-by-breath to second-by-second and 5 s averaged for graphical representation.

during this 5 s BH (**Table 4**). Conversely, Lim et al. (2018), under a similar apnea protocol (5 s), but with free-breathing (25 s), as opposed to regulated breathing of this study, showed lower mean VCO_2 in FLK-BH compared to FLK and higher lactates associated with the higher mean PO (Δ50% vs. Δ20%), suggesting a relative decrease in ventilatory buffering at this much higher relative PO.

To our knowledge, this was the first study that regulated breathing during supra-threshold PO exercise on a cycle ergometer following periods of apnea, to a free-breathing protocol at the same PO similar to the lower extremity work associated with swimming in the prone position. Consequently, mean VO_2 was maintained in these apneic conditions (CONLD-BH and FLK-BH) compared to their free-breathing counterparts (CONLD and FLK, respectively) by greater muscle deoxygenation, despite decreased intramuscular blood perfusion. The regulated f_B and V_E during the 25 s breathing periods of this study eliminated the transient increases in V_E that previously

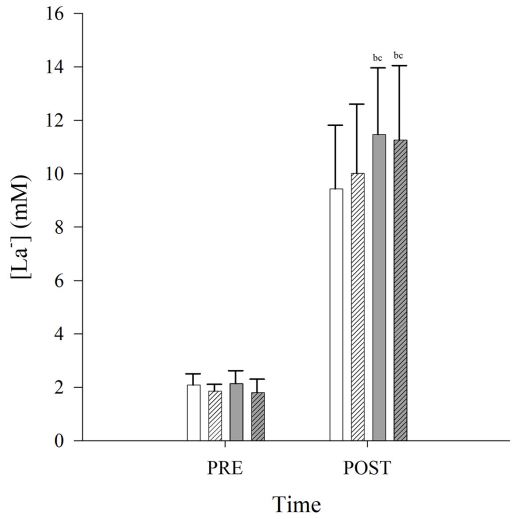

FIGURE 4 | Arterialized capillary lactate concentrations ([La^-]) PRE and POST exercise in CONLD (white), CONLD-BH (white with diagonal lines), FLK (gray) and FLK-BH (gray with diagonal lines). All four POST [La^-] were greater than the PRE [La^-]. [a]Significantly greater than CONLD. [b]Significantly greater than POST CONLD. [c]Significantly greater than POST CONLD-BH.

corresponded with greater post-apneic VO_2 and VCO_2 (Lim et al., 2018). VO_2 was sustained in CONLD-BH compared to CONLD, and in FLK-BH compared to FLK, as a function of the continued pulmonary diffusion during apnea, as is reflected by the immediate increases in $P_{ET}O_2$ post-apnea (**Table 4**). At the level of the muscle, the preserved arterial O_2 content along with the increased muscle deoxygenation facilitated the unchanged VO_2. Although these protocols were designed to replicate the breathing opportunities afforded during regulated compared to free-breathing conditions, the cardiorespiratory responses to facial immersion supine exercise, and the combined arm and leg muscle action specific to swimming (Guyatt et al., 1965; Christie et al., 1990; Leahy et al., 2019) were not studied, due to the inherent difficulties of calculating gas exchange while submerged, and the unavailability of a recumbent ergometer that would interface successfully with our data collection equipment. Additional research is needed to clarify the role of these specific swimming characteristics in these physiological outcomes. Furthermore, our results may not reflect the responses of well-trained competitive swimmer, and as such, future studies should compare the physiological resolution of expert swimmers, compared to non-swimmers, to these breathing restrictions. Finally, only healthy participants were tested; therefore, no comparisons to diseased populations should be made.

Moreover, only male participants were included in this study to best match participant characteristics to the previous study (Lim et al., 2018). Therefore, these observations may not apply to females because of sex differences in body composition, fluctuations in reproductive hormones (estrogen and progesterone) (Arora et al., 1998), and lactate production during exercise (Jurkowski et al., 1981). Also, the sample size (n = 10) of this study adequately powered the statistical analyses used; a larger sample size may be required to generalize these results.

CONCLUSION

The initial and necessary reduction of PO (Δ50%–Δ20%) imposed by the regulated breathing condition demonstrated the severe cardiorespiratory consequences of this regulated breathing protocol compared to the free-breathing paradigm instituted previously by our lab. However, under this reduced PO, mean VO_2 was maintained after the implementation of 5 s apneic periods and 25 s regulated post-BH breathing during supra-threshold exercise. The mechanism for this sustained VO_2 under the apnea condition, with its reduced breathing opportunities, was expounded through an increase in muscle deoxygenation (Δ[HHb]) relative to VO_2 within the constraints of the O_2 conservation or deep diving response (decreased $\Delta[Hb_{tot}]$). This was in contrast with the systematic increases in V_E and unchanged $\Delta[HHb]/VO_2$ observed during free as opposed to regulated breathing conditions, under an otherwise identical apneic protocol compared to this study. From a practical perspective, swimmers competing in the predominantly aerobic front crawl events (400, 800, and 1,500 m) would be advised to increase their minute ventilation by increasing the frequency of breathing to twice per arm cycle as often as is comfortable, increase tidal volumes, or suffer negative performance consequences.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Western University Research Ethics Board for Health Sciences Research Involving Human Participants. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GB and DL conceived, designed the study, collected, and analyzed the data. GB and KG interpreted the results and drafted the manuscript. GB and JM edited the manuscript. All authors contributed to the article and approved the submitted version.

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Extreme Hypoxia Causing Brady-Arrythmias During Apnea in Elite Breath-Hold Divers

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Introduction: The cardiac electrical conduction system is very sensitive to hypoglycemia and hypoxia, and the consequence may be brady-arrythmias. Weddell seals endure brady-arrythmias during their dives when desaturating to 3.2 kPa and elite breath-hold-divers (BHD), who share metabolic and cardiovascular adaptions including bradycardia with diving mammals, endure similar desaturation during maximum apnea. We hypothesized that hypoxia causes brady-arrythmias during maximum apnea in elite BHD. Hence, this study aimed to define the arterial blood glucose (Glu), peripheral saturation (SAT), heart rhythm (HR), and mean arterial blood pressure (MAP) of elite BHD during maximum apneas.

Methods: HR was monitored with Direct-Current-Pads/ECG-lead-II and MAP and Glu from a radial arterial-catheter in nine BHD performing an immersed and head-down maximal static pool apnea after three warm-up apneas. SAT was monitored with a sensor on the neck of the subjects. On a separate day, a 12-lead-ECG-monitored maximum static apnea was repeated dry (n = 6).

Results: During pool apnea of maximum duration (385 ± 70 s), SAT decreased from 99.6 ± 0.5 to 58.5 ± 5.5% (~PaO_2 4.8 ± 1.5 kPa, $P < 0.001$), while Glu increased from 5.8 ± 0.2 to 6.2 ± 0.2 mmol/l ($P = 0.009$). MAP increased from 103 ± 4 to 155 ± 6 mm Hg ($P < 0.005$). HR decreased to 46 ± 10 from 86 ± 14 beats/minute ($P < 0.001$). HR and MAP were unchanged after 3–4 min of apnea. During dry apnea (378 ± 31 s), HR decreased from 55 ± 4 to 40 ± 3 beats/minute ($P = 0.031$). Atrioventricular dissociation and junctional rhythm were observed both during pool and dry apneas.

Conclusion: Our findings contrast with previous studies concluding that Glu decreases during apnea diving. We conclude during maximum apnea in elite BHD that (1) the diving reflex is maximized after 3–4 min, (2) increasing Glu may indicate lactate metabolism in accordance with our previous results, and (3) extreme hypoxia rather than hypoglycemia causes brady-arrythmias in elite BHD similar to diving mammals.

Keywords: junctional rhythm, brady-arrythmia, free-diving, invasive blood pressure, hypoxia induced factor-1 (HIF-1), atrioventricular block, apnea and face immersion, bradycardia

INTRODUCTION

Bradycardia is a well-known consequence of cardiac hypoxia (James et al., 1966; Guilleminault et al., 1983; Kato et al., 1988), but bradycardia is also an underlying oxygen-conserving mechanism in the mammalian diving reflex (Godek and Freeman, 2021). In addition, the mammalian diving reflex includes peripheral vasoconstriction, increased blood pressure, and blood centralized to the brain, lungs, and heart (Foster and Sheel, 2005; Kjeld et al., 2009; Lemaître et al., 2013; **Supplementary Table 1**). The decreasing heart rate during the apnea dives of animals and humans due to the mammalian diving reflex is vagally mediated, and the His bundle conduction system of the heart is very sensitive to hypoxia and hypoglycemia (James et al., 1966; Senges et al., 1980). Hence, hypoxia or hypoglycemia may inhibit normal conduction. Accordingly, during the dives of Weddell seals, where their partial pressure of oxygen (PaO_2) decreases to 3.2 kPa, brady-arrythmias including 2nd degree atrioventricular blockade and junctional rhythm can be observed (Qvist et al., 1986; Davis and Kanatous, 1999; Williams et al., 2015), yet the animals stay conscious. In contrast, blackouts or even death may be the consequence of maximum apneas in elite breath-hold divers (BHD), despite a pronounced diving reflex (Foster and Sheel, 2005; Kjeld et al., 2009; Buzzacott and Denoble, 2018) and also metabolic adaptions to hypoxia and hypercapnia similar to diving mammals (Kjeld et al., 2009, 2018). During maximum apneas of elite BHD, we have previously demonstrated that PET-CT-assessed myocardial blood flow increases markedly (by a factor of 2), whereas both PaO_2 decrease (to 4.3 kPa) and concomitantly circulating lactate levels decrease, indicating lactate metabolism (Kjeld et al., 2021). Lactate metabolism during maximum exercise and apnea can also be demonstrated in high-altitude miners and Sherpas (Ge et al., 1994; Moraga et al., 2019), dwelling at 3,440 to 4,243 m altitudes and having SaO_2 between 88 and 93% (Jansen et al., 2007). However, in contrast to their lowland relatives and diving seals, the Sherpas have no brady-arrythmias during apnea and hypoxia (Busch et al., 2018). Sherpas are constantly exposed to hypoxia, while BHD and seals are exposed to intermittent hypoxia, and both kinds of exposure may lead to increases in the levels of hypoxia-inducible-factor-1-alpha (HIF-1-alpha), a key transcription factor, that regulates cellular adaptions to hypoxia (Li et al., 2009). Skeletal muscle HIF-1-alpha has been found to be cardioprotective in animal models of intermittent hypoxia (Cai et al., 2003). Seal hearts have low levels of HIF-1-alpha whereas the concentration in their skeletal muscles is high (Johnson et al., 2004, 2005). HIF-1 mediates adaptions to hypoxia by downregulating mitochondrial oxygen consumption (Papandreou et al., 2006), and similarly BHD and diving mammals are characterized by low mitochondrial oxygen consumption in their skeletal muscles (Kjeld et al., 2018). HIF-1-alpha do also interrelate with pancreatic beta-cell function: increased levels of HIF-1-alpha improve insulin secretion (Cheng et al., 2010), and according to Dangmann, insulin is theoretically a key factor under hypoxic conditions; it was also concluded that insulin secretion stops during hypoxia (Dangmann, 2015). Sponsiello et al. confirmed this theory in BHD and found a decrease in blood glucose of ∼11.5% with a concomitant increase in insulin of ∼ 55% after 5 dives to 20 m (Sponsiello et al., 2018). Theoretically, the consequence of further repeated dives, for example, in underwater spearfishing, would be fatal hypoglycemia.

In our previous study of elite BHD, we demonstrated both decreased levels of circulating lactate and significantly increased myocardial blood flow during maximum apnea, the latter by a factor of 2 compared to resting conditions (Danad et al., 2014). Hence, maximum apnea in elite BHD can be compared to maximum aerobic exercise. Miller et al. (2002) demonstrated during aerobic exercise and lactate infusion that glucose was stable due to decreased metabolism. Therefore, assuming that the decreasing lactate during maximum apnea is indicative of lactate metabolization (Kjeld et al., 2021), and if glucose is stable in elite BHD during maximum apnea, then hypoxia may be the key factor causing bradycardia, as the His bundle conduction system of the heart is very sensitive to both hypoxia and hypoglycemia (James et al., 1966; Senges et al., 1980).

The aim of the present study was to determine, in elite BHD during maximum apnea, (1) peripheral saturation, (2) blood glucose, (3) cardiac-monitored brady-arrythmias, and (4) invasive measured blood pressure. (5) The resting levels of skeletal muscle HIF-1-alpha in BHD were compared to matched controls.

MATERIALS AND METHODS

Seventeen healthy/non-medicated male non-smoking subjects participated in the study as approved by the Regional Ethics Committee of Copenhagen (H-1-2013-060). All clinical investigations have been conducted according to the principles expressed in the Declaration of Helsinki. Informed consent, written and oral, have been obtained from the participants. Nine subjects were divers (age 43 ± 3 years), and eight judo athletes matched for morphometric variables (age, height, weight, body

TABLE 1 | Subject characteristics.

	Divers	Controls
No. subjects	9 males	8 males
Age (years)	39 ± 10	36 ± 11
Height (cm)	184 ± 6	183 ± 4
Weight (kg)	80.4 ± 6.0	79.9 ± 7.3
Body mass index (kg/m^2)	23.6 ± 1.9	23.6 ± 1.2
Fat mass%	17.7 ± 5.5	15.4 ± 6.2
Fat (kg)	14.2 ± 4.9	12.7 ± 6.0
Maximal oxygen uptake (mL O2/min/kg)	48.6 ± 7.1	47.5 ± 7.1
Hemoglobin (%)	8.9 ± 0.7	8.9 ± 0.8
Static personal best (seconds)	395 ± 48	N/A
Dynamic pool personal best (meters)	171 ± 38	N/A
Dynamic pool no fins personal best (meters)	143 ± 38	N/A

Basic morphometric data. Values are mean ± SD.

mass) and whole-body aerobic capacity (VO_2max, **Table 1**) were chosen for comparison.

All free divers ranked among the national top 10, three of the participating free divers ranked among the World top 10, and one was a 2016 outdoor free-diving World champion, while one reached third place at the same Championship (no limit depth competition), and one was a World record holder.

VO_2 Max and Dual-Energy X-Ray Absorptiometry Scan

Subjects completed a standardized warm-up followed by an incremental cycling test starting at a workload of 150 W and increasing 25 W every minute until voluntary exhaustion. The highest recorded 30 s average oxygen uptake (VO_2) during the test was defined as VO_2max. For recognition of true VO_2max, three of five criteria had to be met: individual perception of exhaustion, respiratory exchange ratio >1.15, plateau of VO_2 curve, heart rate approaching age-predicted maximum, and inability to maintain a pedaling frequency above 70 rpm (**Table 1**).

To describe subjects, an assessment of body composition was determined with a dual-energy X-ray absorptiometry scan (Lunar iDXA; Lunar, Madison, WI, United States) (**Table 1**).

Muscle Biopsies

Subjects were instructed to refrain from exercise and apnea before muscle biopsies were taken. Muscle biopsies were obtained in local anesthesia with lidocaine (5%) from the lateral vastus of the femoral muscle a.m. Bergstroem (Gusba et al., 2008). The biopsies were snap-frozen in liquid N_2 and stored at −80°C. Western blotting was performed as previously described in detail (Krag et al., 2016). Briefly, biopsies were cut on a microtome, the sections were homogenized in sample buffer, and proteins were separated on SDS-page gels and blotted onto a membrane using a Trans-turbo blotter (Bio-Rad, Hercules, CA, United States). The membrane was incubated with antibodies against HIF1-alpha (AF1935, R&D Systems, Minneapolis, MN, United States) and alpha-tubulin (clone 12G10, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, United States), followed by rabbit anti-goat and rabbit anti-mouse horseradish peroxidase-conjugated antibodies. The membrane was developed using Clarity and visualized on a ChemiDoc MP (Bio-Rad). The muscle specimen of one control participant was not sufficient for analyses.

Pool Apneas

The nine elite breath-hold divers were instructed to refrain from caffeine intake and to fast for at least 6 h before the pool apneas. Any strenuous physical activity was discouraged for at least 1 day before the experiment.

A 1.1 mm, 20-gauge catheter was inserted in the radial artery of the non-dominant arm connected to a transducer for continuous flow of saline (3 ml/h; Baxter, Uden, Netherlands) for blood pressure monitoring and for collection of blood glucose. Blood glucose analyses were performed immediately after sampling, using an automated self-calibrating blood gas machine (ABL 725, Radiometer, Copenhagen, Denmark) and evaluated for blood gases as well as described previously (Kjeld et al., 2021).

The BHD performed head immersed maximal static apnea after glossopharyngeal insufflation (GPI) (Seccombe et al., 2006) in a 28°C, 0.8-m-deep indoor pool after a warm-up of three consecutive apneas to maximize the diving response (Kjeld et al., 2009). They were instructed to make a sign with their index finger, just before terminating apnea, so blood glucose sampling could be made just before breathing.

Arterial blood pressures and heart rates were recorded concomitantly using a Lifepack® 20 monitor. To diminish artifacts from water, heart rhythm (HR) was monitored with electrodes for direct current therapy, and hence only one lead was recording (∼ lead II): electrodes were placed at the sternum and under the left arm in an antero-lateral position. All subjects had normal ECG at rest. Peripheral saturation was monitored with a Covidien® saturation sensor placed at the neck of the subjects similarly to avoid artifacts from water, but also to ensure

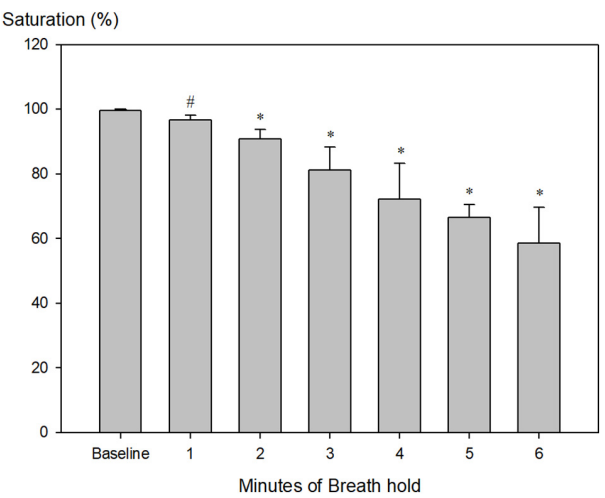

FIGURE 1 | Peripheral measured saturation (sensor placed at neck, $n = 9$) during pool apnea decreased from 99.6 ± 0.5% every minute until termination of breath hold to 58.5 ± 5.5% (#$P = 0.004$, *$P < 0.001$ compared to baseline).

TABLE 2 | Peripheral saturation, blood glucose, heart rate, and mean arterial blood pressure during pool apnea.

	Rest	End BH	After BH
Mean arterial pressure/mmHg	103 ± 11	148 ± 15*	104 ± 31
Heart Rate/beats min^{-1}	86 ± 14	46 ± 10*	64 ± 9*
Peripheral Saturation/%	99.6 ± 0.5	58.5 ± 5.5*	N/A
Blood glucose mmol/l	5.8 ± 0.2	6.2 ± 0.2 #	N/A

*Values are means ± SD; *P < 0.001 vs. rest. #P = 0.009 vs. rest. BH = breath hold.*

valid peripheral saturation, during max apnea and concomitant peripheral vasoconstriction.

Dry Apneas

On a separate day, six of the BHD with the longest self-reported apneas completed a dry apnea after GPI during 12-lead ECG monitoring and after a warm-up of three consecutive apneas to maximize the diving response (Kjeld et al., 2009).

Statistical Analysis

Variables are presented as mean ± standard error of the mean (SEM). Data were analyzed by Sigma-Plot® using one-way repeated-measures ANOVA. The Holm-Sidaks method *post hoc* was used to evaluate differences between the collected data during rest, apnea, and recovery. A *P*-value < 0.05 was considered statistically significant.

RESULTS

At the end of maximum pool apnea (314 ± 64 s), peripheral saturation decreased from 99.6 ± 0.5 to 58.5 ± 5.5% (**Figure 1**; **Table 2**, $P < 0.001$), while blood glucose increased from 5.8 ± 0.2 at rest to 6.2 ± 0.2 mmol/l just before the end of apnea (**Table 2**, $P = 0.009$).

Heart rate decreased from 86 ± 14 beats per minute (bpm) to 46 ± 10 bpm and remained constant after 4 min (**Figure 2**; **Table 2**, $P < 0.001$). Just before the end of apnea, junctional rhythm ($n = 4$), 2nd degree atrioventricular blockade ($n = 1$) and sinus bradycardia ($n = 3$) was observed (**Figures 3A,B** and **Supplementary Datasheet 1**).

Systolic blood pressure increased from 157 ± 7 to a maximum of 239 ± 15 mm Hg after 4 min of apnea (**Figure 4**, $P < 0.001$), whereas diastolic blood pressure increased from 76 ± 3 mmHg to a maximum of 113 ± 5 mm Hg after 3 min of apnea (**Figure 5**, $P < 0.001$).

Mean arterial blood pressure increased from 103 ± 4 to a maximum of 155 ± 6 after 3 min of apnea (**Figure 6**; **Table 2**, $P < 0.001$).

During dry apnea of maximum duration (378 ± 31 s), heart rate decreased from 55 ± 4 to 40 ± 3 bpm ($P = 0.031$). Sinus bradycardia ($n = 3$), 2nd degree atrioventricular dissociation ($n = 1$), and junctional rhythm ($n = 2$) were observed (**Figures 7A,B** and **Supplementary Datasheet 2**). No ST-segment changes were detected.

All BHD remained conscious during both maximum pool and dry static apneas.

Muscle biopsy analyses revealed no differences in HIF-1-alpha between BHD (relative value 0.87 ± 0.09, $n = 9$) and controls (relative value 1.00 ± 0.06, $n = 7$): $P = 0.121$ (**Figure 8**).

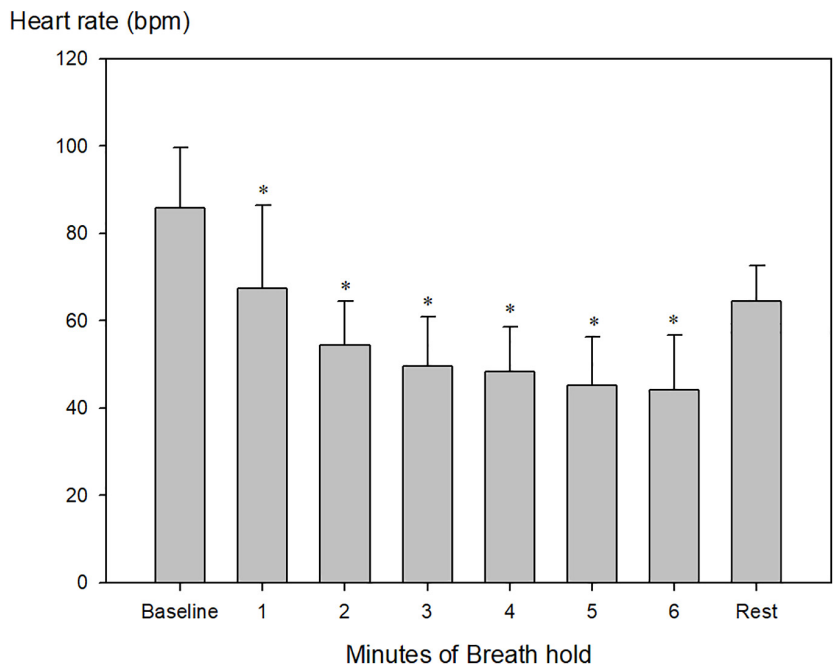

FIGURE 2 | Heart rate during maximum pool apnea. Heart rate decreased from 86 ± 14 beats per minute (bpm) to 46 ± 10 bpm after the first 4 min of apnea compared to baseline and stabilized until termination of breath hold (*$P < 0.001$ compared to baseline, $n = 9$).

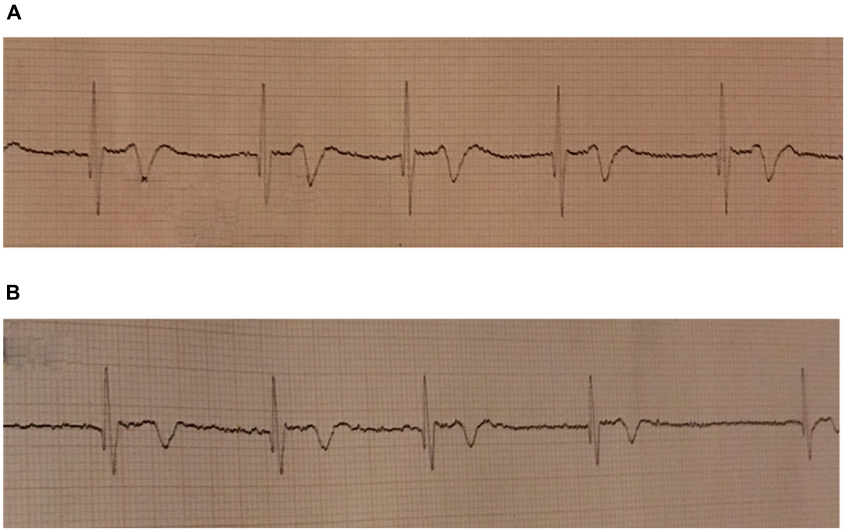

FIGURE 3 | ECG during maximum pool apnea in two subjects. **(A)** Nodal rhythm during maximum dry apnea. Recorded at 25 mm/s. **(B)** Atrioventricular dissociation during maximum pool apnea. Recorded at 25 mm/s.

DISCUSSION

The main and novel findings of our study are:

After a warm-up of three consecutive apneas and at end of maximum pool apnea (314 ± 64 s) after GPI, the elite BHD (1) tolerated peripheral saturation as low as 58.5 ± 5.5% (~PaO_2 4.8 ± 1.5 kPa), (2) had stable or increasing blood glucose, (3) increased in MAP to a maximum after 3 min of apnea without subsequent changing, (4) decreased in heart rate to a minimum of 46 ± 10 bpm and stabilized after 4 min until termination of apnea, (5) junctional rhythm, 2nd degree atrioventricular blockade, and sinus bradycardia were observed concomitantly by 1-lead ECG during maximum pool apnea, and (6) during maximum dry apnea, both junctional rhythm and 2nd atrioventricular dissociations were confirmed with 12-lead ECG. To our knowledge, these combined and detailed hemodynamic observations, and detailed arrythmias in particular have never been demonstrated earlier in elite BHD during static apneas: Although Ferrigno et al. (1997) and Lindholm et al. (2006) reported similar decreases in heart rate and increases in MAP in pressure chamber apnea in two divers and static apnea in competitive divers, respectively, our findings add further

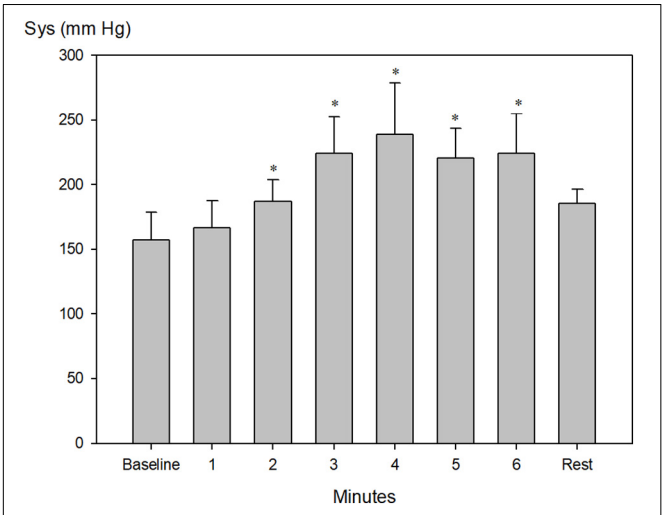

FIGURE 4 | Invasively measured systolic blood pressure (Sys) during pool apnea. Systolic blood pressure increased every minute from 157 ± 7 before apnea (baseline) to a maximum of 239 ± 15 mm Hg after 4 min of apnea (*P < 0.001 compared to baseline, n = 9).

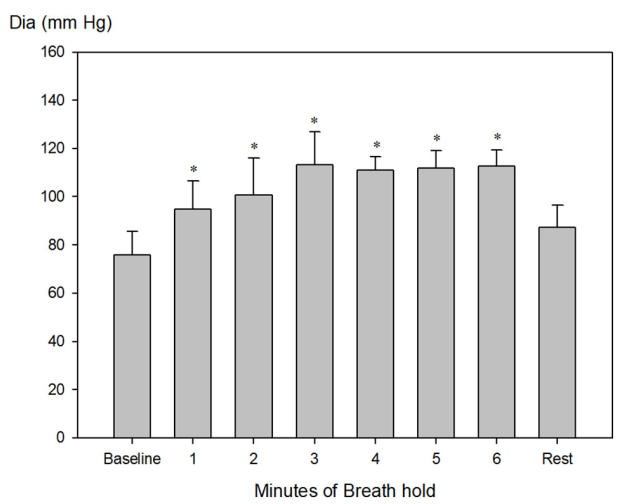

FIGURE 5 | Invasively measured diastolic blood pressure (Dia) during pool apnea. Diastolic blood pressure increased every minute from 76 ± 3 to a maximum of 113 ± 5 mm Hg after 3 min of apnea compared to rest and remained constant hereafter until termination of breath hold (*P < 0.001 compared to baseline, n = 9).

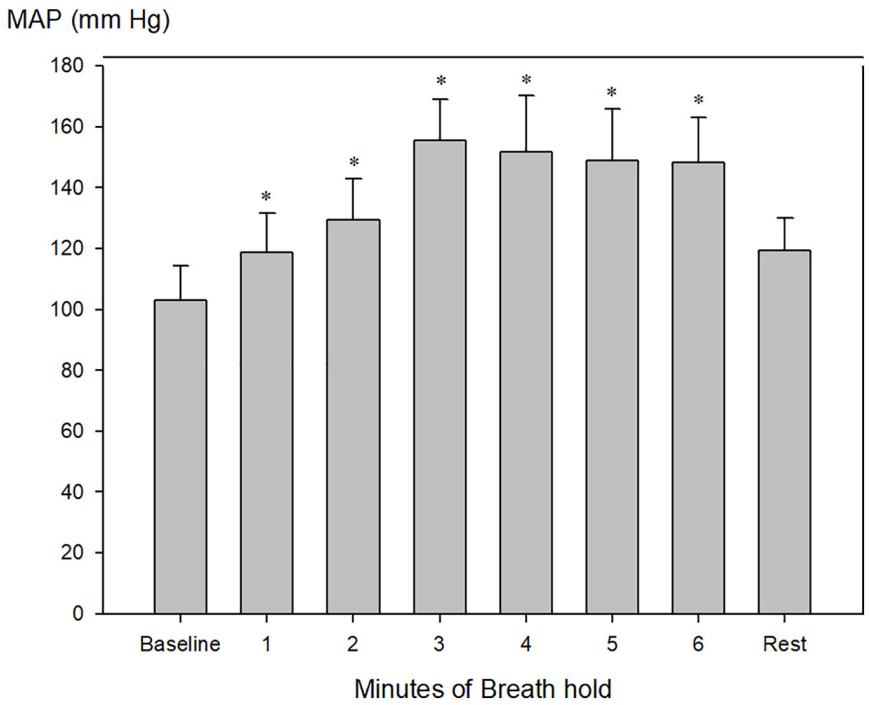

FIGURE 6 | Invasively measured mean arterial blood pressure (MAP) during pool apnea. After 2 min of breath hold mean arterial blood pressure increased every minute compared to rest from 103 ± 4 to a maximum of 155 ± 6 after 3 min of apnea (*$P < 0.001$ compared to baseline, $n = 9$).

information by blood glucose monitoring and continuous ECG recording and analysis. Lemaître et al. reported of junctional rhythm during constant weight 70-m-deep dives (141 s duration, Lemaître et al., 2013), whereas 2nd degree atrioventricular dissociation has not been reported earlier.

Hypoxia as a Cause of Brady-Arrythmias

Multiple factors determine the heart rate and cardiac conduction including sympathetic and parasympathetic activity, but also hydrostatic pressure on myocardial cells, baroreceptors, blood gases, cardiovascular hormones, and pulmonary stretch receptors or pressure changes in lung volume may modify heart rate and cardiac contractility in BHD and diving mammals (Williams et al., 2015). We demonstrated that GPI before apnea is not followed by an initial increase in heart rate, as otherwise expected due to pulmonary stretching in BHD (Heusser et al., 2010; Pinsky, 2018).

Cellular hypoxia along the His bundle, the area of the cardiac conduction system known to be very sensitive to hypoxia, may elicit atrioventricular dissociation (James et al., 1966), and in patients with myocardial infarction during sleep apnea with desaturation below PaO_2 of 85%, sinus arrest and 2nd degree atrioventricular have been demonstrated (Guilleminault et al., 1983; Galatius-Jensen et al., 1994). Sleeping adult seals also experience sleep apnea, but do not increase production of reactive oxygen species (ROS) nor suffer systemic or local oxidative damage in contrast to terrestrial animals with sleep apnea (Vazquez-Medina et al., 2012). Adult seals, compared to pups and juvenile seals, also have an increased relative capacity for mitochondrial respiration (Chicco et al., 2014). Adult diving mammals also have resistance to release of ROS during dives to tolerate ischemia (Vazquez-Medina et al., 2006, 2012). These findings supports that the ability to tolerate hypoxia is an adaption. Similarly, BHD who can endure apnea for more than 4 min have a smaller increase in ischemia-modified albumin (IMA), a marker of the release of ROS and a measure of resistance to hypoxemia, compared to BHD who endure less than 4 min of apnea (Joulia et al., 2015). However, both young and adult seals have been demonstrated to have atrioventricular dissociation during dives (Murdaugh et al., 1961; Williams et al., 2015). In line with this, the hearts of adult seals are also sensitive to localized ischemia (Elsner et al., 1985). Our study of elite BHD during apnea of at least 4 min and a decrease in PaO_2 of ∼ 4.3 kPa demonstrated 2nd degree atrioventricular block, junctional rhythm, and sinus bradycardia similar to diving (juvenile as well as adult) mammals (Williams et al., 2015), and without any association between apnea duration. The consequence of the brady-arrythmias endured by the subjects in our study would, in the general non-diving population if followed by syncope, be treatment with a pacemaker (Brignole et al., 2013).

Cardiac Metabolism During Maximum Apnea

In contrast to BHD and diving mammals, resistance toward brady-arrythmias has been demonstrated in high-altitude Sherpas during high-altitude apnea, whereas a comparable group of lowlanders had junctional rhythm, 3rd degree atrioventricular

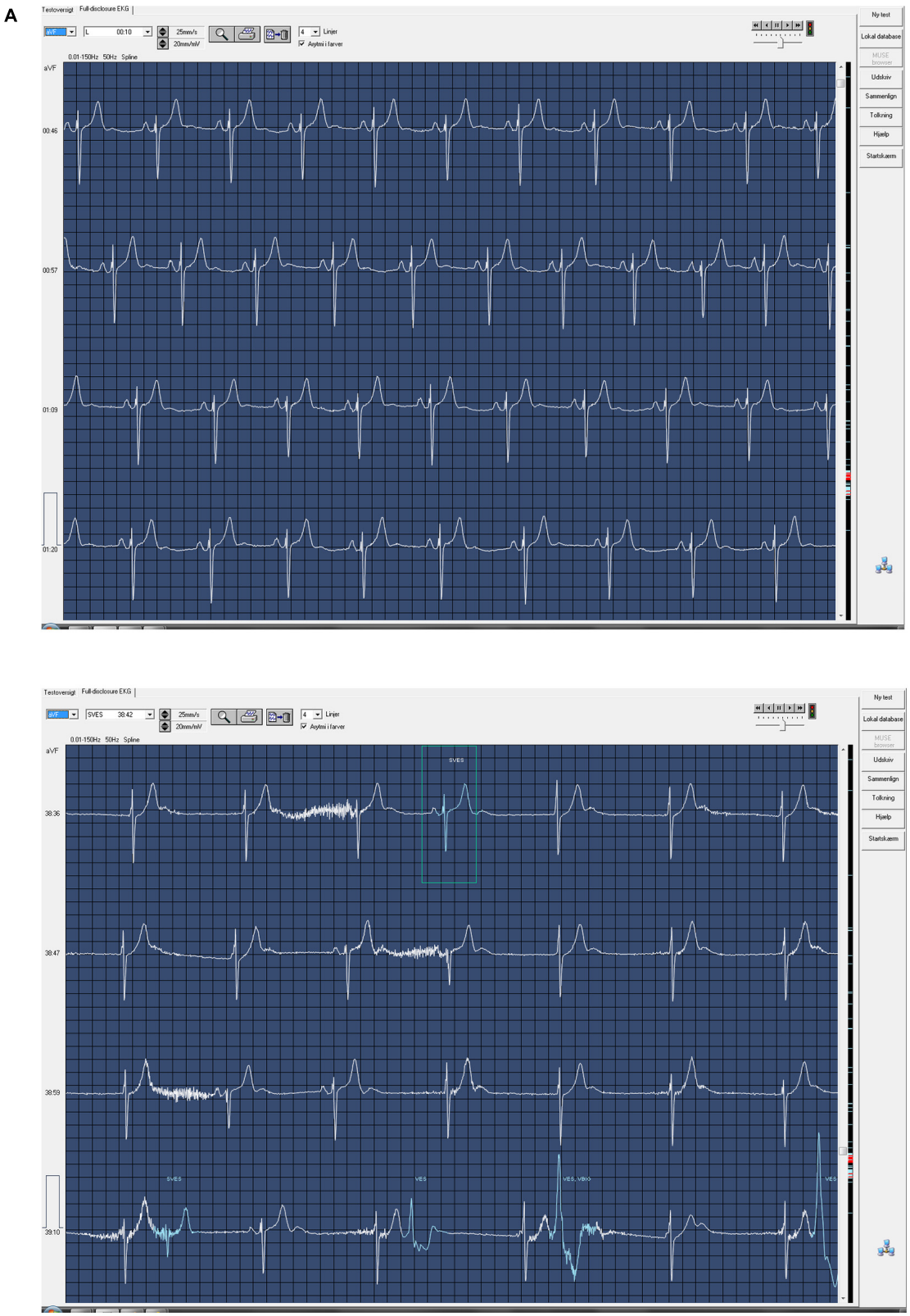

FIGURE 7 | ECG at rest and ECG during maximum dry apnea in two subjects. **(A)** Sinus rhythm at rest (top) and nodal rhythm during maximum dry apnea (bottom). Recorded at 25 mm/s. Continues on next page.

(Continued)

B

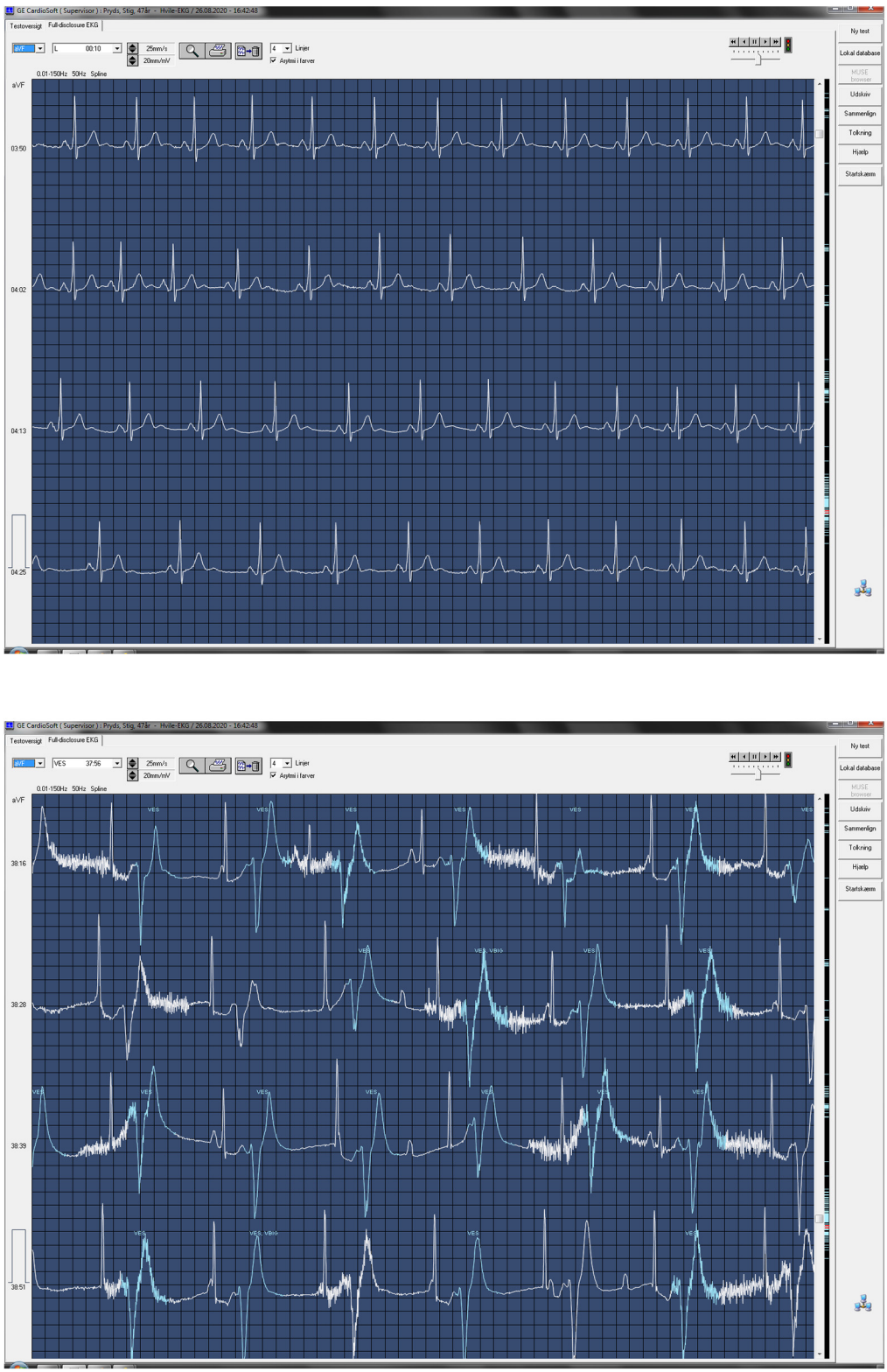

FIGURE 7 | (B) Sinus rhythm at rest (top) and second-degree atrioventricular dissociation during maximum dry apnea (bottom). Recorded at 25 mm/s.

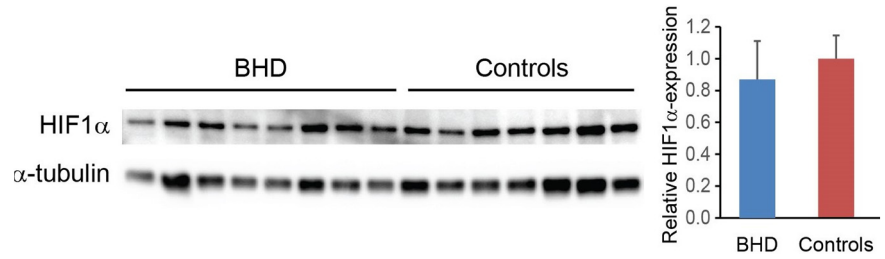

FIGURE 8 | Hypoxia-inducible-factor-1-alpha (HIF1α) expression normalized to α-tubulin shows no difference between breath-hold divers (BHD, $n = 8$) and controls subjects ($n = 6$).

block, and sinus pause (Busch et al., 2018). These Sherpas demonstrate decreasing lactate levels during maximum exercise, indicative of lactate metabolization (Ge et al., 1994). Similarly, we have demonstrated that elite BHD decrease circulating lactate levels during maximum apnea (Kjeld et al., 2021), indicative of a similar cardiac metabolism as found in adult harbor seals (Phoca Vitulina), who possess the highest cardiac lactate dehydrogenase activity, compared to terrestrial animals (Fuson et al., 2003). In the present study, we demonstrate stable blood glucose during maximum apnea in the same population of elite BHD (+1) as described in our previous study (Kjeld et al., 2021). The stable blood glucose in our study during maximum apnea with PaO_2 of ∼ 4.8 kPa may underline that lactate is metabolized, when compared to the similar findings of Miller et al. (2002) demonstrating lactate metabolization during exercise, following decreasing blood glucose metabolization: the pancreatic insulin producing beta-cells are sensitive to moderate hypoxia, causing decreasing insulin production (Sato et al., 2014), and because pancreatic blood supply (from the splenic artery) has been demonstrated to remain stable during apnea (Bakovíc et al., 2003), hypoxia seems to be the main factor affecting the stalled pancreatic insulin production, and hence increasing or more likely stabilizing blood glucose (may be secondarily to peripheral vasoconstriction) during apnea as in our study. Our results contrast with Sponsiello et al. (2018) but are in accordance with Ghiani et al. (2016), who found stable or increasing blood glucose after 30-m-deep apnea diving. Hence, despite the obvious similarities in Sherpa lactate metabolism compared to both seals and BHD, only seals and BHD have atrioventricular dissociation and junctional rhythm during apnea (Murdaugh et al., 1961; Williams et al., 2015). We therefore suggest that metabolism is not a factor causing brady-arrythmias in BHD and diving mammals during apnea, and that the Sherpa resistance towards brady-arrythmia during apnea relies on an adaption to chronic exposure toward hypoxia, whereas the intermittent exposure toward extreme hypoxia of BHD and diving mammals does not protect against brady-arrythmias.

Hypoxia-Inducible-Factor-1-Alpha

Intermittent hypoxia can upregulate HIF-1 and in turn iNOS gene expression in cardiomyocytes, and since skeletal muscle HIF-1-alpha has been found to be cardioprotective in animal models of intermittent hypoxia (Cai et al., 2003), we assumed that skeletal muscle HIF-1-alpha would be higher in BHD as compared to controls. Our study revealed no differences in skeletal muscle HIF-1-alpha between BHD and controls, and hence remote cardioprotection from skeletal muscle HIF-1-alpha could not be demonstrated.

Blood Pressure During Hypoxia and Hypercapnia

Tissue oxygen delivery from Hb depends on 2,3 diphosphoglycerate, pH, and CO_2 (Mairbaurl and Weber, 2012). Partial arterial CO_2 ($PaCO_2$) increases during apnea (Kjeld et al., 2009), and in our latest study of elite BHD increased to 6.7 kPa, in which we also demonstrated that maximum apnea increases myocardial blood flow markedly, whereas cardiac output decreases (Kjeld et al., 2021). In the present study, the blood pressure during maximum apnea in BHD increased to levels comparable to maximum exercise (Caselli et al., 2016): DBP, SYS, and MAP reached a maximum and remained constant after 3 min, and this may depict maximum vasoconstriction as part of the diving response (Ferrigno et al., 1997; Foster and Sheel, 2005). As $PaCO_2$ increases and cardiac output decreases during maximum apnea in BHD (Kjeld et al., 2009, 2021), the consequence could be decreases in MAP. However, MAP remained constant in the present study, and we suggest that despite increases in $PaCO_2$, which also may vasodilate peripherally, the vasoconstriction caused by the diving reflex of the elite BHD overrules the potential peripheral vasodilatation (caused by increases in $PaCO_2$) and decreases in cardiac output, and the consequence is a constant MAP. Of note, HR decreased to a constant level after 4 min of apnea. Hence, we conclude that in elite BHD during maximum apnea and after GPI, the diving response is maximized after 3–4 min and remains constant during apnea.

Summary

In summary, we found that elite BHD similar to diving mammals have brady-arrythmias during maximum apnea enduring hypoxia of PaO_2 of 4.8 kPa, but stable or increasing blood sugar. Our results contrast with previous studies, and the stable blood glucose in this study (1) underlines the possibility of lactate metabolization during maximum apnea in elite BHD as we have sought to determine previously and (2) indicates

that hypoxia rather than hypoglycemia is a factor causing brady-arrythmias during maximum apnea in elite BHD. Furthermore, we conclude that the diving reflex is maximized after 3–4 min of apnea, and extreme hypoxia is the key factor causing brady-arrythmias in elite BHD similar to diving mammals.

Perspectives

A high reliance on cardiac lactate metabolism similar to diving mammals is also part of the high-altitude adaption of the Himalayan plateau Pika (*Ochotona curzoniae*) (Li et al., 2015). The Pika, as compared to mice at sea level, also have higher levels of hypoxia-inducible-factor-1-alpha (HIF-1-alpha). In contrast, seal hearts have low levels of HIF-1-alpha (Johnson et al., 2004, 2005), and since the hearts of seals are also sensitive to localized ischemia (Elsner et al., 1985), it may be the low levels of cardiac HIF-1-alpha that explain why seals have brady-arrythmias during apnea, whereas high-altitude Sherpas, genetically adapted to high levels of HIF-1-alpha do not (Suzuki et al., 2003): HIF-1 has been demonstrated to mediate adaptions to hypoxia by downregulating mitochondrial oxygen consumption (Papandreou et al., 2006). Similarly, BHD and diving mammals have been demonstrated to have low mitochondrial oxygen consumption in their skeletal muscle (Kjeld et al., 2018). We suggest that BHD may have low levels of cardiac HIF-1-alpha similar to adult seals, also explaining the similar brady-arrythmias during apnea and hypoxia.

Future studies may reveal the content of cardiac HIF-1-alpha in BHD. Furthermore, it is speculated that the understanding of these mechanisms may help in developing new treatments of severe clinical conditions caused by extreme hypoxia, e.g., cardiac surgery, organ transplantation, and in the post-resuscitation care setting. Elite sprint athletes, fighter pilots, and mountain climbers may benefit from apnea training.

DATA AVAILABILITY STATEMENT

Data collected in this study are all saved encrypted at hospital servers.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Committee of Copenhagen, Denmark. The participants provided their oral and written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TKj conceived the study and was in charge of the overall direction and planning of the presented ideas and developed the hypothesis. EH, BZ, LG, TKr, JV, and HA carried out the experiments. TKj, EH, AI, KL, TKr, BZ, LG, and HA contributed to sample preparation. All authors provided feedback, assisted in the analysis, critically revised the manuscript, approved the final revised version, and contributed to the interpretation and analysis of the results according to their specific specialized areas.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.712573/full#supplementary-material

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Conflict of Interest: TKj was voluntary (unpaid) board member of the Danish National Diving Federation.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The Human Dive Reflex During Consecutive Apnoeas in Dry and Immersive Environments: Magnitude and Synchronicity

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Introduction: The human dive reflex (HDR), an O_2 conserving reflex, is characterised by an interplay of central parasympathetic and peripheral sympathetic reactions, which are presumed to operate independently of each other. The HDR is fully activated during apnoea with facial immersion in water and complete immersion in water is thought to increase the magnitude of HDR during consecutive apnoeas. A comparison of HDR activity between consecutive apnoeas in full-body immersion with consecutive apnoeas in dry conditions has not been fully explored. Also, the interplay between parasympathetic and sympathetic reactions involved in the HDR has not been thoroughly analysed.

Methods: 11 human volunteers performed 3 consecutive 60 s apnoeas with facial immersion in dry conditions (FIDC) and 3 consecutive apnoeas with facial immersion in full immersion (FIFI). Heart rate (HR), R-R interval (RRI), finger pulse amplitude (FPA), splenic width (SW) and SpO_2 were all measured before, during and after apnoeas. A one-way ANOVA using Dunn's *post hoc* test was performed to assess HDR activity, and a Pearson's correlation test was performed to assess HDR synchronisation between physiological parameters during both conditions.

Results: Although HDR activity was not significantly different between both conditions, HR and RRI showed progressively greater changes during FIFI compared with FIDC, while SW and FPA changes were relatively equivalent. During FIDC, significant correlations were found between SW & SpO_2 and FPA & SpO_2. During FIFI, significant correlations were found between RRI & FPA, SW & FPA, HR & SpO_2 and FPA & SpO_2.

Discussion: While there was no significant difference found between HDR activity during FIDC and FIFI, consecutive apnoeas during FIFI triggered a greater magnitude of cardiac activity. Furthermore, significant correlations between RRI and SW with FPA indicate a crosstalk between parasympathetic tone with splenic contraction and increased peripheral sympathetic outflow during FIFI compared to FIDC. In conclusion, HDR activity during

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consecutive apnoeas does not differ between FIDC and FIFI. There appears to be however a greater level of synchronicity during apnoeas in FIFI compared to FIDC and that this is most likely due to the physiological effects of immersion, which could induce neural recruitment and increased cross talk of HDR pathways.

Keywords: human dive reflex, consecutive apneas, cardiovascular regulation, cardiovascular synchronisation, human physiology

INTRODUCTION

The human dive reflex (HDR), is an innate defensive reflex, which preserves O_2 supply to critical organ systems, such as the brain and heart, during periods of apnoea (Foster and Sheel, 2005). The HDR is composed of 3 basic reflex arcs: sympathetic vasoconstriction, parasympathetic bradycardia and splenic contraction (Panneton and Gan, 2020). All these mechanisms ensure sufficient oxygenation to the brain during apnoea *via* the centralisation of blood volume, maintaining cardiac output and increasing O_2 carrying capacity through increased serum haematocrit concentration (Alboni et al., 2011). The classic response of bradycardia also acts as an O_2 conserving mechanism for the heart, particularly the highly O_2 sensitive myocardial tissue (Marabotti et al., 2013), which ensures continued cardiac function while diving, while minimising O_2 consumption in the periphery, such as muscle tissue.

The HDR is primarily triggered upon sub-maximal inspiration and subsequent facial immersion in water which activate distinct neural network pathways (Panneton, 2013). Upon initiation of apnoea, increased peripheral vasomotor activity, as well as cardio-inhibitory reflexes are initiated in the nucleus tractus solitarius (NTS; Gooden, 1994). Facial immersion in water triggers an increase in vagal activity (Hayashi et al., 1997), which is also controlled from the NTS. The cardio-inhibitory signals received by the NTS are then transmitted to the rostral ventrolateral nucleus (RVL), while activation signals are then transmitted to the nucleus ambiguus, which then the efferent signals are relayed to their respective target systems (Fitz-Clarke, 2018). The simultaneous inhibition/activation of physiological responses, which must be orchestrated properly for the HDR to be effective during periods of apnoea.

Upon activation of the HDR axis, heart rate (HR) decreases, thereby increasing R-R interval (RRI), which are primarily vagally controlled (Foster and Sheel, 2005). Peripherally, increases in peripheral vascular resistance (PVR) in selected arterioles occur (Foster and Sheel, 2005). Splenic contraction also occurs, known also as the haematological sequence of the HDR, which is triggered *via* alpha-adrenergic pathways which stimulate the sympathetic fibres surrounding the splenic capsule (Stewart and McKenzie, 2002). Splenic contraction is theorised to increase haematocrit by 4% in times of physiological stress, which would increase O_2 supply (Stewart and McKenzie, 2002).

Given the common initial neural pathways involved in the HDR (NTS), these aforementioned factors should synchronise, to fully maximise the reflex. Given the inactivation/activation of sympathetic/parasympathetic pathways, there should occur a significant correlation between cardiac, vascular and haematological factors involved in the HDR. Previous studies examining the HDR, appear not to have examined the correlation/synchronisation of these multiple factors during dry or immersive apnoeas (Baković et al., 2003; Schagatay et al., 2004; Baranova et al., 2017; Elia et al., 2020).

The magnitude of the HDR is also dependent upon environmental influences, namely facial immersion in dry or submerged conditions, as well as ambient air and water temperature (Bosco et al., 2018). Complete immersion in water, leads to a centralisation of blood volume due to hydrostatic pressure, even before HDR has been activated (Schipke, 2001; Bosco et al., 2018). Triggering the HDR in immersive environments has the potential to maximise the physiological effect, compared with HDR in dry environments, due to potential neural recruitment, and the physiological priming effects of hydrostatic forces. This is a logical evolutionary advantage, as HDR in immersive environments would allow for deeper and longer dives.

Serial apnoeas lead to consecutive triggering of the HDR, and presumably, the HDR would become progressively stronger with each apnoea cycle. However, previous studies have found that cardiovascular HDR responses do not become progressively stronger during serial apnoeas, whereas splenic contraction does become progressively stronger (Schagatay et al., 2004). To our knowledge, no study has compared cardiovascular and splenic changes during consecutive apnoeas during facial immersion in dry and full-body immersive environments to assess if the magnitude of HDR progressively increases.

Given that the HDR is influenced by environmental conditions, such as the HDR being significantly stronger during apnoea with facial immersion compared with apnoea in dry environments (Hurwitz and Furedy, 1986), we hypothesised that the physiological factors of HDR would be fully activated in full-body immersion apnoeas compared to facial immersion apnoeas in dry environments. Also, these factors should become progressively stronger during consecutive apnoeas in full-body immersion. We further hypothesised that the factors involved in the HDR would show a higher level of synchronisation during immersive apnoeas compared with dry apnoeas, due to the physiological effects of hydrostatic forces augmenting the HDR. The overarching goal of this study was to determine if the magnitude of the factors involved in the HDR during serial apnoeas would differ between dry and immersive environments, as well as determining if the physiological parameters involved in the HDR are synchronised or operate independently during serial apnoeas in both conditions, among non-apnoea trained participants. The results will provide more insights concerning environmental influence and physiological cross talk for future HDR physiological research.

MATERIALS AND METHODS

The study was conducted at the Institute of Physiology, Center for Space Medicine and Extreme Environments, Charité, Universitätsmedizin, Berlin, Germany in the summer of 2015 and was approved by the Charitè ethics committee (EA2/024/15). For this study, 11 participants were recruited. No participants were experienced divers, nor had any participant undergone any apnoea training prior to this study. Participants were asked to provide an estimation of their daily activity level on a scale of 1–10 upon recruitment (1–3 sedentary, 4–5 slightly active, 6–7 moderately active, 8–9 active and 10 very active).

The study was divided into 2 parts: facial immersion in dry conditions (FIDC) and facial immersion in full immersion (FIFI). Both parts were performed in the immersion study room, which contained an 2 × 1 × 1 m immersion tank filled with thermoneutral water. Each participant wore a neoprene short sleeve 3 mm dive-suit during both parts. The arms and legs were exposed for each participant. Also, a square-sized hole was made in the wetsuit, which left the entire left flank exposed on order to minimise interference during splenic measurements. Cardiovascular monitoring equipment consisted of a chest strap with a mobile heart rate monitor capable of measuring RRI (Polar S810, Polar Electro Oy, Kempele, Finland), pulse oximeter worn on the left earlobe and a pulse amplitude analyser worn on the left index finger using piezoelectric material. The Piezoelectric sensor is a polyvinyl difluoride-based system, which reconstructs a pulse wave form. The signal is constructed from converted pressure signals measured from the left index finger arterioles, into a change of strain-dependent resistance which produces a voltage signal in millivolts, observed as a pulse wave (i.e. the dynamic mechanical pressure derived from the pulse wave exerts changes in electrical conductance in the sensor; Wang et al., 2020). This signal was used as a surrogate PVR marker, which was not calibrated prior to testing and will be referred to as finger pulse amplitude (FPA).

HR and RRI from the Polar unit are reported as beats/min and milliseconds. FPA is reported as mV. Splenic measurements were recorded using a GE portable sonography device (GE Logiq e Ultrasound, GE Medical Systems Inc.) and are reported as centimetres. HR, RRI and PVR were recorded *via* Bluetooth using the Heally System (Spacebit GmBH, Wiesbaden, Germany) with Healthlab explorer (Spacebit GmBH, Wiesbaden, Germany) being used to analyse and record data.

For both parts of the study protocol, FIDC and FIFI, 3 consecutive apnoeas were undertaken, in which the participant was requested to hold their breath for as long as they could, for maximal 60 s. Prior to each apnoea, the participant exhaled completely, inhaled to sub-maximal inspiration and then immersed their head in water. A bucket of thermoneutral water was used for FIDC apnoea, while each subject immersed their face in the thermoneutral water in the immersion tank for FIFI. A nose clip was used during the entire study. Prior to apnoeas, a 5-min baseline measurement was performed, as well as a 5-min post apnoea measurement. Between FIDC and FIFI, each participant rested for 2 h. Water temperature in both conditions was kept at thermoneutral temperatures (35°C).

During the first part of the study (FIDC), the participant sat upright, and for each apnoea, the participant lowered his/her head into a water filled basin. Between the 3 consecutive apnoeas, participants performed only one exhalation and one inhalation as preparation for the next apnoea. For the second part of the study (FIFI), each participant immersed themselves up to the neck in water, while sitting in a water-proof chair.

Cardiovascular parameters were monitored continuously during the study. Splenic measurements were performed every 60 s during the pre/post apnoea phase and every 10 s during apnoea. To perform splenic measurements in such short consecutive time slots, our study used measurement of splenic width (SW) as an indicator of splenic activity. The finger and ear sensors were not immersed during both parts and for all apnoeas. The finger of the subject was either resting on a table during FIDC and resting on the side of the immersion tank during FIFI. The sonographic probe was covered in a plastic cover during both measurements, while sonographic gel was used for FIDC measurements. Splenic measurements during FIFI did not require the use of sonographic gel. The researcher performing the splenic measurements was not in the immersion tank with the participant.

For statistical analysis, the median and range for all phases are reported. During apnoea, data from the last 5 s of apnoea were used to reflect maximal HDR effect.

For multiple phase comparisons, a one-way ANOVA was performed, with an emphasis on comparing all values from baseline. Due to the small sample size ($n = 11$) and non-parametric data, a Kruskal-Wallis test was performed with Dunn's *post hoc* test. To test for synchronicity of HDR factors, a correlation matrix was created for all parameters during dry and immersive conditions, using Pearson's correlation test. Statistics were performed using JASP software (JASP Team, Version 0.14.1), with a significance set to $p < 0.05$. Values are reported as median with min-max ranges due to the small sample size and non-normality of data. For correlation analysis, the R^2 value is reported. Graphics were created with Data Graph software (Visual Data Tools, Inc., Version 4.6).

RESULTS

All 11 participants (6 female, 5 male) completed the study protocol without interruption. Demographic information and activity level is presented in **Table 1**. Aside from height, no significant differences were found between females and males. Females reported a median higher daily activity level than the males of the cohort, however, this was not statistically different.

Mean breath-hold time (BHT) for the 3 consecutive apnoeas during FIDC was 45 s ± 5.1, 47 s ± 4.3 and 51 ± 3.4. Median BHT for the 3 consecutive apnoeas during FIFI was 52 s ±4.0, 53 s ±3.3, 54 s ±3.3. BHT between FIDC and FIFI revealed no differences. **Figure 1** shows HDR parameter phase-based activity during the study as box plots.

TABLE 1 | Cohort demographic variables.

Baseline factor	All	Female/Male
Age	24 (21–42)	22 (21–26)/27 (23–42)
Height	178 (163–186)	173 (163–179)/180 (175–186)*
Weight	67 (55–92)	61 (55–70)/76 (58–92)
BMI	21.5 (18.9–26.6)	21.3 (19–24.2)/23.3 (19–26.6)
BSA	1.81 (1.58–2.18)	1.74 (1.58–1.85)/1.95 (1.68–2.18)
Activity level	6 (2–10)	7 (4–10)/4 (2–7)

**Significantly greater.*

HR showed significant decreases from baseline in 2 out of 3 apnoeas during FIDC and during all 3 apnoeas throughout FIFI, with the HR progressively decreasing during FIFI apnoeas. RRI showed significant increases in all 3 apnoeas during both conditions, with RRI during FIFI becoming progressively longer. SW significantly decreased in all 3 apnoeas in FIDC and 2 out of 3 apnoeas in FIFI. FPA amplitude increased significantly during all apnoeas, while also being significantly elevated during immersion prior to apnoea. SpO_2 was significantly decreased in all apnoeas during both conditions. Water immersion did not appear to interfere with signal quality from the HR/RRI monitor. The finger and ear sensors were kept dry during the entire study.

Table 2 displays pairwise correlations between HDR parameters for FIDC and FIFI. The only significant correlations found during FIDC were SW/SpO_2 and FPA/SpO_2. During FIFI, significant correlations were found between HR/SpO_2, RRI/FPA, RRI/SpO_2, SW/FPA and FPA/SpO_2.

DISCUSSION

This study demonstrated that during consecutive apnoeas, the measured physiological parameters involved in HDR exhibited equivalent activity during consecutive apnoeas in FIDC and FIFI. Although no significant differences were found between the two conditions, consecutive apnoeas during FIFI triggered a progressive decrease in HR during all 3 apnoeas with a progressively increasing RRI. FPA was significantly increased equally during both conditions. During FIDC, SW significantly decreased in all 3 apnoeas, whereas this was only present in the last 2 apnoeas during FIFI. Therefore, the components involved in the HDR were activated relatively equally during consecutive apnoeas in both conditions, however, the magnitude of the parasympathetic response seems to be a greater during immersive rather than dry conditions. The synchronisation of the HDR factors exhibited unique significant correlations depending on the environmental condition, with 5 pairwise correlations being observed in FIFI compared to 2 during FIDC. During FIDC, SpO_2 was correlated with SW and FPA, suggesting that the vascular and haematological components of the HDR are at least responding in some part to chemoreflexive stimuli (falling SpO_2). During FIFI, correlations were found between RRI and SW with FPA, which suggest that immersive apnoeas can trigger a synchronisation of parasympathetic tone with peripherally mediated sympathetic tone, which were not present in FIDC. Furthermore, the chemoreflexive correlation between SW was not present in FIFI, rather, SpO_2 was correlated with HR and FPA. The differences in HDR synchronisation are most likely due to the hydrostatic effects of water immersion, which could play a role in heightened neural recruitment.

MECHANISMS OF HDR: NON-IMMERSIVE VS. IMMERSIVE CONDITIONS

Significant changes in HR, RRI, FPA and SW were observed in both conditions, with a tendency for the parasympathetic mechanisms (HR and RRI) during FIFI to show a greater magnitude than in FIDC. This is best explained by the environmental conditions, and not by the actual apnoeas, as the apnoeas were identical in technique and total time during both conditions. Immersion in water triggers an increased intrathoracic volume, and central venous pressure *via* centralised blood shift, leading to an increased cardiac output and stroke volume (Risch et al., 1978). The displacement of this blood volume increases cardiac volume by about 180 ml, which contributes to an increase in atrial volume (Lange et al., 1974). RRI increases in water immersion due to this centralisation of volume (Schipke, 2001), while the hydrostatic pressure leads to venoconstriction, leading to increases in mean arterial pressure and pulse pressure (Pendergast et al., 2015). This physiological change is the equivalent of an autotransfusion, which may negate the need for splenic contraction initially (Pendergast et al., 2015). Due to the physiological effects of water immersion, (centralisation of blood volume), splenic contraction would not necessarily need to occur in the first FIFI apnoea, rather splenic contraction occurs during subsequent apnoeas in water. Other working groups have observed significant splenic contraction during repeated apnoeas (Baković et al., 2003) and that splenic emptying occurs because of repeated apnoeas (Elia et al., 2020). Serial apnoeas in facial immersion alone can elicit an increase in haematocrit, which is attributed to splenic emptying (Stewart and McKenzie, 2002). The observation of no noticeable differences in SW reduction between FIDC and FIFI would suggest that splenic contraction is not necessarily dependent on full water immersion per say, rather due to the triggering of HDR *via* facial immersion alone. Also, splenic contraction in humans during apnoea is triggered by alpha-adrenergic catecholamine stimulation, rather than splenic nerve stimulation (Stewart and McKenzie, 2002). During repeated apnoeas, splenic volume among non-trained breath-hold divers progressively decreased, while all other HDR cardiovascular factors did not show progressive changes, suggesting that these progressive splenic volume decrease enable longer breath-holding for subsequent apnoeas (Baković et al., 2003). Elia et al. recently published their findings showing that during consecutive apnoeas in total body immersion,

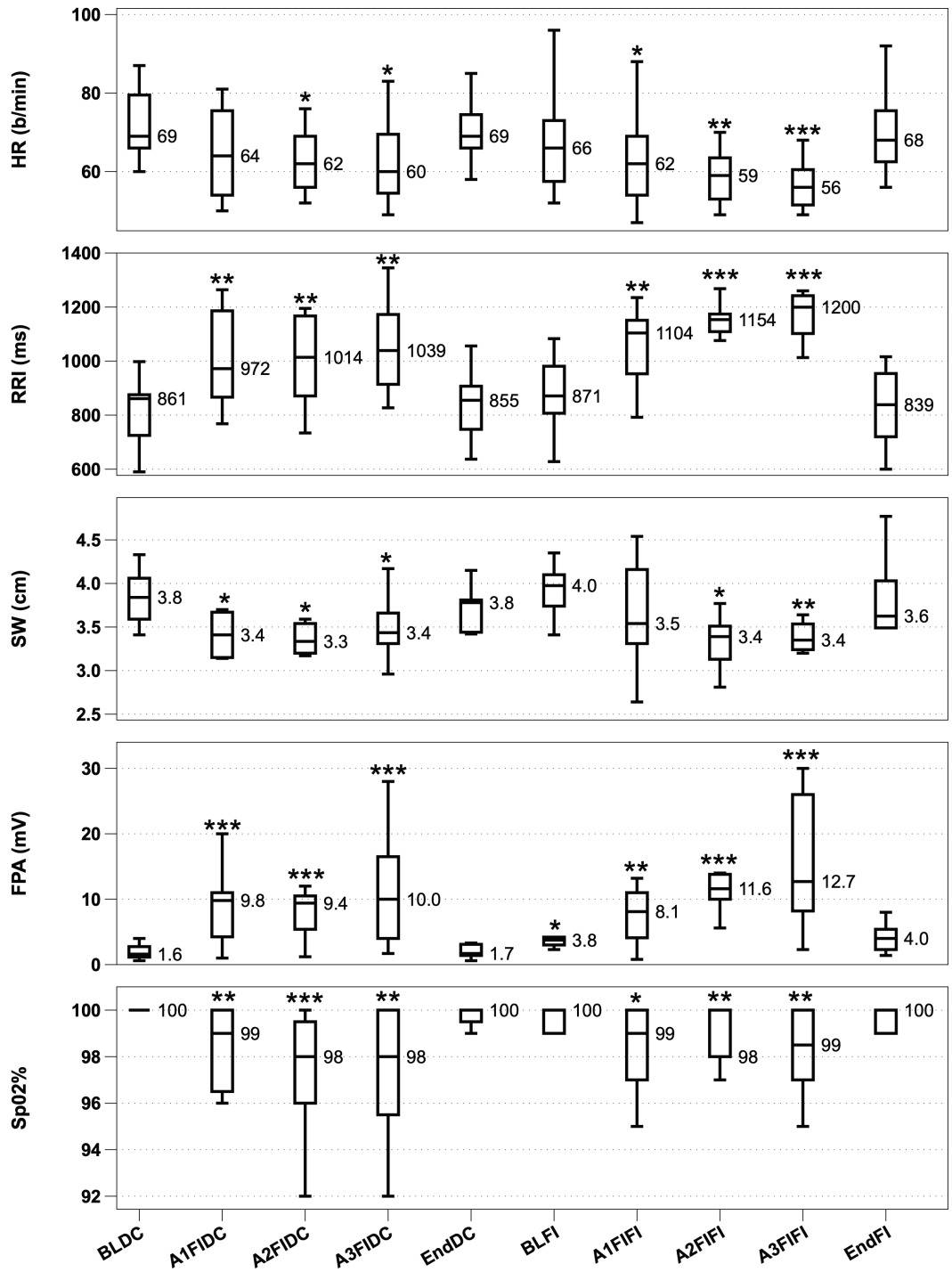

FIGURE 1 | HDR activity from top to bottom with significance denoted. From top to bottom, Heart rate (HR; **A)**, R-R Interval (RRI; **B)**, Splenic width (SW; **C)**, Finger Pulse Amplitude (FPA; **D)** and O_2 saturation (SpO_2; **E)**. Boxplots display interquartile range with a median value reported. *$p<0.05$, **$p<0.01$ and ***$p<0.001$. BLDC (Baseline dry condition), A1-A3 FIDC (Apnoeas 1–3 facial immersion dry condition), EndDC (end of apnoea sequence dry condition), BLFI (Baseline full immersion), A1-A3 FIFI (Apnoeas 1–3 full immersion, facial immersion) and EndFI (End of Full immersion).

non-trained breath-hold divers exhibited non-significant decreases in HR, whereas splenic volume did significantly decrease in 3 out of 5 apnoeas. They concluded that the reduction in splenic volume is due to hypoxic stress, and therefore, the spleen is triggered by chemoreflexive, rather than baroreflexive mechanisms (Elia et al., 2020). The resulting splenic contraction after serial apnoeas does appear to significantly increase plasma erythrocyte count in trained and

TABLE 2 | Pairwise correlations (*R* and *p* values) between HDR parameters during FIDC (right) and FIFI (left).

HDR pair	Pearson's *R* FIDC	*p* value FIDC	Pearson's *R* FIFI	*p* value FIFI
HR/SW	0.05	0.72	−0.06	0.68
HR/FPA	0.05	0.7	−0.01	0.94
HR/SpO_2	−0.04	0.75	0.3	0.03*
RRI/SW	−0.04	0.81	−0.13	0.39
RRI/FPA	0.08	0.57	0.33	0.02*
RRI/SpO_2	−0.02	0.86	−0.48	<0.001***
SW/FPA	−0.03	0.84	−0.39	<0.01**
SW/SpO_2	0.4	<0.01**	−0.01	0.95
FPA/SpO_2	−0.48	<0.001***	−0.33	0.02*

*Significance is denoted as *p<0.05; **p<0.01; and ***p<0.001.*

non-trained apnoea, although this increases in much more pronounced in trained apnoea divers (Baković et al., 2005). Our study did not measure directly erythrocyte counts, so no conclusion can be made if in fact this splenic contraction seen in our study impacted haematological values.

Results from earlier studies observing the differences in cardiovascular mechanisms during prolonged apnoeas in dry vs. immersive environments have found that end-systolic volume is greater in immersion apnoea than dry apnoea (Marabotti et al., 2013), while no differences in HR changes were found. Decreases in HR and increases in arterial pressure have also been found to be no different during apnoeas in dry vs. immersive environments, if apnoea is performed with full facial immersion in water (de Bruijn et al., 2009). Our findings can confirm these findings, as facial immersion alone appears to be an adequate HDR trigger; however, the environmental condition can affect the magnitude of the HDR response regardless of the environmental condition (de Bruijn et al., 2009).

The main reasons as to why HR and RRI exhibited a greater response magnitude during FIFI is most likely due to the physiological effects of water immersion (fluid shifts), as water immersion stimulates an increase in RRI, which is primarily parasympathetically modulated (Schipke, 2001), although in our cohort, immersion alone did not seem to effect RRI. Apnoeas in FIFI have been observed to trigger higher vagal tone, leading to greater HR decreases (Foster and Sheel, 2005). In a previous study, during 3 repeated maximal apnoeas, HR, MAP and skin blood flow, although significantly different from baseline, did not progressively change, whereas splenic volume did appear to progressively decrease, along with increasing haematocrit (Schagatay et al., 2004). Therefore, based on these previous findings, it is thought that splenic emptying acts as a promotor for increasing BHT and O_2 conserving ability, while the cardiovascular mechanisms of the HDR do not differ during serial apnoeas.

In this study, FPA increased significantly throughout apnoeas in both conditions equally. Peripherally mediated vasoconstrictive reflex of the HDR only requires facial immersion and/or apnoea for activation; however, FPA was also significantly increased upon immersion, which would suggest that immersion alone can activate the peripheral mechanism of the HDR prior to apnoea onset. Heightened arteriolar resistance has been recorded during breath holds while completely immersed compared to dry condition apnoeas (Craig, 1963). Water immersion can trigger baroreflex stimulation, which would explain the initial significant increase in FPA during immersion baseline, and the subsequent FPA increases during FIFI (Lindholm and Lundgren, 2009). Thermoneutral water induces significant venodilation in the lower extremities, which can further induce a refractory arteriole constriction, which would explain the increased FPA during immersion (Beliard et al., 2017). Immersion causes a shift of 500 ml of blood flow to the intrathoracic circulation, which would induce an increase in vascular activity, as well as affecting the degree of vagal tone and vasomotor activity during apnoeas in immersive environments (Bosco et al., 2018). Finally, vascular activity during immersion is highly selective, with increased vasomotor activity in the periphery and vasodilation in the visceral organs, which may account for a transient decrease in overall systemic vascular resistance (Krasney, 2011).

Costalat et al. found that the O_2 conserving breaking point for non-trained breath-holding divers occurs halfway through apnoea time, which is at the time point where HDR activity rapidly increases (Costalat et al., 2016). In our cohort, BHT was not different between FIDC and FIFI, suggesting that full immersion does not play a role in enabling longer breath-holding, and rather, individual factors involved in the HDR activity play a role in influencing BHT to 60 s, as well as participants presumably becoming more accustomed to subsequent breath holds. Total immersion apnoea in elite divers triggered a maximal HR decrease and RRI increase at 60 s breath hold, which plateaued afterwards, meaning that 60 s appears to be adequate to trigger full HDR cardiac activity (Kiviniemi et al., 2012). Had our cohort performed apnoeas for longer than 60 s, a possible higher magnitude of HDR activity may have been observed; however, this is speculation. On the other hand, HR responses during HDR activation plateau from 30 s from start of apnoea (Yamaguchi et al., 1993).

The HDR is also highly individual-dependent, with non-uniform responses among humans, which suggests genetic polymorphism. In a study with 80 participants, consecutive FIDC apnoeas did not significantly decrease HR, but triggered uniform increases in PVR. The intensity of this vascular response was found to be highly dependent upon genotype influencing the renin-angiotensin and kinin systems (Baranova et al., 2017). Other findings have indicated that the HR decreases during apnoea are ubiquitous among all humans and are not individually dependent, which would counter the findings of Baranova et al. (Ferretti and Costa, 2003). This study could show that the measured vascular component (FPA) was consistently active during all apnoeas regardless of conditions, whereas HR activity showed progressive declines during FIFI, thereby supporting the findings of Baranova et al.

Finally, although breath-hold time between FIDC and FIFI was not statistically different, there was a tendency for BHT to progressively increase for subsequent apnoeas. This trend has also been recorded previously and is explained in partly due to participants getting accustomed to longer breath holds, while O_2 debt seems to increase during each subsequent apnoea (Heath and Irwin, 1968). The slightly longer, albeit, non-significant differences between BHT during FIFI compared

to FIDC is possibly due to immersion causing a delay in CO_2 build-up (Sterba and Lundgren, 1985).

SYNCHRONISATION OF THE HDR: FIDC VS. FIFI

The results of this study found that significant correlations exist between the vascular pathway of the HDR (FPA) with vagal tone (RRI) and splenic contraction during FIFI compared with FIDC. This suggests that apnoeas in immersive environments induce a cross talk between these systems, allowing for a higher level of synchronisation during FIFI than FIDC. While FIDC apnoeas did trigger a significant change in FPA, HR, RRI, SW and SpO_2 these factors appear to operate independently from each other. The only significant correlations were between SpO_2/FPA and SpO_2/SW. This suggests that during apnoeas in dry environments, the vascular mechanisms are stimulated *via* chemoreflexive pathways due to falling SpO_2. This has also been confirmed by prior breath-hold studies, which found that the increases in vascular resistance and bradycardia are most likely mediated by chemoreflexive pathways *via* breath-holding (Brick, 1966). Also, previous studies have shown that mild hypoxia can elicit splenic emptying (Stewart and McKenzie, 2002). The splenic expulsion of stored red blood cells can lead to a 10% increase in arterial O_2 content (Stewart and McKenzie, 2002), which could explain also the positive significant correlation between SW and SpO_2 during FIDC.

During FIFI, significant correlations were found between HR/SpO_2, RRI/FPA, RRI/SpO_2, SW/FPA and FPA/SpO_2. Apnoea in immersive environments seems to allow for a greater neural network synchronisation of the sympathetically mediated vasoconstriction and the parasympathetically mediated vagal tone. While no correlation was found between HR and PVR, FPA and RRI did show a moderate significant correlation, which would indicate that the interplay between central parasympathetic and peripheral sympathetic drive coordinate during underwater diving. The moderate significant negative correlation between FPA and SW does suggest that splenic activity is linked to sympathetic vasoactive inputs, rather than vagal tone as evidenced by previous research (Espersen et al., 2002).

The greater synchronisation of HDR factors during FIFI can also be explained by a greater central integration of the HDR. This central integration relay station, situated in the dorsal medullary horn of the trigeminal nerve, receives the peripheral afferent signals of apnoea/environmental cues and the following efferent output activates the HDR to a greater extent than in dry conditions (Panneton, 2013). This could also explain the greater synchronisation of HDR factors during FIFI. Water immersion, compared to dry environments, leads to an increase in plasma volume *via* the translocation of intracellular to intravascular volume to the thoracic cavity (Gauer and Henry, 1976; Pendergast et al., 2015). Afferent signals from the periphery are received at the nucleus solitarius which is embedded in the medulla oblongata, and consequently, 2 distinct pathways are activated. One pathway is mediated by the nucleus ambiguus, which triggers the parasympathetic arm of the HDR (HR, RRI). The other arm flows to the rostral ventral lateral medulla complex, which efferently activates the peripheral sympathetic drive of the HDR (increase of PVR). Both pathways lead to an increase of mean arterial pressure (MAP). It is of interest that these two arms do not appear to intersect, beyond their origination point in the nucleus ambiguus (Choate et al., 2014). One would expect to see at least a moderate correlation between HR and FPA based on the common dual cholinergic pathways to the cardiac system and the adrenergic pathways to the vasomotor system, as these factors have been shown to independently increase BHT during apnoeas (Baranova et al., 2020). However, no correlation could be found between these 2 factors, although a correlation could be found between FPA and RRI during FIFI. These findings would suggest that despite cross talk with vagal tone and sympathetic HDR factors, heart rate seems to operate independently.

Finally, the link between the cardiovascular and haematological responses during apnoea is thought to not be linked, but consist of independent reflexes (Schagatay et al., 2007). This appears to be supported during FIDC, but not FIFI, where the significant correlation between SW and FPA exhibits some form of communication between these responses. Whether it is a purely independent operation or a neurally linked one remains to be investigated.

LIMITATIONS

The primary limitation is that of a small sample size ($n = 11$). Also, our study included only non-apnoea trained participants, as apnoea trained individuals exhibit a specific HDR than non-apnoea trained divers, which includes a more pronounced bradycardia and increases in SVR activity (Tocco et al., 2012), as well as a increased splenic contraction (Prommer et al., 2007). Thus, the results of this study were from a homogenous cohort of non-apnoea trained divers, and the inclusion of apnoea trained divers may have impacted the results. Also, the specific goal of this study was to specifically examine non-apnoea trained individuals so that the results would apply to a broader segment of the population. A further limitation is that the apnoea protocol did not allow for maximal apnoeas and that apnoea time was held to maximal 60 s in order to maintain uniformity in the protocol. While the HDR could be induced in these 60-s intervals, longer apnoeas may have induced a stronger HDR responses. Another limitation of this study is the use of only SW to indicate splenic activity. This was done due to the narrow time window of maximal apnoeas, thereby negating the need to identify the caudal-cranial length of the spleen, which can be made difficult due to costal shadowing. Also, during immersion in apnoeas, the cranial border of the spleen was not visible in all subjects during the last 5 s of apnoea making complete splenic measurements difficult. Also, doppler views of the splenic vasculature would have been useful to solidify correlations between FPA and splenic contraction. Advanced cardiovascular monitoring may have led to more insights, as the availability of better methods could have enabled stroke volume and cardiac output monitoring.

Finally, although cold water can induce a stronger HDR, specifically HR decreases (Gooden, 1994), thermoneutral water was used in the study as the immersion pool at our facility did not allow for water cooling. Furthermore, cold water contact with the trigeminal nerve can elicit the cardiovascular effects of the HDR (bradycardia, lengthening RRI and vascular resistance increases) even without apnoea (Johnson et al., 2017). The sole use of trigeminal stimulation *via* facial immersion in cold water without apnoea has been used for autonomic testing (Khurana and Wu, 2006) or as a countermeasure to orthostatic stress (Johnson et al., 2017). The intent of this study, however, was to utilise thermoneutral water to isolate the HDR solely based on apnoea and bypass the cold water HDR activation. Also, in order to isolate the mechanisms of the HDR triggered solely by facial immersion and apnoea would trigger splenic emptying and enable a correlation analysis. It remains to be explored if splenic emptying can be elicited solely by cold water trigeminal stimulation without apnoea. Also, had cold water been used either during FIDC or FIFI, the HDR effects would have been much more pronounced (Andersson et al., 2000). Finally, baseline physical activity was not uniform among the participants. Physical conditioning can affect autonomic functioning, as well as the HDR (Manley, 1990), which may have influenced our findings. A larger cohort, as well as grouping for physical activity, or explicitly examining a homogenous group of subjects with regards to physical activity may lead unique HDR activation and activity.

SUMMARY AND FUTURE OUTLOOK

This study showed that the mechanisms of the HDR were stimulated nearly equally during consecutive apnoeas in both dry and immersive conditions. HR and RRI did seem to show a greater magnitude in immersive conditions, suggesting that the HDR is stronger during apnoeas in aquatic environments. The synchronicity of HDR factors were greater in number in immersive rather than dry conditions, and this is due to the physiological effects of water immersion, leading to increased neural recruitment and neural network cross talk among HDR pathways. The results from this small cohort do shed more light on the environmental influence and synchronicity of the HDR mechanisms in humans. Beyond that of purely physiological data, the consecutive apnoeas to elicit the HDR could be used prior to anaesthesia induction in order to promote splenic emptying and increase O_2 availability, as well as increasing blood pressure during anaesthesia induction. Triggering the HDR could further prolong apnoea time in high-risk patient and could possibly maintain blood pressure during the induction period. Furthermore, clinical applications include autonomic testing to determine parasympathetic/sympathetic reactivity (Macartney et al., 2020) and potential HDR training prior to SCUBA or apnoea diving. In summary, the results of this study do shed light on the interplay of the HDR factors active in both conditions and warrant further investigations into these neural pathways supporting O_2 conservation among humans in aquatic environments and beyond.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Charité-Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MN, AS, and OO performed the study. H-CG provided the research facility. MN and AS performed the statistical analysis. MN, AS, RB, and OO wrote and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Exponential Relationship Between Maximal Apnea Duration and Exercise Intensity in Non-apnea Trained Individuals

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It is well known that the duration of apnea is longer in static than in dynamic conditions, but the impact of exercise intensity on the apnea duration needs to be investigated. The aim of this study was to determine the relationship between apnea duration and exercise intensity, and the associated metabolic parameters. Ten healthy active young non-apnea trained (NAT) men participated in this study. During the first visit, they carried out a maximum static apnea (SA) and a maximal progressive cycle exercise to evaluate the power output achieved at peak oxygen uptake (PVO_2peak). During the second visit, they performed four randomized dynamic apneas (DAs) at 20, 30, 40, and 50% of PVO_2peak (P20, P30, P40, and P50) preceded by 4 min of exercise without apnea. Duration of apnea, heart rate (HR), arterial oxygen saturation (SpO_2), blood lactate concentration [La], rating of perceived exertion (RPE), and subjective feeling were recorded. Apnea duration was significantly higher during SA (68.1 ± 23.6 s) compared with DA. Apnea duration at P20 (35.6 ± 11.7 s) was higher compared with P30 (25.6 ± 6.3 s), P40 (19.2 ± 6.7 s), and P50 (16.9 ± 2.5 s). The relationship between apnea duration and exercise intensity followed an exponential function ($y = 56.388e^{-0.025x}$). SA as DA performed at P20 and P30 induces a bradycardia. Apnea induces an SpO_2 decrease which is higher during DA (−10%) compared with SA (−4.4%). The decreases of SPO_2 recorded during DA do not differ despite the increase in exercise intensity. An increase of [La] was observed in P30 and P40 conditions. RPE and subjective feeling remained unchanged whatever the apnea conditions might be. These results suggest that the DA performed at 30% of VO_2peak could be the best compromise between apnea duration and exercise intensity. Then, DA training at low intensity could be added to aerobic training since, despite the moderate hypoxia, it is sufficient to induce and increase [La] generally observed during high-intensity training.

Keywords: aerobic training, dynamic apnea, oxygen uptake, exponential function, oxygen saturation, lactatemia, RPE, subjective feeling

INTRODUCTION

Hypoxia is commonly used as a training modality in many sports. To avoid going to altitude or using expensive devices simulating a hypoxic environment, other alternatives have emerged such as training with voluntary hypoventilation (at low pulmonary volumes) (Woorons et al., 2007, 2008, 2010) or even apnea. Indeed, apnea certainly exists as a sport, but it also appears as a new modality of training in water sports but also on land. Apnea is then considered as a model of decreased O_2 availability that can spontaneously be compared with hypoxia as a model of hypoxic stimulus. While apnea training could be an effective alternative to hypobaric or normobaric hypoxia to increase aerobic and/or anaerobic performance (Lemaître et al., 2010), this type of training is most often used empirically. Apnea causes a well-known cardiovascular adaptation called the "diving reflex" (Lindholm and Lundgren, 2009). In humans, the diving response includes bradycardia, peripheral vasoconstriction, increased arterial blood pressure, reduced cardiac output and blood flow, and increased sympathetic activity triggered in response to cessation of ventilation (Sterba and Lundgren, 1988; Gooden, 1994; Foster and Sheel, 2005; Lindholm and Lundgren, 2009). An active contraction of the spleen was also considered as part of this diving response (Hurford et al., 1990). The diving response, which would aim to save O_2, causes a distribution of pulmonary and blood O_2 stocks preferentially toward the heart and the brain (Lindholm and Lundgren, 2009) and can, therefore, be considered as an important mechanism of defense against damage from hypoxia (Alboni et al., 2011).

During physical exercise, the energy is mainly produced through the use of O_2, and many studies have shown that the main factor involved in physical performance is the ability of the body to bring oxygen to the muscles and also to the brain. For equivalent apnea durations, there is a greater decrease in arterial oxygen saturation (SpO_2) (Delahoche et al., 2005) during dynamic apnea (DA) (Ahn et al., 1989; Lindholm et al., 1999, 2002; Joulia et al., 2009; Breskovic et al., 2011; Guimard et al., 2014, 2017, 2018) than during static apnea (SA) (Palada et al., 2007; Andersson et al., 2008; Kiviniemi et al., 2012; Costalat et al., 2013; Engan et al., 2013). This drop is greater in free divers due to the longer duration of the apnea (Palada et al., 2007).

More broadly, if apnea alone (i.e., in the air) is sufficient to trigger the diving response, the observed response is modulated according to various factors such as immersion, water temperature, hypoxia, or even training (Stromme et al., 1970; Schuitema and Holm, 1988; Lindholm and Lundgren, 2009). It is important to note that the longest apneas and the most pronounced cardiovascular adjustments were observed in trained participants (Andersson and Schagatay, 1998), suggesting a link between the duration of the apnea and the importance of the diving reflex (Caspers et al., 2011).

For a training purpose, the coaches empirically carry out exercises of varying intensity and duration of apnea, i.e., either exercises at very high intensity therefore of shorter duration of exercise and apnea or exercises at lower intensity therefore of longer duration of exercise and apnea. It is well known that the duration of apnea is longer in static than in dynamic conditions, but the impact of exercise intensity on the apnea duration needs to be investigated. This interaction is still poorly understood because very less was studied. Wein's team studied the physiological effects of dynamics apneas as a discipline in recreational or competitive breath-hold diving (Wein et al., 2007). For this, participants trained in apnea performed maximum apnea with their face submerged at rest and simultaneously with exercises of different intensities (40, 80, and 120 W) on an ergocycle. The results seem to indicate that the duration of apnea decreased with the exercise power according to an exponential function (data not mentioned) of the maximal apnea duration. The precise knowledge of this interaction could be used as a basis for the development of apnea training, because it is likely to increase induced cellular hypoxia and therefore the adaptive responses to training. In addition, it might be interesting to test this interaction on untrained participants in apnea and in the air for wider application in the field of training. Thus, the aim of this study was to determine the relationship between apnea duration and exercise intensity, and the apnea exercise metabolic effects. We hypothesized that there is an optimal exercise intensity during DA to induce metabolic effect and an exponential relationship between exercise intensity and duration of apnea.

MATERIALS AND METHODS

Participants

The experimental group consisted of 10 healthy male students: 21 ± 3.3 years (weight: 69.3 ± 5.9 kg and height 176.4 ± 5.3 cm). None of them had been trained in apnea. However, participants were not naïve to apnea and well-trained in physical activities. All participants were students at the Faculty of Sport Sciences. During their studies, it was mandatory to practice swimming and water rescue that are activities known to require apnea phases. Furthermore, during the recruitment, all the volunteers were asked if they feel comfortable with apnea. Only the one comfortable participated in this study. Finally, the participants who consent to participate indicated that they were able to maintain at least 1 min of SA. All of them were non-smokers, and none of them were taking any medication or had a family history of cardiac, respiratory, or metabolic pathology. After being informed of the nature of the experiments, all the participants gave their informed consent to participate in the protocol, and all procedures were designed according to the declaration of Helsinki and approved by the Committee for the Protection of Persons Tours—Région Centre—Ouest 1.

Abbreviations: DA, dynamic apnea; FS, feeling scale; HR, heart rate; [La], blood lactate concentration; NAT, non-apnea trained; P20, 20% of the power output achieved at peak oxygen uptake; P30, 30% of the power output achieved at peak oxygen uptake; P40, 40% of the power output achieved at peak oxygen uptake; P50, 50% of the power output achieved at peak oxygen uptake; PVO_2peak, power output achieved at peak oxygen uptake; RPE, rating of perceived exertion; SA, static apnea; SpO_2, arterial oxygen saturation; SpO_2min, minimal value of arterial oxygen saturation; VO_2peak, peak oxygen uptake.

Procedures

Participants performed, after a day off from intense exercise, different tests on two visits separated by 48 h. The participants consumed no caffeine or alcohol during the preceding 24 h of the experiment.

During the first visit, the participants were informed about the experiment. After the medical consultation that included anthropometric measurements and basal data of cardiovascular parameters such as heart rate (HR), each subject performed on an ergocycle (Ergoline 200) a maximum SA preceded by 5 min of rest, followed by an incremental exercise test. The latter consisted of 5 min of rest sitting on the same cycle ergometer followed by 3 min warm up at 60 W and by an intensity increase of 30 W every 2 min (60 rpm) until exhaustion to determine the peak oxygen uptake (VO_2peak) and the power output achieved at VO_2peak (PVO_2peak). This maximal exercise test was followed by 6 min of passive sitting recovery. These two tests were separated by 15 min of passive recovery.

During the second visit (**Figure 1**), participants started with 5 min of rest. Then, they performed four blocks of exercise at 60 rpm and at a power corresponding to 20, 30, 40, and 50% of PVO_2peak (P20, P30, P40, and P50, respectively). Each block consisted of 4 min of exercise without apnea at a specific power to reach a steady state followed by a maximum DA (i.e., the longest time that a participant could endure in apnea at that power). Each block was separated from the following one by 15 min of passive recovery. The order of the blocks was randomized. All the apneas were performed after a maximal inspiration.

Measurements

Gas exchanges were continuously measured for the analysis during the incremental cycle exercise by a breath-by-breath gas exchange measurement system (Jaeger; CareFusion, Germany). Each apnea duration was measured using a manual stopwatch. HR was continuously monitored and recorded at rest, during exercise, and during the recovery period (PhysioFlow®, Manatec type PF05L1, Paris, France). In addition, the arterial O_2 saturation (SpO_2) was continuously evaluated using a pulse oximeter with an ear sensor of the same device at rest for each apnea condition, during SA and DA, and during the post-exercise rest period in visit 2. The minimal value of SpO_2 was determined (SpO_2min) during SA and DA and during the 30 s after the end of each apnea condition. For the measurement of lactatemia ([La]) (ABL 700 Radiometer), 10 μl of capillary blood was drawn from the earlobe at rest, 3 min after the end of incremental exercise (visit 1) and 2 min after the end of SA and DA (visits 1 and 2).

The rating of perceived exertion (RPE) scores was estimated by the participants using the 15-grade (from 6 to 20) Borg RPE Scale (Borg, 1970; Shephard et al., 1992) 1 min after the end of the incremental exercise (visit 1) and the end of SA and DA (visits 1 and 2). Two minutes after the end of SA and DA (visits 1 and 2), the participants also estimated their subjective feeling (feeling of pleasure or displeasure) about the exercise performed using a Feeling Scale (FS) (Hardy and Rejeski, 1989) consisting of 11 grades between −5 and +5, combined with verbal information (ranging from "Very bad" for −5 to "Very good" for +5).

Statistical Analysis

Since we test the same group in different conditions and for different variables, we performed a Friedman ANOVA. Then, we used a Wilcoxon matched-pair signed-rank test for intragroup comparisons (condition 1 vs. condition 2). P-values < 0.0125 were considered significant since we used a Bonferroni correction. Analyses were performed with Statistica 6.0 software.

RESULTS

Table 1 shows data at maximal exercise obtained during the incremental test and used to determine P20, P30, P40, and P50.

Apnea duration was higher during SA (68.1 ± 23.6 s) than during DA, and apnea duration at P20 (35.6 ± 11.7 s) was higher than that at P30 (25.6 ± 6.3 s), P40 (19.2 ± 6.7 s), and P50 (16.9 ± 2.5 s). Apnea duration at P30 was higher than that at P40, and no difference was noted between P40 and P50. The relationship between apnea duration and exercise intensity followed an exponential function ($y = 56.388e^{-0.025x}$; **Figure 2**).

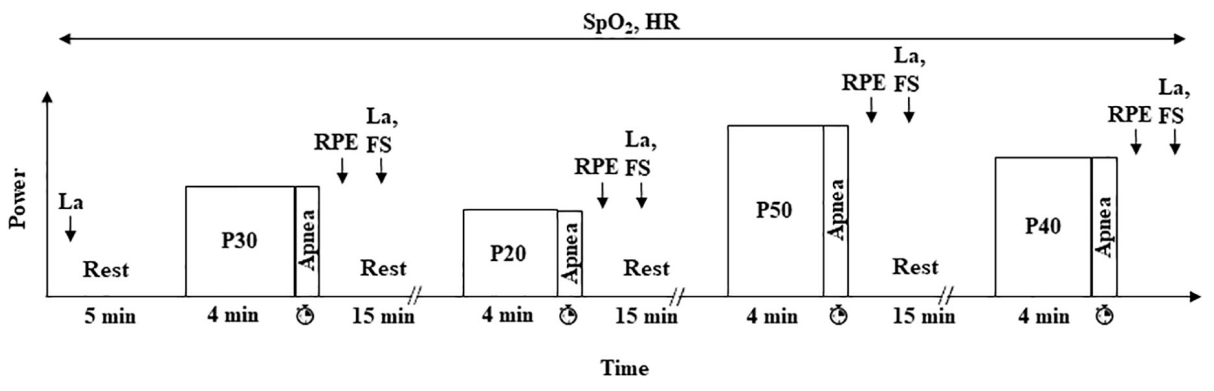

FIGURE 1 | Experimental protocol (visit 2). Participants performed four randomized exercises at 20, 30, 40, and 50% of PVO_2peak (P20, P30, P40, and P50, respectively) followed by a maximal dynamic apnea at the same power. HR and SpO_2 were continuously monitored, and [La] was measured at rest and 2 min after the end of each dynamic apnea. RPE and FS were estimated 1 and 2 min after exercise, respectively. The duration of each apnea was measured.

TABLE 1 | Measurements taken during the incremental test (visit 1) and used for the dynamic apneas (visit 2).

VO_2peak (ml.min^{-1}.kg^{-1})	VEpeak (l.min^{-1})	HRmax (bpm)	[La] (mmol.l^{-1})	RPE	PVO_2peak (W)	P20 (W)	P30 (W)	P40 (W)	P50 (W)
47.5 ± 8.2	128.6 ± 28.2	189 ± 7.7	14.9 ± 2.9	16 ± 1.7	232 ± 29.7	46 ± 6.1	70 ± 9.1	93 ± 11.8	116 ± 14.9

Peak oxygen uptake (VO_2peak), maximal ventilation (VEpeak), maximal heart rate (HRmax), and blood lactate concentration ([La]) 3 min after the end of the exercise, rating of perceived exertion (RPE), power output achieved at peak oxygen uptake (PVO_2peak), and power corresponding to 20, 30, 40, and 50% of PVO_2peak (P20, P30, P40, and P50, respectively).

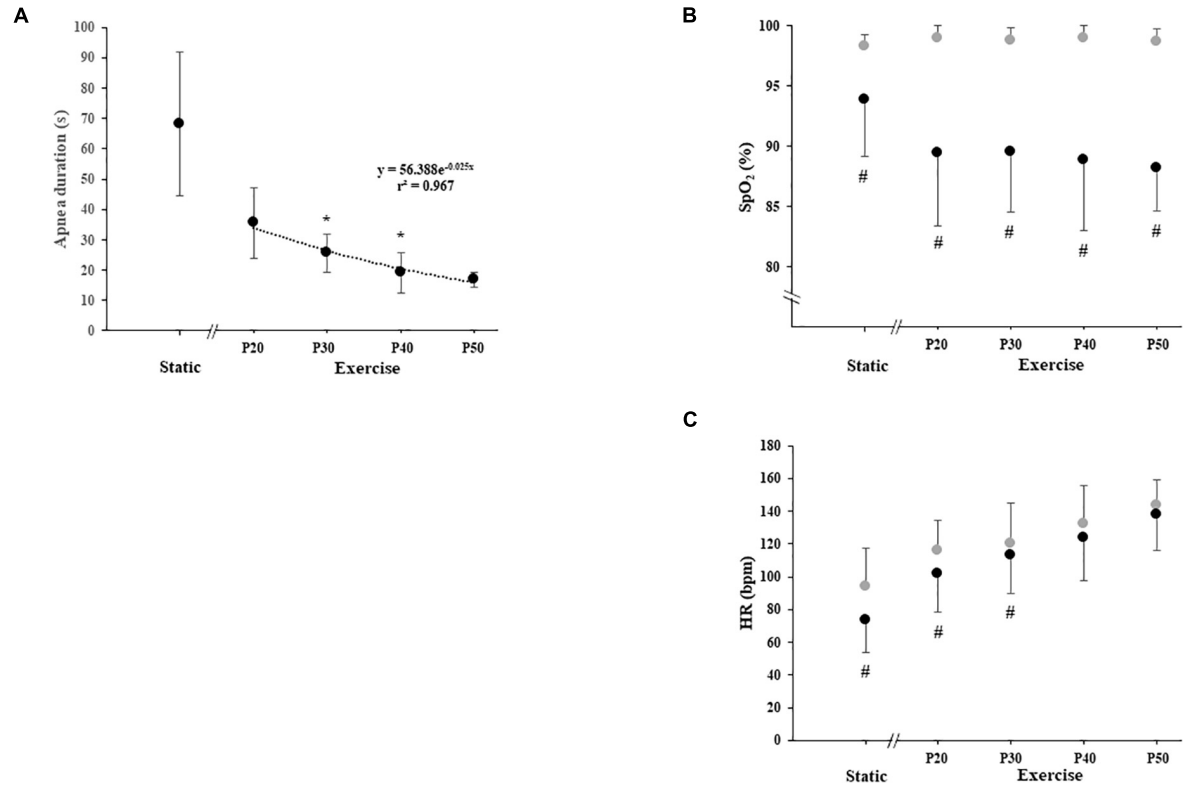

FIGURE 2 | (A) Duration of static and dynamic apnea, **(B)** arterial oxygen saturation (SpO_2), and **(C)** heart rate (HR) with apnea (black circle) and without apnea (gray circle) during static and exercise. The exercise was carried out at 20, 30, 40, and 50% of power output achieved at peak oxygen uptake (P20, P30, P40, and P50, respectively). Significant difference with the apnea duration of the previous power output: *$P < 0.0125$ (for panel **A**). Significant difference between with and without apnea: #$P < 0.0125$ (for panels **B** and **C**).

Static apnea induces a decrease in HR (−22%; $P = 0.0051$). DA performed at P20 and P30 induced a decrease in HR compared with exercise without apnea (−13%, $P = 0.0081$; −6%, $P = 0.007$, respectively). We did not find any apnea effect on HR when dynamic exercises were performed at P40 and P50 (**Figure 2**).

Static apnea and DA performed at P20 did not modify [La] ($P = 0.26$ and $P = 0.063$, respectively). We found an increase of [La] when we compared rest with P30 values (+97%; $P = 0.0077$) and rest with P40 values (+165%; $P = 0.0051$); but, since we have some missing values (three missing values), despite a very high increase of [La] recorded in seven participants (+247%), we did not find a difference between rest and P50 ($P = 0.018$) (**Table 2**).

Static apnea induces a decrease in SpO_2 (−4.4%; $P = 0.0117$). DA induces a decrease in SpO_2 compared with exercise without apnea at P20 (−9.6%, $P = 0.0051$), P30 (−9.5%; $P = 0.0051$), P40 (−10%; $P = 0.0070$), and P50 (−10.5%; $P = 0.0051$). The SpO_2 decreases observed during the different DA conditions were always higher compared with SA but were not dependent on the level of exercise intensity (**Figure 2**). We did not find any effect of experimental conditions on RPE and FS (**Table 2**).

DISCUSSION

To the best of our knowledge, this is the first study comparing different exercise intensities during DAs, relationship between duration and exercise intensity, and the associated metabolic parameters. The main result is that the relationship between apnea duration and exercise intensity followed an exponential function. Our outcomes suggest that the best compromise between apnea duration and exercise intensity is around 30% of VO_2peak exercise in order to induce a sufficient stimulus to lead to hypoxia and [La] increase which can be used during aerobic training.

TABLE 2 | Rating of perceived exertion (RPE), feeling scale (FS), respectively, 1 and 2 min after the end of static apnea (SA) and dynamic apnea (DA) carried out at 20, 30, 40, and 50% of power output achieved at peak oxygen uptake (P20, P30, P40, and P50, respectively), blood lactate ([La]) at rest, 2 min after the end of SA and DA carried out at P20, P30, P40, and P50.

	Rest SA	SA	Rest DA	P20	P30	P40	P50
RPE	/	12.6 ± 3.5	/	11.0 ± 2.2	11.5 ± 2.7	13.6 ± 2.6	13.3 ± 1.9
FS	/	1.1 ± 2.7	/	1.7 ± 1.9	1.2 ± 1.7	0.1 ± 1.9	0.4 ± 2.4
[La] ($mmol.l^{-1}$)	0.96 ± 0.3	0.94 ± 0.3	0.92 ± 0.4	1.20 ± 0.5	1.82 ± 0.5*	2.44 ± 0.8*	3.20 ± 0.7

*Significant difference between rest and apnea conditions: *P < 0.0125.*

Stopping ventilation has been shown to initiate the diving response at rest as during exercise (Lin et al., 1983; Lindholm et al., 1999; Lindholm and Lundgren, 2009). Apnea alone is sufficient to trigger the diving response, and it could be modulated by immersion, water temperature, hypoxia, physical activity, and training (Stromme et al., 1970; Schuitema and Holm, 1988; Foster and Sheel, 2005; Andersson and Evaggelidis, 2009; Joulia et al., 2009; Lindholm and Lundgren, 2009). The mechanisms involved are more intense during dynamic than during static conditions (Butler and Woakes, 1987), justifying our interest to compare SA and DA modalities.

The low durations of maximal SA and DA observed during our study confirmed that our participants did not regularly practice apnea. The duration of apnea is determined by the interactions of mechanical, chemical, and psychological factors (Hentsch and Ulmer, 1984; Schagatay, 2009). The longest apneas occur when the most diving response is pronounced (Schagatay and Andersson, 1998). Thus, elite free divers are known to maintain long apnea and to exhibit an accentuated diving reflex (Joulia et al., 2009; Rodriguez-Zamora et al., 2018). The diving response has been shown partly genetically defined (Baranova et al., 2017; Ilardo et al., 2018), but an accentuated diving response can be obtained after a DA training at low exercise intensity in apnea naïve athletes (Joulia et al., 2003). It is very difficult to maintain DA for non-apnea trained (NAT) participants, and if we want to develop apnea as a new modality of training as previously suggested (Lemaître et al., 2010), we need to adapt apnea training to NAT participants. Then, it is necessary to determine the best compromise between apnea duration and the intensity of exercise to limit the duration of the struggle phase of apnea. Later, we observed a significant decrease in the DA durations compared with SA when the intensity of exercise increased (P20: −48%, P30: −60%, and P40: −70%).

Lactatemia was not modified by SA nor by P20. It has been previously shown that SA and DA could induce an increase of lactatemia in trained and NAT participants (Joulia et al., 2003). However, to obtain this increase, the durations of SA and DA needed to induce sufficient hypoxia, and these two conditions were not present in SA and P20 conditions. The diving reflex has been described as accentuated in free divers compared with NAT subjects. Since the diving reflex is an oxygen preservation mechanism, during apneas, free divers are presenting lower O_2 uptake and CO_2 production compared with NAT (Joulia et al., 2003). Consequently, the short duration of apneas observed in this study can be partly explained by the higher oxygen uptake and CO_2 production in our participants and their difficulties to maintain the struggle phase (Hentsch and Ulmer, 1984). Despite very short apnea durations in this study, we observed increases of [La] at P30 (+97%), P40 (+165%), and P50 (+247%) even though for this last condition some missing values did not permit to have significant results. Then, P30 intensity of exercise seems sufficient to stimulate anaerobic glycolysis, justifying the difficulty for participants to maintain apnea for a longer period.

During apnea despite stopping the ventilation, the oxygen uptake was likely unchanged. Then, an SpO_2 decrease was observed during SA depending on the duration or during DA depending on the duration and the intensity of the exercise (Fitz-Clarke, 2018). In this study, despite a decrease in SpO_2 during each DA condition, there is no effect of the intensity exercise on SpO_2. It suggests that the durations are too short to induce a different SpO_2 decrease. We also know that SpO_2 is reduced differently depending on the level of apnea practice (Joulia et al., 2015). This study has shown that in order to maximize the apnea stimulus in untrained participants, we should exercise with maximum SA or DA durations. Then, the performed DA training at very low intensity is interesting for NAT participants compared with SA because it is permitting to obtain a greater desaturation suggesting a higher hypoxic stimulus.

Diving bradycardia was the first component of the diving reflex studied (Bert, 1870). It is a primordial oxygen-conserving reflex since less the myocardial tissue oxygen uptake is more the apnea duration will be important (Hoiland et al., 2017). In agreement with previous studies, the greatest bradycardia is observed during SA (−22% vs. rest). This decrease was lower compared with elite free divers since the maximal durations are too short to induce the full development of the bradycardia (Jung and Stolle, 1981), and the bradycardia is known to be more pronounced in elite free divers (Baković et al., 2003; Joulia et al., 2013; Bain et al., 2018). Bradycardia during DA conditions is less marked compared with SA since there is an antagonist effect of exercise on apnea response (Joulia et al., 2009; Alboni et al., 2011). The decrease is also noticeable when comparing DA with the previous exercise performed at the same intensity in normal breathing and because of the effect of exercise and apnea duration on the bradycardias are more marked at P20 (−13%) than at P30 (−6%) confirming Jung and Stolle observations suggesting a minimum of 30 s in apnea to obtain the full development of the diving response (Jung and Stolle, 1981). Likewise, bradycardias were not observed from P40 and P50 since the diving response is limited due to the short apnea time and the intensity of the exercise high enough to counterbalance the effects of apnea in NAT participants.

It has been noted that RPE scores positively correlate with the total immersion time and inversely correlate with the minimum and average HR (Rodríguez-Zamora et al., 2014). Then, we would expect to observe a modified RPE depending on the apnea conditions. Indeed, apnea involves stopping a vital function and constitutes a real "effort" in that psycho-physical stress appears, especially as it is paired with intense exercises (Rodríguez-Zamora et al., 2014). We expect that the stress induced by the apnea associated with the higher intensity of exercise would modify the part of the struggle phase in the apnea, but probably due to the too short duration of the apneas, participants did not feel unpleasant sensations. Since the participants were not apnea trained, the exercise effect is dominant compared with the apnea effect, and participants stopped the apnea at the end of the easy-going phase since unpleasant sensations appear (Hentsch and Ulmer, 1984). FS is poorly used in training sessions. However, an exercise can be felt as hard without being unpleasant, which justifies the addition of an assessment of feeling experienced using an FS. FS values remained unchanged whatever the apnea conditions might be and remained neutral or even had positive values (between "neutral" and "slightly good to good"). The SA and DA were not considered unpleasant. The latter would thus make it possible to carry out an exercise in "bearable" conditions while having an effective stimulation. A strong stimulation of anaerobic glycolysis could take place while limiting the unpleasant effect, often felt during fractional work (HITT) (Saanijoki et al., 2015). Moreover, some non-free-diver athletes also use apnea at the end of training (carried out without necessarily having included apnea in the body of the session) to relax and recover during the so-called calm down phase. It is well known that in free diving training, breathing techniques are derived from pranayama yoga. Since yoga respiration training induces long-lasting modifications of the ventilatory pattern (Villien et al., 2005), we can consider that learning to control the breathing of an individual is also a way of relaxing.

The main limitation of this study is the short durations of the apneas performed by the participants and their number. These short durations are the consequence of the non-apnea practice of the participants, but if a DA training wants to be performed by non-apnea participants, it was necessary to test the different DA conditions in NAT participants.

This study showed that the best compromise between maximal apnea and exercise intensity is around 30% of VO_2peak exercise in order to induce the higher stimulus for aerobic training. The use of the apnea modality shows that despite the low-intensity exercise and the short duration, we can obtain measurable metabolic modifications. It could be used in sport activities where fast modification of blood flow distributions can appear as during the transitions in triathlon or during isometric phases as downhill mountain bike causing a hypoxia or an ischemia in some territories. Since a low-intensity training associated with apnea induces a metabolic response, it could be interesting to propose this kind of training in rehabilitation after an injury. Moreover, apnea could be an easy way to induce hypoxic preconditioning, which is known to increase the endurance capacity (Wang et al., 2019). Finally, in the field of physical activity conditioning, this modality of exercise would both potentiate the effects of exercise while optimizing the time spent and vary the exercise (which can also be experienced as a challenge). Faster progression is a major source of motivation for patients (Kraemer et al., 2002).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Local Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AG, FP, and FJ conceived and designed the project. KH, AG, and FL performed the data collection. KH, AG, FP, and FJ performed the data analysis and the interpretation of data. All authors contributed to the preparation, critically revised the manuscript, and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Effects of Extended Underwater Sections on the Physiological and Biomechanical Parameters of Competitive Swimmers

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Despite changes in the underwater sections of swimming races affecting overall performance, there is no information about the effects of the apnea-induced changes on the physiological state of competitive swimmers. The aim of the present research was to examine the effect of changes in the underwater race sections on the physiological [blood lactate concentration, heart rate, and rating of perceived exertion (RPE)] and biomechanical (underwater time, distance, and velocity) parameters of competitive swimmers. Twelve youth competitive swimmers belonging to the national team (706 ± 28.9 FINA points) performed 2 × 75 m efforts under three different conditions, while maintaining a 200 m race pace: (1) free underwater sections, (2) kick number of condition 1 plus two kicks, and (3) maximum distance underwater. Overall performance was maintained, and underwater section durations increased from condition 1 to 3 as expected according to the experimental design. Heart rate and blood lactate concentration values did not show differences between conditions, but the RPE values were significantly greater ($F_{2, 36} = 18.00$, $p = 0.001$, η^2: 0.50) for the constrained (conditions 2 and 3) vs. the free underwater condition. Underwater parameters were modified within the 75 m efforts (lap 1 to lap 3), but the magnitude of changes did not depend on the experimental condition (all lap × condition effects $p > 0.05$). Controlled increases of underwater sections in trained swimmers can led to optimizing performance in these race segments despite small increases of perceived discomfort.

Keywords: apnea, underwater undulatory swimming, swimming start, swimming turn, breath-holding, RPE, elite swimmers, dolphin kick

INTRODUCTION

The underwater segments of swimming races have reunited the interest of coaches and researchers in the last years, according to the increasing number of publications on the topic. These race segments represent the periods where swimmers stay underwater in apnea conditions after they dive or push off the starting or turning wall and before they emerge at the water's surface

for mid-pool swimming. According to FINA rules (FINA, 2021), the maximum distance that swimmers can travel underwater before breaking the water's surface is 15 m from the wall.

There are several reasons that explain the increasing importance of the underwater race parts for overall swimming performance. When underwater, swimmers encounter much less hydrodynamic drag resistance (Marinho et al., 2009) and therefore achieve faster forward velocities than when surface swimming (Takeda et al., 2009). The loss of forward velocity in the different race laps seems to be less in the underwater vs. the surface-swimming segments (Veiga and Roig, 2016). In addition, the underwater swimming segments have a positive effect on the forward velocity of the subsequent surface swimming. Indeed, the first strokes after the starting or turning movements have been reported to be faster than mid-pool swimming, and this is believed to occur due to previous momentum from underwater swimming (Veiga and Roig, 2017).

In line with the aforementioned factors, analysis of the 100 m and 200 m races at the World Swimming Championships revealed that small changes in the underwater distance and velocity traveled by elite swimmers could have a great impact on the overall race results (Veiga et al., 2016). Indeed, the relative contribution of the underwater parts to the overall swimming race distances has increased considerably over the last 20 years (Veiga et al., 2014), and the percentage of non-swimming time (underwater) can even discriminate between swimmers of different skill levels (Pla et al., 2021). Accordingly, in recent years, procedures for swimming race analysis in elite competitions have incorporated a more comprehensive approach, where both times at fixed distances (i.e., 15 m start or turn times) and also individual underwater distances traveled by swimmers have been measured. In this way, race analysts can perform a more specific evaluation of the underwater race parts (Veiga et al., 2013b).

However, despite it being well-known that changes in underwater swimming segments can influence overall race velocity, the consequences of the apnea periods on the physiological state of swimmers during competitions are unknown. When underwater, swimmers have to deal with a well-known cardiovascular response that aims for a distribution of pulmonary and blood oxygen stocks preferentially to the heart and the brain (Lindholm and Lundgren, 2009) to limit the effect of a possible hypoxia (Alboni et al., 2011). This so-called diving response is characterized by bradycardia, peripheral vasoconstriction, and increased blood pressure, reduced cardiac output and blood flow, and increased sympathetic activity (Sterba and Lundgren, 1988; Foster and Sheel, 2005). Also, apnea leads to hypercapnia (Qvist et al., 1985), which has many consequences particularly on tissue oxygenation (Woorons et al., 2010), lactatemia (Graham et al., 1980), and probably discomfort (Woorons et al., 2007). The diving response (and the bradycardic phenomenon) is somehow in contradiction with exercise, which implies an increase in heart rate (Alboni et al., 2011), and this is why it is difficult to identify physiological patterns for apnea in the context of exercise performance and particularly in swimming. For example, in artistic swimming, Rodríguez-Zamora et al. (2012) described a rapidly developing tachycardia up to maximal levels during technical routines with interspersed periods of marked bradycardia in the exercise bouts performed in apnea.

There is a precedent in the swimming literature (Rozi et al., 2018) that compared the results of an anaerobic capacity test performed with two protocols, one with underwater start sections of 15 m for all participants and one with no underwater start sections. No differences were observed in swimming performance, lactate concentration, or heart rate. During surface swimming, Guimard et al. (2014, 2018) examined the effect of an apnea condition on short distances and observed a decrease in the arterial oxygen saturation and an increase in the RPE values of swimmers in their 400 m race pace. However, the physiological cost of the underwater race sections of competitive swimming that represent the longest apnea periods during races is still unclear. Therefore, the aim of the present research was to examine the effect of changes in the underwater race sections on the physiological and biomechanical parameters of competitive swimmers. It was hypothesized that despite providing an advantage in terms of average velocity, the extended underwater sections would lead to an alteration in the physiological parameters of competitive swimmers and a decrease in the performance of the last laps of simulated race distances.

MATERIALS AND METHODS

Twelve competitive swimmers (five males: 184.2 cm and 70.9 kg, and seven females: 170.1 cm and 58.9 kg) belonging to the national youth swimming team were recruited to participate in the present study. All of them competed for the national team in youth international competitions, and their personal-best times at their preferred stroke were, on average, 706 ± 28.9 FINA points. Their parents or legal tutors gave written consent for the experiment, and all procedures were designed according to the declaration of Helsinki and approved by the local university's ethics committee.

The experiment was performed in a 25 m pool with a water temperature of 26°C and all test were conducted in the same training session. After a standardized warm-up consisting of 1,500 m with drills, some aerobic descending work and 25 m speed repeats, swimmers performed a testing set at their 200 m race pace designed to evaluate the role of underwater swimming on biomechanical and physiological parameters. The set consisted of two repeats of 75 m with a dive start at their preferred stroke (four swimmers at front crawl, four swimmers at backstroke, and four swimmers at butterfly) and with a one-and-a-half-minute rest between the first and the second repeat. The 200 m pace was calculated for each swimmer in relation to their personal best, and reference times for the 75 m repeats were given to swimmers before the commencement of the experiment. If variations greater than 1% in swimming velocity were observed in the first of the two 75 m repeats (compared to goal times), then some feedback was given to swimmers for the second repeat. Only data from the second repeat were collected. The set (2 × 75 m) was repeated three times in a fixed order with a minimum of 15 min of active rest between

each set. Before the commencement of the second and third block, different indications were given to swimmers in relation to the underwater segments for each block. Details can be seen in **Table 1**.

The experimental protocol was part of a national team training camp, and dedicated staff guaranteed that swimmers maintained the same routine (sleep, diet, and lifestyle) for the 24 h preceding the test. Right after the completion of each set (around 2 s after the end of the set), swimmers' heart rates were measured with a Garmin HRM Tri (Garmin Ltd., United States) monitor for a period of 30 s. The highest heart rate during that period was defined as the peak heart rate. To minimize potential instrumentation bias, this procedure was tested before the study protocol, and the swimmers were familiarized with this technique. Around 1 min after the completion of the set, capillary samples for blood lactate were collected from the ear lobe with Lactate Pro2 (Arkray Factory, Inc., Japan). According to the authors' experience, for young swimmers performing a non-maximal effort of 75 m, 1 min post-exercise could be the most representative moment to estimate lactate peak. Around 30 s after the lactate measurements, for each swimmer, a rating of perceived exertion (RPE) was used to estimate the perception of effort with the Borg CR-10 category-ratio scale (Borg, 1982), which has been suggested as being efficient to estimate swimming intensity for swimmers (Psycharakis, 2011).

Two video cameras (Sony DCR-HC20E) recording at a frame rate of 50 Hz and situated on the public stands 5 m from the starting or turning wall (approximately 4 m above and 10 m away from the swimmers lane) were employed to record the experiment. Videos were analyzed with Kinovea software 0.8.27 (Copyright © 2006–2011, Joan Charmant & Contrib.), including the manual digitization of the swimmers' head emersion from underwater by an experienced observer. Six control points represented by colored floating buoys surrounding the swimming-pool lanes were employed for calibration purposes, as previously done by Veiga et al. (2010). This allowed converting two-dimensional screen coordinates into real-space coordinates of the plane formed by the water's surface. Two experienced coaches using a manual stopwatch Seiko SVAS003 (Seiko Watch Corporation, Japan) collected total time in seconds of each swimming repeat. The distance traveled underwater (m) in each of the three swimming laps was calculated from the starting or turning wall to the point of the swimmers' head emersion, underwater time (s) was calculated from wall contact to head emersion, average underwater velocity (m/s) was obtained by dividing underwater distance and underwater time, and also the number of underwater undulatory kicks (*n*) was counted from the trials' video footage. Finally, average surface swimming velocity (m/s) was computed from the total time and distance of the repeats minus the underwater segments.

The distribution of data was examined for normality using the Shapiro–Wilk test, and all parameters were graphed for visual inspection to screen for outliers. Two outliers were presented in the total underwater distance, and they were then excluded for statistical analyses. A repeated-measures ANOVA was performed according to the swimming condition (set 1, set 2, or set 3) and swimming lap (lap 1, lap 2, or lap 3). Bonferroni's *post hoc* tests were used to verify localized differences. Data were expressed as mean ± SD, and the magnitude of differences (if they existed) was calculated by eta-squared effect sizes. All statistical analyses were conducted using R version 4.1.0. Significance was set at $p < 0.05$.

TABLE 1 | Experimental protocol of competitive swimmers to evaluate the role of the underwater swimming on biomechanical and physiological parameters.

Free underwater condition	Set 1	2 × 75 dive on 2′30	200 m pace (swimmers asked to count the underwater kicks per lap)
Constrained underwater conditions	Set 2	2 × 75 dive on 2′30	200 m pace with two additional underwater kicks in each underwater section after start and turns
	Set 3	2 × 75 dive on 2′30	200 m pace with the maximum distance covered in each underwater section after start and turns

RESULTS

As designed in the experimental protocol, overall swimming times did not present a significant main effect between the three different conditions ($F_{2,\ 22} = 0.86$, $p = 0.43$, η^2: 0.05), but this was observed in the total underwater distance, underwater time, and underwater velocity ($F_{2,\ 20} = 19.55$, $p = 0.001$, η^2: 0.52 for distance, $F_{2,\ 22} = 10.94$, $p = 0.001$, η^2: 0.50 for time, and $F_{2,\ 20} = 4.25$, $p = 0.03$, η^2: 0.30 for velocity) as well as the surface swimming velocity ($F_{2,\ 20} = 3.86$, $p = 0.04$, η^2: 0.28) traveled by swimmers. *Post hoc* test revealed longer distances and times underwater in the constrained (conditions 2 and 3) vs. the free underwater conditions, slower underwater velocity in the maximum underwater condition and slower surface velocity in condition 2 than the rest of conditions (**Table 2**). In particular, there was a main effect for the total number of underwater kicks ($F_{2,\ 22} = 38.70$, $p = 0.001$, η^2: 0.68) with significant differences in each of the experimental conditions.

TABLE 2 | Performance, underwater kicking, and physiological parameters of competitive swimmers in different underwater swimming conditions.

	Condition 1	Condition 2	Condition 3
Swimming time (s)	49.99 ± 5.77	49.83 ± 5.52	49.69 ± 5.58
Total number of kicks (*n*)	16.0 ± 8.1[b,c]	20.7 ± 8.9[a,c]	25.4 ± 11.1[a,b]
Total underwater distance (m)	26.13 ± 3.50[b,c]	30.94 ± 4.98	32.21 ± 6.40
Total underwater time (s)	14.23 ± 3.99[b,c]	16.74 ± 4.56	18.06 ± 5.47
Total underwater velocity (m/s)	1.86 ± 0.24	1.84 ± 0.24	1.76 ± 0.24[a,b]
Total surface swimming velocity (m/s)	1.45 ± 0.14	1.43 ± 0.15[a,c]	1.45 ± 0.14
Heart rate (bpm)	174.5 ± 10.3	176.1 ± 9.9	177.0 ± 10.7
Blood lactate (Mmol/L)	9.35 ± 2.55	8.96 ± 3.84	10.31 ± 3.97
RPE	7.61 ± 0.82[b,c]	8.29 ± 0.81	8.62 ± 0.81

[a,b,c]*Statistically different from the first, second, or third underwater swimming conditions, respectively.*

In relation to the physiological measures, there were no main effects for the heart rate values ($F_{2, 22} = 0.83$, $p = 0.44$, η^2: 0.07) or the blood lactate concentration ($F_{2, 22} = 2.83$, $p = 0.08$, η^2: 0.20) of swimmers in the different experimental conditions, despite a tendency for blood lactate in condition 3 to be greater than in condition 2. However, there was a significant effect for the RPE ($F_{2, 22} = 18.00$, $p = 0.001$, η^2: 0.50) with values in the constrained conditions being greater than the free underwater condition. Individual variation of physiological and biomechanical parameters is displayed in **Figures 1, 2**.

Finally, the evolution of the biomechanical parameters on the 75 m repetitions presented a significant effect for the number of underwater kicks ($F_{2, 88} = 55.35$, $p = 0.001$, η^2: 0.43) and also in the underwater distance ($F_{2, 88} = 203.81$, $p = 0.001$, η^2: 0.74), underwater time ($F_{2, 80} = 26.05$, $p = 0.001$, η^2: 0.32), underwater velocity ($F_{2, 80} = 29.37$, $p = 0.001$, η^2: 0.42), and surface swimming velocity ($F_{2, 80} = 74.36$, $p = 0.001$, η^2: 0.65) from lap 1 to lap 3. However, the magnitude of changes in the biomechanical parameters did not depend on the experimental condition (all lap × condition effects $p > 0.05$). Pairwise comparisons in the underwater segments between laps 1, 2, and 3 are presented in **Figure 3**.

DISCUSSION

The present research aimed to examine the effect of extended underwater race sections on the physiological and biomechanical parameters of competitive swimmers. Despite underwater sections of the start and turns being the fastest parts of swimming races and their relative contribution increasing at the elite level, there is no information in the literature about the effects of the apnea periods on the physiological state of competitive swimmers. Our results indicate an increased rate of perceived exertion when swimming for the same overall time with extended underwater swimming conditions but non-significant differences

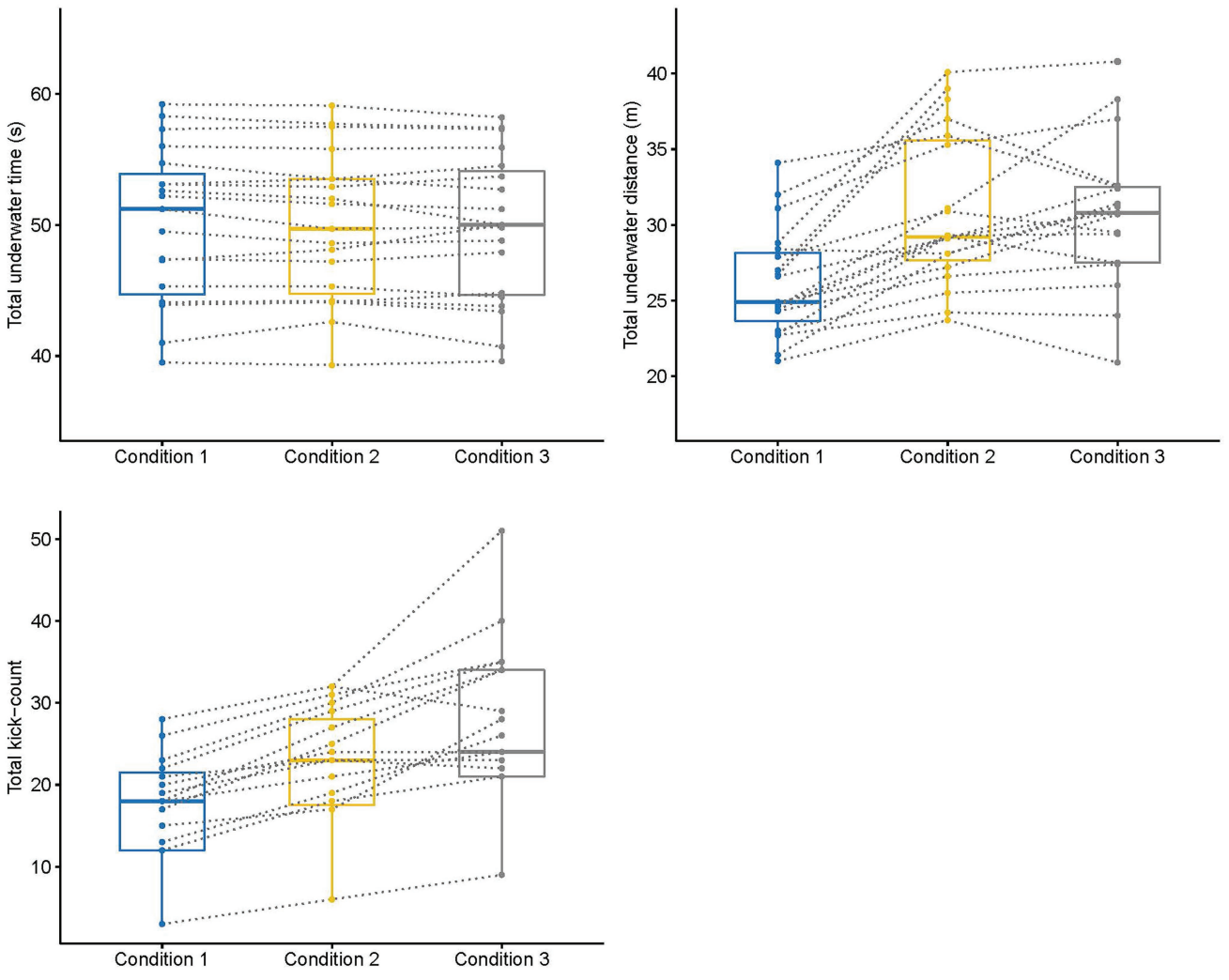

FIGURE 1 | Change of underwater parameters of competitive swimmers from free (condition 1) to constrained (conditions 2 and 3) underwater swimming conditions. Box plots indicate minimum, first quartile, median, third quartile, and maximum of the data, respectively.

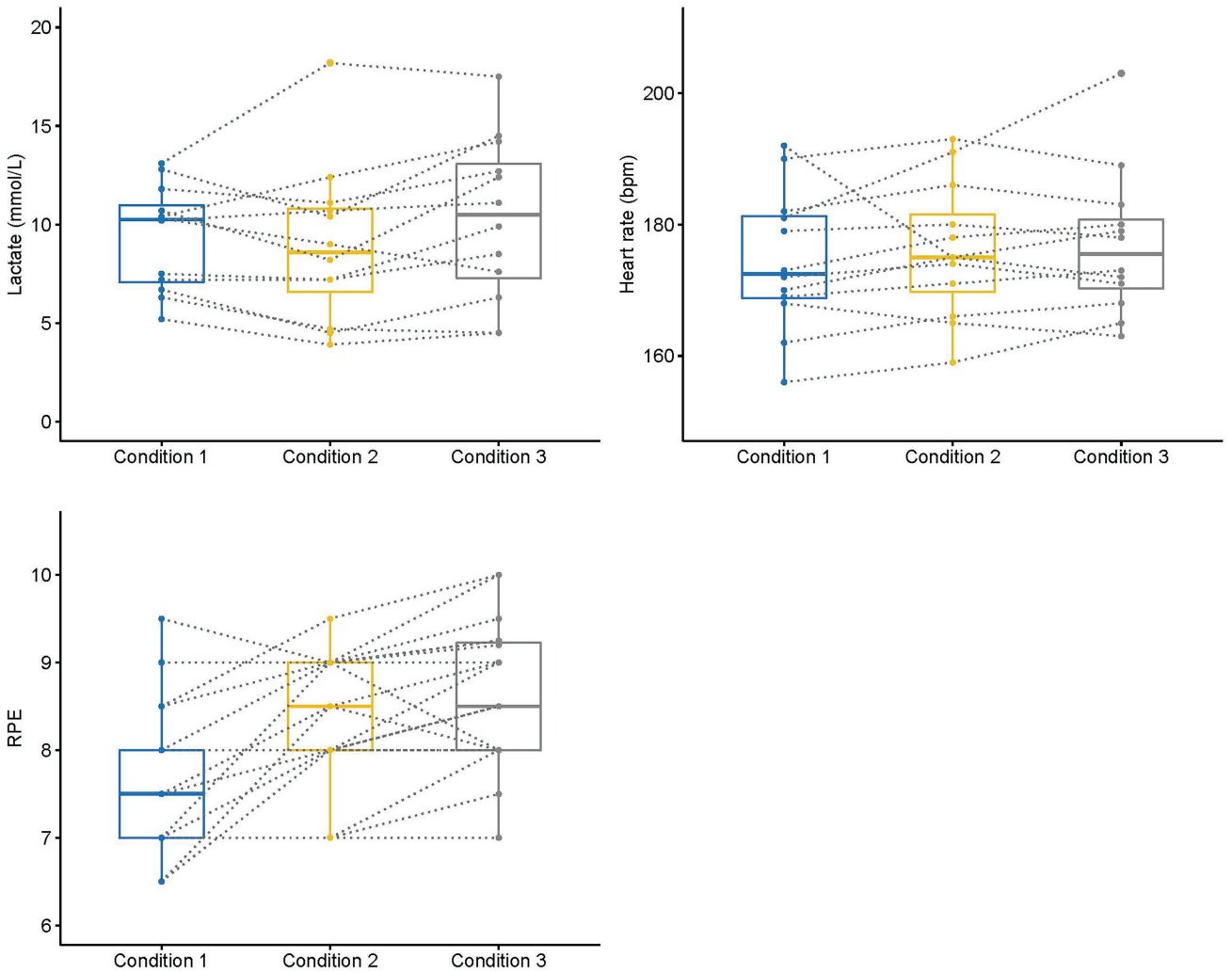

FIGURE 2 | Change of physiological parameters and perceived exertion of competitive swimmers from free (condition 1) to constrained (conditions 2 and 3) underwater swimming conditions. Box plots indicate minimum, first quartile, median, third quartile, and maximum of the data, respectively.

in the physiological parameters and similar modifications in the underwater segments from lap to lap. This is the first evidence in the literature for how changes in underwater swimming can affect competitive swimming performances.

As a first point of interest, the measurement of underwater sections in the different experimental conditions provided insightful data compared to previous research. When youth swimmers (male and females) spontaneously distributed their effort between underwater and surface sections (condition 1), they performed between one and two kicks less and traveled underwater distances around 1 m shorter than those reported for elite swimmers in the 200 m World Championships races (Veiga and Roig, 2016; Veiga et al., 2016). However, their average velocity during underwater swimming was considerably slower than that of male elite swimmers, who maintain values above 2 m/s (Pla et al., 2021). As expected, the average underwater velocity tended to decrease in the experimental conditions, where underwater sections lasted longer, but interestingly swimmers maintained their underwater velocity when they were constrained to adding only two underwater kicks per lap (condition 2). This could have strategic implications for swimmers and coaches, as athletes should aim to extend the underwater section provided the forward momentum does not fall too much (Veiga and Roig, 2017). When underwater distances were fully extended, while maintaining the total 75 m time (condition 3), the underwater distances of youth swimmers reached around 10 m with seven underwater kicks and altogether comprised 36% of the total swimming time. These values reached a similar contribution for the non-swimming times of international and national level male swimmers when at front-crawl events (Pla et al., 2021). Regardless of the experimental condition, distances traveled underwater from a dive start (lap 1) were 3–3.5 m longer than when pushing off the turning wall (laps 2 and 3). This represents a greater difference than the typical flight distances observed in elite youth swimmers during dive starts (Qiu et al., 2021), but differences were

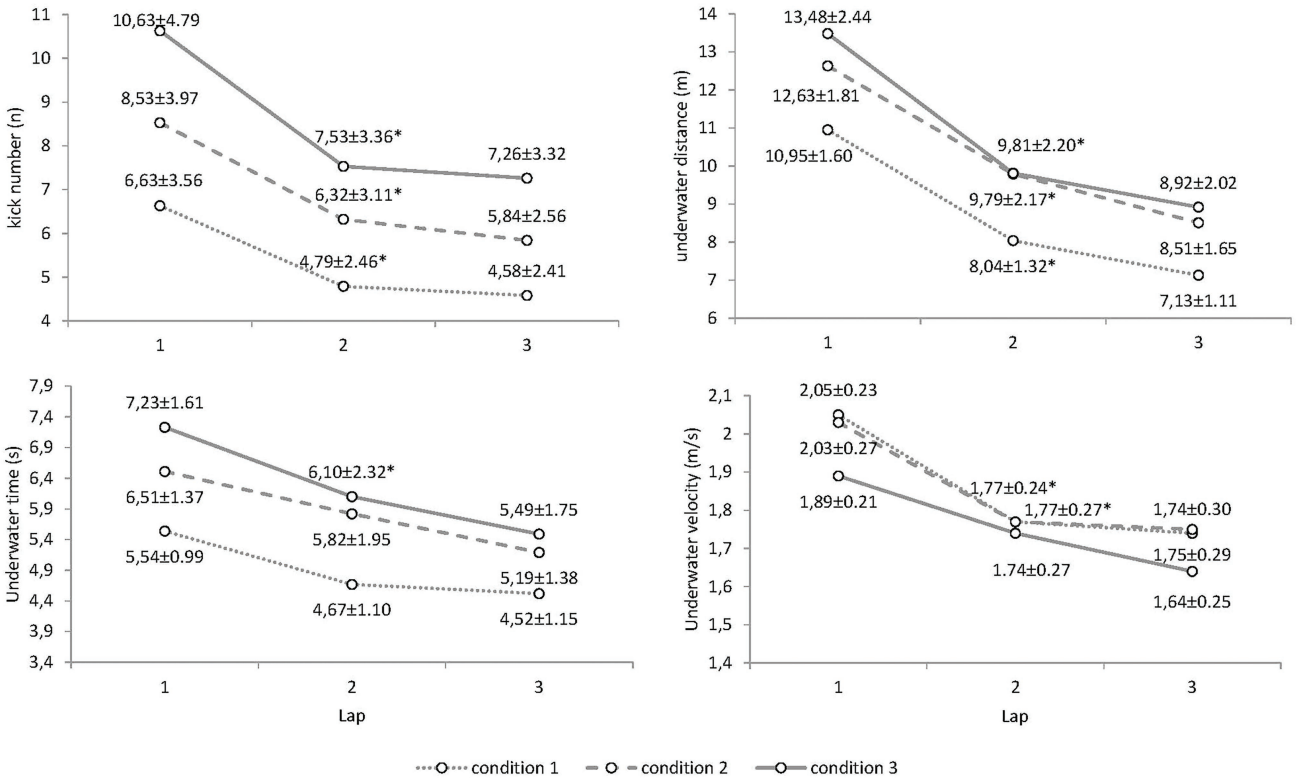

FIGURE 3 | Evolution of the underwater parameters of competitive swimmers in different underwater swimming conditions.

confirmed by the greater number of underwater kicks observed in lap 1 vs. laps 2 and 3 (**Figure 3**).

The evolution of underwater parameters displayed a typical decrease in distances and time spent underwater from lap to lap (**Figure 3**), probably explained by a continuous rise in blood lactate concentration during the race coupled with an increased over-time hypoxemic stimulus that probably increased the perception of effort (RPE). The time of the apnea and the exercise preceding the underwater swimming was also longer; however, when following a turn (laps 2 and 3) than during the dive start (lap 1), which could have a cumulative effect on the 75 m efforts. Youth swimmers in the present research were able to maintain the kick number and the underwater distances in the last turn in all the experimental conditions, as previously observed in elite swimmers (Veiga and Roig, 2016). In addition, underwater velocity values displayed practically no decrease in the last lap (**Figure 3**). This is something previously observed in elite races that confirms the importance of underwater sections to compensate for the loss of velocity during the surface swimming laps (Veiga and Roig, 2016). However, an important finding of the present research was that the changes of underwater sections did not depend on the apnea condition. Despite extended underwater sections revealing an increase in the rate of perceived exertion (**Table 2**), swimmers were able to maintain similar underwater changes from lap to lap when extending the underwater sections. Previous research on pacing has reported a lower performance level at the end of races when athletes have expended too much energy in the first part (Rodriguez and Veiga, 2018). However, our results indicate that when focusing on the underwater kick number, swimmers were able to improve their underwater performance despite an unavoidable increase in discomfort. Maybe the switching of attention from an internal focus (the difficulty and discomfort of apnea) toward an external focus (kick number) could represent a useful resource for improving performance (Lohse and Sherwood, 2011; Becker and Fairbrother, 2019) in swimming. Of course, simulations of race conditions in the present research occurred in 75 m efforts, where the number of consecutive turns was lower than in real competitions. However, this is the first study in the literature that examines underwater sections in 25 m pool races except that by Veiga et al. (2013a), which examined the 200 m backstroke event only.

For the heart rate values, no significant differences were observed between the experimental conditions. It is well-known that the magnitude of the diving response (and the consequent bradycardia) does not depend on the depth of diving but rather the length of the apnea (Schagatay and Andersson, 1998; Schagatay et al., 2000; Bosco et al., 2008). Therefore, it could be assumed that longer underwater swimming sections may imply a decrease in heart rate because of a more pronounced immersion time. This may be true for the present study, but the fact that heart rate was collected at the end of the 75 m efforts (and not at the end of the underwater sections) may

hinder this conclusion. It could happen that bradycardia occurring during extended underwater sections may be counterbalanced by a bigger increase in heart rate after the underwater part, leading to similar heart rate values at the end of the effort. Only if checking the instantaneous heart rate during efforts, as previous studies have done for artistic swimming (Rodríguez-Zamora et al., 2012) and surface swimming (Guimard et al., 2014, 2017, 2018), could it be possible to draw solid conclusions about this. Also, another factor that could hinder conclusions is the large individual variability observed in the bradycardia response to apnea, as found in the study by Lindholm et al. (2002). The decrease in heart rate can vary from 15 to 40%, but a small proportion of healthy individuals can develop bradycardia up to 20 beats per minute (Alboni et al., 2011). It is interesting to note that previous studies on swimming have shown that the expected bradycardia was only observed in athletes unable to maintain their performance in apnea conditions (referred to as "with a bad apnea capacity") during either 4 × 25 m (Guimard et al., 2014) or 50 m with fins (Guimard et al., 2017). Considering the high level of the swimmers, the present study was probably carried out by athletes "with a good apnea capacity" (Guimard et al., 2017), capable of maintaining an optimal cardiac output under conditions of intense dynamic apnea and thus their performance. Indeed, this could be confirmed by the similar relative decrease in underwater parameters lap to lap regardless of the longer apnea times requested (**Figure 3**).

Since some studies have found an increase in lactatemia in dynamic apnea (Matheson and McKenzie, 1988; Kume et al., 2013, 2016), one might logically expect that blood lactate concentration would be increased under conditions, where the duration of apnea is longer. However, there is some controversy in the literature as some swimming studies and other exercise modalities have never found an increase in lactatemia in apnea (Kjeld et al., 2009; Guimard et al., 2014, 2017, 2018). The present study presented no statistical differences between conditions but an increasing tendency of blood lactate concentration when underwater distances were maximum (condition 3). This could be explained by the swimmers' effort regulation between the underwater and surface segments of 75 m laps. Indeed, swimmers had to keep the same overall time in the 75 m efforts, while modifying the extension of the underwater sections. Therefore, they could have adjusted the underwater over the surface swimming to complete the trial in the required pace. Results indicate that, in condition 2, swimmers maintained their underwater velocities but they slowed down on the surface swimming compared to the free underwater (condition 1). On the other hand, in condition 3, swimmers decreased their underwater velocities but maintained the swimming pace on the surface compared to condition 1 (**Table 2**). We can hypothesize that if swimmers had to swim the 75 m distances at a maximal effort with constrained underwater swimming, they probably would not be able to sustain this effort and maybe some differences in lactate concentration could be observed. In addition, the fact that lactatemia in the blood reflects the production but also the use of lactate as an energy substrate in apnea (Joulia et al., 2002) could hinder some differences in the lactatemia between conditions. Another suggestion is that in apnea the hypercapnia, i.e., the accumulation of CO_2 (and therefore H^+), could limit the output of muscle lactate (accompanied by H^+; Lancha et al., 2015). Nevertheless, in the present study, only one blood lactate sample was taken for each swimmer at 1 min post-exercise and this may be too early. The procedure was employed to avoid interruptions on the training session and also based on the authors' experience with young swimmers performing a non-maximal effort, where 1 min post-exercise samples were considered the most representative to estimate lactate peak. However, the phenomenon of vasoconstriction caused by the diving response could induce a delayed release of muscle lactates into the blood stream (Guimard et al., 2014). In a further study, it would be interesting to collect lactatemia at different times after the end of the race in order to identify the kinetics of changes and the peak of lactatemia.

Despite the lack of changes in the physiological markers, competitive swimmers reported a higher RPE in the constrained (conditions 2 and 3) vs. the free underwater swimming condition, and this would reveal a higher intensity perceived by the swimmers when they performed longer underwater sections. A previous study had reported higher RPE in swimmers in apnea than in normal breathing conditions when swimming at a 400 m race velocity (Guimard et al., 2018). The reason for this is the apnea-induced hypercapnia associated with prolonged exercise duration (Woorons et al., 2007), which causes discomfort, and it could also be the reason for swimmers perceiving a higher level of effort during longer underwater swimming. Lack of differences in RPE between the conditions 2 and 3 (where swimmers performed maximum underwater distances) could be explained by the lack of differences in the total underwater distances between conditions (**Table 2**), despite the tendency for swimmers to perform one more kick in condition 3. Swimmers tried to maximally extend the underwater swimming in condition 3, but their underwater kicking was probably not efficient enough to maintain the distance per kick at the end of underwater sections (Zamparo et al., 2012). Therefore, swimmers should probably try to extend underwater swimming provided: (1) the underwater kicking efficiency is keeping at the same level, (2) the average underwater velocity does not slow down, (3) the evolution from lap to lap is maintained, and (4) the physiological parameters and RPE do not present large modifications. In this context, the use of an RPE-type scale seems to constitute an interesting tool to manage the internal load related to the different apnea conditions while swimming.

Results of the present research involve some limitations that should be acknowledged in order to adequately draw conclusions. First, 75 m distances were not swum at a maximal effort, which may allow swimmers to regulate their effort between the underwater and surface swimming segments. Future studies where maximal efforts are performed by competitive swimmers could inform about the overall effect of changes in underwater parameters. Second, the data collection about the physiological parameters was after the end of the event and not after the

end of the underwater part. This could explain why few differences in lactatemia or heart rate were observed between conditions considering that the final time was the same for each condition. Also, a measurement of gas exchange and blood samples right after each underwater section could inform in particular about the kinetics of O_2 and CO_2 to further clarify the effects of the experiment. Third, the fixed order on the experimental conditions could have some influence on reported data. This experimental design allowed to examine the spontaneous underwater pattern of swimmers during set 1, but it could have also affected reported values of RPE after the first set. Finally, data collection was performed during 75 m efforts at the swimmers' preferred stroke that did not represent an official competitive distance, although it allowed for the research purposes to repeat different experimental conditions within the same experiment.

CONCLUSION

The 200 m race pace efforts of competitive swimmers displayed an increased rate of perceived exertion when increasing the underwater swimming duration but non-significant differences in the physiological parameters. In addition, the evolution of the underwater parameters from lap to lap displayed similar changes in free vs. extended underwater swimming conditions. Effort regulation between the underwater and surface sections could explain the lack of differences in the physiological parameters of swimmers despite a higher effort perceived during longer underwater swimming. The control of the underwater kick number was revealed as a useful resource to optimize the underwater swimming performances provided the average underwater velocity was maintained. Also, the use of an RPE-type scale could represent an interesting tool to manage the internal load in different apnea conditions while swimming. However, in order to fully understand the changes in underwater sections on performance, testing on maximal swimming efforts should be conducted.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comité Etico Universidad Politécnica de Madrid. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

SV and DB contributed to the conception and design of the study. DB and XQ organized the database. XQ performed the statistical analysis. SV, XQ, RP, and AG performed the data analysis and the interpretation of the data. SV, RP, and AG contributed to the preparation and critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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