Six weeks of static apnea training does not affect Hbmass and exercise performance.

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Abstract

Purpose: Acute apnea is known to induce decreases in oxyhemoglobin desaturation (SpO_2) and increases in erythropoietin concentration ([EPO]). This study examined the potential of an apnea training program to induce erythropoiesis and increase hematological parameters and exercise performance.

Methods: Twenty-two male subjects were randomly divided into an apnea and control group. The apnea group performed a 6-week apnea training program consisting of a daily series of 5 maximal static apneas. Before and after training, subjects visited the lab on three test days to perform 1) a ramp incremental test measuring $\dot{V}O_{2peak}$, 2) CO-rebreathing for Hbmass determination and a 3-km time trial and 3) an apnea test protocol with continuous finger SpO_2 registration. Venous blood samples were drawn before and 180 minutes after the apnea test for analysis of [EPO].

Results: Minimal SpO_2 reached during the apnea test protocol was 91 ±7% pre and 82 ±7% post apnea training. The apnea test protocol did not elicit an acute increase in [EPO] (p=0.685) before nor after the training program. Consequently, resting [EPO] (p=0.170), Hbmass (p=0.134), $\dot{V}O_{2peak}$ (p=0.796) and 3-km cycling time trial performance (p=0.509) were not affected either.

Conclusion: The apnea test and training protocol, consisting of 5 maximal static apneas, did not induce a sufficiently strong hypoxic stimulus to cause erythropoiesis and therefore did not result in an increase in resting [EPO], Hbmass, $\dot{V}O_{2peak}$ or time trial performance. Longer and/or more intense training sessions inducing a stronger hypoxic stimulus are probably needed to obtain changes in hematological and exercise parameters.

New and noteworthy

Apnea training has been suggested as a promising method to improve exercise performance for over a decade. However, this study is the first to evaluate its value on both hematological parameters and exercise performance, including Hbmass and a control group. No changes in Hbmass nor exercise performance were observed. Contradicting previous research, no acute increase in [EPO] following apnea was observed either, indicating that more intense protocols are needed, at least in non-apnea trained individuals.

Key words

Apnea training, desaturation, EPO, hemoglobin mass, performance

Running title

Static apnea training, Hbmass and performance

Introduction

Oxygen (O_2) supply and transport capacity to the working muscle are important determinants for exercise performance, especially in activities with predominantly aerobic energy turnover. Most of the O_2 in the blood is bound to hemoglobin (Hb), an iron-containing globular protein within the red blood cells, while the amount of O_2 dissolved in the plasma is only trivial (1). The oxygen transport capacity of the body is therefore largely determined by the amount of Hb. Indeed, Schmidt and Prommer (2) have shown a strong correlation between hemoglobin mass (Hbmass) and maximal oxygen uptake ($\dot{V}_{O_2}$max) with an increase of 1 g in Hbmass corresponding to an increase of 4 mL.min^{-1} in $\dot{V}_{O_2}$max. This relationship has been confirmed experimentally by showing that lowering Hb through phlebotomy and blood donation decreases oxygen uptake and performance (3–6) while increasing Hb enhances $\dot{V}_{O_2}$max and exercise performance (4–7).

In this context, apnea training has been proposed as a new training method to improve exercise performance (8). Indeed, cross-sectional data show acute increases in erythropoietin concentration ([EPO]), the main hormone regulating the volume of red blood cells (9), in response to different apnea protocols in trained and/or elite breath-hold divers (10–12). Additionally, baseline [EPO] levels are elevated in patients suffering from sleep apnea compared to healthy controls (13).The lack of breathing during apnea causes a depletion of the oxygen stores of the body and leads to decreases in arterial oxygen saturation (SaO_2) (19). This may therefore act as a stimulus for an acute increase in EPO production, with the post-apneic [EPO] value seemingly dependent on the level of hypoxia (11). Serum EPO levels therefore increase in a dose-dependent manner in response to reductions in blood oxygenation (16–18). However, acute effects of apnea on [EPO] have barely been investigated in individuals naïve to

breath-holding, with the only study to date failing to observe any differences (11). Notably, apnea duration was very short and arterial oxygen saturation barely decreased in that study compared to training research in similar populations (20, 21).

Contrastingly, there are some longitudinal indications that an apnea training program might improve hemoglobin content in non-apnea trained subjects. First, apnea duration increases quickly during a static training study in naïve subjects (20, 21) leading to stronger desaturation after only two weeks of apnea training (20). Second, an increase in reticulocytes, which are immature red blood cells, has been observed after two weeks of static (20) and six weeks of dynamic apnea training (22) in naïve subjects. The effect on hemoglobin concentration ([Hb]) on the other hand, shows mixed results with some studies observing no changes (20, 22) while one study observed a small albeit significant increase in [Hb] following 8 weeks of apnea training (21). Third, improvements in $\dot{V}_{O_2}$peak (23) and lung function tests (23, 24) have been observed after either three months of dynamic or four weeks of static apnea training. However, these improvements did not translate into improved swimming performance.

Although these studies reported some promising data suggesting potential beneficial effects of apnea training on hematological parameters and exercise performance, some methodological precautions should be considered. Up to date, only changes in [Hb] have been reported which is known to be highly influenced by changes in plasma volume through body position (25), exercise and hydration status (26). As venous blood sampling is often not standardized in these studies, interpreting data is difficult (27). This indicates the need for the use of Hbmass to evaluate the hematological effects of apnea training. Additionally, the lack of a control group makes it difficult to evaluate the importance of longitudinal effects.

79 The aim of the study is therefore twofold: (1) to examine the effects of a six week static apnea
80 training program on hematological parameters and its possible relationship with peak oxygen
81 uptake and exercise performance compared to a control group and (2) to unravel the possible
82 underlying physiological mechanisms. We hypothesized that 1) apnea training sessions would
83 lead to an acute increase in [EPO] which would be enhanced after training and therefore 2)
84 Hbmass would increase during the training period which would improve $\dot{V}_{O_2}$peak and 3km time
85 trial performance. These parameters are expected to remain stable in the control group.

Methods

Ethical approval

All protocols and procedures were conform to the Declaration of Helsinki. Ethical approval was obtained from the local ethical committee of the Ghent University Hospital (EC UZG 2018/0221). Each participant was informed about the procedure and the aim of the study and gave their written informed consent. Subjects underwent a medical examination including a medical history questionnaire prior to the study. All volunteers were declared to be in good health.

Subjects

Twenty-two male subjects naïve to breath-holding volunteered to participate in this study. Inclusion criteria for selection were: age (18-35 years old), sex (male), being physically active and in good health. Exclusion criteria were: smoking, blood donation and residence at high altitude within 3 months prior to the study, experience with breath-holding and clinically low iron values. Participants were 27.1 ± 3.5 years old with an average height of 180.1 ± 8.5 cm and weight of 72.5 + 8.3 kg and practiced physical activity in a wide variety of sports on a self-reported basis of 7.0 ± 2.6 hours a week. Subjects were instructed not to serve as blood donor nor travel to high altitude and were instructed to maintain the same level of physical exercise during the intervention period. Due to national Covid-19 restrictions, not all subjects were able to maintain their physical activity. The effect of these restrictions on their physical activity has been monitored through a questionnaire.

Procedures and measurements

Overview

The study covered a period of 11 weeks and consisted of two phases (Figure 1): a familiarization and intervention phase. All test sessions took place in the Sport Science Laboratory Jacques Rogge (Ghent, sea level) with constant ambient air temperature of 18°C and relative humidity of 48%. In the first phase, subjects visited the laboratory two times separated by two weeks for familiarization for 3km time trials (TT). This method of two prior familiarizations has been shown to lead to a typical error varying between 1.7 and 3.5 s or 0.5 and 1.3% in our lab. At the end of the first phase, subjects were randomly assigned to either the control or experimental group in a counterbalanced fashion in order to match both groups for age, length, height and weight. The second phase was the intervention phase (week 5 – 12). Subjects visited the laboratory 5 (control) or 8 (apnea group) times: in the week before (PRE), after three (MID) and six weeks (POST) of intervention. Tests in the PRE and POST week were identical. On test day A, venous blood values and Hbmass were measured and a 3km TT was performed. On test day B, a maximal incremental exercise test was performed, while test day C was only for the experimental group who performed an apnea test analyzing the acute EPO response. During the MID test, venous blood parameters and Hbmass were analyzed for the control group (Test day A), while an additional control condition for EPO response was performed for the apnea group to control for possible diurnal changes in [EPO]. Test days were in a random order with each type of test on the same time of the day and the same day of the week for each individual. Subjects were instructed not to perform exercise nor consume alcoholic beverages and maintain a similar diet 24h before each test.

Apnea training protocol

The training protocol consisted of a six week daily static apnea training program that subjects performed at home. The protocol was similar to our previous training study (21) and designed

to be practically feasible in combination with regular training and to obtain decent training adherence. Subjects daily performed a series of 5 maximal static breath-holds with 30-s rest intervals. Subjects were not allowed to hyperventilate and instructed to inhale deeply but not maximal and hold their breath as long as possible. Subjects registered their apnea times in an online diary which was used to track the training adherence and progress of the subjects. The control group was instructed to continue their normal physical activities. Training continued during MID test week. In this period, training sessions were performed at home after the tests in the laboratory to avoid the effect of the training session on the test results.

Test day A

Hb and Hbmass

Venous blood samples (K3 EDTA 4mL, Vacutest Kima, Azergrande PD, Italy) were drawn from the V Mediana Cubiti at the PRE, MID and POST test. Venous blood samples were always drawn at the same time of the day and same day of the week and in accordance with WADA guidelines: sampling occurred after 15 minutes of seated rest and tourniquet application did not exceed 60 seconds in order to minimize changes in plasma volume (28). Blood samples were kept cool (2-12°C) during storage and transport and were analyzed within 48 hours. Samples were analyzed at a WADA accredited laboratory (Doping Control Laboratory, Ghent) for analysis of [Hb] and Hct, reticulocytes and immature reticulocyte fraction (IRF) using an automated hematology analyzer (SYSMEX XN 1000, Sysmex Corporations, Kobe, Japan) according to WADA guidelines (29).

Hemoglobin mass was measured at the PRE, MID and POST test with the optimized carbon monoxide (CO) rebreathing method as described by Schmidt and Prommer (30, 31). Application of this method in our lab has a typical error of 15.4 g or 1.76%. The volume of CO that subjects

had to inhale was calculated as 1.0 mL x (kg body mass)$^{-1}$. After fully exhaling, subjects inhaled the CO bolus followed by 3L of medical oxygen through a glass spirometer (SpicCo, BloodTec, Bayreuth, Germany) and after a 10 second breath-hold rebreathed for 2 minutes. End tidal CO was measured before and 4 minutes after the start of inhalation (Draeger PAC 6000, Draeger, Luebeck, Germany). Capillary blood samples were collected from the fingertip before and 6 and 8 minutes after start of CO inhalation and analyzed for carboxyhemoglobin (COHb in %) using Radiometer ABL 90 Flex (Radiometer, Copenhagen, Denmark). Hemoglobin mass was calculated according to Equation 1 (30):

(1) $\text{Hbmass (in grams)} = K \times MCO \times 100\ (\Delta COHb\% \times 1.39)^{-1}$

With K calculated as current barometric pressure in mmHg x 760^{-1} x (0.003611 x current temperature), MCO calculated as the volume of CO administered minus the sum of the volume of CO not taken up by the body and the volume of CO exhaled after rebreathing. ΔCOHb% is the difference of the average COHb% obtained 6 and 8 minutes after inhalation and COHb% before administration. 1.39 is Huffners number (Gorelov 2004).

3km time trial

During the two familiarization trials, subjects performed a 3km cycling TT at a self-selected gear and pacing strategy. Experimental 3km TT were performed PRE and POST training and combined with Hbmass determination. After a standardized warm up (10 minutes at GET with three intervals of 1 minute at RCP as calculated from their ramp incremental exercise test), subjects started the 3km time trial 50 minutes after the start of CO inhalation. Time, continuous and average power and cadence were recorded by the Cyclus 2 Ergometer Software.

Test day B: Ramp incremental exercise

Ramp incremental exercise tests were performed PRE and POST training on an electromagnetically braked ergometer (Lode Excalibur Sport, Groningen, The Netherlands). After 3 minutes of cycling at 50W, work load consistently increased with 30 W . min^{-1}. Subjects were instructed to keep cadence constant between 80 and 100 rpm and consistent between tests. The test was finished at volitional exhaustion: when subjects were not able to maintain cadence above 80 rpm for more than 5 seconds. Pulmonary gas exchange was measured on a breath-by-breath basis (Metalyzer 3B, Cortex, Leipzig, Germany). Maximal power output (P_{Max}) was defined as the highest values obtained during the ramp test, while $\dot{V}_{O_2}$peak was defined as the highest 30 s average observed during the ramp incremental exercise test. GET and RCP were derived from the maximal incremental test by three independent researchers. GET was defined as the point where [1] $\dot{V}_{CO_2}$ increases disproportionate to $\dot{V}_{O_2}$, [2] the first deviation of the linear increase in minute ventilation (VE) occurs and [3] an increase in VE/$\dot{V}_{O_2}$ without simultaneous increase in VE/$\dot{V}_{CO_2}$ is observed. RCP was defined as the point where [1] the second deviation of the linear increase in VE occurs, [2] an increase in VE/$\dot{V}_{CO_2}$ is observed (32). These breakpoints were corrected for the $\dot{V}_{O_2}$ mean response time (33).

Test day C: Apnea test protocol

Subjects in the experimental group performed the first (PRE) and last (POST) apnea training in the lab. Subjects performed the same protocol as during training: a series of 5 maximal seated static breath-holds interspersed with 30s rest intervals. Subjects were not allowed to hyperventilate and instructed to inhale deeply but not maximally and hold their breath as long as possible. Subjects were notified 10 s prior to each breath-hold and started inhaling after a 5 s countdown. Apnea duration was continuously monitored using a stopwatch, while oxygen saturation (Beurer PO80, Beurer Medical, Ulm, Germany) was measured at the left index finger.

Venous blood samples (Vacutainer gel and clot activator, 3,5 mL, Kima, Azergrande PD, Italy) were drawn from an antecubital vein before apnea and 150 minutes after the last apnea. All samples were drawn after 15 minutes of seated rest. In order to assess the natural diurnal variation of the subject, a control condition was performed during the MID test. Venous blood samples were taken at the same time of day as the PRE test, while the subject remained seated and no apneas were performed. Blood samples were centrifuged for 5 min at 3500 rpm at 4°C. Serum was stored at -20°C and analyzed within three months of sample collection. After thawing and homogenization, the samples were analyzed via a chemiluminiscent immunoassay designed for EPO (ADVIA Centaur XP, Siemens Healthineers, Germany). Maximal apnea time during the apnea protocol at PRE and POST test was defined as the group average of the duration of the longest of the 5 apnea bouts for each individual. Minimal oxyhemoglobin desaturation (SpO_2) was defined as the lowest value of SpO_2 from the start of the apnea protocol till 1 min after completion of the last apnea. Δ[EPO] was calculated by subtracting [EPO] at 150 min after apnea from the baseline [EPO] value. Average training breath-hold time was calculated for each week as the average of all breath-holds performed during that week (7 days of 5 breath-holds).

Statistical analysis

All data were expressed as mean ± SD. IBM SPSS statistics 26 package was used for the statistical analyses. Shapiro-Wilks tests indicated a normal distribution for all parameters, while Mauchly's test of Sphericity was used to test for sphericity. When sphericity was violated, Hyun-Feld correction was applied. Homogeneity of variance was tested by a Levene's test. Statistical significance was set at $p < 0.05$ for all tests.

A one way Manova was used to check for differences between both groups (apnea and control) in subject characteristics for the dependent variables age, height, weight, physical activity and $\dot{V}_{O_2}$peak. A 2x2 Repeated Measures Anova (Test Day x Group) was used to check if the physical activity changed throughout the protocol (PRE and POST training) and whether this was different between groups (apnea and control).

A 1x6 Repeated Measures Anova was used to evaluate the week-by-week evolution in average training breath-hold times over the six week period (Within subject factor: week 1 to 6). A 1x2 Repeated Measures Manova was used to analyze the acute changes (BL and 150 min post apnea) in [EPO] following an apnea protocol before (PRE) and after (POST) training and a diurnal control protocol. A 2x6 and 2x7 repeated measures Manova (Test Day x Time Point) was used to evaluate differences in evolution of breath-hold time and SpO_2 during the series of apneas (baseline, apnea 1 to 5 and minimal value) and between PRE and POST tests. Least square differences (LSD) and two by two analysis were applied as post hoc tests to assess the differences between apneas and PRE and POST.

Training effects on blood values [Hb], Hct, Ret, IRF and Hbmass were analyzed using a 3x2 Repeated Measures Manova (Test day x Group) with Test Day (PRE, MID, POST) as a within subject factor and Group (Control, Apnea) as between subject factor. For baseline [EPO], $\dot{V}_{O_2}$peak and performance variables (, P_{Max}, 3km time trial time and power), a 2x2 Repeated Measures Manova (Test day x Group) was used with Test Day (PRE and POST) as within and Group (Control and Apnea) as between subject factor.

Results

Subject characteristics

Subject characteristics are displayed in Table 1. The one way Manova revealed that age (F = 0.096, p=0.760), height (F = 0.062, p = 0.806), weight (F = 0.651, p = 0.429), self-reported physical activity (F = 0.077, p = 0.784) and $\dot{V}_{O_2}$peak (F = 0.004, p = 0.953) did not differ between groups (Table 1). A significant main effect (F = 7.120, p = 0.017) showed that physical activity decreased during the training period from 7.0 ± 2.6 to 5.9 ± 3.5 hours per week in the control group (CO) and 7.1 ± 2.7 to 5.6 ± 2.9 in the apnea group (AP). This change was not different among groups (F = 0.660, p = 0.429).

Training data

Subjects (apnea training group) reached a good training adherence, completing 85 ± 17% of their training sessions. Average training breath-hold times for the five attempts increased during the 6-week training period from 88 ± 26 s in the first week to 110 ± 36 s in the last week of training (F = 8.809, p = 0.004, Fig 2).

Apnea test

Apnea tests were only performed by the apnea training group. No Test Day x Time Point interaction effect could be observed for apnea duration (F = 1.658, p = 0.296) indicating that the duration of apneas during the protocol evolved similarly PRE and POST training. Main effects for Test Day (F = 45.975, p < 0.001) and Time Point (11.260, p = 0.009) were found to be significant. The longest recorded breath-holds during the PRE and POST test were attained at either the fourth or fifth apnea. Apnea duration increased from the first to the longest apnea, both PRE (p=0.003, from 67± 19 s to 118 ± 54, Fig 3A) and POST (p = 0.002, from 109 ± 37 s to 155 ± 53 s) training (Fig 3A). Each apnea from the POST test was significantly longer than the respective apnea from the PRE training test (p < 0.05).

Test Day x Time Point interaction effect only showed a trend to significance (F = 5.857, p < 0.001) suggesting that saturation decreased stronger during the breath-hold series in the POST compared to the PRE test. Significant main effects were found for both Test Day (F = 21.753, p < 0.001) and Time Point (F = 8.757, p < 0.001). The lowest observed saturation during the series was usually obtained during the fourth or fifth breath-hold and decreased from 98 ± 1% to 91 ± 7% PRE training (p = 0.016) and from 98 ± 1% to 82 ± 7% POST training (p < 0.001, Fig 3B).

Saturation significantly decreased compared to BL in all five breath-holds during the POST test ($p < 0.05$) but only during the second and fifth in the PRE test ($p < 0.05$) while it tended to be lower at the end of the third and fourth breath-hold ($p = 0.074$ and $p = 0.061$).

The apnea test protocol did not change [EPO] values from BL to 150 min after apnea before training (PRE, $F = 1.318$, $p = 0.281$), after training (POST, $F = 1.098$, $p = 0.322$) nor during the diurnal control condition during the MID test ($F = 0.010$, $p = 0.924$, Fig 4A).

Training effect on blood values

Baseline blood values are summarized in Table 2. No significant interaction effects (Test day x Group) were observed for the blood values [Hb] ($F = 0.588$, $p = 0.560$) Hct ($F = 1.288$, $p = 0.287$), Ret ($F = 2.492$, $p = 0.096$), baseline [EPO] ($F = 0.002$, $p = 0.963$, Fig 4B) nor Hbmass ($F = 0.712$, $p = 0.497$, Fig 5A) indicating that there was no effect of the apnea training intervention on these parameters. Additionally, no main effects were found for the blood values [Hb] ($F = 0.675$, $p = 0.515$), Hct ($F = 0.239$, $p = 0.788$), Ret ($F = 0.553$, $p = 0.580$), IRF ($F = 1.831$, $p = 0.173$), [EPO] ($F = 3.564$, $p = 0.074$) and Hbmass ($F = 2.547$, $p = 0.092$, Fig 5A) indicating that blood values did not change throughout the training period. However, the evolution in IRF differed between groups ($F = 3.142$, $p = 0.043$, Fig 5B) with a significant increase in the apnea group from MID to POST ($F = 6.807$, $p = 0.026$, from 6.6 ± 1.6 to 8.1 ± 2.0%) and decrease in the control group from PRE to MID ($F = 5.239$, $p = 0.045$, from 8.4 ±3.9 to 7.0 ± 1.2%).

Training effect on maximal incremental exercise test and 3km time trial

No interaction effects were observed for absolute $\dot{V}_{O_2 peak}$ ($F = 2.309$, $p = 0.145$), P_{Max} ($F = 0.051$ $P = 0.824$), 3km TT time ($F = 0.010$, $p = 0.923$) nor 3km TT power ($F = 0.425$, $p = 0.522$) indicating that there was no effect of the apnea training intervention on these parameters. Main effects however, showed that P_{Max} decreased from 375 ± 41 to 367 ± 45 W ($F = 9.083$, $p = 0.007$,) and that absolute $\dot{V}_{O_2 peak}$ values for the total population decreased from 3.73 ± 0.52 to 3.63 ± 0.59 L . min^{-1} ($F = 5.960$, $p=0.025$, Fig 6A). Additionally, main effects showed that there was no difference in 3km TT time (262.3 ± 14.0 s PRE and 263.0 ± 14.4 s POST, $F = 0.454$, $p = 0.509$, Fig 6B) nor average power output (328.5 ± 49.1 W PRE and 328.3 ± 50.6 W POST, $F < 0.000$, $p = 0.998$) before and after the intervention.

Discussion

This study is the first to holistically examine the impact of 1) an apnea training session on the acute [EPO] response PRE and POST training and 2) a six week apnea training protocol on both hematological and performance parameters. The novelty of the study lies first within the holistic approach of combining hematological and performance parameters which allows us to interpret both the effect on performance and the underlying physiological mechanisms starting from arterial desaturation over EPO, IRF, reticulocytes and Hbmass to $\dot{V}_{O_2 peak}$. Second, this study addresses methodological concerns in previous research concerning blood values by standardization of venous blood sampling (27), by measuring Hbmass and by the addition of a control group.

Despite good training adherence, the effectiveness of the training in improving breath-hold time and the ability to reach lower SpO_2 values during apnea following training, no acute increases in [EPO] in response to an apnea training session were observed. Accordingly, no increases in Hbmass, nor improvements in $\dot{V}_{O_2 peak}$ or 3km time trials were observed. This suggests that 6 weeks of static apnea training consisting of a daily protocol of 5 maximal breath-holds is not sufficient to improve hematological parameters and exercise performance in naïve subjects.

Contrary to our first hypothesis and previous research (10, 11), the applied apnea protocol consisting of 5 maximal static apneas did not induce an acute increase in [EPO], neither before, nor after training. In order to cause an acute increase in [EPO], the apnea protocol needs to cause a sufficiently strong hypoxic condition to stimulate the hypoxia-inducible factor (HIF) complex. Measures of HIF signaling would therefore be informative in providing important

321 insight into the lack of the expected [EPO] response. HIF's are known to coordinate the
322 transcriptional responses during hypoxia in the cell at different levels, with HIF-2α being the
323 main factor involved in the regulation of [EPO] (14, 15). Under normal, well-oxygenated
324 conditions, HIF-2α is degraded in a series of biochemical pathways. Under hypoxic conditions
325 however, this degradation is inhibited, which stabilizes the HIF complex and allows for
326 transcriptional activation and ultimately the stimulation of EPO production, the primary
327 regulator of erythrocyte formation (9). In this study, subjects reached on average a minimal
328 SpO_2 of 91 ± 7% and 82 ± 7% over the entire series in the PRE and POST-test respectively, while
329 previous studies which observed increases in [EPO], reported average minimal post-apneic
330 values of 72 ± 11% and 76 ± 5% (10, 22). These results suggest that in order to induce an acute
331 increase in [EPO], a strong decrease in SpO_2 is essential. Second, the total time spend in hypoxia
332 might also be important to induce a sufficient hypoxic stimulus. The total length of the apnea
333 protocol including rest was on average around 10 minutes before and 12 minutes after the 6-
334 week apnea training program. For instance, in the study of de Bruijn et al. (10) subjects
335 averaged 12 minutes beneath an SpO_2 of 85% spread over 15 breath-holds, leading to an [EPO]
336 increase of 24%. The observed increases in [EPO] following acute breath-holding are in strong
337 contrast to current guidelines (34) in high altitude research, recommending a hypoxic exposure
338 of 20 to 22 hours a day for three to four weeks at an altitude between 2000 and 2500 m (35)
339 for optimal hematological effects, although shorter protocols have also been recommended
340 (36). However, Balestra et al. (37) suggested that relative changes in oxygen availability, rather
341 than a constant steady-state hypoxic state are important in HIF transcriptional effects,
342 explaining how intermittent hypoxia (38) and apnea protocols (10, 11) elevate [EPO] despite
343 the relatively short time spent in hypoxia. The current protocol probably did not elicit a
344 sufficient change in oxygen availability as shown by the modest decrease in SpO_2, or the total

time spend in hypoxia was insufficient or a combination of both. Therefore, heavier protocols eliciting longer, stronger and more frequent decreases in SpO_2 seem to be needed to obtain acute changes in [EPO].

Our second hypothesis was directly influenced by the ability of the apnea protocol to elicit an acute increase in [EPO]. Therefore, it is unsurprising that, despite a good training adherence of 85%, there was no effect of the 6-week apnea training program on resting [EPO] levels, [Hb], Hct nor Hbmass. Additionally, $\dot{V}O_{2peak}$ and performance on a 3-km time trial were not influenced by the training program either. The lack of change in hematological parameters is conflicting with previous research which had shown increases in reticulocytes of 15% after two (20) and 26% after six weeks of training (22). However, both studies imposed a stronger hypoxic stimulus with subjects reaching lower minimal SpO_2 values (20) and performing more and longer apneas (20, 22). Interestingly, neither of these studies observed changes in [Hb] nor measures Hbmass. Additionally, [EPO] only increased acutely PRE training while the training session induced no acute [EPO] changes after 3 and 6 weeks of dynamic apnea training (22). Lastly, Bouten et al. (21) found an increase in [Hb] of 3.3% but did not find changes in reticulocytes nor Hct after 8 weeks of apnea training. As our apnea training protocol is similar to that of Bouten et al. (21), we suspect that the increase in [Hb] in the previous study can probably be attributed to plasma and blood volume changes as only [Hb] was measured and not Hbmass.

IRF showed positive changes to the current apnea training program compared to the control group. This increase could be of significance as an elevated proportion of immature reticulocytes is an early indicator of increased erythropoietic response in the bone marrow and may precede a measurable increase in the absolute reticulocyte count (39, 40). Notably, the 13% increase in IRF occurred from MID to POST. This could mean that, as the subjects were

able to complete 31% longer apneas and reach a lower minimal SpO_2 throughout the program, the hypoxic stimulus increased in the second part of the training period. However, caution is warranted when ascribing the differences in IRF to the apnea training protocol. First, an increase in IRF should be preceded by an increase in erythropoietic activity as IRF can be seen as an indicator of erythropoietic activity (41). However, in the present study there was no indication of increased erythropoietic activity as measured by the acute response in [EPO]. Second, the observed increase in IRF did not result in an increase in reticulocytes nor [Hb] and Hbmass, while maturation of IRF and reticulocytes, once released into the bloodstream is usually completed within one day (39). And last, the coefficient of variation (CV) for IRF in our lab is 24%, which indicates very large intra-individual variability. It is therefore likely that, despite statistical significance, this change does not reflect a true physiological adaptation.

The results of this study suggest that the use of an apnea training protocol to stimulate erythropoiesis is not yet a viable method to improve sports performance in individuals naïve to breath-holding. First of all, most athletes who are not used to perform apneas, are probably unable to reach sufficiently low SpO_2 levels to stimulate erythropoiesis. Second, using the current protocol, well-trained individuals novice to breath-holding are still not able to reach sufficiently low SpO_2 levels after six weeks of training. A first limitation of the study is the lack of control of the six week apnea training due to self-report of the home based training. A second limitation of this study design is that only one training protocol (5 maximal static apneas with 30 second rest intervals) was used and that this remained unaltered during the six week training period. A specific designed and periodized apnea training protocol with more variation in modalities (i.e. volume, intensity, recovery, static, dynamic) and an increase in apnea duration over time could lead to better improvements, due to a higher hypoxic stimulus. Additionally, dynamic apneas might be better suited to obtain lower SpO_2 values although these apneas are

shorter (11). The exact magnitude of the hypoxic stimulus necessary to increase these hematological parameters remains unclear but a training schedule which consist of ≥10 maximal apnea bouts per session and is able to cause a significant decrease in the SpO_2 should probably be preferred. Future research should therefore focus the optimal modalities to induce a large hypoxic dose and investigate over a longer training program, as well as the practical applicability to integrate this within athletes training schedule. Lastly, further research should also investigate the longevity of the potential increase in resting [EPO] and hematological parameters caused by apnea training.

Conclusion

This study was the first to provide longitudinal data on the impact of apnea training on both the hematological parameters and its effect on exercise performance. Additionally, it was the first to include a control group and to measure hemoglobin mass, which addressed important methodological limitations of previous research. An apnea protocol consisting of 5 maximal static apneas, separated by 30 second intervals did not seem to cause a sufficiently strong hypoxic stimulus to induce an acute increase in [EPO]. Consequently, resting EPO concentration, [Hb], Hbmass, Hct, $\dot{V}O_{2peak}$ and performance on a 3km cycling time trial had not improved either. This suggests the need for a stronger and more intense training protocol, preferably periodized with the aim of improving the ability to increase duration and reach low SpO_2 values in order to evoke a stronger, longer and/or more frequent desaturation to stimulate erythropoiesis.

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There were no conflicts of interest in this study.

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Figure captions

Figure 1: Overview of the protocol. AP = apnea group, CO = control group. Test days (A, B, C) were performed in a randomized order.

Figure 2. Average (black line) and individual (gray lines, n = 11) training breath-hold duration per training week for the apnea training group.
* Indicates significant difference from week 1 at $p < 0.05$.

Figure 3. Apnea duration (panel A) and arterial oxygen saturation (SpO_2, panel B) for the apnea training group during apnea tests at the PRE (black line) and POST (gray line) test for each corresponding apnea (A1 – A5) and the maximal or minimal value during the series. * Indicates significant differences from BL, $ indicates differences from A1 and # indicates differences between PRE and POST at $p < 0.05$

Figure 4. Acute changes in [EPO] (panel A) in the apnea group after an apnea protocol before (PRE) and after six weeks of training (POST) and in a diurnal control condition (CONTROL) and chronic changes in baseline [EPO] (panel B) in the apnea (AP) and control (CO) group.

Figure 5. Evolution of Hbmass (panel A) and immature reticulocyte fraction (IRF, panel B) before (PRE), after three weeks (MID) and eight weeks (POST) of intervention for the apnea (AP) and control group (CO). $ indicates significant interaction effect for Group X Test day at $p < 0.05$. * Indicates significantly different from PRE to MID or MID to POST at $p < 0.05$

Figure 6. Maximal oxygen uptake ($\dot{V}_{O_2}$peak, panel A) and 3km time trial time (panel B) before (PRE) and after six weeks (POST) of intervention for the apnea (AP) and control (CO) group.

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Table captions

Table 1. Subject characteristics.

* Indicates significant differences between control (CO) and apnea (AP) group at $p < 0.05$

Table 2. Averages and SD for baseline blood values before (PRE), after three (MID) and six weeks (POST) of intervention for the apnea (AP) and control group (CO).
[EPO] = erythropoietin concentration, Hbmass = hemoglobin mass, [Hb] = hemoglobin concentration, Hct = hematocrit, Ret = reticulocytes, IRF = immature reticulocyte factor

[a] indicates significant differences from PRE at $p < 0.05$
[b] indicates significant differences from MID at $p < 0.05$