# Senzitivnost perifernih kemoreceptora te obrazac aktivacije simpatičkog živčanog sustava tijekom zadržavanja daha pri različitim volumenima pluća u ronilaca na dah

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SVEUČILIŠTE U SPLITU
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# SENZITIVNOST PERIFERNIH KEMORECEPTORA TE OBRAZAC AKTIVACIJE SIMPATIČKOG ŽIVČANOG SUSTAVA TIJEKOM ZADRŽAVANJA DAHA PRI RAZLIČITIM VOLUMENIMA PLUĆA U RONILACA NA DAH

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## 2. POPIS OZNAKA I KRATICA

| | |
|---|---|
| AP | akcijski potencijal |
| au | arbitrarna jedinica (engl. *arbitrary unit*) |
| $B_f$ | frekvencija disanja (engl. *breathing frequency*) |
| CO | srčani minutni volumen (engl. *cardiac output*) |
| $CO_2$ | ugljični dioksid |
| DBP | dijastolički tlak arterijske krvi (engl. *arterial diastolic blood pressure*) |
| $H^+$ | vodikov ion |
| HR | frekvencija rada srca (engl. *heart rate*) |
| MAP | srednji arterijski tlak (engl. *mean arterial pressure*) |
| MSNA | mišićna simpatička živčana aktivnost (engl. *muscle sympathetic nerve activity*) |
| $MSNA_f$ | frekvencija simpatičkih izbijanja |
| $MSNA_i$ | incidencija simpatičkih izbijanja |
| $MSNA_t$ | ukupna MSNA |
| $O_2$ | kisik |
| OSA | opstruktivna apneja u spavanju (engl. *obstructive sleep apnea*) |
| $PetO_2$ | vršna koncentracija $O_2$ u izdahnutom zraku (engl. *peak end-tidal $PO_2$*) |
| Pp | parcijalni tlak (engl. *partial pressure*) |
| $SaO_2$ | saturacija arterijske krvi kisikom |
| SBP | sistolički tlak arterijske krvi (engl. *arterial systolic blood pressure*) |
| SSNA | kožna simpatička živčana aktivnost (engl. *skin sympathetic nerve activity*) |
| SV | udarni volumen srca (engl. *stroke volume*) |
| TLC | ukupni kapacitet pluća (engl. *total lung capacity*) |
| $V_E$ | minutna ventilacija |
| $V_T$ | volumen udisaja (engl. *tidal volume*) |

# 3. PREGLED OBJEDINJENIH RADOVA

Ova doktorska disertacija temelji se na objedinjenim sljedećim znanstvenim radovima:

1. Brešković T, Valić Z, Lipp A, Heusser K, Ivančev V, Tank J, i sur. *Peripheral chemoreflex regulation of sympathetic vasomotor tone in apnea divers*. Clin Auton Res. 2010;20:57-63.
2. Brešković T, Ivančev V, Banić I, Jordan J, Dujić Ž. *Peripheral chemoreflex sensitivity and sympathetic nerve activity are normal in apnea divers during training season*. Auton Neurosci. 2010;154:42-7.
3. Brešković T, Steinback CD, Salmanpour A, Shoemaker JK, Dujić Ž. *Recruitment pattern of sympathetic neurons during breath-holding at different lung volumes in apnea divers and controls*. Auton Neurosci. 2011. [u tisku]

## 3.1. Uvod

Osnovna uloga disanja je održavanje odgovarajućih koncentracija kisika ($O_2$) ugljičnog dioksida ($CO_2$) i vodikovih iona ($H^+$) u tkivima. Sposobnost živčanog sustava u prilagođavanju alveolarne ventilacije trenutačnim potrebama organizma je, uslijed dobre usklađenosti središnje i periferne regulacije disanja, razvijena do te mjere da se parcijalni tlakovi (Pp) $O_2$ i $CO_2$ vrlo malo mijenjaju. Do značajnijih promjena u koncentracijama otopljenih plinova u krvi i acido-baznom statusu ne dolazi ni tijekom stanja povećane energetske potrošnje u organizmu.

Povećana koncentracija $CO_2$ i $H^+$ iona u krvi djeluje izravno na centralne kemoreceptore, smještene u ventralnom dijelu produljene moždine, pojačavajući ventilacijski odgovor[1]. Naglašen je akutni učinak promjene koncentracije $CO_2$ na središnju regulaciju disanja, dok je kronični učinak neznatan zbog prilagodbe. Nasuprot tome, $O_2$ nema snažan izravni učinak na središnju regulaciju disanja, nego djeluje na periferne kemoreceptore u karotidnim i aortalnim tjelešcima, koji dalje šalju informacije u dišni centar[2]. Međutim, jedan od rezultata aktivacije centralnih i perifernih kemoreceptora je periferna vazokonstikcija posredovana porastom aktivnosti simpatičkog živčanog sustava[3;4].

Određivanje aktivnosti autonomnog živčanog sustava u ljudi je izrazito složeno. Do sada najčešće korištene metode su uključivale snimanje aktivnosti različitih organa poput frekvencije rada srca, protoka krvi, tlaka arterijske krvi i produkcije znoja te su se na temelju tih indirektnih pokazatelja donosili zaključci o radu autonomnog živčanog sustava. Mikroneurografija je, za sada, jedina metoda koja omogućuje direktnu kvantifikaciju adrenergičke aktivnosti u ljudi[5–8]. Tehnika se izvodi koristeći mikroelektrode približnog

promjera 100 μm i promjera vrha od 1 do 5 μm. Pomoću tehnike mikroneurografije moguće je zabilježiti aktivnost postganglijskih simpatičkih neurona koji inerviraju krvne žile u mišićima (mišična simpatička živčana aktivnost; engl. *muscle sympathetic nerve activity*; MSNA) ili koži (kožna simaptička živčana aktivnost; engl. *skin sympathetic nerve activity*; SSNA). Aktivnost simpatičkih neurona koji inerviraju otporničke krvne žile u skeletnim mišićima predstavlja čimbenik regulacije protoka krvi u periferiji i ukupnog perifernog otpora krvožilja. Izbijanja zabilježena ovom metodom u simpatičkom sustavu su sinkrona s frekvencijom rada srca, te stoga zabilježeni broj impulsa u minuti ne može biti veći od broja otkucaja srca u minuti. Razina bazalne simpatičke živčane aktivnosti se definira kao broj izbijanja u simpatičkom sustavu tijekom 100 srčanih otkucaja. Ovaj oblik kvantifikacije živčane aktivnosti predstavlja način na osnovu kojeg se ona može usporediti između više skupina ispitanika. Najvažnija prednost ove tehnike je mogućnost kontinuiranog praćenja promjena u simpatičkoj aktivnosti tijekom različitih podražaja.

Izraženi primjer međusobnog utjecaja prenaglašene senzitivnosti perifernih kemoreceptora na simpatičku živčanu aktivnost je opisan u bolesnika s opstruktivnom apnejom u spavanju (OSA). Osnovno svojstvo ovog poremećaja je pojava prekida spontanog disanja tijekom spavanja u trajanju od 10 ili više sekundi. Broj ovakvih prekida u disanju može dosegnuti 300 do 500 tijekom spavanja, rezultirajući s približno 20% vremena spavanja provedenog u apneji. Učestale nevoljne apneje u bolesnika s OSA izlažu te bolesnike učestalim hipoksično/hiperkapničnim podražajima koji za posljedicu imaju razvoj prenaglašene senzitivnosti perifernih kemoreceptora[9;10]. Hipersenzitivni periferni kemoreceptori povećavaju eferentnu simpatičku živčanu aktivnost i ukupni periferni otpor, što naposljetku rezultira razvojem arterijske hipertenzije[4;11]. Nasuprot tome, dosadašnje studije su pokazale da je senzitivnost centralnih kemoreceptora u ovih bolesnika očuvana[10]. Navedeni poremećaji su kronično prisutni u ovih bolesnika i kada se nalaze u budnom stanju[12;13]. Prenaglašena simpatička živčana aktivnost i posljedična arterijska hipertenzija te endotelna disfunkcija u ovih bolesnika predstavljaju faktore rizika za razvoj bolesti srca i krvožilnog sustava[14;15].

Daljnja istraživanja[16–18] pokazala su da voljno zadržavanje daha u laboratorijskim uvjetima te izlaganje laboratorijskih životinja isprekidanoj hipoksiji uzrokuju kratkotrajne i dugotrajne promjene u regulaciji autonomnog sustava. Isprekidana hipoksija u trajanju od 20 do 30 minuta predstavlja podražaj koji uzrokuje privremeni porast MSNA i arterijskog tlaka[17].

Ronioci na dah se uzimaju kao primjer „zdravih“ ljudi koji se učestalo voljno izlažu višeminutnim prekidima u disanju – apneji. Jedna od najraširenijih aktivnosti koja uključuje apneju je podvodni ribolov. Mnogo više ljudi prakticira podvodni ribolov iz rekreacijskih nego li zbog komercijalnih ili natjecateljskih pobuda. Posebnu skupinu ronilaca na dah sačinjavaju trenirani ronioci na dah, koji se bave isključivo apnejaškim natjecateljskim disciplinama. Ronioci na dah sudjeluju u natjecanjima u nekoliko disciplina. Između ostalih to su statička odnosno dinamička apneja te ronjenje uz konstantno opterećenje (engl. *constant weight*). Tijekom izvođenja statičke apneje, ronilac mirno pluta na površini, s licem uronjenim u vodu, s ciljem postizanja maksimalnog mogućeg vremena trajanja apneje. Prilikom izvođenja dinamičke apneje, ronilac nastoji preroniti maksimalnu moguću udaljenost. Ronjenje na dah uz konstantno opterećenje se izvodi tako da ronilac uz pomoć peraja roni na maksimalnu moguću dubinu uzduž okomito spuštenog konopca. Usprkos izrazito ekstremnim uvjetima koji vladaju u ovom sportu rekordi se konstantno popravljaju. Trenutni svjetski rekordi u statičkoj apneji iznose 11 min 35 s za muškarce i 8 min 23 s za žene, u dinamičkoj apneji 250 m za muškarce odnosno 214 m za žene i u apneji s konstantnim opterećenjem 122 m za muškarce, odnosno 101 m za žene. Trenirani ronioci na dah su izloženi ekstremnoj hipoksiji/hiperkapniji tijekom izvođenja maksimalnih apneja. Po završetku maksimalne apneje, $PpO_2$ u plućnim alveoalama može iznositi 20 – 30 mmHg (2,5 – 4 kPa), uz saturaciju arterijske krvi kisikom ($SaO_2$) od približno 50%[19;20]. Prilikom izvođenja statičke i dinamičke apneje, ronioci su izloženi progresivnoj hipoksičnoj hiperkapniji. Nasuprot tome, tijekom izvođenja discipline uz konstantno opterećenje, tijekom većeg dijela zarona ronioci su izloženi hiperoksičnoj hiperkapniji zbog porasta tlaka u alveolama radi pritiska povećanog hidrostatskog tlaka na stijenku prsnog koša[21].

Maksimalna voljna apneja predstavlja podražaj koji za rezultat ima izrazito povećavanje simpatičke živčane aktivnosti koje spada među najviše zabilježene poraste mišićne simpatičke živčane aktivnosti opisane u literaturi. U netreniranih kontrolnih ispitanika, porast simpatičke živčane aktivnosti tijekom apneje u odnosu na bazalno stanje je približno četverostruk. Međutim, u treniranih ronilaca na dah povećanje iznosi preko 20 puta[22]. Uzroci za porast aktivacije simpatičkog živčanog sustava tijekom apneje su višestruki. Između ostalog, simpatički odgovor je uvjetovan početnim volumenom pluća na kojem se započinje zadržavanje daha. Odgovor simpatičkog živčanog sustava tijekom zadržavanja daha pri ukupnom kapacitetu pluća (TLC) je bifazičan[22]. U prvoj fazi zadržavanja daha (u prosjeku unutar prvih 30 s), odgovor simpatičkog živčanog sustava oponaša onaj zabilježen prilikom izvođenja Valsalvinog manevra. Povećani intratorakalni tlak koji se javlja prilikom

zadržavanja daha pri TLC rezultira smanjenjem venskog priljeva u srce, uz smanjenje arterijskog tlaka te se posljedično aktiviraju „niskotlačni“ kardio-pulmonalni baroreceptori te arterijski baroreceptori, što rezultira refleksnim porastom simpatičke aktivnosti. Nakon normalizacije arterijskog tlaka, simpatička aktivnost se linearno povećava uslijed promjena u $PpO_2$ i $PpCO_2$ te izostanku inhibitornog djelovanja disanja na simpatičku živčanu aktivnost. Nasuprot navedenom, zadržavanje daha pri funkcionalnom rezidualnom kapacitetu pluća (FRC) nema za posljedicu porast intratorakalnog tlaka te posljedičnu aktivaciju barorefleksa. U ovom slučaju, simpatička aktivnost je regulirana isključivo porastom aktivnosti kemoreceptora uslijed metaboličkog nagomilavanja $CO_2$ i smanjenja $O_2$ te odsustva inhibitornog učinka ventilacije na simpatičku živčanu aktivnost.

Uzevši u obzir promjene do kojih dolazi prilikom izlaganja učestaloj i izraženoj hipoksiji, primjerice u bolesnika s OSA, postavlja se pitanje javljaju li se slični kronični poremećaji u osoba koji se izlažu učestalim produljenim voljnim apnejama? U tom slučaju, u ronilaca na dah bi se manifestirao porast bazalne MSNA koja bi uzrokovala porast vazomotornog tonusa i posljedično razvoj hipertenzije u tih ljudi. U slučaju pronalaska takvih poremećaja u ronilaca na dah, potrebno bi bilo utvrditi koliko dugo te promjene opstaju nakon završetka perioda intenzivnih treninga. Naime, osim natjecatelja u ronjenju na dah, u svijetu postoji značajan broj zdravih i relativno mladih ljudi koji se bave sportovima koji uključuju dugotrajno voljno zadržavanje daha. Primjer takvih sportova su: plivanje, sinkronizirano plivanje, podvodni hokej i u konačnici podvodni ribolov koji je raširen u cijelom svijetu.

Točan obrazac aktivacije postganglijskih simpatičkih neurona i na taj način kontroliranje razine simpatičkog odgovora do danas nisu u potpunosti istraženi. Tehnika koja omogućava dobivanje uvida u promjenu obrasca okidanja postganglijskih simpatičkih neurona je metoda mikroneurografije pojedinačnih simpatičkih vlakana. Međutim, najveći nedostatak navedene metode je što se istovremeno može snimati aktivnost samo jednog simpatičkog neurona. Pomoću te tehnike pretpostavljeno je nekoliko scenarija promjena u obrascu okidanja simpatičkih postganglijskih neurona koji se ne mogu detektirati pomoću mikroneurografskog snimanja simpatičke živčane aktivnosti više živčanih jedinica. Ukratko, predloženi obrasci aktivacije simpatičkog živčanog sustava uključuju: porast frekvencije okidanja pojedinog simpatičkog neurona, pojavu ponavljanog okidanju istog neurona za vrijeme jednog simpatičkog izbijanja te regrutiranje novih neurona koji su do podražaja bili neaktivni ili iznimno rijetko aktivni[23]. Štoviše, dokazano je da su u nekim patološkim stanjima koji za posljedicu imaju kronično bazalno povećanu simpatičku aktivnost (OSA, zatajivanje srca)

navedene promjene češće prisutne u obrascu okidanja simpatičkih neurona u odnosu na zdravo stanje[23–26].

Znanstveni radovi objedinjeni u ovoj disertaciji testiraju sljedeće hipoteze:

i) U treniranih ronilaca na dah koji se nalaze u fazi višemjesečnih intenzivnih apnejaških treninga, bazalna mišićna simpatička živčana aktivnost je viša od one u kontrolnih ispitanika.

ii) U treniranih ronilaca na dah koji se nalaze u fazi višemjesečnih intenzivnih apnejaških treninga, slično kao i u bolesnika s OSA, odgovor simpatičkoga živčanog sustava na podražaj hipoksijom je prenaglašen u odnosu na kontrolne ispitanike.

iii) U treniranih ronilaca na dah, nakon prestanka intenzivnih treninga u trajanju od minimalno mjesec dana dolazi do normaliziranja bazalne mišićne simpatičke živčane aktivnosti.

iv) U treniranih ronilaca na dah, nakon prestanka intenzivnih treninga u trajanju od minimalno mjesec dana dolazi do normaliziranja odgovora simpatičkoga živčanog sustava na podražaj hipoksijom.

v) Izraženije povećanje simpatičke živčane aktivnosti u treniranih ronilaca na dah tijekom apneje je postignuto sličnim obrascem aktivacije simpatičkih postganglijskih neurona kao i u kontrolnih ispitanika.

vi) Obrazac aktivacije postganglijskih simpatičkih neurona je različit ovisno o tome radi li se o apneji pri FRC ili TLC.

## 3.2. Pregled metodologije objedinjenih radova

### 3.2.1. Ispitanici

Ispitivanu skupinu su sačinjavali trenirani ronioci na dah. Kontrolnu skupinu su sačinjavali ispitanici podjednakih karakteristika kao sudionici ispitivane skupine, osim treniranja apneje. U istraživanje su uključeni ispitanici od 18 do 35 godina starosti. Primarne natjecateljske discipline ronilaca na dah bile su statička i/ili dinamička apneja, uz prosječan intenzitet treniranja od 2-3 apnejaška treninga tjedno, u trajanju od minimalno 1 sat. Za potrebe studije 1, nakon završenog perioda intenzivnih treninga u trajanju od minimalno 2 mjeseca, ispitanici nisu smjeli imati apnejaške treninge minimalno mjesec dana prije testiranja. U studiji 2 i 3 ispitanici su se nalazili u fazi intenzivnih treninga koja je do trenutka testiranja u laboratoriju trajala minimalno 2 mjeseca. Ronioci na dah nisu smjeli biti natjecatelji u disciplini apneje uz konstantno opterećenje ili se intenzivno baviti podvodnim ribolovom. Svi su ispitanici u trenutku testiranja bili zdravi, u anamnestičkim podacima nisu imali težih bolesti ili ozljeda.

U prijašnjoj studiji[27], bazalna frekvencija izbijanja mišićne simpatičke živčane aktivnosti u kontrolnih ispitanika je iznosila 33 izbijanja/min, uz standardnu devijaciju od 13 izbijanja/min. Uz predodređenu snagu studije od 0,75 i definiranu vjerojatnost alfa-pogreške ($\alpha$=0,05), statističkom je analizom određen minimalni potreban broj od 10 ispitanika po skupini da bi se mogla dokazati razlika među skupinama u frekvencijama izbijanja simpatičke živčane aktivnosti od minimalno 50%. U obzir smo uzeli limitirajući broj dostupnih treniranih ronilaca na dah koji zadovoljavaju kriterije uključenja u studiju, te opaženu razliku među skupinama u frekvenciji izbijanja mišićne živčane simpatičke aktivnosti u sličnoj studiji[10]. Navedena studija je uključivala bolesnike s OSA (bazalna frekvencija izbijanja simpatičkog sustava u ovih bolesnika je bila približno 100% veća u odnosu na kontrole). Stoga, minimalna razlika frekvencije izbijanja simpatičke aktivnosti od 50%, koju je bilo u stanju detektirati ovo istraživanje, se smatra relevantnom. Iz tog razloga, u svim znanstvenim radovima koji sačinjavaju ovu doktorsku disertaciju uključeno je po 20 dobrovoljnih ispitanika. Skupine je sačinjavalo 10 profesionalnih ronilaca na dah te jednak broj kontrolnih ispitanika.

### 3.2.2. Mjerenja

*Antropometrija.* Svakom ispitaniku je izmjerena tjelesna visina i težina te je na osnovu dobivenih podataka izračunat indeks tjelesne mase (engl. *body mass index*). Kaliperom su

izmjereni kožni nabori na tri mjesta (nadlaktica, trbuh, natkoljenica) te je uz pomoć formule Jacksona i Pollocka[28] izračunat indeks tjelesne masti (engl. *body fat index*).

*Spirometrija.* Ispitanicima je napravljena dinamička spirometrija u stojećem položaju, sukladno preporukama Američkog torakalnog društva (engl. *American Thoracic Society*)[29]. U tu svrhu koristio se uređaj Quark PFT (Cosmed, Rim, Italija).

*Mjerenje zasićenosti hemoglobina kisikom u arterijskoj krvi.* Ovo mjerenje je obavljeno infracrvenim senzorom za pulsnu oksimetriju (Poet II, Criticare Systems, Waukesha, WI, SAD), postavljenim na prst ispitanika.

*Hemodinamički parametri.* Za kontinuirano mjerenje arterijskog tlaka i bilježenje frekvencije rada srca koristio se uređaj Finometer (Finapress Medical Systems, Arnhem, Nizozemska). Isti uređaj je mjereći svojstva vala arterijskog pulsa zabilježenog u manžeti postavljenoj na prst ispitanika, kontinuirano određivao promjene u vrijednosti udarnog volumena srca koristeći unaprijeđenu Wesselingovu metodu (Modelflow program)[30]. Ispitanicima se također postavio jednokanalni EKG uređaj (Dual Bio Amp/Stimulator, ADInstruments, Castle Hill, Australija).

*Snimanje mišićne simpatičke živčane aktivnosti.* Za direktno snimanje simpatičke aktivnosti koristila se tehnika mikroneurografije[6]. Mikroelektroda visoke impedancije, napravljena od volframa (FHC Inc., Bowdoin, ME, SAD) uvela se u peronealni živac ispitanika dok se druga mikroelektroda uvela pod kožu ispitanika u krugu 5 cm od mjesta uvođenja prve mikroelektrode služeći kao referentna elektroda. Dobiveni signal se pojačao 100.000 puta. Nakon toga se signal pojasno filtrirao u rasponu od 0,7 do 2,0 kHz, ispravio te integrirao koristeći vremensku konstantu od 0,1 s (662C-4, Nerve Traffic Analysis System, Bioengineering, The University of Iowa, Iowa City, IA, SAD).

*Identifikacija izbijanja u zapisu mišićne simpatičke živčane aktivnosti.* Izbijanja u integriranom zapisu neurograma simpatičke živčane aktivnosti su morala zadovoljiti sljedeće uvjete: 1) omjer signal-šum > 2; 2) sinkronizacija s arterijskim pulsom; 3) latencija u odnosu na R zubac u EKG zapisu od 0,9 do 1,5 s; 4) primjereno trajanje izbijanja (kratki = artefakt, dugi = SSNA); 5) vidljiv porast aktivnosti nakon manevara koji povećavaju intratorakalni tlak (npr. Valsalvin manevar); 6) izostanak porasta aktivnosti nakon podražaja glasnim zvukom ili mentalnim opterećenjem.

*Identifikacija pojedinačnih akcijskih potencijala iz „sirovog" neurograma mišićne simpatičke živčane aktivnosti.* Nakon pojačanja, „sirovi" zapis mišićne simpatičke živčane aktivnosti se pojasno filtrirao u rasponu od 0,7 do 2,0 kHz te pohranio u računalo. Dobivena računalna datoteka se analizirala pomoću posebno razvijene aplikacije[31] (APD v 1.0., Aryan

Salmanpour, Neurovascular Research Laboratory, School of Kinesiology, University of Western Ontario, London, Ontario, Kanada). Aplikacija koristi tehniku „*continuous wavelet transform*“ za detekciju pojedinačnih akcijskih potencijala. Na taj način određen je broj akcijskih potencijala (AP) i amplituda svakog akcijskog potencijala u zapisu, te je, zavisno o veličini amplitude, svaki AP svrstan u pojedini skup (engl. *cluster*). Nadalje, određena je latencija svakog detektiranog akcijskog potencijala u odnosu na R zubac u EKG-u te ukupan broj AP unutar svakog pojedinog izbijanja mišićne simpatičke živčane aktivnosti.

Signali iz svih uređaja bili su povezani na analogno-digitalni pretvarač (Powerlab/16SP, ADInstruments, Castle Hill, Australija) te pohranjeni u osobno računalo. Podaci su uzorkovani frekvencijom od 1 kHz (studija 1 i 2) odnosno 10 kHz (studija 3) pomoću računalnog programa Chart (ADInstruments, verzija 5.5.6.7) te naknadno analizirani.

### 3.2.3. Eksperimentalni protokol

Istraživanje je provedeno u Zavodu za integrativnu fiziologiju Medicinskog fakulteta Sveučilišta u Splitu. Svi eksperimentalni postupci izvedeni su u suglasnosti s Helsinškom deklaracijom i odobreni od strane fakultetskog Etičkog povjerenstva za biomedicinska istraživanja. Studija se provodila u jutarnjim satima radi toga da bi ispitanici mogli doći u laboratorij na tašte. Ispitanici su bili upozoreni da ne konzumiraju kofeinske proizvode, alkohol i ostale stimulanse najmanje 12 sata prije testiranja u laboratoriju. Ispitanice su bile testirane za vrijeme folikularne faze menstruacijskog ciklusa[32–34].

Po dolasku u laboratorij ispitanicima su bili pojašnjeni postupci istraživanja. Po potpisivanju obrasca o informiranom pristanku te uzimanja antropometrijskih podataka, ispitanici su postavljeni u ležeći položaj i opremljeni mjernim uređajima. Potom se pristupilo izvođenju tehnike snimanja mišićne simpatičke živčane aktivnosti. U slučaju nemogućnosti pronalaženja optimalnog signala mišićne simpatičke živčane aktivnosti ispitanik je bio isključen iz studije, a u suprotnom nastavilo se s izvođenjem pokusa. U nastavku istraživanja za potrebe studija 1 i 2 ispitanik je izložen normokapničnoj hipoksiji. Po završetku hipoksičnog podražaja ispitanik je nastavio i dalje mirno ležati te su se svi fiziološki parametri nastavili bilježiti sljedećih 30 minuta.

Za potrebe studije 3, po pronalaženju neurograma zadovoljavajuće kvalitete, ispitanik je mirno ležao 15 minuta. Nakon toga, ispitanik je maksimalno zadržavao dah pri razini FRC. Nakon perioda oporavka od apneje u trajanju od 15 minuta, ispitanik je još jednom zadržao dah, ali ovaj put pri razini TLC. Po završetku druge apneje, svi fiziološki parametri su se bilježili sljedećih 15 minuta.

### 3.2.4. Statistički postupci

*Kvantifikacija izbijanja mišićne simpatičke živčane aktivnosti.* Aktivnost simpatičkog sustava iz integriranog signala se kvantificirala na nekoliko načina: 1) frekvencija izbijanja ($MSNA_f$) – ukupan broj izbijanja u 1 minuti; 2) incidencija izbijanja ($MSNA_i$) – broj izbijanja tijekom 100 otkucaja srca; 3) amplituda pojedinog izbijanja – izračunata je površina ispod krivulje (engl. *area under the curve*) za pojedino izbijanje i normalizirana u odnosu na najveće izbijanje u zapisu; 4) ukupna MSNA ($MSNA_t$) – zbroj površina svih izbijanja u jednoj minuti. Nakon identifikacije AP u zapisu mišićne simpatičke živčane aktivnosti podaci su se kvantificirali na sljedeći način: 1) broj AP u jednom izbijanju; 2) ukupan broj AP u 1 min (frekvencija AP); 3) učestalost izbijanja AP koji pripadaju pojedinom skupu (ukupno 20 skupova) za različite faze apneje; 4) broj aktivnih skupova AP u jednom izbijanju.

*Kvantifikacija senzitivnosti utjecaja perifernih kemoreceptora na simpatički sustav.* Izračunata je kao promjena $MSNA_t$ u odnosu na promjenu $SaO_2$ za vrijeme hipoksičnog podražaja.

*Kvantifikacija senzitivnosti utjecaja perifernih kemoreceptora na ventilacijski odgovor.* Izračunata je kao promjena u minutnoj ventilaciji ($V_E$) u odnosu na promjenu $SaO_2$ za vrijeme hipoksičnog podražaja.

Za potrebe studije 1 i 2 podaci su analizirani u 7 točaka protokola: 1) tijekom trominutnog perioda prije započinjanja hipoksije; 2) tijekom tri minute kada je saturacija hemoglobina kisikom u arterijskoj krvi približno 90%; 3) tijekom zadnje tri minute perioda kada je saturacija hemoglobina kisikom u arterijskoj krvi približno 80%; 4) tijekom tri minute nakon prestanka udisanja hipoksične smjese; tijekom tri minute u 5) 10.; 6) 20. i 7) 30. minuti perioda oporavka.

Za potrebe studije 3 podaci su analizirani u 5 točaka protokola: 1) tijekom trominutnog perioda prije započinjana apneje pri FRC; 2) tijekom apneje pri FRC; 3) tijekom trominutnog perioda prije započinjanja apneje pri TLC; 4) tijekom prvih 30 s apneje pri TLC; 5) tijekom zadnjih 30 s apneje pri TLC.

Svi izračunati podaci su prikazani kao aritmetička sredina s 95%-tnim intervalima pouzdanosti. Vrijednost $P<0,05$ predstavlja granicu statističke značajnosti. U studijama 1 i 2 bazalne vrijednosti, vrijednosti u istim mjernim točkama i senzitivnosti kemoreceptora između dvije skupine su uspoređene Studentovim *t*-testom za nezavisne uzroke. Promjene uzrokovane utjecajem hipoksije na pojedini parametar u istoj skupini su testirane ANOVA-om za ponavljana mjerenja. Bonferronijev test je korišten kao *post hoc* test. Interakcije

odgovora pojedinih parametara između dvije skupine su određene koristeći *general linear model* za ANOVA-u za ponavljana mjerenja. U studiji 3, zbog relativno malog broja ispitanika, korištene su neparametrijske inačice statističkih testova. Razlike između skupinama u istim mjernim točkama uspoređene su Mann-Whitney U testom. Promjene u različitim parametrima prije i tijekom apneje pri FRC su uspoređene Wilcoxonovim testom, dok su promjene prije i u različitim mjernim točkama za trajanja apneje pri TLC uspoređene Friedmanovom ANOVA-om. U slučaju značajnosti, Wilcoxonov test je korišten kao *post hoc* test. Nagibi krivulja koje opisuju promjene u simpatičkoj živčanoj aktivnosti u jedinici vremena su određene korištenjem linearne regresije. Statistička analiza svih podataka je napravljena koristeći računalnu aplikaciju Statistica (verzija 7.0; Statsoft Inc., Tulsa, OK, SAD).

## 3.3. Sažeti pregled rezultata objedinjenih radova

### 3.3.1. Rad 1

Bazalne vrijednosti MSNA, $V_E$ i frekvencija rada srca (HR) su bile slične među skupinama. Sistolički (SBP), dijastolički (DBP) i srednji arterijski tlak (MAP) u mirovanju su bili povišeni u treniranih ronilaca na dah, međutim razlika nije bila statistički značajna. Početne vrijednosti različitih fizioloških parametara u obje skupine ispitanika prikazane su u Tablici 1.

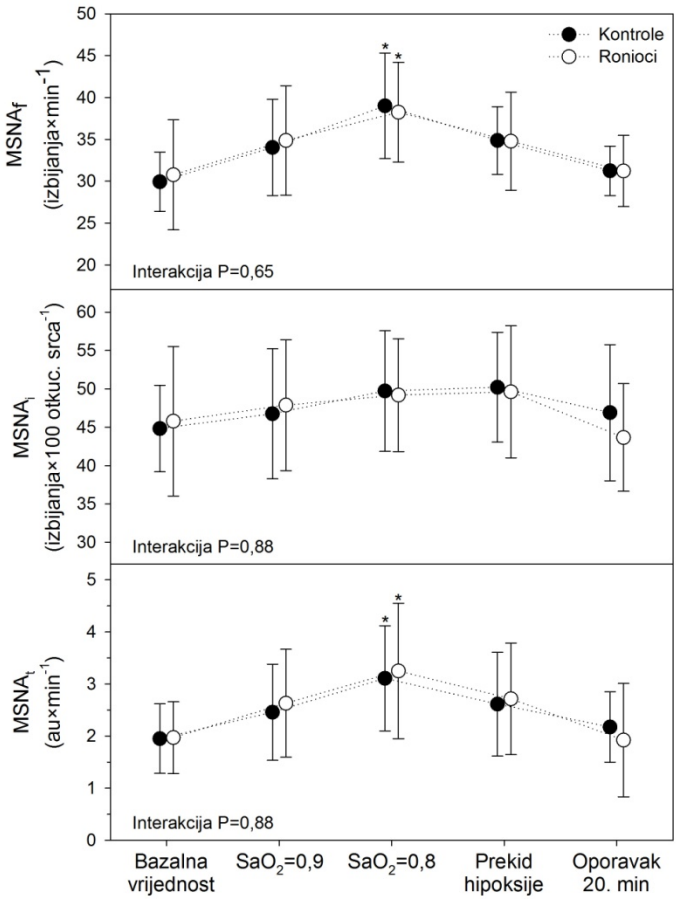
**Tablica 1.** Bazalne vrijednosti mjerenih fizioloških parametara u skupini kontrolnih ispitanika te u skupini treniranih ronilaca na dah. Prikazane su izračunate *P* vrijednosti statističkih usporedbi između dviju skupina za svaki parametar.

| | **Kontrole (n=11)** | **Ronioci (n=11)** | ***P*** |
|---|---|---|---|
| $MSNA_f$ (izbijanja×$min^{-1}$) | 29,9±3,5 | 30,8±6,6 | *0,83* |
| $MSNA_t$ (au×$min^{-1}$) | 2,0±0,7 | 2,0±0,7[a] | *0,97* |
| $V_E$ (l×$min^{-1}$) | 7,8±1,2 | 7,6±0,8 | *0,78* |
| HR ($min^{-1}$) | 67,7±6,1 | 68,1±3,0 | *0,91* |
| MAP (mmHg) | 95,8±4,4 | 101,4±4,6 | *0,10* |
| SBP (mmHg) | 128,0±5,7 | 136,9±6,5 | *0,057* |
| DBP (mmHg) | 77,4±3,6 | 81,6±3,3 | *0,11* |

Vrijednosti su aritmetičke sredine±95%-tni intervali pouzdanosti. $MSNA_f$ – frekvencija simpatičkih izbijanja; $MSNA_t$ - ukupna MSNA; $V_E$ - minutna ventilacija; HR – frekvencija rada srca; MAP - srednji arterijski tlak; SBP – sistolički arterijski tlak; DBP – dijastolički arterijski tlak. [a] – podatak je izračunat u 10 ispitanika.

Slika 1 prikazuje odgovor MSNA na podražaj normokapničnom hipoksijom. U obje skupine zabilježen je sličan porast u $MSNA_f$ te u $MSNA_t$ (29,5±10,6%, odnosno 65,9±26,8% u kontrola; 27,4±10,9%, odnosno 59,8±22,0% u ronilaca). Povišene vrijednosti MSNA su se normalizirale nakon 20 min od prestanka udisanja hipoksične smjese.

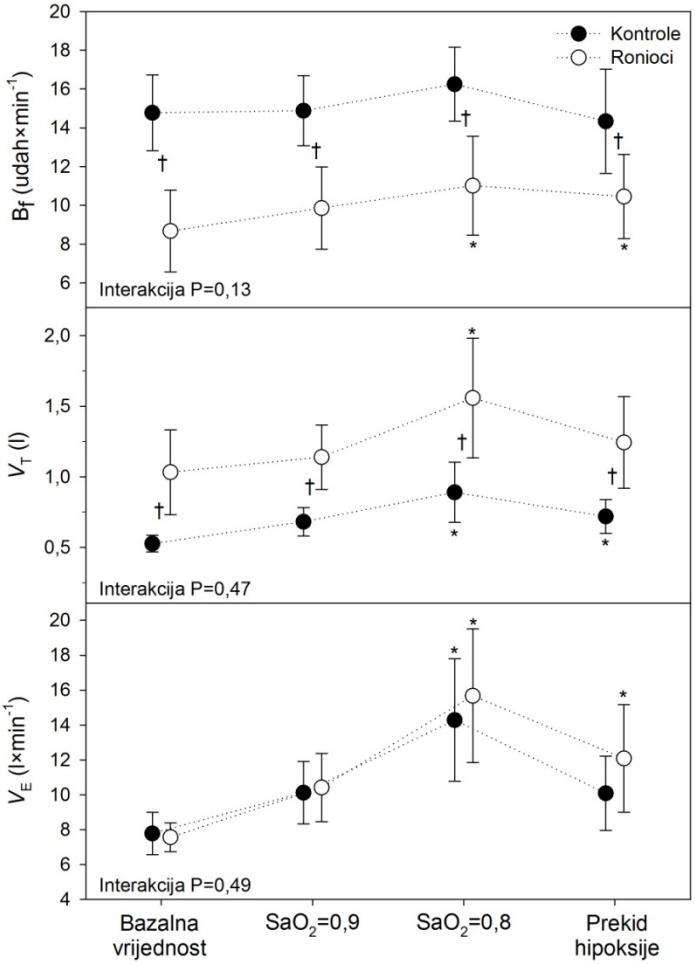
Nagib krivulje porasta $MSNA_t$ tijekom hipoksije nije se razlikovao između skupina (0,06±0,03 au/min/1% promjene $SaO_2$ u kontrola, te 0,07±0,03 au/min/1% promjene $SaO_2$ u ronilaca; *P=0,86*).




**Slika 1.** Promjene u mišićnoj simpatičkoj živčanoj aktivnosti (MSNA) u skupini kontrolnih ispitanika te u skupini treniranih ronilaca na dah tijekom svih faza hipoksičnog protokola. Kružići predstavljaju prosječne vrijednosti, okomite linije označavaju 95%-tne intervale pouzdanosti, zvjezdica (*) označava statistički značajnu (*P<0,05*) promjenu unutar skupine u odnosu na bazalnu vrijednost. *P* vrijednost interakcije između odgovora skupina naznačena je za svaki mjereni parametar. Statistički značajne (*P<0,05*) razlike među skupinama u istim mjernim točkama nisu zabilježene. $MSNA_f$ - frekvencija simpatičkih izbijanja; $MSNA_i$ - incidencija simpatičkih izbijanja; $MSNA_t$ - ukupna MSNA; $SaO_2$ – saturacija arterijske krvi kisikom.

Ronioci na dah su održavali bazalnu $V_E$ s većim volumenom udisaja ($V_T$) (0,5±0,1 l, odnosno 1,0±0,3 l) te nižom frekvencijom disanja ($B_f$) (14,8±2,0 /min, odnosno 8,7±2,1 /min) u odnosu na kontrole. Podražaj hipoksijom uzrokovao je porast $V_E$ u obje skupine, prvenstveno utječući na porast $V_T$ (66,3±32,9% u kontrola; 60,3±33,5% u ronilaca). Tijekom udisanja hipoksične smjese ronioci su također povećali i $B_f$ (30,3±14,6%) dok se u kontrolnih ispitanika ona nije značajno mijenjala (11,8±11,6%) (Slika 2).

Nagib krivulje porasta $V_E$ uslijed disanja hipoksične smjese je bio sličan između skupina (0,35±0,16 l/min/1% promjene $SaO_2$ u kontrola; 0,49±0,23 l/min/1% promjene $SaO_2$ u ronilaca; *P=0,35*).




**Slika 2.** Ventilacijski odgovor u skupini kontrolnih ispitanika te u skupini treniranih ronilaca na dah tijekom svih faza hipoksičnog protokola. Kružići predstavljaju prosječne vrijednosti, okomite linije označavaju 95%-tne intervale pouzdanosti, zvjezdica (*) označava statistički značajnu (*P<0,05*) promjenu unutar skupine u odnosu na bazalnu vrijednost. *P* vrijednost interakcije između odgovora skupina naznačena je za svaki mjereni parametar. Statistički značajne (*P<0,05*) razlike među skupinama u istim mjernim točkama označene su križićima (†). $B_f$ – frekvencija disanja; $V_T$ – volumen udisaja; $V_E$ – minutna ventilacija; $SaO_2$ – saturacija arterijske krvi kisikom.

3.3.2. Rad 2

Pregled bazalnih vrijednosti mjerenih fizioloških parametara nalazi se u Tablici 2. Ronioci na dah imali su nešto nižu $MSNA_f$, međutim, izmjerena razlika nije bila statistički značajna. Početna $MSNA_t$, te $MSNA_i$ su bile slične među skupinama.

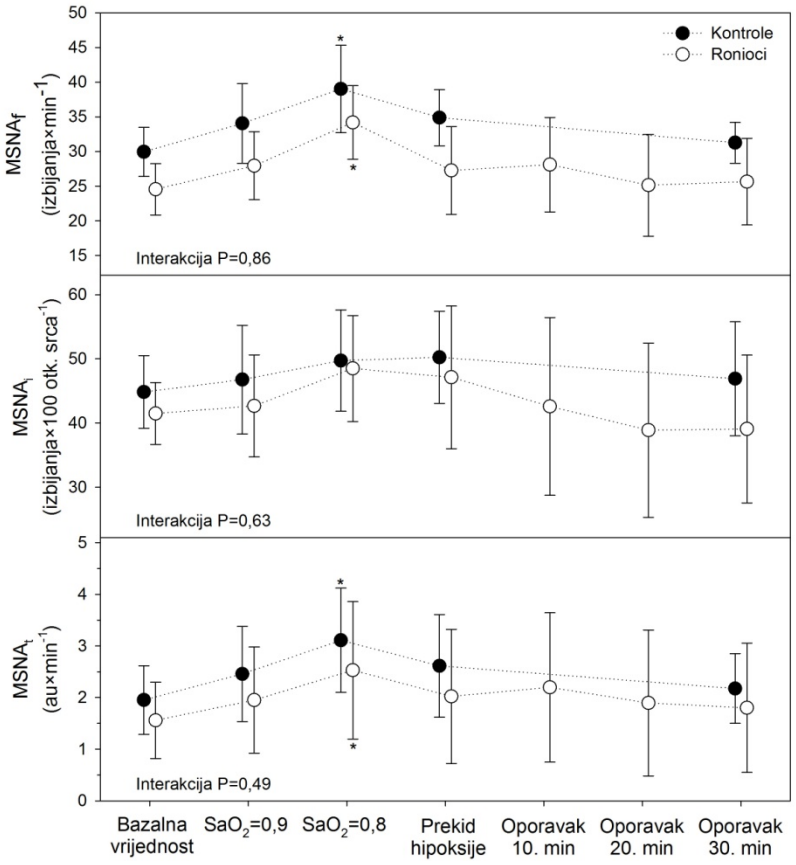
**Tablica 2.** Bazalne vrijednosti mjerenih fizioloških parametara u skupini kontrolnih ispitanika te u skupini treniranih ronilaca na dah. Prikazane su izračunate *P* vrijednosti statističkih usporedbi između dviju skupina za svaki parametar.

| | **Kontrole (n=11)** | **Ronioci (n=10)** | ***P*** |
|---|---|---|---|
| $MSNA_f$ (izbijanja×$min^{-1}$) | 29,9±3,5 | 24,5±3,7 | *0,053* |
| $MSNA_i$ (izbijanja×100 otkuc. $srca^{-1}$) | 44,8±5,7 | 41,5±4,8 | *0,39* |
| Površina ispod krivulje izbijanja (au) | 21,2±3,6 | 26,5±3,9 | *0,07* |
| $MSNA_t$ (au×$min^{-1}$) | 2,0±0,7 | 1,6±0,7 | *0,45* |
| $V_E$ (l×$min^{-1}$) | 7,8±1,2 | 7,2±0,8 | *0,49* |
| HR ($min^{-1}$) | 67,7±6,1 | 59,1±4,6 | *0,043* |
| MAP (mmHg) | 95,8±4,4 | 93,9±5,8 | *0,60* |

Vrijednosti su aritmetičke sredine±95%-tni intervali pouzdanosti; $MSNA_f$ – frekvencija simpatičkih izbijanja; $MSNA_i$ – incidencija simpatičkih izbijanja; $MSNA_t$ - ukupna MSNA; $V_E$ - minutna ventilacija; HR – frekvencija rada srca; MAP – srednji arterijski tlak.

Udisanje hipoksične smjese uzrokovalo je približno jednak porast u $MSNA_f$ te $MSNA_t$ u obje skupine (29,5±10,6 odnosno 65,9±26,8% u kontrola; 42,8±22,7%, odnosno 60,0±27,3% u ronilaca). Simpatička aktivnost se postupno normalizirala u obje skupine nakon 30-minutnog perioda oporavka. $MSNA_i$ se nije mijenjala tijekom cijelog protokola u obje skupine (Slika 3).

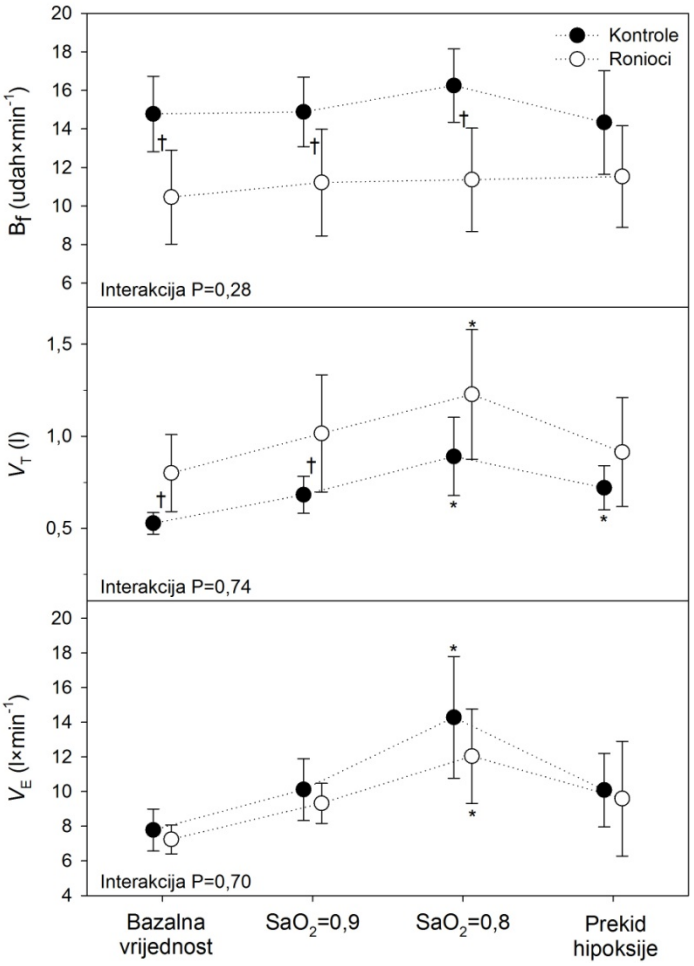
Nagib krivulje porasta $MSNA_t$ tijekom hipoksije nije se razlikovao između skupina (0,06±0,03 au/min/1% promjene $SaO_2$ u kontrola, te 0,05±0,04 au/min/1% promjene $SaO_2$ u ronilaca; *P=0,69*).




**Slika 3.** Promjene u mišićnoj simpatičkoj živčanoj aktivnosti (MSNA) u skupini kontrolnih ispitanika te u skupini treniranih ronilaca na dah tijekom svih faza hipoksičnog protokola. Kružići predstavljaju prosječne vrijednosti, okomite linije označavaju 95%-tne intervale pouzdanosti, zvjezdica (*) označava statistički značajnu (*P<0,05*) promjenu unutar skupine u odnosu na bazalnu vrijednost. *P* vrijednost interakcije između odgovora skupina naznačena je za svaki mjereni parametar. Statistički značajne (*P<0,05*) razlike među skupinama u istim mjernim točkama nisu zabilježene. $MSNA_f$ - frekvencija simpatičkih izbijanja; $MSNA_i$ - incidencija simpatičkih izbijanja; $MSNA_t$ - ukupna MSNA; $SaO_2$ – saturacija arterijske krvi kisikom.

Ronioci su održavali svoju bazalnu $V_E$ značajno višim $V_T$ (0,5±0,1 l, odnosno 0,8±0,2 l) te nižom $B_f$ (14,8±2,0 /min, odnosno 10,5±2,4 /min) u usporedbi sa kontrolnim ispitanicima. Udisanje hipoksične smjese uzrokovalo je porast $V_E$ u obje ispitivane skupine (66,3±32,9% u kontrola; 61,5±44,3% u ronilaca) (Slika 4).

Nagib krivulje porasta $V_E$ uslijed disanja hipoksične smjese je bio sličan između skupina (0,35±0,16 l/min/1% promjene $SaO_2$ u kontrola; 0,27±0,16 l/min/1% promjene $SaO_2$ u ronilaca; *P=0,48*).




**Slika 4.** Ventilacijski odgovor u skupini kontrolnih ispitanika te u skupini treniranih ronilaca na dah tijekom svih faza hipoksičnog protokola. Kružići predstavljaju prosječne vrijednosti, okomite linije označavaju 95%-tne intervale pouzdanosti, zvjezdica (*) označava statistički značajnu (*P<0,05*) promjenu unutar skupine u odnosu na bazalnu vrijednost. *P* vrijednost interakcije između odgovora skupina naznačena je za svaki mjereni parametar. Statistički značajne (*P<0,05*) razlike među skupinama u istim mjernim točkama označene su križićima (†). $B_f$ – frekvencija disanja; $V_T$ – volumen udisaja; $V_E$ – minutna ventilacija; $SaO_2$ – saturacija arterijske krvi kisikom.

3.3.3. Rad 3

Kontrolni ispitanici su imali značajno kraće vrijeme trajanja apneje pri FRC u odnosu na trenirane ronioce na dah (27,7±5,5 s, odnosno 60,4±26,1 s; *P=0,006*). Prosječno trajanje apneje pri TLC je bilo slično između ispitivanih skupina (156,5±50 s, odnosno 214,0±41,4; *P=0,07*).

*Analiza integriranog signala*

Bazalna $MSNA_f$ je u prosjeku bila niža u kontrolnih ispitanika (13±5 izbijanja×min$^{-1}$ *vs.* 20±4 izbijanja×min$^{-1}$; *P=0,039*). Bazalna $MSNA_i$ je bila slična među skupinama (23±6 *vs.* 30±15 izbijanja×100 otkuc. srca$^{-1}$; *P=0,09*).

Slika 5a prikazuje promjene zabilježene analizom integriranog signala MSNA prije i za vrijeme trajanja apneje pri FRC. Ukupni porast $MSNA_t$ tijekom apneje pri FRC je bio viši u skupini ronilaca na dah. Međutim, nagib krivulje koja opisuje porast $MSNA_t$ u jedinici vremena trajanja apneje pri FRC nije se značajno razlikovao među skupinama (32,9±16,2 au×min$^{-2}$ u kontrola, odnosno 30,2±10,6 au×min$^{-2}$ u ronilaca; *P=0,79*).

Promjene u MSNA uzrokovane zadržavanjem daha na razini TLC prikazane su na Slici 5b. Tijekom prvih 30 s trajanja apneje pri TLC u ronilaca na dah zabilježen je naglašeniji porast u $MSNA_t$ nego u kontrolnih ispitanika. Nagib krivulje koja opisuje porast $MSNA_t$ tijekom prvih 30 s apneje pri TLC je dvostruko veći u ronilaca na dah (52,9±31,3 au×$min^{-2}$, odnosno 115,3±34,6 au×$min^{-2}$; *P=0,03*). $MSNA_i$ tijekom prvih 30 s apneje pri TLC nije se značajno razlikovala među skupinama (50±16 u kontrola *vs.* 73±13 izbijanja×100 otkuc. $srca^{-1}$ u ronilaca; *P=0,09*). Nije bilo značajnijih razlika u normaliziranoj amplitudi simpatičkih izbijanja između skupina. Na kraju apneje pri TLC obje skupine su dosegle slične razine ukupne MSNA.

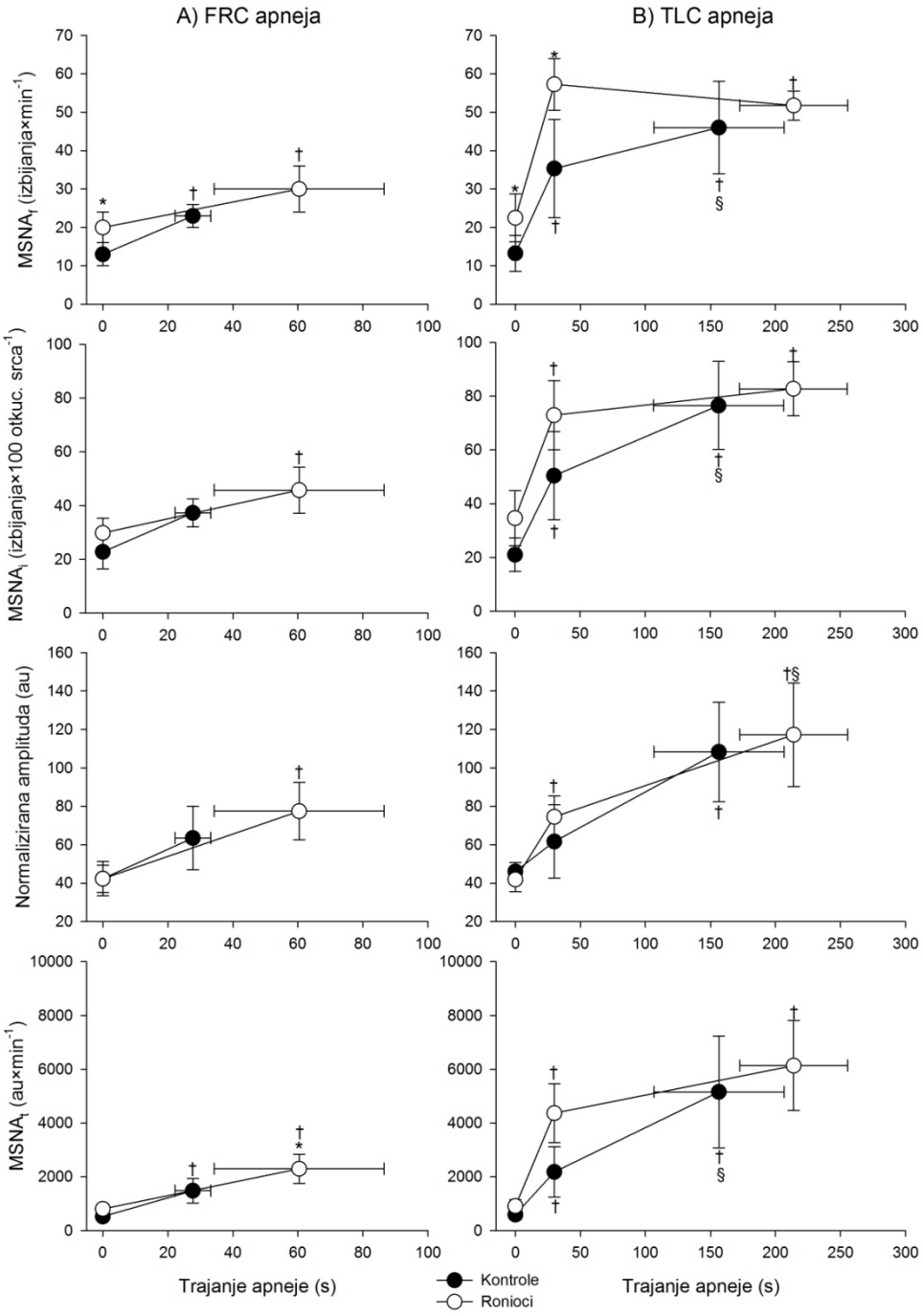
*Analiza simpatičkih akcijskih potencijala*

Podskupine ispitanika u kojih su analizirana svojstva simpatičkih postganglijskih AP nisu su razlikovale u trajanju apneja pri FRC (32±4 s kontrole, odnosno 69±41 s ronioci; *P=0,09*) ni TLC (176±67 s kontrole, odnosno. 234±59 s ronioci; *P=0,20*).

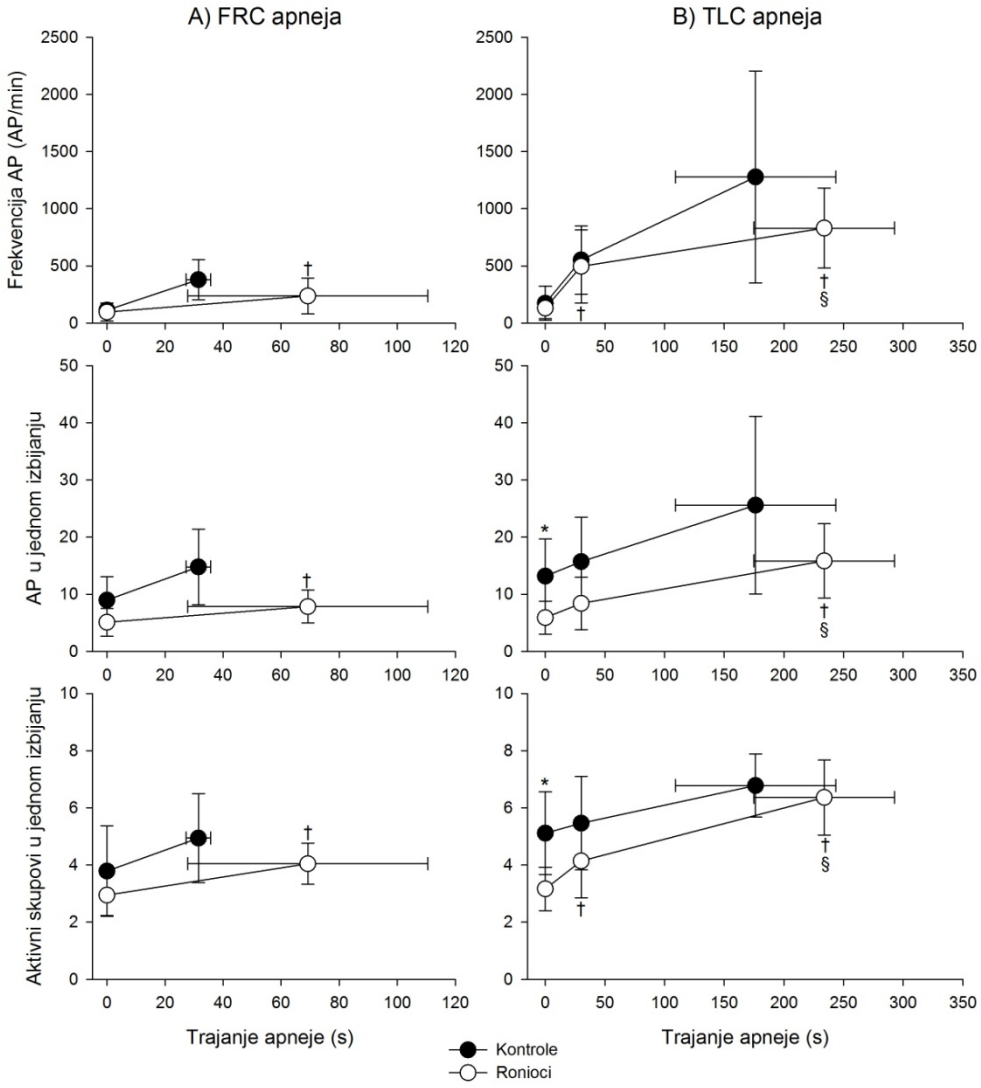
U usporedbi s kontrolnim ispitanicima ronioci na dah su se prezentirali s prosječno manjim brojem AP unutar jednog simpatičkog izbijanja (13±7, odnosno 6±3 AP/izbijanje; *P=0,05*). Uz navedeno, ronioci su također imali manji broj aktivnih skupova u jednom izbijanju (5±1, odnosno 3±1 skup/izbijanje; *P=0,05*). Međutim, ukupan bazalni broj AP po minuti nije se razlikovao među skupinama (173±149 AP/min u kontrola te 131±92 AP/min u ronilaca; *P=0,62*).

Promjene svojstava simpatičkih AP kao posljedica zadržavanja daha na razini FRC i TLC prikazane su na Slici 6a odnosno Slici 6b.

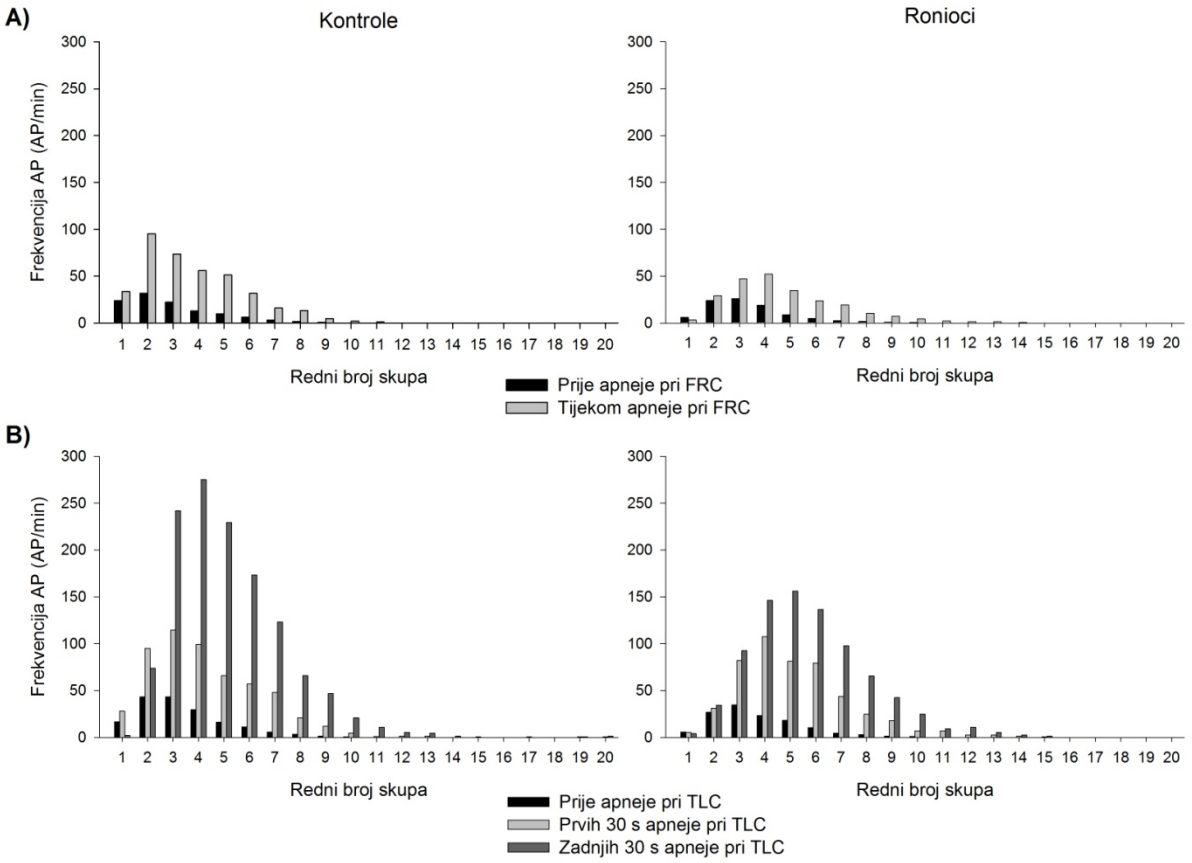
U oba tipa zadržavanja daha (FRC i TLC) te u obje skupine ispitanika zamijećen je porast aktivnosti simpatičkih AP većih amplituda tj. onih koji se pripisuju višem rednom broju skupa (Slika 7).




**Slika 5.** Promjene zabilježene analizom integriranog zapisa mišićne simpatičke živčane aktivnosti (MSNA) tijekom različitih faza apneja pri FRC (panel A) te TLC (panel B). Crni kružići označavaju prosječne vrijednosti za kontrolne ispitanike dok bijeli kružići za ronioce. Okomite i vodoravne linije označavaju 95%-tne intervale pouzdanosti. FRC – funkcionalni rezidualni kapacitet pluća; TLC – ukupni kapacitet pluća; * – statistički značajna razlika (*P<0,05*) između skupina u istoj mjernoj točki; † – statistički značajna razlika (*P<0,05*) u odnosu na početnu vrijednost unutar iste skupine; § – statistički značajna razlika (*P<0,05*) u odnosu na prvih 30 s apneje pri TLC unutar iste skupine; $MSNA_f$ – frekvencija simpatičkih izbijanja; $MSNA_i$ – incidencija simpatičkih izbijanja; $MSNA_t$ – ukupna MSNA.




**Slika 6.** Promjene zabilježene analizom svojstava akcijskih potencijala (AP) postganglijskih simpatičkih neurona tijekom različitih faza apneja pri FRC (panel A) te TLC (panel B). Crni kružići označavaju prosječne vrijednosti za kontrolne ispitanike dok bijeli kružići za ronioce. Okomite i vodoravne linije označavaju 95%-tne intervale pouzdanosti. FRC – funkcionalni rezidualni kapacitet pluća; TLC – ukupni kapacitet pluća; * – statistički značajna razlika (*P<0,05*) između skupina u istoj mjernoj točki; † – statistički značajna razlika (*P<0,05*) u odnosu na početnu vrijednost unutar iste skupine; § – statistički značajna razlika (*P<0,05*) u odnosu na prvih 30 s apneje pri TLC unutar iste skupine.




**Slika 7.** Distribucija frekvencija izbijanja akcijskih potencijala (AP) postganglijskih simpatičkih neurona razdijeljenih prema veličini amplitude u pripadajuće skupove tijekom različitih faza apneja pri FRC (panel A) i TLC (panel B). FRC – funkcionalni rezidualni kapacitet pluća; TLC – ukupni kapacitet pluća.

## 3.4. Rasprava

### 3.4.1. Senzitivnost perifernih kemoreceptora u treniranih ronilaca na dah

Rezultati znanstvenih radova broj 1 i 2 pokazali su da ne dolazi do akutnih (za trajanja perioda intenzivnih apnejaških treninga) ni kroničnih (nakon više od mjesec dana od prestanka intenzivnih treninga) promjena u vidu prenaglašenosti aktivacije simpatičkog živčanog sustava i prenaglašenog ventilacijskog odgovora nakon podražaja hipoksijom u treniranih ronilaca na dah, što bi upućivalo na pojavu pojačane senzitivnosti perifernih kemoreceptora.

U prethodnim istraživanjima, kao posljedica učestalih izlaganjima hipoksiji, u bolesnika s OSA opažena je hipersenzitivnost perifernih kemoreceptora uz očuvanu normalnu aktivnost centralnih kemoreceptora[10]. Očuvana periferna i centralna kemosenzitivnost u treniranih ronilaca na dah vjerojatno predstavlja zaštitni mehanizam pomoću kojeg se održava normalna moždana perfuzija tijekom izražene asfiksije uslijed dugotrajnog zadržavanja daha, kakvim je često izložena ova populacija ljudi. Naime, povišene vrijednosti $CO_2$ te snižene vrijednosti $O_2$ imaju izrazit vazodilatacijski učinak na krvožilni sustav. Stoga bi se povišena simpatička živčana aktivnost direktno suprotstavljala spomenutim vazodilatacijskim učincima.

Ispitanici u našim istraživanjima su bili trenirani ronioci na dah čije primarne natjecateljske discipline su statička i/ili dinamička apneja. Upravo prilikom izvođenja te dvije vrste apnejaških disciplina ronioci su najintenzivnije izloženi izraženoj i dugotrajnoj hipoksiji i hiperkapniji. Od naših ispitanika iz skupine ronilaca na dah 60% je tijekom svoje ronilačke karijere doživjelo gubitak svijesti, skoro isključivo uzrokovan ekstremnom hipoksijom. Dvojica ispitanika u anamnestičkim podacima imaju višestruke epizode hipoksičnih gubitaka svijesti. Iz navedenih podataka može se zaključiti da su naši ispitanici bili redovito izlagani ozbiljnim razinama moždane hipoksije. Trenirani ronioci na dah s vremenom razvijaju potencijalne adaptacijske fiziološke mehanizme koji im omogućuju bolje kompenziranje ekstremne hipoksije/hiperkapnije. Među spomenute potencijalne adaptacijske mehanizme, između ostalih, ubrajaju se: povećani volumen pluća čime se povećava količina zalihe zraka prilikom ronjenja te se ujedno povećava dilucijski prostor koji usporava porast $CO_2$; pojačana simpatička i parasimpatička aktivacija tijekom zadržavanja daha pomoću kojih se postiže efikasnija centralizacija krvotoka te usporava frekvencija rada srca, a time i metaboličke potrebe organizma, a sve u svrhu smanjivanja potrošnje zaliha $O_2$; te povećana produkcija laktata uz pojavu retencije $CO_2$ u tkivima[22;35–40]. Štoviše, u ronilaca na dah zabilježena je

manja acidoza arterijske krvi i smanjena pojava oksidacijskog stresa nakon zadržavanja daha kao i nakon tjelovježbe u usporedbi s kontrolnim ispitanicima[41].

U protokolu istraživanja primijenili smo normokapničnu hipoksiju. Smatramo da smo kontroliranjem razine vršnog tlaka $CO_2$ na kraju izdisaja ($PetCO_2$) minimalizirali utjecaj $CO_2$ na centralne kemoreceptore i na moždano krvožilje. Stimulacija perifernih kemoreceptora hipoksijom uzrokuje umjereni porast ventilacije. Nagib krivulje koja opisuje porast $V_E$ tijekom promjena u $SaO_2$ za trajanja hipoksičnog podražaja je bio sličan između ispitivanih skupina. Navedeno bi upućivalo na nepromijenjenu osjetljivost regulacije disanja u ronilaca na dah. Međutim, rezultati nekih prijašnjih studija ukazuju na postojanje oslabljenog ventilacijskog odgovora na hipoksiju u osoba koje se bave podvodnim sportovima poput sinkroniziranog plivanja[42] i u japanskih Ama – žena koje rone na dah[43]. Oslabljeni ventilacijski odgovor na hipoksični podražaj mogao bi predstavljati još jedan potencijalni adaptacijski mehanizam koji bi omogućio produljenje vremena zadržavanja daha u ovih ljudi.

Odgovor simpatičkog živčanog sustava na hipoksiju je također bio sličan u obje ispitivane skupine. U našim studijama zabilježen je očit porast razine simpatičke živčane aktivnosti pri $SaO_2$ od 90%. Međutim, navedeni porast je dosegao statističku značajnost tek prilikom dostizanja vrijednosti od 80% $SaO_2$. U nekoliko prethodnih studija zabilježeni porast razine MSNA se dogodio tek pri dostizanju još nižih razina $SaO_2$[44]. Magnituda simpatičkog odgovora na podražaj hipoksijom određena je kako „dubinom" hipoksije tako i trajanjem hipoksije. U naših ispitanika hipoksija je izazivana postupno i dugotrajno, te su vjerojatno na taj način stvoreni uvjeti za porast MSNA i pri višim vrijednostima $SaO_2$ (od približno 90%).

Ukupni hemodinamički odgovor na hipoksiju je rezultat direktnog utjecaja hipoksije na krvne žile i na kontraktilnost srca te na autonomni živčani sustav, bilo direktno centralno ili putem različitih refleksnih putova. Postoje brojne studije koje su istraživale utjecaj hipoksije na krvne žile. U ispitanika izloženih sniženom tlaku donje polovice tijela (engl. *lower body negative pressure*) koji su pri tome udisali hipoksičnu mješavinu, bile su potrebne dvostruko veće doze noradrenalina i angiotenzina da bi se održao vaskularni tonus sličan onome u normoksiji[45]. Nadalje, tijekom farmakološke blokade α-adrenoreceptora, periferna vazodilatacija je bila značajno produljena nakon izlaganja hipoksiji[46]. Obje navedene studije sugeriraju da je vazodilatacija uzrokovana hipoksijom „maskirana" pojačanom aktivacijom simpatičkog sustava, a koja je posredovana kemorefleksom. Uz navedeno, hipoksija također ima direktni negativni inotropni učinak na srčani mišić[47].

U našim studijama simpatička živčana aktivnost počela se normalizirati nakon 20 – 30 minuta po završetku udisanja hipoksične mješavine. U istraživanju Xie i suradnika[48] MSNA

odgovor na hipoksiju se normalizirao tek nakon 60 min. Razlike u odnosu na naše rezultate mogu se objasniti razlikama u trajanju te intenzitetu hipoksičnih podražaja. U naših ispitanika tijekom izlaganja hipoksiji zamijećen je izraženiji porast u $MSNA_t$ nego u $MSNA_f$. Navedeno bi moglo upućivati na pojavu „regrutiranja" novih simpatičkih postganglijskih neurona[25].

Suprotno zapažanjima dobivenim u studijama koje uključuju bolesnike s OSA, trenirani ronioci na dah nisu kronično izloženi prenaglašenoj aktivnosti simpatičkog živčanog sustava. Moguće obrazloženje za postojanje razlika u razini simpatičke aktivnosti u te dvije populacije su razlike u trajanju i modalitetu izloženosti hipoksiji te razlike u populacijskim karakteristikama. Ronioci na dah izloženi su hipoksiji za vrijeme voljnih apneja na kraju inspirija, dok su bolesnici s OSA hipoksiji izloženi za vrijeme nevoljnih apneja na kraju ekspirija. Nadalje, ronioci na dah imaju apnejaške treninge 3 – 4 puta tjedno u prosječnom trajanju od 1 – 1,5 h; bolesnici s OSA imaju ponavljane epizode apneje skoro svaki put prilikom spavanja. Stoga, ukupno vrijeme trajanja apneje tj. izloženosti hipoksiji višestruko je veće u bolesnika s OSA nego u treniranih ronilaca na dah. Starosna dob također predstavlja moguću varijablu koja pridonosi razlici između spomenutih skupina. Trenirani ronioci na dah u našim istraživanjima su bili relativno mladi. Nasuprot tome, u većini studija bolesnici s OSA su pripadali starijim dobnim skupinama. Naposljetku, bolesnici s OSA često imaju druge komorbiditete poput patološke debljine, bolesti srca i dijabetesa. Svako od tih stanja dokazano uzrokuje ili potencira porast bazalne simpatičke živčane aktivnosti. Nasuprot tome, ispitivana skupina ronilaca na dah, najčešće je bila skupina zdravih mladih ljudi.

### 3.4.2. Obrazac aktivacije simpatičkog živčanog sustava u apneji

Rezultati 3. znanstvenog rada pokazuju da je obrazac porasta aktivacije simpatičkog živčanog sustava prilikom zadržavanja daha na razini FRC i TLC u treniranih ronilaca na dah identičan onome u kontrolnih ispitanika. Nadalje, uz podjednako vremensko trajanje zadržavanja daha (u ovoj studiji približno 3 min) odnosno uz podjednaku razinu kemorefleksnog stresa, razina simpatičke živčane aktivnosti među ispitivanim skupinama je slična. Međutim, zamijećena je razlika u mehanizmu postizanja iste bazalne razine simpatičke aktivnosti između dvije skupine. Trenirani ronioci na dah imali su u prosjeku manji broj AP unutar pojedinačnog simpatičkog izbijanja, ali uz povišenu vrijednost $MSNA_i$ i $MSNA_f$ ukupna dosegnuta simpatička aktivnost (broj AP u jedinici vremena) je bila slična među skupinama.

*Analiza integriranog signala*

*FRC protokol.* Prosječno trajanje apneje pri FRC je bilo znatno dulje u skupini ronilaca na dah nego u kontrolnih ispitanika. Posljedično, ronioci na dah su bili izloženi višoj razini kemorefleksnog stresa, te je stoga kao posljedica apneje pri FRC zabilježen značajno viši porast MSNA u treniranih ronilaca na dah. Međutim, nagibi krivulja koje opisuju porast u MSNA u jedinici vremena trajanja apneje pri FRC su gotovo identični između skupina. Dobiveni podatak potvrđuje rezultate ranijih istraživanja o nepostojanju promjena u regulaciji rada autonomnog sustava u treniranih ronilaca na dah u usporedbi s kontrolnim ispitanicima[27;49–51]. Stoga, ova studija također isključuje postojanje utjecaja učestalih, produljenih, voljnih apneja na senzitivnost kemoreceptora kakva je opisana u bolesnika s OSA[10].

*TLC protokol.* Budući da je u ovom istraživanju prosječno vrijeme trajanja apneje pri TLC bilo slično između skupina, zabilježen je sličan porast u MSNA u obje skupine. Međutim, zamijećena je razlika u obrascu porasta MSNA. Unutar prvih 30 s trajanja zadržavanja daha pri razini TLC pluća zabilježen je naglašeniji porast MSNA u treniranih ronilaca na dah u odnosu na onaj zabilježen u kontrolnih ispitanika. U isto vrijeme, u hemodinamskim parametrima treniranih ronilaca na dah zabilježen je izraženiji pad MAP-a i SBP-a, uz tendenciju sniženja DBP-a, udarnog volumena srca (SV) te srčanog minutnog volumena (CO). Stoga, izrazitiji porast MSNA tijekom prvih 30 s apneje pri TLC u skupini ronilaca može se pripisati pojačanoj aktivaciji baroreceptora, u svrhu kompenziranja nastalih hemodinamičkih promjena uzrokovanih dubokim udahom prilikom započinjanja zadržavanja daha.

Opažene razlike u navedenim fiziološkim parametrima između skupina vjerojatno su uzrokovane različitim dubinama udaha prije zadržavanja daha. Naime, trenirani ronioci na dah imaju sposobnost uzimanja dubljih udaha prije samog čina zadržavanja daha, što je posljedica bolje kontrole nad respiracijskim mišićima i/ili veće popustljivosti prsnog koša kao posljedica treninga[52]. Posljedično, nagib krivulje koji opisuje porast MSNA tijekom prvih 30 s apneje pri TLC je bio dvostruko veći u skupini ronilaca na dah. Također je važno napomenuti da je izrazito brzi porast u ukupnoj MSNA tijekom prvih 30 s apneje pri TLC bio prvenstveno uzrokovan porastom $MSNA_f$, dok je vrlo mala promjena zabilježena u amplitudi izbijanja i prosječnom broju AP unutar jednog izbijanja.

*Analiza simpatičkih akcijskih potencijala*

Iako se zabilježeni obrazac porasta simpatičke živčane aktivnosti tijekom apneje pri FRC i TLC nije znatno razlikovao između ispitivanih skupina, identifikacijom postganglijskih simpatičkih AP opaženo je da ronioci na dah imaju značajno manji prosječan broj AP unutar jednog simpatičkog izbijanja te manji prosječni broj aktivnih skupova AP. U ovom trenutku postojeća znanstvena saznanja nisu u mogućnosti objasniti uzrok ovog opažanja. Moguće je da se radi o modulaciji obrasca aktivnosti simpatičkog sustava kao posljedice izlaganja učestalim i dugotrajnim simpatoekscitacijskim podražajima (u ovom slučaju zadržavanjem daha), kakvim su ovi ispitanici redovito izloženi tijekom svojih treninga. Isto tako, moguće je da opažene razlike u obrascu aktivnosti simpatičkog živčanog sustava predstavljaju „samo" populacijsku karakteristiku.

Ovo istraživanje je ukazalo na postojanje razlike u obrascu simpatičkog odgovora koje ovise o vrsti podražaja. U prethodnoj studiji Steinbacka i sur.[53] utvrđeno je postojanje uređenog redoslijeda aktiviranja simpatičkih neurona tijekom progresivnog porasta kemorefleksnog stresa. Obrazac regrutacije neurona podsjećao je na Hannemanov „princip veličine" opisan u motoneurona[54]. Ranije studije koje su koristile tehniku snimanja pojedinačnih simpatičkih neurona također su pretpostavile ovakav scenarij aktivacije simpatičkih neurona[55]. U ovoj studiji, apneja pri FRC te kasnija faza apneje pri TLC predstavljaju periode kada su ispitanici izloženi progresivnom porastu kemorefleksong stresa. Tijekom ovih faza protokola zabilježen je porast u frekvenciji AP koji je bio posljedica kako porasta $MSNA_i$ tako i porasta prosječnog broja AP unutar jednog simpatičkog izbijanja. Zabilježeni porast u broju AP unutar jednog izbijanja je korelirao s porastom aktivnih skupova AP unutar jednog izbijanja.

Navedeni rezultati ukazuju na porast vjerojatnosti okidanja simpatičkih postganglijskih neurona te na vjerojatno regrutiranje prethodno neaktivnih (ili manje aktivnih) neurona većeg promjera i brže provodljivosti. Ovaj zaključak je u skladu s rezultatima ranijih istraživanja[53;55]. Za trajanja apneje pri FRC, porast u frekvenciji AP potpomognut je proporcionalnim porastom $MSNA_f$ (porast od ~ 100%) te porastom broja AP unutar jednog simpatičkog izbijanja (porast od ~ 50%). Međutim, u drugoj polovici apneje pri TLC $MSNA_f$ je već bila izrazito visoka, stoga porast frekvencije AP u ovoj fazi je bio posljedica prvenstveno porasta broja AP unutar jednog izbijanja (porast od ~ 100%). Navedeni rezultati za drugu polovicu apneje pri TLC ukazuju na izraženu pojavu regrutiranja simpatičkih neurona te moguću pojavu ponavljanog okidanja simpatičkih neurona tijekom istog

simpatičkog izbijanja, a koje je bilo popraćeno slabim porastom $MSNA_f$ (porast od ~ 5 – 25%).

Početnih 30 s zadržavanja daha na TLC razini pluća karakterizirano je sniženjem MAP-a, SV-a i CO-a koje posljedično uzrokuje aktivaciju barorefleksa. Iako je i ova faza protokola predstavljala izrazit simpatoekscitacijski podražaj, zabilježeni obrazac aktivacije simpatičkog živčanog sustava je bio različit od onoga uzrokovanog progresivnim kemorefleksnim stresom. Tijekom prvih 30 s apneje pri TLC zabilježen je izrazit porast $MSNA_f$ (porast od ~ 200%) koji je bio popraćen relativno malim porastom prosječnog broja AP unutar pojedinog simpatičkog izbijanja (porast od ~ 30%). Uzrok pojavi različitog obrasca aktiviranja simpatičkog živčanog sustava nakon aktivacije barorefleksa u odnosu na kemorefleks je za sada nepoznat. Jedno od objašnjenja bi moglo biti postojanje određenog praga podražaja koji uzrokuje pojavu regrutiranja simpatičkih postganglijskih neurona u obrascu simpatičke aktivacije. U tom slučaju, rezultati ove studije bi se mogli objasniti postojanjem potrebe za dosezanjem relativno višeg intenziteta pojedinačnog podražaja za prelaženje navedenog praga za berorefleks nego li je to potrebno za kemorefleksni podražaj.

Moguća potvrda ove tvrdnje leži u činjenici da su u našem istraživanju, u skupini ronilaca na dah, zabilježeni rezultati koji bi mogli upućivati na pojavu regrutiranja simpatičkih postganglijskih neurona tijekom aktiviranja barorefleksa. Naime, zabilježen je trend porasta broja AP unutar jednog izbijanja te broja aktivnih skupova AP u ronilaca, ali ne i u kontrolnih ispitanika. Zbog dubljeg udaha prije započinjanja zadržavanja daha te posljedično tome sniženjem MAP-a (od ~ 35%) i SV-a (od ~ 40%), moguće je da su ronioci na dah dosegli tzv. prag podražaja potreban za pojavu regrutiranja simpatičkih neurona.

Studija koju su napravili Salmanpour i sur.[56] koristila je istu metodologiju detekcije simpatičkih AP kao i naša studija. U toj studiji ispitanici su bili izloženi negativnom tlaku donje polovice tijela (sve do -60 mmHg) u svrhu aktiviranja barorefleksa. Njihova studija je također pokazala da je porast aktivnosti simpatičkog sustava uslijed aktivacije barorefleksa bio uzrokovan isključivo porastom $MSNA_f$ i $MSNA_i$ bez promjena u broju AP unutar simpatičkih izbijanja koji bi upućivali na pojavu regrutiranja dodatnih simpatičkih neurona.

## 3.5. Zaključci

Trenirani ronioci na dah koji se učestalo izlažu produljenim, voljnim zadržavanjima daha imaju normalan odgovor simpatičkog živčanog sustava te ventilacijski i hemodinamčki odgovor na podražaj intermitentnom hipoksijom/hiperkapnijom. Poremećaji regulacije autonomnog živčanog sustava nisu prisutni nakon mjesec dana od prestanka učestalih treninga ni tijekom intenzivnih višemjesečnih perioda apenjaških treninga. Ova saznanja upotpunjuju ona dobivena ranijim istraživanjima koja su pokazala normalnu senzitivnost centralnih kemoreceptora[27] te normalnu cerebrovaskularnu reaktivnost[57] u ovoj populaciji. Može se zaključiti da u odsustvu faktora rizika poput arterijske hipertenzije, intolerancije glukoze i hiperlipidemije, voljno izlaganje učestaloj hipoksiji i hiperkapniji nema trajan efekt na regulaciju simpatičke aktivnosti, te na regulaciju ventilacije i krvožilnog sustava.

Produljeno, voljno zadržavanje daha rezultira značajnim porastom mišićne simpatičke živčane aktivnosti. Navedeni porast se doseže sljedećim mehanizmima: 1) porastom incidencije simpatičkih izbijanja (povećanjem frekvencije okidanja postganglijskih simpatičkih neurona), 2) regrutiranjem novih, prethodno neaktivnih ili manje aktivnih simpatičkih neurona koji se prezentiraju akcijskim potencijalima veće amplitude (ukazuje na veći promjer neurona i veću brzinu provođenja impulsa), te 3) vrlo vjerojatno pojavom opetovanih okidanja simpatičkih neurona unutar jednog simpatičkog izbijanja.

Obrazac aktivacije simpatičkog živčanog sustava uvjetovan je tipom provokacijskog faktora koji ga je izazvao. Aktivacija kemorefleksa koja se javlja tijekom apneje pri FRC i u drugom dijelu apneje pri TLC uzrokuje porast vjerojatnosti okidanja simpatičkih postganglijskih neurona i pojavu regrutiranja dodatnih simpatičkih neurona većeg promjera. Porast simpatičke aktivnosti zbog aktivacije barorefleksa u ovom istraživanju prvenstveno je bilo uzrokovano povećanjem frekvencije i incidencije simpatičkih izbijanja, te nije predstavljalo izrazit poticaj za regrutiranje dodatnih simpatičkih postganglijskih neurona.

Moguće je da redovito izlaganje simpatoekscitacijskim podražajima kakvi se javljaju tijekom zadržavanja daha (posebice u apneji pri TLC) predstavlja potencijalni mehanizam koji uzrokuje promjenu bazalnog obrasca aktivnosti simpatičke živčanog sustava, a koji se manifestira smanjenjem broja akcijskih potencijala unutar jednog simpatičkog izbijanja te porastom frekvencije simpatičkih izbijanja.

## 3.6. Sažetak

Trenirani ronioci na dah učestalo su izloženi ponavljanim, izrazitim smanjenjima saturacije arterijske krvi kisikom koji mogu dovesti do poremećaja regulacije kemorefleksa. Iako je voljno zadržavanje daha već ranije opisano kao izraziti simpatoekscitacijski podražaj, obrazac aktiviranja postganglijskih simpatičkih neurona kojima se određuje odgovor simpatičkog živčanog sustava u čovjeka su vrlo malo istraženi.

Cilj ove doktorske disertacije je pokazati mogu li učestala, voljna izlaganja izrazitoj hipoksiji, kakvoj su izloženi trenirani ronioci na dah, uzrokovati poremećaj autonomne regulacije u vidu pojave hipersenzitivnosti perifernih kemoreceptora. Nadalje, utvrditi postoje li navedeni poremećaji kemorefleksa i nakon više od mjesec dana od prestanka intenzivnih apnejaških treninga. Naposljetku, posljednji cilj ove disertacije je odrediti obrazac aktiviranja simpatičkog živčanog sustava tijekom zadržavanja daha te rezultate usporediti između skupina treniranih ronilaca na dah i zdravih kontrolnih ispitanika.

U tu svrhu, te dvije skupine ispitanika su bile izložene udisanju normokapnične hipoksične plinske smjese do smanjenja saturacije arterijske krvi kisikom do razine od oko 80%. Pri tome ispitanicima se mjerila mišićna simpatička živčana aktivnost (MSNA) u peronealnom živcu korištenjem tehnike mikroneurografije. Također se mjerio ventilacijski odgovor te različiti hemodinamski parametri. Isti parametri su mjereni i u drugom eksperimentalnom protokolu koji je uključivao maksimalno zadržavanje daha pri funkcionalnom rezidualnom kapacitetu pluća (apneja pri FRC) te na ukupnom kapacitetu pluća (apneja pri TLC). Dobiveni mikroneurografski zapis se analizirao računalnom aplikacijom koja omogućava identifikaciju pojedinačnih simpatičkih akcijskih potencijala (AP) te određuje njihova svojstava.

Rezultati znanstvenih radova broj 1 i 2 pokazali su da ne dolazi do akutnih (za trajanja perioda intenzivnih apnejaških treninga) ni kroničnih (nakon više od mjesec dana od prestanka intenzivnih treninga) promjena u vidu prenaglašenosti aktivacije simpatičkog živčanog sustava i prenaglašenog ventilacijskog odgovora nakon podražaja hipoksijom u treniranih ronilaca na dah, a što bi upućivalo na pojavu pojačane senzitivnosti perifernih kemoreceptora. Može se zaključiti da u odsustvu faktora rizika kao što je arterijska hipertenzija, intolerancija glukoze i hiperlipedemija, voljno izlaganje učestaloj hipoksiji i hiperkapniji nema dugotrajan efekt na regulaciju simpatičke aktivnosti, te na regulaciju ventilacije i krvožilnog sustava.

Rezultati 3. znanstvenog rada pokazuju da je obrazac porasta aktivacije simpatičkog živčanog sustava prilikom zadržavanja daha pri razinama FRC (izolirana stimulacija kemorefleksa) i TLC (kombinacija stimulacije barorefleksa i kemorefleksa) u treniranih ronilaca na dah identičan onome u kontrolnih ispitanika. Zabilježen je različit obrazac aktivacije simpatičkog živčanog sustava ovisno o tipu provokacijskog faktora koji ga je izazvao (barorefleks *vs.* kremorefleks). Zamijećena je i razlika u mehanizmu postizanja iste bazalne razine simpatičke aktivnosti između dvije skupine. Trenirani ronioci na dah imali su u prosjeku manji broj akcijskih potencijala unutar pojedinačnog simpatičkog izbijanja, ali uz povišenu incidenciju i frekvenciju simpatičkih izbijanja ukupna bazalna dosegnuta simpatička aktivnost (broj akcijskih potencijala u jedinici vremena) je bio sličan među skupinama. Navedeno sugerira na postojanje mogućeg adaptacijskog mehanizma promjene bazalnog obrasca aktivnosti simpatičkog živčanog sustava zbog izlaganja učestalim simpatoekscitacijskim podražajima poput zadržavanja daha.

## 3.7. Summary

**Sensitivity of peripheral chemoreceptors and activation pattern of sympathetic nervous system during breath-holding at different lung volumes in apnea divers**

Elite breath-hold divers are regularly exposed to repeated massive arterial oxygen desaturations, which can perturb chemoreflexes. Voluntary breath-holding has already been recognized as a pronounced sympathoexcitatory stimulus; however pattern of activation of postganglionic sympathetic neurons which determine the level of sympathetic outflow in men is poorly investigated.

Aim of this doctoral dissertation is to evaluate the influence of frequent, voluntary exposures to profound hypoxia, occurring in trained breath-hold divers, on autonomic regulation characterized by hypersensitivity of peripheral chemoreceptors. The existence of these chemoreflex disorders was tested after one-month cessation period of apnea trainings. Finally, the last aim of this dissertation is to assess the activation pattern of sympathetic nervous system during breath-holding and to compare the responses between the groups of elite breath-hold divers and healthy control subjects.

These two groups were exposed to breathing of normocapnic hypoxic gas mixture until reaching 80% of arterial oxygen saturation. Simultaneously, muscle sympathetic nerve activity (MSNA) was assessed in the peroneal nerve using the technique of microneurography. Ventilation and various hemodynamic physiological parameters were measured as well. Similar set of measurements was used in the second experimental protocol. This protocol included breath-holding at different lung volumes (functional residual capacity (FRC) and total lung capacity (TLC), respectively). Microneurographic recordings were analyzed using custom made computer software that enabled identification of individual sympathetic action potentials (APs) and determination of their characteristics.

The results of studies no. 1 and 2 discarded the existence of acute (throughout the intensive apnea trainings period) or chronic (after at least one-month training cessation period) increase in basal level of MSNA and excessive ventilatory response following exposure to hypoxia in trained breath-hold divers, suggesting normal peripheral chemoreceptor sensitivity. Consequently, it can be concluded that in the absence of additional risk factors like hypertension, glucose intolerance or hyperlipidemia, voluntary exposure to intermittent hypoxia/hypercapnia may not have a negative impact on autonomic, ventilatory, and cardiovascular regulation.

The results of study no. 3 showed similar recruitment patterns of sympathetic neuron activity during FRC (isolated chemorefelex stimulation) and TLC breath-holds (combined activation of baroreflex and chemoreflex) in breath-hold divers and controls. Different patterns of activation of postganglionic sympathetic neurons were observed depending on provocation factor (baroreflex *vs.* chemoreflex). The mechanism by which the same sympathetic response was elicited during the breath-holds differed between the two groups. Specifically, the divers exhibited fewer APs per burst at rest but a pronounced increase in the burst incidence in this group achieved the same overall sympathetic response (number of APs per unit-time). This observation suggests possible existence of adaptation mechanism by which the pattern of basal sympathetic outflow can be altered due to regular and frequent exposures to the level of sympathoexcitation attained during breath-holding.

## 3.8. Životopis

### TONI BREŠKOVIĆ


| | |
|---|---|
| **Zanimanje:** | Doktor medicine, znanstveni novak |
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#### OBRAZOVANJE

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- 08. 06. – 12. 06. 2009. – Cardiovascular Ultrasound Summer School, Research Institute for Sport and Exercise Sciences, Liverpool John Moores University, Liverpool, Ujedinjeno kraljevstvo
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**OBJAVLJENE PUBLIKACIJE**

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**POZNAVANJE STRANIH JEZIKA**

- Engleski – razina B2
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RESEARCH ARTICLE

# Peripheral chemoreflex regulation of sympathetic vasomotor tone in apnea divers

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**Abstract**
*Objectives* Involuntary apnea episodes in obstructive sleep apnea patients result in selective potentiation of peripheral chemoreceptor regulation of sympathetic vasomotor tone. Breath-hold diving is associated with repeated "voluntary" apnea episodes and massive arterial oxygen desaturation, which could also perturb chemoreflex function.
*Methods* We measured ventilation, heart rate, blood pressure, cardiac stroke volume, and muscle sympathetic nerve activity (MSNA) during isocapnic hypoxia in 11 breath-hold divers and eleven matched control subjects. The study was carried out at least 1 month after intense apnea training.
*Results* Baseline MSNA frequency was 30 ± 4 bursts/min in control subjects and 31 ± 7 bursts/min in divers (ns). During hypoxia MSNA frequency and total activity increased similarly in both groups (30 and 66% in controls and 27 and 60% in divers, respectively). MSNA remained increased after termination of hypoxia and approached baseline measurements after 20 min. Hypoxia-induced stimulation of minute ventilation was similar in both groups, although in divers it was maintained by higher tidal volumes and lower breathing frequency compared with control subjects. In both groups, hypoxia-induced tachycardia drove an increase in cardiac output whereas total peripheral resistance decreased. Blood pressure remained unchanged.
*Interpretation* We conclude that after the end of intensive training/competition periods, apnea divers show normal peripheral chemoreflex regulation of ventilation and sympathetic vasomotor tone. Although voluntary apnea may not lead to sustained changes in sympathetic nervous system regulation, we cannot exclude the possibility that repeated sympathetic activation elicited by voluntary apnea imposes a burden on the cardiovascular system.

**Keywords** Isocapnic hypoxia · MSNA · Apnea · Breath-holding · Detraining

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## Introduction

Peripheral chemoreceptors, located in the carotid bodies, primarily respond to hypoxia [30], whereas central chemoreceptors on the ventral surface of the medulla selectively respond to hypercapnia [6]. Both chemoreceptor types regulate respiration [21, 24]. Furthermore, central and peripheral chemoreceptor activation elicits a sympathetically mediated pressor response [22, 26]. Involuntary

apnea in obstructive sleep apnea patients is associated with augmented peripheral chemoreflex sensitivity [10, 20] that may predispose to cardiovascular disease. In contrast, central chemoreflex regulation appears to be normal in these patients [20]. These observations are a matter of concern given the increasing number of recreational athletes practicing "voluntary apnea", such as water hockey players, synchronized swimmers, and breath-hold divers. Elite breath-hold divers are an extreme example for voluntary apnea. After maximal apnea, alveolar oxygen partial pressure can be as low as 30–40 mmHg with arterial oxygen saturation around 50% [3, 5]. Typically, spear-fishing competitions last for approximately 5 h with cumulative apnea duration of around 1 h. Voluntary apnea under laboratory conditions, and intermittent hypoxemia, cause acute, as well as sustained, changes in cardiovascular autonomic regulation [2, 9, 15, 17, 18]. Twenty to thirty minutes of intermittent hypoxia are sufficient to raise muscle sympathetic nerve activity (MSNA) and blood pressure [3, 15]. Since, obstructive sleep apnea patients and breath-hold divers are exposed to comparable levels of intermittent hypoxia/hypercapnia at least during the diving season, we expected to observe similar abnormalities in autonomic cardiovascular control. Previously, we observed that central chemoreflex control of respiration and sympathetic activity is maintained in breath-hold divers [3]. In the current study, we tested the hypothesis that in a manner that is similar to obstructive sleep apnea patients, peripheral chemosensitivity is abnormal in breath-hold divers after a detraining period. We measured autonomic, ventilatory, and hemodynamic responses to isocapnic hypoxia in divers and matched control subjects.

## Methods

### Subjects

We included 22 healthy male ($N = 17$) and female ($N = 5$) volunteers in our study. Of those, 11 were experienced elite breath-hold divers (BHD) (8 males and 3 females) while 11 untrained subjects served as controls (9 males, 2 females). Anthropometric and pulmonary function data are given in Table 1. All experimental procedures in this study were performed in accordance with the Declaration of Helsinki and were approved by the ethical committee of the University of Split School of Medicine. Informed, written consent was obtained from each subject.

### Protocol

We conducted our studies in December 2007/January 2008. The study was carried out approximately 1 month after the majority of divers (6 out of 11) had finished their most intensive apnea training; in 5 participants the period after termination of intensive apnea trainings was 2 months. During the detraining period, apnea divers significantly reduce their apnea trainings to an average of once per week in form of "non-formal trainings" (for example, training at home and individually in the pool, or activities such as spear-fishing). All experiments were carried out in a climatized room in the morning hours. Participants were instructed not to eat at least 4 h before the arrival to the laboratory. Female subjects participated in the experiment during follicular phase of their menstrual cycle.

**Table 1** Anthropometric characteristics of subjects

| | Controls ($N = 11$) | BHD ($N = 11$) | $P$ |
|---|---|---|---|
| Age (years) | 25.5 ± 4.4 | 27.7 ± 4.1 | 0.24 |
| Mass (kg) | 82.5 ± 14.1 | 81.6 ± 12.7 | 0.87 |
| Height (m) | 1.83 ± 0.07 | 1.82 ± 0.08 | 0.76 |
| BMI | 24.5 ± 3.5 | 24.5 ± 2.4 | 0.96 |
| BFI (% body fat/kg) | 21.1 ± 7.6 | 20.1 ± 8.9 | 0.79 |
| FVC (% predicted) | 105.8 ± 13.6 | 132.5 ± 16.2 | <0.001 |
| $FEV_1$ (% predicted) | 105.2 ± 9.2 | 109.9 ± 14.4 | 0.41 |

Values are means ± SD. Differences between groups were analyzed by unpaired Student $t$ test

*BMI* body mass index, *BFI* body fat index (calculated by Jackson and Pollock three site measurement), *FVC* forced vital capacity, $FEV_1$ forced expiratory volume in first second

Before instrumentation subjects underwent dynamic spirometry (Quark PFT, Cosmed, Rome, Italy) while standing after which their anthropometry measurements were made. Subsequently, the participants assumed the supine position where they were instrumented for the hypoxic test.

An infrared probe was positioned on the middle finger to monitor arterial oxygen saturation (Poet II, Criticare Systems, Waukesha, USA). Beat-by-beat blood pressure and heart rate were measured using a finger cuff (Finometer, Finapress Medical Systems, Arnhem, Netherlands) and electrocardiography, respectively. Multiunit muscle sympathetic nerve activity (MSNA) of postganglionic sympathetic activity was recorded from the right peroneal nerve with a unipolar tungsten electrode as described previously [3]. The nerve signal was amplified 100,000 times. Afterwards signal was band-pass filtered (0.7–2.0 kHz), rectified and integrated using 0.1 s time constant (662C-4, Nerve traffic analysis system, Bioengineering, The University of Iowa, USA).

Subjects were breathing from a mouthpiece connected to a non-rebreathing Y-valve (Hans-Rudolph 2730 Series, Large 2-way, NRBV, Y Shape, K.C., MO, USA) whose inspiratory port was connected to a three-way valve (Hans Rudloph 4000 Series Large non mixing, 3-way "Y"

Stopcock, K.C., MO, USA) allowing switching between room air and gas-reservoir. The spirometer (Harvard apparatus, Student model, Holliston, MA, USA) acted as reservoir for the gas mixture whose composition was regulated by a blender. Blender was attached to three gas cylinders (compressed air, 100% $N_2$ and 100% $CO_2$) thus enabling to produce different hypoxic gas mixtures. The gases were sampled breath-by-breath at the mouth using a respiratory analyzer (Quark $b^2$, Cosmed, Rome, Italy). Before each trial, control data were collected by having participants breathe room air for 3–5 min while monitoring the $PetCO_2$ concentration to establish his or her normoxic level. This was followed by progressive normocapnic hypoxia introduced in two steps by increasing the $N_2$ concentration until the required $SaO_2$ was achieved. Mean $SaO_2$ levels of $\sim$0.9 lasted for 3 min, and $\sim$0.8 for 5 min. Normocapnic $PetCO_2$ was maintained by adding $CO_2$ to the inspired gas, thus, minimizing central chemoreceptor engagement. After cessation of hypoxia testing, subjects were switched to breathe room air while measurements were continued for another 20–25 min.

### Data acquisition and analysis

All data were acquired using an analog-to-digital converter (Powerlab/16SP, ADInstruments, Castle Hill, Australia) interfaced with PC. Data were sampled at 1 kHz and stored for subsequent analysis using Chart software (ADInstruments, version 5.5.6.7). MSNA bursts were identified according to following criteria: (1) signal to noise ratio that was >2; (2) latency limit; (3) burst width limit (short duration = artifact, long duration = skin sympathetic nerve activity or afferent activity; (4) no preceding premature beats [29]. MSNA activity was expressed as frequency of bursts per minute (burst frequency) and per 100 heart beats (burst incidence). The amplitude and area of each burst was calculated. Total MSNA was calculated as the sum of all burst areas per minute. We obtained good quality MSNA recordings in 10 out of 11 breath-hold divers. In one diver the electrode shifted during the protocol and total MSNA was not calculated for that participant. Recordings of all participants in the control group were suitable for analysis. We quantified sympathetic chemoreflex sensitivity and ventilatory responses to incremental hypoxia as the change in sympathetic nerve activity or ventilation per change in $SaO_2$ during hypoxic protocol. Changes in left ventricular stroke volume were estimated by pulse wave analysis using an improved method of Wesseling (Modelflow program) [12]. The data were analyzed during 3-min period before hypoxia, 3 min during $SaO_2 = 0.9$, last 3 min of hypoxia trial while $SaO_2 = 0.8$, during 3 min upon cessation of hypoxia and 3 min after 20-min recovery period.

### Statistical analysis

All data were expressed as means ± 95% confidence intervals (95% CI). Baseline values, values at the same time points and chemoreflex sensitivities were compared using unpaired Student *t* test. The effects of hypoxia on all measured variables within groups were determined using repeated-measures ANOVA. A Bonferroni test was used as post hoc test. Interactions among responses were assessed using a general linear model for repeated-measures ANOVA. All analyses were performed with Statistica 7.0 software (Statsoft, Inc., Tulsa, USA).

## Results

Baseline sympathetic nerve activity, minute ventilation ($V_E$) and heart rate (HR) were similar between the groups. Systolic (Sys AP), diastolic (Dia AP) and mean arterial pressure (MAP) were elevated in BHD but the values did not reach statistical significance (Table 2).

Figure 1 presents an individual response to hypoxic stimulus in one control subject and one BHD. In both groups burst frequency and total MSNA increased during hypoxia (29.5 ± 10.6 and 65.9 ± 26.8% in control group; 27.4 ± 10.9 and 59.8 ± 22.0% in divers, respectively). These measurements remained slightly elevated during the first 3 min after termination of hypoxia but became completely normalized after 20-min recovery period. Burst incidence did not change throughout hypoxia testing in both groups (Fig. 2).

Sympathetic chemoreflex sensitivity was similar between groups (0.06 ± 0.03 au/min per 1% change in $SaO_2$ in controls and 0.07 ± 0.03 au/min per 1% change in $SaO_2$ in divers; $P = 0.86$).

**Table 2** Baseline data

| | Controls ($N = 11$) | BHD ($N = 11$) | $P$ |
|---|---|---|---|
| $MSNA_f$ (bursts $min^{-1}$) | 29.9 ± 3.5 | 30.8 ± 6.6 | 0.83 |
| $MSNA_t$ (au $min^{-1}$) | 2.0 ± 0.7 | 2.0 ± 0.7[a] | 0.97 |
| $V_E$ (l $min^{-1}$) | 7.8 ± 1.2 | 7.6 ± 0.8 | 0.78 |
| HR (beats $min^{-1}$) | 67.7 ± 6.1 | 68.1 ± 3.0 | 0.91 |
| MAP (mmHg) | 95.8 ± 4.4 | 101.4 ± 4.6 | 0.10 |
| Sys AP (mmHg) | 128.0 ± 5.7 | 136.9 ± 6.5 | 0.057 |
| Dia AP (mmHg) | 77.4 ± 3.6 | 81.6 ± 3.3 | 0.11 |

Values are means ± 95% CI. Differences between groups were analyzed by unpaired Student *t* test

*$MSNA_f$* burst frequency, *$MSNA_t$* total MSNA, *$V_E$* minute ventilation, *HR* heart rate, *MAP* mean arterial pressure, *Sys AP* systolic arterial pressure, *Dia AP* diastolic arterial pressure

[a] Data analyzed in 10 subjects

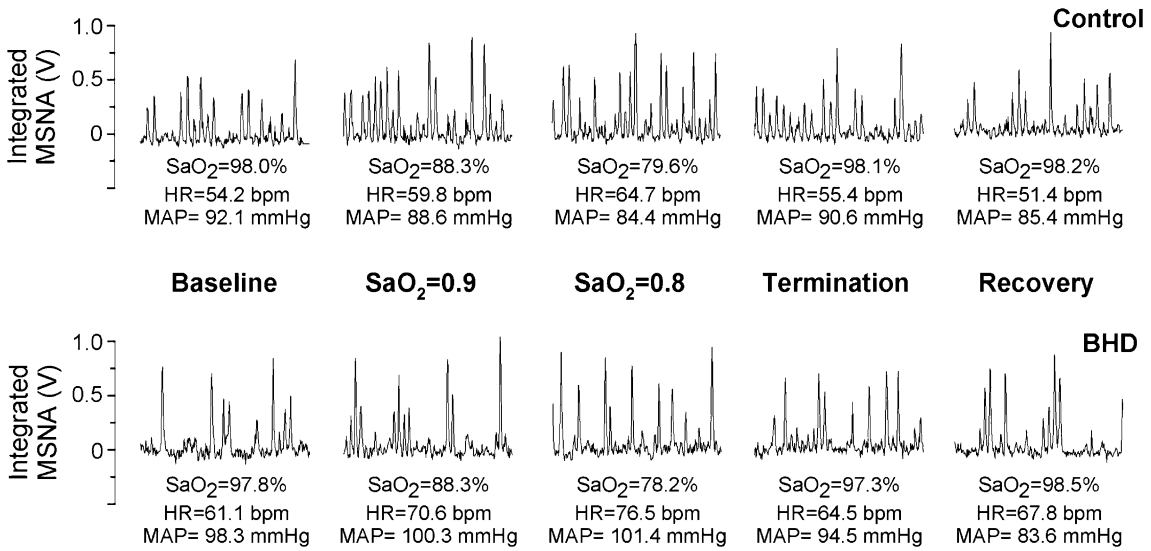



**Fig. 1** Representative 30-s fragments of integrated MSNA neurograms during different phases of the hypoxia protocol in one control participant and one breath-hold diver (*BHD*). Bellow each neurogram are the average values for $SaO_2$, HR and MAP. $SaO_2$ arterial oxygen saturation, *HR* heart rate, *MAP* mean arterial pressure

In the BHD group $V_E$ was maintained by a significantly larger tidal volume ($V_T$) (0.5 ± 0.1 l in controls vs. 1.0 ± 0.3 l in divers, respectively; $P < 0.05$) and lower breathing frequency (14.8 ± 2.0 in controls vs. 8.7 ± 2.1 breaths/min in divers, respectively; $P < 0.05$) compared to the control group. Hypoxic stimulation caused augmentation of $V_E$ for subjects in both groups primarily by increasing $V_T$ (66.3 ± 32.9% in controls; 60.3 ± 33.5% in divers). Divers increased significantly their $B_f$ by 30.3 ± 14.6% while $B_f$ did not change in the control group (11.8 ± 11.6%) (Fig. 3).

There was no difference among groups in ventilatory responses to incremental hypoxia. Compared with the control group (0.35 ± 0.16 l/min per 1% change in $SaO_2$), the change in ventilation not different in BHD (0.49 ± 0.23 l/min per 1% change in $SaO_2$; $P = 0.35$).

During the hypoxic protocol MAP, Sys AP, Dia AP and stroke volume (SV), did not change in either group. Heart rate (HR) and cardiac output (CO) increased as hypoxia progressed. Total peripheral resistance decreased during hypoxia in the control group only. The hemodynamic response to hypoxia was similar in both groups (Fig. 4).

## Discussion

The main finding of our study is that autonomic, ventilatory, and cardiovascular responses to hypoxia are virtually identical in elite divers after a detraining period and in control subjects. Similar to previous studies, divers showed normal baseline levels of sympathetic nerve activity [3]. Nevertheless, arterial blood pressure was increased in BHD group by an average of 5 mmHg; however, the difference was not statistically significant. The observation that peripheral and central chemosensitivity are preserved is consistent with previous findings [3]. Contrary to obstructive sleep apnea patients, breath-hold divers may not be exposed to excessive sympathetic activation. Normal peripheral and central chemosensitivity may serve as a protective mechanism for maintaining cerebral perfusion during extreme asphyxia associated with long breath-holds, since increased $CO_2$ and decreased $O_2$ have major vasodilatatory effect on this vascular bed, whereas increased peripheral sympathetic nerve traffic during hypoxia in divers and controls counteracts direct vasodilatatory effects.

The pattern of the sympathetic neural activity response to hypoxia was very similar in both groups. We observed an apparent, although non-significant, increase in MSNA already when subjects' arterial oxygen saturation reached 0.9. In several previous studies, the onset of MSNA increase as a response to hypoxic stimulus occurred on lower arterial saturations [23]. The magnitude of sympathetic response to the hypoxic stimulus is determined by severity of hypoxia and its duration. Induction of hypoxia to our subjects was long-lasting and gradual, thus possibly allowing development of a slight MSNA increase even at $SaO_2$ around 0.9.

Breath-hold divers compete in different disciplines such as static apnea, dynamic apnea, and constant weight diving. Static apnea divers float motionless face down in a pool for as long as possible. These different events produce varying degrees of hypoxic and hypercapnic stress. In dynamic apnea diving, the goal is to attain maximal underwater swimming distances. Finally, constant weight divers swim

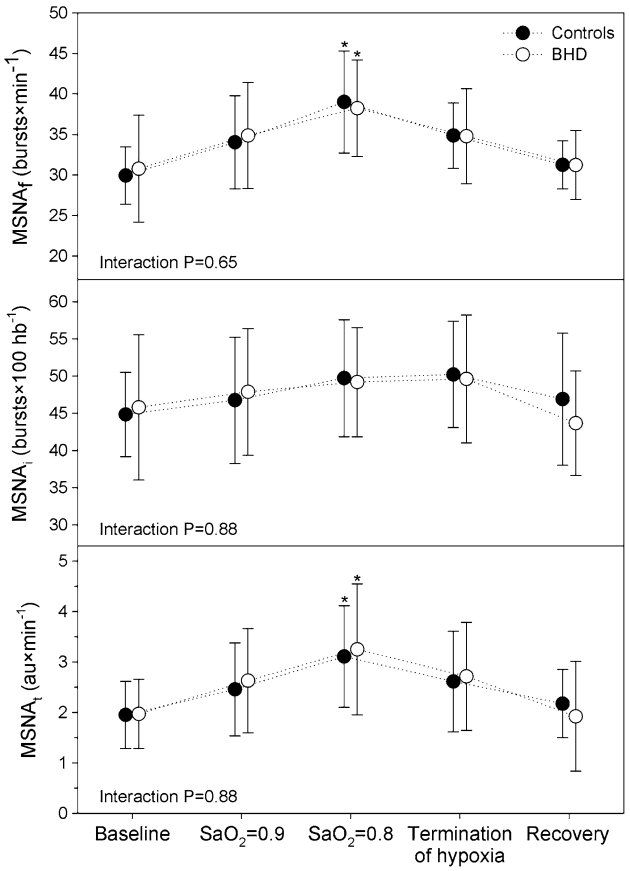



**Fig. 2** Changes in MSNA in both groups during all phases of hypoxic protocol. *Circles* represent means, *error bars* denote 95% confidence intervals. Significant changes within single group were determined using repeated-measures ANOVA with Bonferroni test as post hoc test (*$P < 0.05$). Interactions among responses of the two groups were tested using the general linear model for repeated-measures ANOVA. Differences between groups in the same time point were not observed (unpaired Student *t* test)

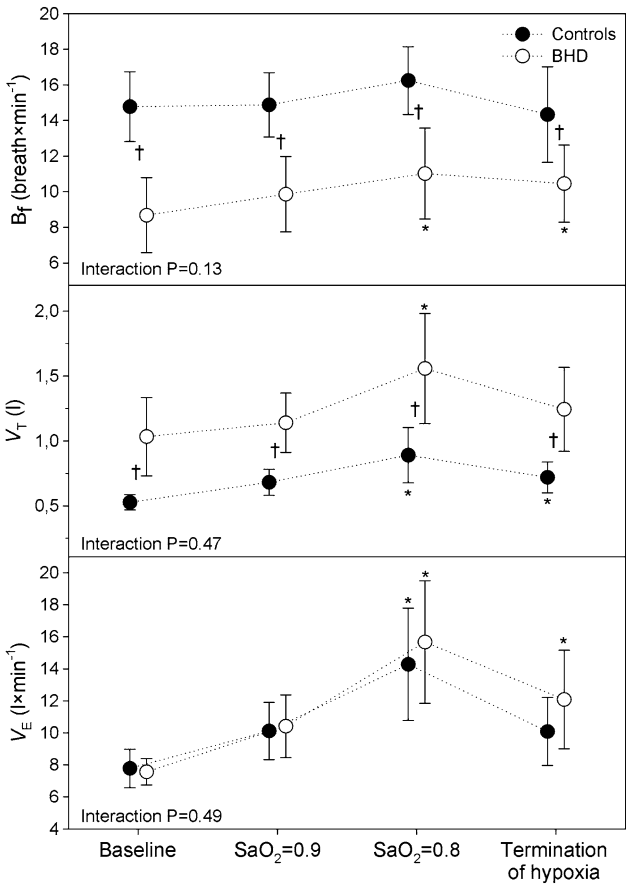



**Fig. 3** Ventilatory responses in the two groups during all phases of hypoxic protocol. *Circles* represent means, *error bars* denote 95% confidence intervals. Significant changes within a single group were determined using repeated-measures ANOVA with Bonferroni test as post hoc test (*$P < 0.05$). Interactions among responses of the two groups were tested using general linear model for repeated-measures ANOVA. Differences between groups in the same time point were identified with unpaired Student *t* test (†$P < 0.05$)

down as deeply as possible along a vertically suspended rope using fins. During static and dynamic apnea diving, divers are exposed to progressively increasing hypercapnic hypoxia such as obstructive apnea patients. During constant weight diving, divers are exposed to hyperoxic hypercapnia during the descent and at the bottom due to hydrostatic pressure-induced compression of the chest wall [19]. Hypoxia is experienced only during the last phase of the ascent when the rapid reduction of hydrostatic pressure causes lung expansion and extreme hypoxia can cause blackouts. Despite extreme conditions of the sport world records are constantly improved. For example, the current record in static apnea is 10.2 min in men and 8 min in women. Elite breath-hold divers are adapted to extreme hypoxia/hypercapnia due to ventilatory, cardiovascular and cerebrovascular adaptations, such as decreased ventilatory sensitivity to $CO_2$, increased lung volume, enhanced peripheral sympathetic and parasympathetic activation, increased lactate production among others [27]. Furthermore, they have reduced post-apnea as well as post-exercise blood acidosis and oxidative stress, mimicking the responses of diving animals [13].

In our study, we applied isocapnic hypoxia testing. In the event of isocapnia, end tidal $CO_2$ was well maintained throughout the test. We are confident that we minimized influences of $CO_2$ changes on central chemoreceptors and cerebral vasculature. Stimulation of the peripheral chemoreflex elicits a moderate increase in ventilation. Similar to the MSNA response, the increase in minute ventilation began during first phase of hypoxia protocol (while $SaO_2$ was around 0.9) and became statistically elevated when arterial oxygen saturation reached 0.8, resulting in a doubling of ventilation in both groups. The slope of the hypoxic ventilatory response was similar in divers and in control subjects, suggesting unchanged ventilatory drive to hypoxia. Previously, the ventilatory response to hypoxia

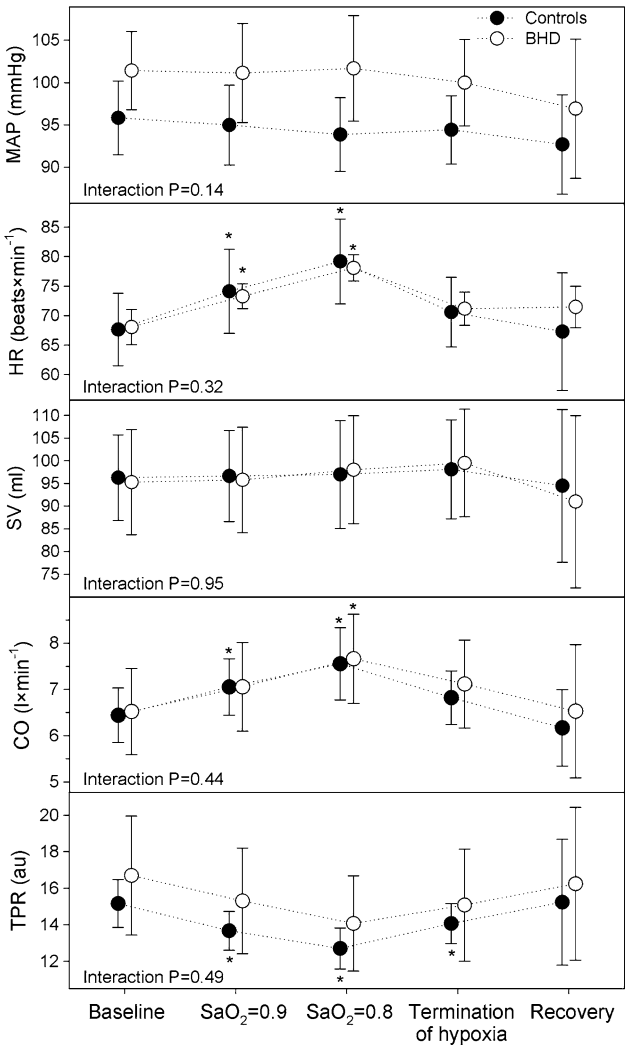



**Fig. 4** Hemodynamic responses in the two groups during all phases of hypoxic protocol. *Circles* represent means, *error bars* denote 95% confidence intervals. Significant changes within single group were determined using repeated-measures ANOVA with Bonferroni test as post hoc test (*$P < 0.05$). Interactions among responses of the two groups were tested using general linear model for repeated-measures ANOVA. Differences between groups in the same time point were not observed (unpaired Student *t* test)

was shown to be blunted in persons engaging in underwater sports such as synchronized swimmers [1] and in the Japanese Ama [16]. The blunted ventilator response of this earlier study suggests adaptations that may conserve oxygen, thus, prolonging maximal asphyxia during long breath-holds. In contrast, obstructive sleep apnea patients feature excessive increases in ventilation, blood pressure, and heart rate to hypoxic breathing [20]. The discrepancies between divers and obstructive sleep apnea patients are difficult to reconcile. Possibly, differences in quality and quantity of the breathing challenge (poikilocapnic vs. isocapnic hypoxia) and gender may be involved.

The overall cardiovascular response to hypoxia may result from direct influences on vascular tone and cardiac contractility together with centrally and reflex-mediated changes in autonomic nervous system activity. Hypoxia exerts a direct negative inotropic effect on the myocardium [8]. In our study, isocapnic hypoxia did not affect mean arterial pressure or cardiac stroke volume in either group. Heart rate, and consequently cardiac output, increased as hypoxia progressed while peripheral resistance decreased and remained reduced over 3 min after the cessation of hypoxia. Several previous studies investigated the influence of hypoxia on peripheral vasodilatation. When subjects were exposed to lower body negative pressure during hypoxia, norepinephrine and angiotensin doses had to be doubled to restore vascular tone to levels occurring during normoxia [7]. Moreover, during pharmacological α-adrenoreceptor blockade, peripheral vasodilation was massively prolonged after cessation of hypoxia [28]. In our study, sympathetic vasomotor tone approached baseline levels 20 min after cessation of hypoxia. In another study, sympathetic activation outlasted hypoxia up to 60 min [31]. Together, these observations suggest that hypoxia-induced vasodilation is masked by chemoreflex-mediated sympathetic activation. The substantial increase in sympathetic vasomotor tone is not sufficient to completely abrogate the vasodilator response. Remarkably, total sympathetic activity increased more markedly than burst frequency. The observation is consistent with increased recruitment of efferent sympathetic neurons [4].

The hypoxic stimulus did not cause significant changes in arterial pressure in either group. However, we have observed somewhat higher baseline arterial pressure in BHD group. Although, we observed the same levels of basal MSNA in BHD group and in controls, there can be several reasons for increased MAP in BHD. Perhaps in divers the peripheral response to sympathetic neural stimulation is augmented or there is a disturbance in counterbalancing vasodilating mediators [14]. This potential blood pressure-raising impact of apnea diving requires further examination as it could pose a significant risk for development of cardiovascular diseases in these individuals.

An important limitation of our study is that measurements of the ventilatory, cardiovascular and autonomic responses to isocapnic hypoxia were only performed when participants were awake, and not during an actual apnea dive. Apnea per se should potentiate the sympathetic response to hypoxia, since stimulation of pulmonary stretch receptors and baroreceptors affects sympathetic activity [25]. We investigated our divers at least 1 month after intensive diving training/competition periods, when physiological adaptations to diving may have been attenuated. Further studies are needed to investigate training and detraining-induced changes in ventilatory, cardiovascular

and autonomic variables and apneic performances in trained and/or untrained subjects.

In summary, peripheral chemosensitivity to hypoxia after a detraining period, central chemosensitivity [3], and cerebrovascular reactivity [11] are normal in elite apnea divers. These observations suggest that repeated voluntary exposure to intermittent hypoxia/hypercapnia in the absence of additional risk factors like hypertension, glucose intolerance or hyperlipidemia may not have a negative long-term impact on autonomic, cardiovascular and ventilatory regulation. Although elite divers are not exposed to sustained changes in sympathetic nervous system regulation, we cannot exclude that acute sympathetic activation during diving has harmful effects on cardiovascular health.

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# Peripheral chemoreflex sensitivity and sympathetic nerve activity are normal in apnea divers during training season

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ABSTRACT

Apnea divers are exposed to repeated massive arterial oxygen desaturation, which could perturb chemoreflexes. An earlier study suggested that peripheral chemoreflex regulation of sympathetic vasomotor tone and ventilation may have recovered 4 or more weeks into the off season. Therefore, we tested the hypothesis that peripheral chemoreflex regulation of ventilation and sympathetic vasomotor tone is present during the training season. We determined ventilation, heart rate, blood pressure, cardiac stroke volume, and muscle sympathetic nerve activity (MSNA) during isocapnic hypoxia in 10 breath hold divers and 11 matched control subjects. The study was carried out at the end of the season of intense apnea trainings. Baseline MSNA frequency was $30 \pm 4$ bursts/min in control subjects and $25 \pm 4$ bursts/min in breath hold divers ($P = 0.053$). During hypoxia burst frequency and total sympathetic activity increased similarly in both groups. Sympathetic activity normalized during the 30-minute recovery. Hypoxia-induced stimulation of minute ventilation was similar in both groups, although in divers it was maintained by higher tidal volumes and lower breathing frequency compared with control subjects. In both groups, hypoxia increased heart rate and cardiac output whereas total peripheral resistance decreased. Blood pressure remained unchanged.
We conclude that peripheral chemoreflex regulation of ventilation and sympathetic vasomotor tone is paradoxically preserved in apnea divers, both, during the off and during the training season. The observation suggests that repeated arterial oxygen desaturation may not be sufficient explaining sympathetic reflex abnormalities similar to those in obstructive sleep apnea patients.

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## 1. Introduction

Peripheral and central chemoreceptors are primarily involved in regulation of respiration (O'Donnell et al., 1996), but can also elicit a sympathetically mediated pressor response (Sapru, 1996; Somers et al., 1989). Frequent involuntary apneas in obstructive sleep apnea patients (OSA) may result in augmented peripheral chemoreflex sensitivity (Imadojemu et al., 2007; Narkiewicz et al., 1999), whereas central chemoreflex regulation is unimpaired (Narkiewicz et al., 1999).

Elite breath hold divers are exposed to extreme hypoxia/hypercapnia during maximal apneas lasting for several minutes. After maximal apnea, alveolar oxygen partial pressure can be as low as 20–30 mm Hg with arterial oxygen saturation around 50% (Lindholm and Lundgren, 2006; Overgaard et al., 2006). Breath hold divers compete in different disciplines such as static apnea, dynamic apnea, and constant weight. During static apnea, divers float motionless face down in a pool, while during dynamic apnea, the goal is to attain maximal underwater swimming distances. Finally, during constant weight, divers swim down as deeply as possible along a vertically suspended rope using fins. With static and dynamic apnea, divers are exposed to progressively increasing hypercapnic hypoxia. During constant weight diving, subjects are exposed during descent and at the bottom to hyperoxic hypercapnia due to hydrostatic pressure-induced compression of the chest wall (Muth et al., 2003).

Voluntary apnea under laboratory conditions and intermittent hypoxemia causes acute as well as sustained changes in cardiovascular autonomic regulation (Cutler et al., 2004; Leuenberger et al., 2005; Morgan et al., 1995). Twenty to 30 min of intermittent hypoxia is sufficient to raise muscle sympathetic nerve activity (MSNA) and blood pressure (Leuenberger et al., 2005). Previously, we observed that central chemoreflex control of respiration and sympathetic activity is maintained in breath hold divers (Dujic et al., 2008) and that, after detraining, they have normal peripheral chemoreflex regulation of ventilation and sympathetic vasomotor tone (Breskovic et al., in press), indicating no sustained autonomic impairment. Now, we investigated the hypothesis whether during training season the peripheral chemosensitivity is abnormal in breath hold divers as was previously shown for OSA patients. We measured autonomic, ventilatory, and hemodynamic responses to isocapnic hypoxia in divers and matched control subjects at the end of several months intensive training period.

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## 2. Methods

### 2.1. Subjects

We included 21 healthy men ($N=16$) and women ($N=5$) in our study. Of those, ten were experienced elite breath hold divers (BHD) (3 women) while eleven matched, untrained subjects served as controls (2 women). All divers were competitors in static and/or dynamic apnea. Seven out of ten divers were also non-competitively practicing constant weight apnea discipline but considerably less frequently than their primary competitive discipline(s). Average personal best results for divers were $340\pm59$ s in static apnea and $157\pm31$ m in dynamic apnea. Six out of ten divers previously had episodes associated with hypoxia in range from mild disorientations to blackouts with or without convulsions or loss of motor control. On average, divers had apnea trainings thrice weekly. Apnea training duration ranged between 1 and 1.5 h. We conducted the study in accordance with the Declaration of Helsinki and after approval by the ethical committee of the University of Split School of Medicine. We obtained written consent from each subject.

### 2.2. Protocol

We conducted our studies in December 2008, at the end of apnea season. The study was carried out within one month after the last major static and dynamic apnea competition of the season. We asked divers to continue their training with the same intensity until they were evaluated in the laboratory. The period between testing in the laboratory and last training was maximally 2 days. All experiments were carried out in a climatized room in the morning hours. Participants were instructed not to eat at least 4 h before the arrival to the laboratory. We studied women during the follicular phase of the menstrual cycle.

Before instrumentation subjects underwent dynamic spirometry (Quark PFT, Cosmed, Rome, Italy) while standing and afterwards their anthropometric measurements were taken. Then, they were asked to lie down and were instrumented for the hypoxic test.

We applied an infrared probe on the middle finger to monitor arterial oxygen saturation (Poet II, Criticare Systems, Waukesha, USA). Beat-by-beat blood pressure and heart rate were measured using a finger cuff (Finometer, Finapress Medical Systems, Arnhem, Netherlands) and electrocardiography, respectively. Multiunit muscle sympathetic nerve activity (MSNA) of postganglionic sympathetic activity was recorded from the right peroneal nerve with a unipolar tungsten electrode as described previously (Dujic et al., 2008). The nerve signal was amplified 100,000 times. Afterwards signal was band-pass filtered (0.7–2.0 kHz), rectified and integrated using 0.1 s time constant (662C-4, Nerve traffic analysis system, Bioengineering, The University of Iowa, USA).

Subjects were breathing from a mouthpiece connected to a non-rebreathing Y-valve (Hans-Rudolph 2730 Series, Large 2-way, NRBV, Y Shape, K.C., MO, USA) whose inspiratory port was connected to a three-way valve (Hans-Rudolph 4000 Series Large non mixing, 3-way "Y" Stopcock, K.C., MO, USA) allowing switching between room air and gas-reservoir. The spirometer (Harvard apparatus, Student model, Holliston, MA, USA) acted as reservoir for the gas mixture whose composition was regulated by a blender. Blender was attached to three gas cylinders (compressed air, 100% $N_2$ and 100% $CO_2$) thus enabling to produce different hypoxic gas mixtures. The gases were sampled breath-by-breath at the mouth using a respiratory analyzer (Quark $b^2$, Cosmed, Rome, Italy). Before each trial, control data were collected by having subjects breathe room air for 3 to 5 min while monitoring the $PetCO_2$ concentration to establish his or her normoxic level. This was followed by progressive normocapnic hypoxia introduced in two steps by increasing the $N_2$ concentration until the required $SaO_2$ was achieved. Mean $SaO_2$ level of ~0.9 was maintained for 3 min, and ~0.8 for 5 min. Normocapnic $PetCO_2$ was regulated by adding $CO_2$ to the inspired gas, as required, thus minimizing central chemoreceptor engagement. After cessation of hypoxia testing, subjects were switched to breathe room air while measurements were continued for another 30 min.

### 2.3. Data acquisition and analysis

All data were acquired using an analog to digital converter (Powerlab/16SP, ADInstruments, Castle Hill, Australia) interfaced with PC. Data were sampled at 1 kHz and stored for subsequent analysis using Chart software (ADInstruments, version 5.5.6.7). MSNA bursts were identified according to the following criteria: (1) signal to noise ratio >2; (2) latency limit; (3) burst width limit (short duration = artifact, long duration = skin sympathetic nerve activity or afferent activity; (4) no preceding premature beats (Tank et al., 2001). MSNA activity was expressed as frequency of bursts per minute (burst frequency) and per 100 heart beats (burst incidence). Amplitude and area of each burst was calculated. Total MSNA was calculated as the sum of all burst areas per minute. We obtained good quality MSNA recordings in all subjects. We quantified sympathetic chemoreflex sensitivity and ventilatory responses to incremental hypoxia as change in sympathetic nerve activity or ventilation per change in $SaO_2$ during hypoxic protocol. Changes in left ventricular stroke volume were estimated by pulse wave analysis using an improved method of Wesseling (Modelflow program) (Jellema et al., 1999). The data were analyzed during 3 min period before hypoxia, 3 min during $SaO_2=0.9$, last 3 min of hypoxia trial while $SaO_2=0.8$, during 3 min upon cessation of hypoxia and during 3 min periods in 10th, 20th and 30th minute of recovery period.

### 2.4. Statistical analysis

All data were expressed as means ± 95% confidence intervals (95% CI). Baseline values, values at the same time points and chemoreflex sensitivities were compared using unpaired Student *t*-test. The effects of hypoxia on all measured variables within group were determined using

**Table 1**
Anthropometric characteristics of subjects.

| | Controls ($N=11$) | BHD ($N=10$) | *P* |
|---|---|---|---|
| Age (years) | 25.5 ± 2.6 | 27.0 ± 3.4 | 0.50 |
| Mass (kg) | 82.5 ± 8.3 | 76.0 ± 5.4 | 0.22 |
| Height (m) | 1.83 ± 0.04 | 1.83 ± 0.04 | 0.94 |
| BMI | 24.5 ± 2.1 | 22.6 ± 1.0 | 0.12 |
| BFI (%, body fat/kg) | 21.1 ± 4.7 | 22.7 ± 3.9 | 0.60 |
| FVC (l) | 5.9 ± 0.7 | 6.9 ± 0.9 | 0.09 |
| $FEV_1$ (l) | 4.9 ± 0.5 | 5.5 ± 0.9 | 0.27 |

Values are means ± 95% CI; differences between groups analyzed by unpaired Student *t*-test; BMI — body mass index; BFI — body fat index (calculated by Jackson and Pollock three site measurement); FVC — forced vital capacity; $FEV_1$ — forced expiratory volume in 1st second.

**Table 2**
Baseline data.

| | Controls ($N=11$) | BHD ($N=10$) | *P* |
|---|---|---|---|
| $MSNA_f$ (bursts × $min^{-1}$) | 29.9 ± 3.5 | 24.5 ± 3.7 | 0.053 |
| $MSNA_i$ (bursts × 100$hb^{-1}$) | 44.8 ± 5.7 | 41.5 ± 4.8 | 0.39 |
| Burst area (au) | 21.2 ± 3.6 | 26.5 ± 3.9 | 0.07 |
| $MSNA_t$ (au × $min^{-1}$) | 2.0 ± 0.7 | 1.6 ± 0.7 | 0.45 |
| $V_E$ (l × $min^{-1}$) | 7.8 ± 1.2 | 7.2 ± 0.8 | 0.49 |
| HR (beats × $min^{-1}$) | 67.7 ± 6.1 | 59.1 ± 4.6 | 0.043 |
| MAP (mmHg) | 95.8 ± 4.4 | 93.9 ± 5.8 | 0.60 |

Values are means ± 95% CI; differences between groups analyzed by unpaired Student *t*-test. $MSNA_f$ — burst frequency; $MSNA_i$ — burst incidence; $MSNA_t$ — total MSNA; $V_E$ — minute ventilation; HR — heart rate; MAP — mean arterial pressure.

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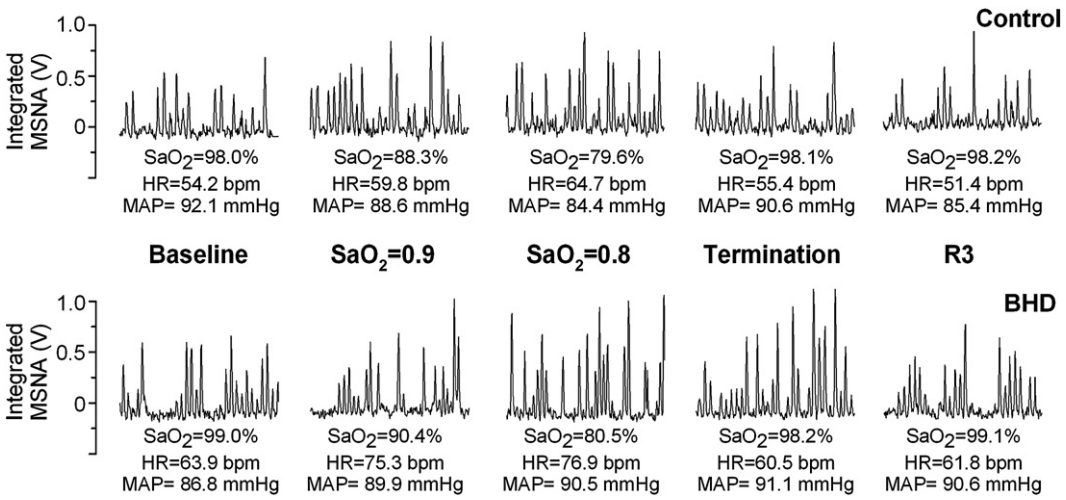



**Fig. 1.** Representative 30 s fragments of integrated MSNA neurograms during different phases of the hypoxia protocol in one control subject and one breath hold diver (BHD). Below each neurogram are the average values for $SaO_2$, HR and MAP. $SaO_2$ — arterial oxygen saturation; HR — heart rate; MAP — mean arterial pressure.

repeated-measures ANOVA. A Bonferroni test was used as *post hoc* test. Interactions among responses between the groups were assessed using a general linear model for repeated-measures ANOVA. All analyses were performed with Statistica 7.0 software (Statsoft, Inc., Tulsa, USA).

## 3. Results

Anthropometric and pulmonary function data of the subjects are presented in Table 1. Participants in both groups were acceptably

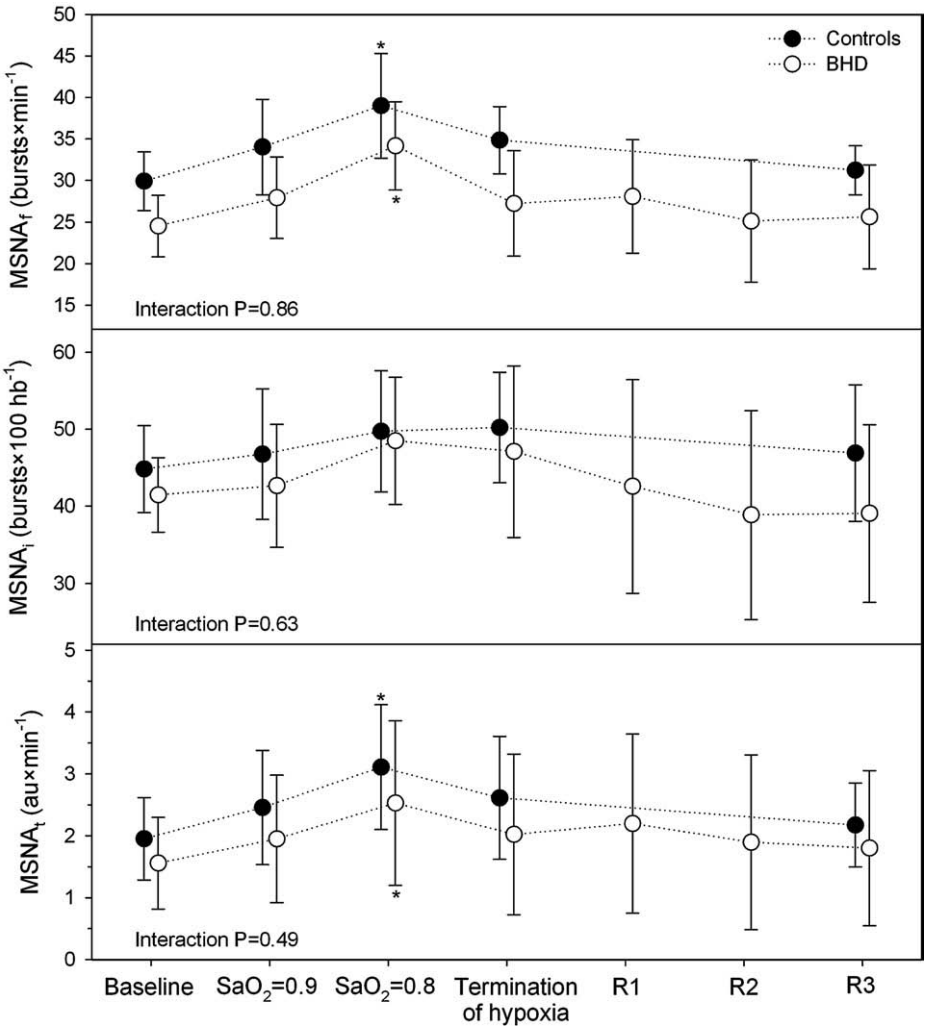



**Fig. 2.** Changes in MSNA in both groups during all phases of hypoxic protocol. Circles represent means, error bars denote 95% confidence intervals. Significant changes within single group were determined using repeated-measures ANOVA with Bonferroni test as *post hoc* test (*$P<0.05$). Interactions among responses of the two groups were tested using general linear model for repeated-measures ANOVA. Differences between groups in the same time point were not observed (unpaired Student *t*-test).

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matched. Even though divers had higher pulmonary function parameters, absolute values didn't reach statistical significance, however, relative predicted values did. Forced vital capacity in controls was 105.8 ± 8.9 vs. 131.9 ± 7.1% predicted in divers ($P<0.001$), and forced expiratory volume in 1st second in controls was 105.2 ± 6.0 vs. 123.7 ± 11.0% predicted in divers ($P=0.013$).

Divers had slightly lower baseline burst frequency ($MSNA_f$) and higher burst area, although the difference was not statistically significant. Total sympathetic activity ($MSNA_t$) and burst incidence ($MSNA_i$) was comparable among the groups. Divers had lower heart rate (HR). Baseline minute ventilation ($V_E$) and mean arterial pressure (MAP) were similar (Table 2).

Fig. 1 presents an individual response to hypoxia in one control subject and one diver. In both groups $MSNA_f$ and $MSNA_t$ increased during hypoxia (29.5 ± 10.6% and 65.9 ± 26.8% in control group; 42.8 ± 22.7% and 60.0 ± 27.3% in divers, respectively). These measurements started to normalize after termination of hypoxia and completely normalized during the 30 min recovery period. Burst incidence did not change throughout hypoxia testing in both groups (Fig. 2).

Sympathetic chemoreflex sensitivity was similar between groups (0.06 ± 0.03 au/min per 1% change in $SaO_2$ in controls and 0.05 ± 0.04 au/min per 1% change in $SaO_2$ in divers; $P=0.69$).

In divers $V_E$ was maintained by significantly larger tidal volume ($V_T$) (0.5 ± 0.1 l in controls vs. 0.8 ± 0.2 l in divers; $P=0.019$) and lower breathing frequency (14.8 ± 2.0 breaths/min in controls vs. 10.5 ± 2.4 breaths/min in divers; $P=0.013$) compared to control subjects. Hypoxic stimulation augmented $V_E$ in both groups primarily by increasing $V_T$ (66.3 ± 32.9% in controls; 61.5 ± 44.3% in divers) (Fig. 3).

During hypoxia, ventilation increased 0.35 ± 0.16 l/min per 1% change in $SaO_2$ in the control group and BHD 0.27 ± 0.16 l/min per 1% change in $SaO_2$ in divers ($P=0.48$).

With hypoxia, MAP and stroke volume (SV) did not change in either group. Heart rate (HR) and cardiac output (CO) increased as hypoxia progressed. Total peripheral resistance (TPR) decreased during hypoxia trial in both groups, although in divers the reduction reached statistical significance. The hemodynamic response to hypoxia was similar in both groups (Fig. 4).

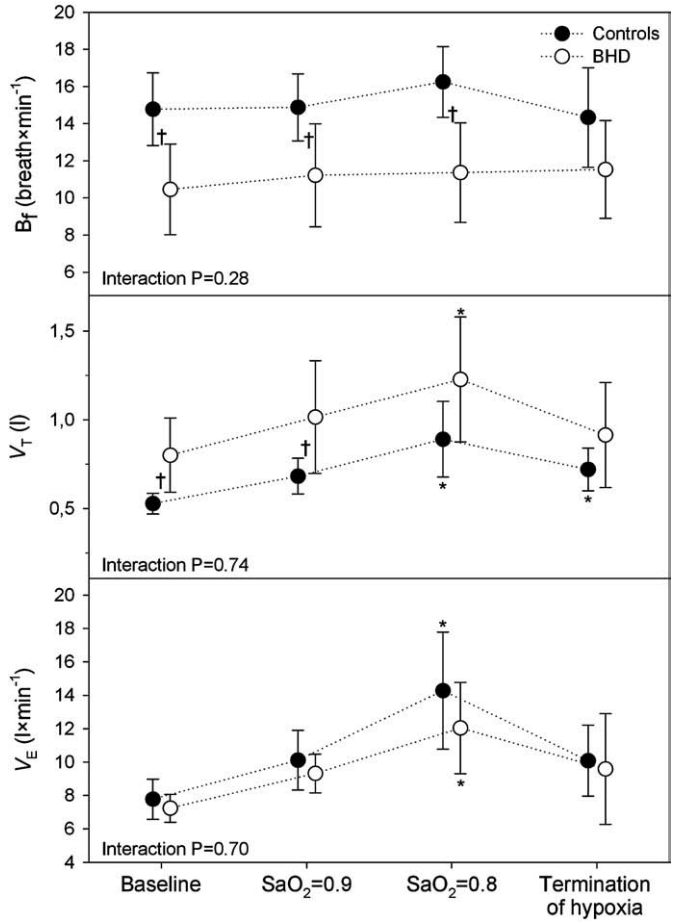



**Fig. 3.** Ventilatory responses in two groups during all phases of hypoxic protocol. Circles represent means, error bars denote 95% confidence intervals. Significant changes within single group were determined using repeated-measures ANOVA with Bonferroni test as *post hoc* test (*$P<0.05$). Interactions among responses of the two groups were tested using general linear model for repeated-measures ANOVA. Differences between groups in the same time point were identified with unpaired Student *t*-test (†$P<0.05$).

## 4. Discussion

The main finding of our study is that autonomic, ventilatory, and cardiovascular responses to hypoxia are normal in elite divers during intensive apnea trainings. Divers showed normal baseline blood pressure and sympathetic activity. The observation that peripheral and central chemosensitivity are preserved is reassuring (Breskovic et al., in press; Dujic et al., 2008). Normal peripheral and central chemosensitivity in elite breath hold divers may serve as a protective mechanism for maintaining cerebral perfusion during extreme asphyxia associated with long breath holds, since increased $CO_2$ and decreased $O_2$ have major vasodilatatory effect on this vascular bed, whereas increased peripheral sympathetic nerve traffic during hypoxia in divers counteracts direct vasodilatatory effects.

We studied divers competing in static and dynamic apnea since they are most intensively exposed to hypoxia. Despite extreme conditions of the sport, world records are constantly improved. For example current record in static apnea is 11.6 min in men and 8.4 min in women. Elite breath hold divers are adapted to extreme hypoxia/hypercapnia due to ventilatory, cardiovascular, and cerebrovascular adjustments, such as decreased ventilatory sensitivity to $CO_2$, increased lung volume, enhanced peripheral sympathetic and parasympathetic activation, and increased lactate production among others (Bakovic et al., 2003; Heusser et al., 2009; Palada et al., 2007). Furthermore, they show reduced post-apnea as well as post-exercise blood acidosis and oxidative stress, mimicking the responses of diving animals (Joulia et al., 2002). We tested our divers at the end of the competitive apnea season after divers underwent numerous intensive apnea trainings and competitions lasting for more than half of a year. Sixty percent of our subjects had during their career hypoxia related loss of consciousness. Moreover, two of them had multiple occurrences of such episodes, suggesting that our subjects have been regularly exposed to severe hypoxia.

We applied isocapnic hypoxia testing. In the event, end tidal $CO_2$ was well maintained throughout the test. We are confident that we minimized influences of $CO_2$ changes on central chemoreceptors and cerebral vasculature. Stimulation of peripheral chemoreflex elicited a moderate increase in ventilation. The slope of the hypoxic ventilatory response was similar in divers and in control subjects, suggesting unchanged ventilatory drive to hypoxia. Previously, the ventilatory response to hypoxia was shown to be blunted in persons engaging in underwater sports such as synchronized swimmers (Bjurstrom and Schoene, 1987) and in the Japanese Ama (Masuda et al., 1981). The observation suggests adaptations that may conserve oxygen, thus, prolonging maximal asphyxia during long breath holds.

The overall cardiovascular response to hypoxia may result from direct influences on vascular tone and cardiac contractility together with centrally and reflex mediated changes in autonomic nervous system activity. Several previous studies investigated the influence of hypoxia on peripheral vasodilatation. In subjects exposed to lower body negative pressure during hypoxia, norepinephrine and angiotensin doses had to be doubled to restore vascular tone to levels occurring during normoxia (Heistad and Wheeler, 1970). Furthermore, during pharmacological α-adrenoreceptor blockade, peripheral

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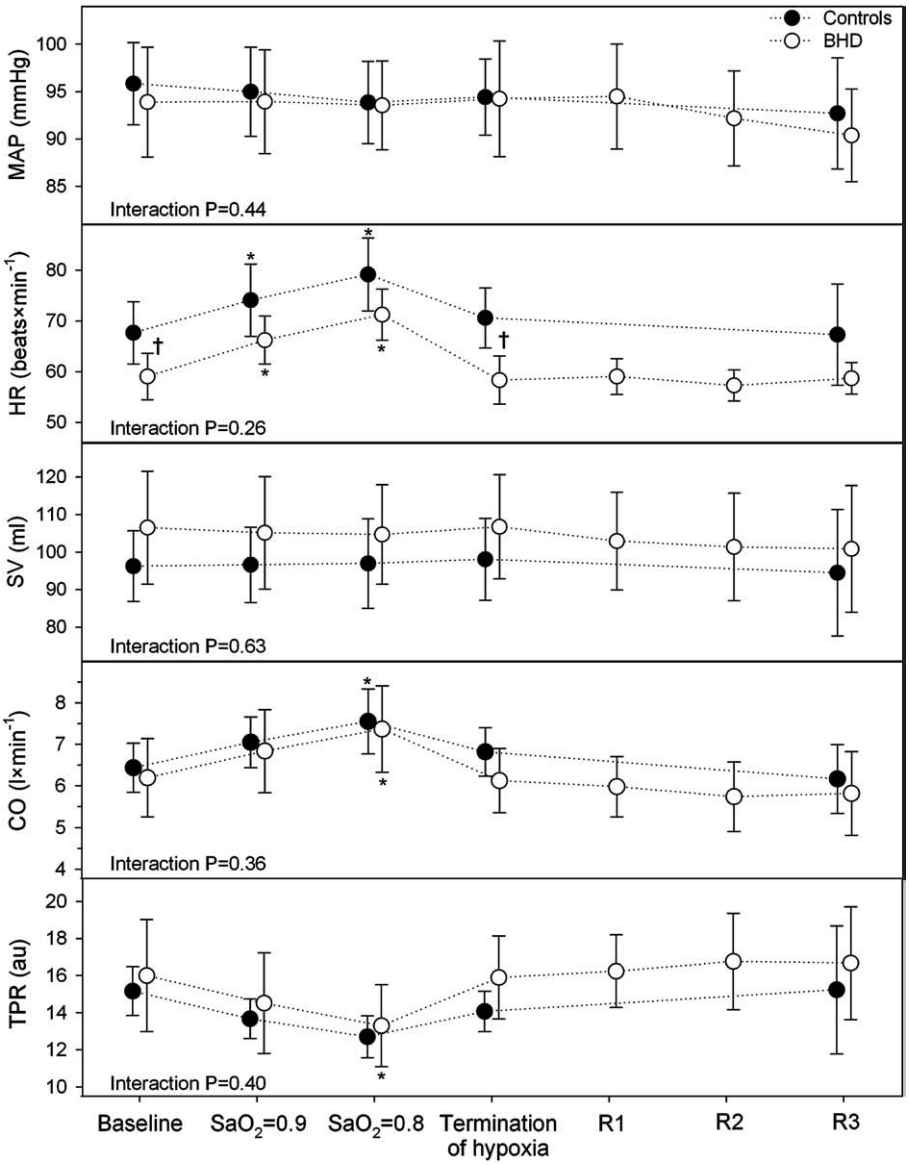



**Fig. 4.** Hemodynamic responses in two groups during all phases of hypoxic protocol. Circles represent means, error bars denote 95% confidence intervals. Significant changes within single group were determined using repeated-measures ANOVA with Bonferroni test as *post hoc* test (*$P<0.05$). Interactions among responses of the two groups were tested using general linear model for repeated-measures ANOVA. Differences between groups in the same time point were identified with unpaired Student *t*-test (†$P<0.05$).

vasodilation was massively prolonged after cessation of hypoxia (Tamisier et al., 2004). Both studies suggest that hypoxia-induced vasodilation is masked by chemoreflex mediated sympathetic activation. Additionally, hypoxia also exerts a direct negative inotropic effect on the myocardium (Henderson and Brutsaert, 1973). In our study, sympathetic vasomotor tone started to normalize after termination of hypoxia and returned to baseline 20 to 30 min after the end of the hypoxic protocol. Xie et al. (Xie et al., 2001) observed increased sympathetic activity which outlasted hypoxia up to 60 min. Differences between studies may be due to various duration and magnitude of hypoxia. The increase in sympathetic vasomotor tone was not sufficient to completely abrogate the vasodilator response to hypoxia. Arterial pressure was maintained through augmented cardiac output. More marked increase in total sympathetic activity than in burst frequency suggests increased recruitment of efferent sympathetic neurons (Elam et al., 2003).

Contrary to obstructive sleep apnea patients, breath hold divers are not exposed to excessive sympathetic activation. One possible explanation for the discrepancy could be a difference in the time course of hypoxic episodes between groups. Breath hold divers are exposed to hypoxia during voluntary end-inspiratory apnea while in OSA patients the apneas are involuntary and end-expiratory. Additionally, divers usually train 3 or 4 times per week for 1–1.5 h while OSA patients have repetitive apneic episodes almost every time when they are asleep. Therefore, total apnea time and exposure to hypoxia may be greater in OSA patients than in elite apnea divers. Age may be another variable explaining the discrepancy between divers and OSA patients in terms of chemoreflex regulation. Our divers were relatively young. In contrast, most OSA patients are older people. Finally, OSA patients often have multiple comorbidities like obesity, heart disease and diabetes, among others which can all potentiate increased sympathetic activation in this population. On contrary, apnea divers are, usually, group of healthy, young individuals.

One potential limitation of our study is that ventilatory, cardiovascular, and autonomic measurements to isocapnic hypoxia were only obtained during wakefulness, and not during apnea. Apnea *per se* may

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potentiate the sympathetic response to hypoxia, since stimulation of pulmonary stretch receptors and baroreceptors affects sympathetic activity (Somers et al., 1995).

In summary, our observations suggest that repeated voluntary exposure to intermittent hypoxia/hypercapnia in the absence of additional risk factors like hypertension, glucose intolerance or hyperlipidemia may not have a negative impact on autonomic, cardiovascular and ventilatory regulation. Although elite divers are not exposed to sustained changes in sympathetic nervous system regulation, we cannot exclude that acute sympathetic activation during prolonged breath hold has harmful effects on cardiovascular health.

## Acknowledgments

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# Recruitment pattern of sympathetic neurons during breath-holding at different lung volumes in apnea divers and controls

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ABSTRACT

We tested the hypothesis that breath-hold divers (BHD) attain higher level of sympathetic activation than controls due to the duration of breath-hold rather than a different recruitment strategy. In 6 control subjects and 8 BHD we measured muscle sympathetic neural activity (MSNA) prior to and during functional residual capacity (FRC) and total lung capacity (TLC) breath-holding. On a subset of subjects we applied a new technique for the detection of action potentials (APs) in multiunit MSNA. Compared with controls, BHD group had lower burst AP content (13 ± 7 *vs.* 6 ± 3 AP/burst; P = 0.05) and number of active clusters (5 ± 1 *vs.* 3 ± 1 clusters/burst; P = 0.05) at baseline. However, the overall sympathetic AP/unit-time was comparable between the groups (131 ± 105 *vs.* 173 ± 152 AP/min; P = 0.62) due to increased burst frequency in BHD group (20 ± 4 bursts/min) *vs.* controls (13 ± 3 bursts/min) (P = 0.039). The achieved level in total MSNA during FRC breath-holds was higher in divers (2298 ± 780 *vs.* 1484 ± 575 a.u./min; P = 0.039). Total MSNA at the end of TLC breath-hold was comparable between the groups (157 ± 50 (controls) *vs.* 214 ± 41 s (BHD); P = 0.61). FRC and TLC breath-holds increased AP frequency, burst AP content and active clusters/bursts in both groups but the response magnitude was determined by the type of the breath-hold. The divers used fewer number of APs/burst and active clusters/burst. In both groups breath-holds resulted in similar increases in MSNA which were reached both by an increase in firing frequency and by recruitment of previously silent, larger (faster conducting) sympathetic neurons, and possibly by repeated firing within the same burst.
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## 1. Introduction

Trained breath-hold divers (BHD) are capable of enduring extremely long periods of apnea. The current world record for static apnea for males is 11 min 35 s (AIDA international). After such a maximal apnea, alveolar oxygen partial pressure can be as low as 20–30 mm Hg with arterial oxygen saturation around 50% (Lindholm and Lundgren, 2006; Overgaard et al., 2006). Such periods of oxygen desaturation are also correlated with increases in muscle sympathetic nerve activity (MSNA) (Heusser et al., 2009). Further, Heusser et al. (2009) showed that, when compared to baseline, the overall increase in MSNA during breath-holding in trained BHD is >20-fold, a level that is ~5 times higher than observed in untrained control subjects. Under such conditions, increases in the action potential (AP) firing frequency and/or the recruitment of postganglionic sympathetic neurons may be necessary in order to enable such a large augmentation in MSNA. However, sympathetic neural firing patterns and strategies of activation of sympathetic nervous system, to date, are not clarified completely.

To address the issue of postganglionic recruitment strategies previous studies examined single-unit recordings and showed that sympathetic neurons, when active, fire predominantly once with a given burst of activity (~70% of occurrences) (Macefield et al., 1994; Macefield and Wallin, 1999) with the probability of multiple firings of the same neuron within a burst increasing during voluntary apnea (Macefield and Wallin, 1999) and with certain pathologies (Elam et al., 2003). A different but complementary approach is needed if one aims to determine discharge patterns of the multi-unit MSNA neurogram and to see if new action potentials appear in the recording.

To expand on this single neuron approach, and examine how the multi-unit signal changes with reflex activation, we have used a new spike detection algorithm that uses the continuous wavelet transform approach that enables determination of the number of sympathetic APs contributing to the multiunit MSNA (Salmanpour et al., 2010). Steinback et al. (2010b) recently applied this technique to analyze sympathetic activity in BHD during a prolonged, maximal end-inspiratory breath-hold. This earlier study suggested that large sympathetic neurons, which generate larger APs and faster conduction velocities, may be silent at rest

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but may be recruited during chemoreceptor-induced increases in sympathetic drive at the end of maximal end-inspiratory apneas.

Breath-holds starting at different lung volumes elicit increase in sympathetic neural traffic through different mechanisms. The sympathetic response to a breath-hold starting at functional residual capacity (FRC) of the lungs appears to be controlled principally by arterial oxygen desaturation and increasing levels of blood $CO_2$ (Somers et al., 1989), representing a dominant influence of chemoreflex stress. However, the increase in sympathetic nervous traffic during total lung capacity (TLC) breath-hold is driven by diverse stimuli present at different phases of the TLC breath-hold, causing a biphasic response in MSNA. During the initial 30 s of TLC breath-hold the SNA response resembles that observed during a Valsalva maneuver (Heusser et al., 2009). This neural response likely is due to the high intrathoracic pressure that, in turn, reduces venous return and cardiac output eliciting unloading of low and high-pressure baroreceptors (Ferrigno et al., 1986; Macefield and Wallin, 1995). After the initial phase of the TLC breath-hold, blood pressure stabilizes but MSNA continues to increase linearly towards the end of the breath-hold. The underlying mechanisms for the increase in MSNA during the latter phase of the breath-hold must include an increase of chemoreflex stress (Somers et al., 1989; Morgan et al., 1993; Heusser et al., 2009) and the lack of ventilatory MSNA inhibition (Somers et al., 1989).

The purpose of the present study was to test the hypothesis that the sympathetic response to either FRC or TLC apneas would be greater in BHD compared with controls due simply to the ability of the divers to sustain a greater duration of breath-hold and the consequent magnitude of chemoreflex stress, rather than a training-induced difference in sympathetic AP recruitment.

## 2. Materials and methods

### 2.1. Subjects

For the purposes of this study we recruited 19 healthy, male subjects (nine elite breath-hold divers and 10 matched control subjects). After receiving verbal and written instructions outlining the experimental procedures, and having providing informed written consent, subjects underwent the protocol. In fourteen (6 control subjects and 8 breath-hold divers), good quality MSNA recordings were obtained. Anthropometrics and diving history are presented in Table 1.

All participants were non-smokers and none had any history of cardiovascular or respiratory disease. Participants arrived at the lab at least 2 h postprandial and having abstained from caffeine, alcohol, or other stimulants for 12 h. Participants voided their bladder immediately prior to testing. The study was performed at the University of Split School of Medicine. We conducted the study in accordance with the Declaration of Helsinki and it was approved by research ethics board at The University of Split School of Medicine in Croatia.

**Table 1**
Anthropometric characteristics of the subjects.

| | Controls (N = 6) | Divers (N = 8) |
|---|---|---|
| Age (years) | 23 ± 1 | 25 ± 4 |
| Height (cm) | 184.8 ± 7.2 | 184.9 ± 5.0 |
| Weight (kg) | 82.5 ± 5.9 | 83.9 ± 5.2 |
| BMI | 24.2 ± 0.3 | 24.6 ± 1.8 |
| Years practicing apnea (years) | N/A | 4.6 ± 2.2 |
| Personal best static apnea (s) | N/A | 337.0 ± 41.9 |
| Time since last training (days) | N/A | 9.4 ± 10.2 |

Values are means ± SD. BMI, body mass index.

### 2.2. Experimental protocol

We applied an infrared probe on the middle finger to monitor arterial oxygen saturation (Poet II, Criticare Systems, Waukesha, USA). Mean arterial blood pressure was measured on a beat-by-beat basis from the blood pressure waveform using finger photoplethysmography (Finometer; Finapres Medical Systems, The Netherlands). Heart rate was calculated from a standard electrocardiogram. MSNA was assessed in the right fibular (peroneal) nerve by microneurography (Hagbarth and Vallbo, 1968). A tungsten microelectrode (35 mm long, 200 μm in diameter, and tapered to a 1–5 μm uninsulated tip) was inserted percutaneously into the fibular nerve posterior to the fibular head. A reference electrode was positioned subcutaneously 1–3 cm from the recording site. A suitable sympathetic nerve site was searched for by manually manipulating the microelectrode until a characteristic pulse-synchronous burst pattern was observed. Confirmation that the recorded signal represented MSNA was determined by the absence of skin paresthesia and a signal that increased in response to voluntary apnea but not during arousal to a loud noise (Delius et al., 1972). The MSNA neurogram was amplified 1000× through a pre-amplifier and 100× by a variable-gain, isolated amplifier. The amplified, raw MSNA signal was band-pass filtered at a bandwidth of 700–2000 Hz, sampled at 10,000 Hz and stored offline for further analysis.

APs were detected and extracted from the filtered raw MSNA signal using the techniques reported previously (Salmanpour et al., 2010; Steinback et al., 2010b). Using this technique, individual spikes were detected from the raw MSNA signal using a continuous wavelet transform approach (Salmanpour et al., 2010). There is concern about the reliability of the current method to efficiently extract APs from the background of Gaussian noise in recordings with lower signal-to-noise ratio (SNR) (Salmanpour et al., 2010). Therefore, we restricted the analysis to subjects with higher SNR (≥3). The SNR for a period of data was determined as the amplitude of the negative peak of the mean AP over the standard deviation of the background noise (*i.e.* during sympathetic silence). Using this criterion raw neurograms of 9 subjects (4 control subjects and 5 divers) were included in the analysis. Consequently in these subjects we were able to identify, on average, 178 bursts of sympathetic activity per subject throughout the whole protocol (ranging from 88 to 253 bursts) and to detect and analyze an average of 1931 APs (ranging between 743 and 4190 APs) per subject.

After instrumentation, subjects were instructed to lie quietly for 15 min allowing normalization of hemodynamic parameters. After a period of basal normal ventilation subjects were instructed to exhale until reaching lung FRC and to cease breathing as long as possible. After 15 min of recovery subjects were asked to take a couple of deep breathes and inspire to TLC and perform a maximal breath-hold. Afterwards, subjects were monitored for additional 15 min.

### 2.3. Data acquisition and analysis

All data were acquired using an analog to digital converter (Powerlab/16SP, ADInstruments, Castle Hill, Australia) interfaced with a PC. Data were sampled at 10,000 Hz and stored for subsequent analysis using Chart software (ADInstruments, version 5.5.6.7).

Changes in left ventricular stroke volume were estimated by pulse wave analysis using an improved method of Wesseling (Modelflow program) (Jellema et al., 1999).

Integrated bursts of MSNA were identified as exhibiting pulse-synchrony, having a SNR of least 2:1 with respect to the previous period of neural silence between bursts, having characteristic of rising and falling slopes, and increasing in incidence and size during end-expiratory apneas but not startle. Burst occurrence was confirmed by visually inspecting the corresponding raw neurogram.

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Integrated MSNA activity was expressed as frequency of bursts per minute (burst frequency) and per 100 heart beats (burst incidence). Amplitude of each burst was calculated and normalized to the largest burst amplitude in the corresponding baseline period. Total MSNA was calculated as the sum of all normalized amplitudes per minute.

The average raw MSNA SNR for the control group was $3.8 \pm 0.4$ and $3.6 \pm 0.4$ in divers, respectively. Based on simulation results (Salmanpour et al., 2010), we expect that these levels of SNR would produce a 95% correct detection rate for AP and a rate of false positive detection of 2.9% for controls and a 90% correct detection and 3.1% false positive rate for divers, respectively. Raw MSNA was quantified as the number of APs per minute and number of APs in each burst. Extracted APs were then ordered based on peak-to-peak amplitude and histogram analysis was performed to separate APs into amplitude-based clusters. Cluster bin widths were defined individually in each subject by dividing the peak-to-peak amplitude range into 20 clusters. As such, the number of total clusters was the same between each subject.

The data were analyzed during a 3 minute baseline period before FRC and TLC breath-hold. Furthermore, the data were analyzed throughout duration of FRC breath-hold, and during first and last 30 second periods of TLC breath-hold. Additionally, hemodynamic parameters were calculated during the period of maximal blood pressure drop at the beginning of TLC breath-hold.

### 2.4. Statistical analysis

All data were expressed as means with 95% confidence interval (95% CI) ranges. Differences between groups at the same time points, as well as breath-hold times, were compared using Mann–Whitney U test. Differences within certain group for the FRC breath-hold were compared using Wilcoxon test. For the TLC breath-hold Friedman's ANOVA was used and if significant, a Wilcoxon *post hoc* test was used. Slopes representing the change in MSNA *vs.* time were calculated using linear regression. The level of statistical significance was set at $P = 0.05$. All analyses were performed with Statistica 7.0 software (Statsoft, Inc., Tulsa, USA).

## 3. Results

Controls performed FRC breath-holds which lasted 27.7 (22.2–33.2) s, significantly shorter than the FRC breath-holds in the BHD group (60.4 (34.3–86.5) s ($P = 0.006$)). Breath-hold duration at TLC lasted on average 156.5 (106.5–206.5) s in the control group and 214.0 (172.6–255.4) s in the BHD group ($P = 0.07$).

### 3.1. Integrated burst analysis

At baseline, controls had an average burst frequency of 13 (8–18) bursts per minute while baseline burst frequency in divers was 20 (16–24) bursts per minute, respectively ($P = 0.039$). Baseline burst incidence was similar between the groups. Burst incidence in controls was 23 (17–29) bursts per 100 heart beats while the divers had 30 (15–35) bursts per 100 heart beats, respectively ($P = 0.09$).

Changes in integrated MSNA during the FRC breath-hold are presented in Fig. 1a. The cessation of breathing during FRC breath-holding caused an increase in all measured MSNA parameters in the BHD group while in controls the increase reached significance for burst frequency and total activity, while burst incidence and normalized amplitude showed a tendency of increase ($P = 0.07$). Overall, the increase in total MSNA during the FRC breath-hold was higher in divers compared to controls. The slopes representing change in total MSNA activity *vs.* FRC breath-hold duration were similar between the control (32.9 (16.7–49.1) a.u. $\times$ min$^{-2}$) and BHD (30.2 (19.6–40.8) a.u. $\times$ min$^{-2}$) groups ($P = 0.79$).

Fig. 1b depicts changes in integrated MSNA during the TLC breath-hold. Again, this type of breath-hold caused a significant increase in muscle sympathetic traffic in both groups. A more abrupt increase in burst frequency and total activity in first 30 s of TLC breath-hold was observed in divers compared with controls. The slope representing the change in total MSNA *vs.* time for first 30 s of breath-hold was more than twofold steeper in divers compared to controls (115.3 (80.7–149.9) a.u. $\times$ min$^{-2}$ *vs.* 52.9 (21.6–84.2) a.u. $\times$ min$^{-2}$ respectively; $P = 0.03$). Burst incidence in first 30 s was 73 (60–86) bursts/100 heart beats in the BHD group and 50 (34–66) in the controls ($P = 0.09$). There was no difference between the groups in normalized amplitude. At the end of TLC breath-hold controls and divers reached similar levels of total MSNA.

### 3.2. Action potential analysis

Subgroups of subjects in which we analyzed raw MSNA neurograms for AP discharge patterns did not achieve different duration of FRC (32 (28–36) s *vs.* 69 (28–110) s, respectively; $P = 0.09$) nor TLC (176 (109–243) s *vs.* 234 (175–293) s, respectively $P = 0.20$) breath-holds.

Compared with controls (13 (6–20) AP/burst) divers had lower AP content per burst at baseline (6 (3–9) AP/burst) ($P = 0.05$) as well as a lower number of active clusters per burst (5 (4–6) *vs.* 3 (2–4) clusters/burst; controls *vs.* BHD, respectively; $P = 0.05$). However, the overall sympathetic AP content per unit time was comparable between the divers (131 (39–223) AP/min) and controls (173 (24–322) AP/min; $P = 0.62$) due to increased burst frequency in the BHD group.

Compared to baseline, FRC breath-hold caused an increase in AP frequency, number of APs per burst and number of active clusters per burst (Fig. 2a) in divers. The same pattern of change was observed for all measured AP parameters in control group but these did not reach statistical significance ($P = 0.07$).

In the divers group, AP frequency and the average number of active clusters in a single burst were increased significantly throughout the TLC breath-hold, compared to the 30 s baseline period. A further increase in these AP parameters was observed towards the end of the breath-hold. A similar trend was observed in controls (Fig. 2b).

Fig. 3 represents differentiation of detected APs by clusters. In both groups, there was a trend towards an increased presence of APs assigned to larger clusters. The increase was seen towards the end of both the FRC (panel A) and TLC (panel B) breath-holds.

### 3.3. Hemodynamics

Changes in hemodynamic parameters during FRC and TLC breath-holds are presented in Table 2. Physiological responses to FRC breath-hold were similar between the groups, except arterial oxygen saturation which tended to be lower at the end of breath-hold in divers ($P = 0.05$). After the first 30 s of the TLC breath-hold mean arterial pressure (MAP) and systolic blood pressure (SBP), but not DBP, were lower in the divers. ($P = 0.09$). At the point in which arterial blood pressure was lowest (BP nadir) stroke volume (SV) and cardiac output (CO) tended to be lower in divers compared with controls ($P = 0.09$ and $P = 0.05$, respectively). At the end of the TLC breath-hold measured hemodynamic parameters were comparable among the groups; at this stage arterial hemoglobin oxygen saturation tended to be lower in the divers ($P = 0.07$).

## 4. Discussion

The main finding of our study is that the levels and recruitment patterns of sympathetic neuron activity during FRC (isolated chemoreflex stimulation) and TLC breath-holds (combined activation of baroreflex and chemoreflex) are similar in BHD *vs.* control

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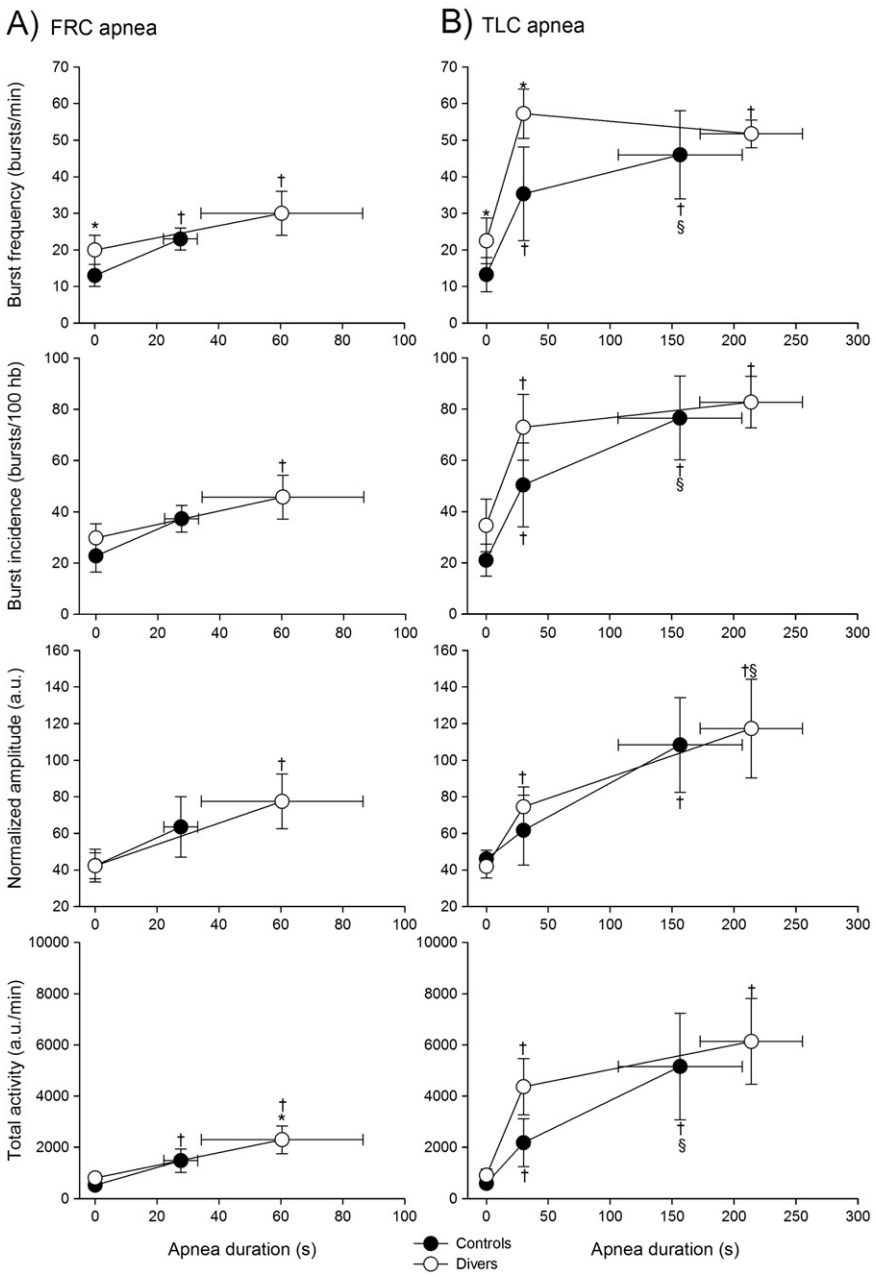



**Fig. 1.** Changes in the properties of integrated neurogram during various stages of FRC (panel A) and TLC (panel B) breath-hold. Black circles represent controls and white circles represent divers. Values are means, bars represent 95% confidence intervals. FRC, functional residual capacity; TLC, total lung capacity; *, $P<0.05$ between groups; †, $P<0.05$ compared to baseline; §, $P<0.05$ compared to first 30 s of breath-hold.

subjects if the breath-hold is similar in duration. In other words, if control subjects are brought to the comparable level of chemoreflex stress as BHD by performing a prolonged breath-hold of at least several minutes (3 min in current study), similar sympathetic activation occurs. However, the second major finding was that the mechanism by which the same sympathetic response was elicited during the breath holds differed between the two groups. Specifically, the divers exhibited fewer APs per burst and fewer active clusters per burst at rest but a pronounced increase in the burst incidence in this group achieved the same overall sympathetic response as the control group. Thus, the analysis of both integrated MSNA neurogram as well as the AP distribution within the neurogram exposed not only variations in recruitment patterns, but also compensatory responses that appear to develop in the trained BHD group.

### 4.1. Integrated burst analysis

#### 4.1.1. FRC protocol

In the current study a greater increase in MSNA at end of the breath-hold was observed in divers compared to controls. When using the integrated MSNA signal, the augmented sympathetic drive during breath holds was related to chemoreflex stress. In this interpretation, the FRC breath-hold duration was significantly longer in the BHD group, hence, causing a higher level of chemoreflex stress. However, the slope of the change in total MSNA *vs.* breath-hold duration is almost identical between the groups. This finding confirms that chemoreflex sensitivity is unchanged in BHD compared to controls, as shown previously (Dujic et al., 2008; Breskovic et al., 2010a; Breskovic et al., 2010b; Steinback et al., 2010a) and that different sympathetic responses between groups were due to variations in the duration of

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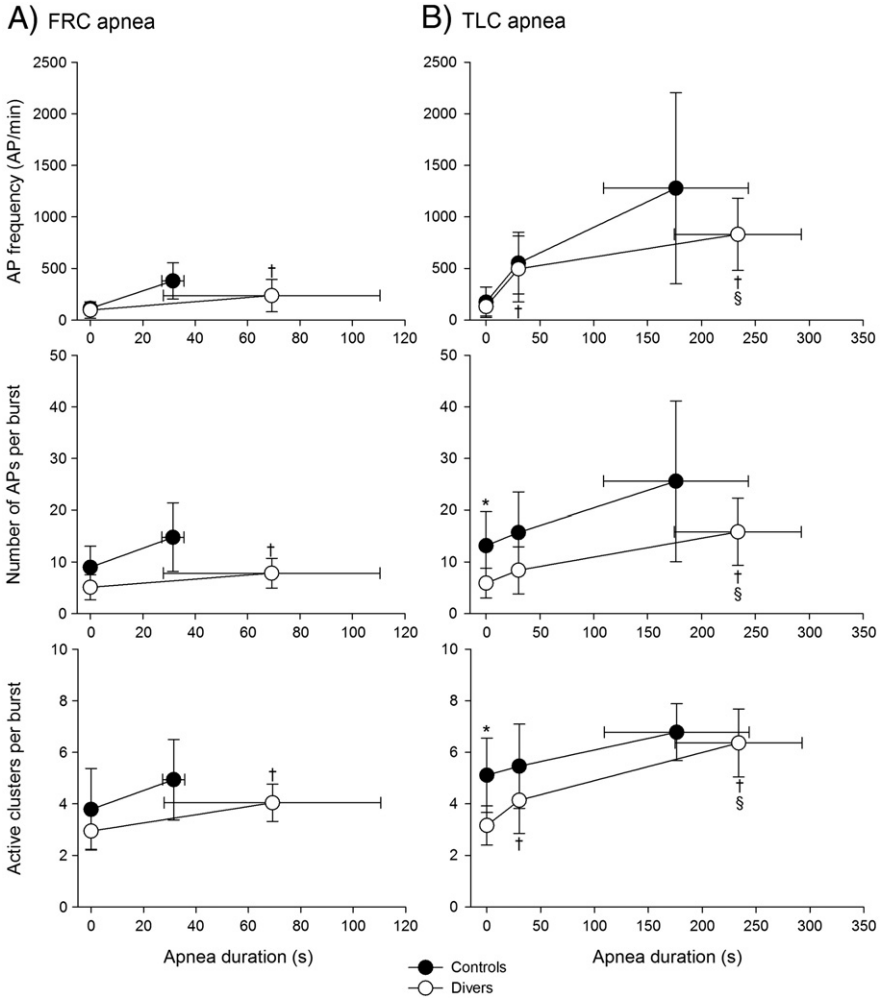



**Fig. 2.** Changes in action potential (AP) properties during various stages of FRC (panel A) and TLC (panel B) breath-hold. Black circles represent controls and white circles represent divers. Values are means, bars represent 95% confidence intervals. FRC, functional residual capacity; TLC, total lung capacity; *, $P<0.05$ between groups; †, $P<0.05$ compared to baseline; §, $P<0.05$ compared to first 30 s of breath-hold.

the breath-hold. Therefore, there is no influence of repeated prolonged apneas on chemoreflex sensitivity as seen in obstructive sleep apnea patients (Narkiewicz et al., 1999).

*4.1.2. TLC protocol*

Since, in this study, there was not much difference in duration of the TLC breath-hold in the two groups, the overall increase in MSNA for all measured parameters was comparable among the groups. Nevertheless, a different pattern of increase in MSNA was observed throughout the TLC breath-hold. During the initial 30 s of TLC breath-hold there is a more pronounced increase in MSNA in divers compared to controls. Hemodynamic parameters indicate a marked and greater decrease in MAP and SBP, with a tendency towards lower DBP, SV, and CO in the divers compared with controls. Thus, the greater MSNA response in the first 30 s of the TLC breath-hold likely was due to greater unloading of baroreceptors. The between-group hemodynamic difference was probably caused by the ability of divers to inhale deeper before the breath-hold as a function of improved respiratory muscle control and/or rib-cage compliance as a result of training (Hentsch and Ulmer, 1984). Therefore, the slope representing the change in total MSNA *vs.* time for the first 30 s of the TLC breath-hold is more than twofold steeper in divers compared to controls. Importantly, the rapid increase in total MSNA for the first 30 s of the TLC breath-hold can be credited to an increase in burst frequency with little impact of a change in burst amplitude or AP/burst.

*4.2. Action potential analysis*

Based on the quantification of bursts in the integrated MSNA signal, this study did not show a difference between breath-hold divers and control subjects in the pattern of sympathetic activation during the FRC and TLC breath-holds. However, the numbers of APs/burst as well as active clusters/burst at baseline were greater in controls compared to divers. The reason for fewer APs/burst but higher frequency of integrated bursts per minute in the divers is unknown. Perhaps there has been some modulation of either AP recruitment or population characteristic to compensate for the frequent and prolonged sympathetic activations these individuals elicit during training.

Furthermore, it is possible to distinguish different properties of sympathetic neuron activity depending of the type of stimulus. As shown previously, (Steinback et al., 2010b) there seems to be an ordered pattern of activation of sympathetic neurons during progressive chemoreflex stress, resembling Henneman's size principle observed in skeletal motor system (Henneman et al., 1965). Previous studies involving single-unit recordings of sympathetic neurons portended this observation (Macefield and Wallin, 1999). In this study, FRC breath-hold and the later phase of the TLC breath-hold represent periods during which the subjects are exposed to rising chemoreflex stress. During these periods of the experimental protocol an increase in total sympathetic AP frequency was observed that was

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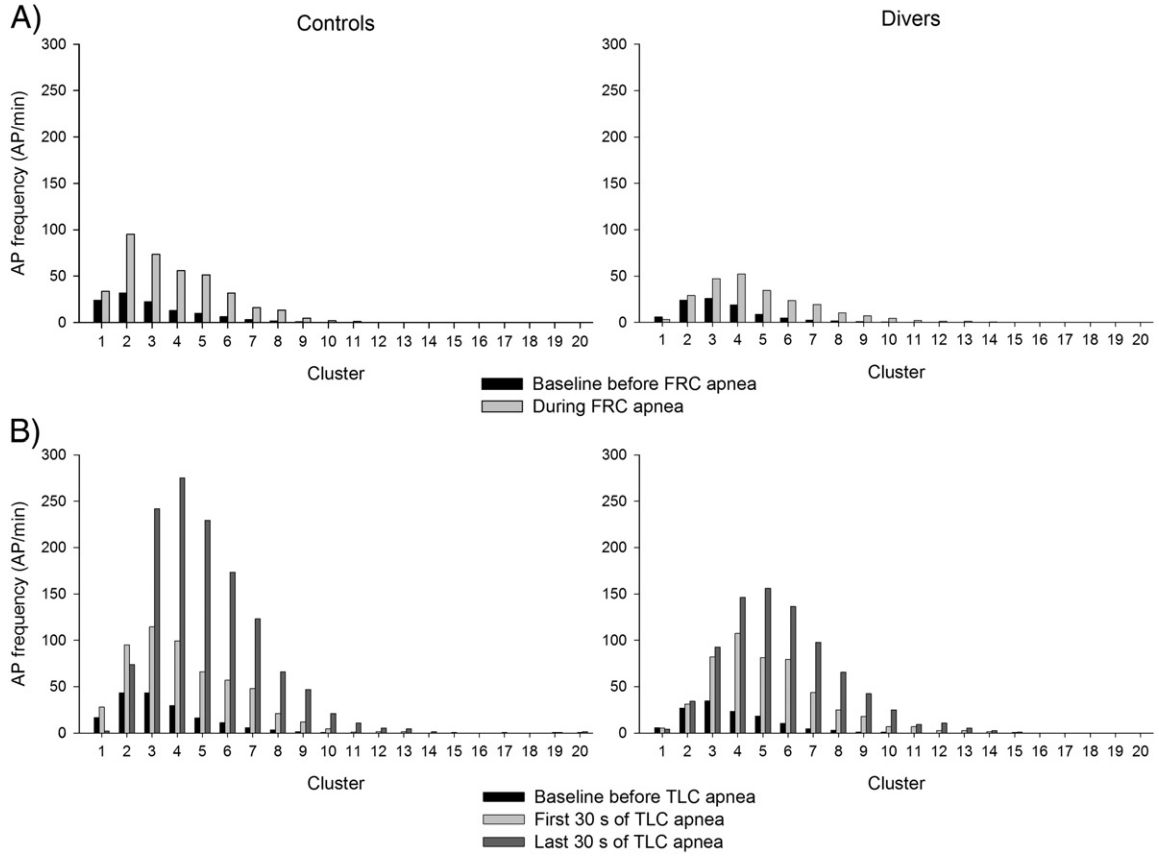



**Fig. 3.** Histograms representing changes in frequency of action potentials (AP) of sympathetic neurons differentiated by peak to peak amplitude size to corresponding clusters for various periods of FRC (panel A) and TLC breath-hold (panel B). FRC, functional residual capacity; TLC, total lung capacity.

caused by increases in both the percentage of cardiac cycles accompanied by bursts and by an increase in the average number of APs within a single burst. The rise in APs/burst was associated with a simultaneous increase in the number of active clusters per burst. These findings suggest the occurrence of increased firing probability as well as the possible recruitment of larger, faster conducting sympathetic neurons, as reported previously (Macefield and Wallin, 1999; Steinback et al., 2010b). Throughout the FRC breath-hold there was a proportional contribution to increase in total AP frequency by increases in burst frequency and number of APs/burst (~100% increase in burst frequency *vs.* ~50% increase in APs content within burst). However, as the burst frequency was rather high after the initial 30 s of the TLC breath-hold, especially in the divers, the increase in AP frequency in the later part of TLC breath-hold can be attributed

**Table 2**
Hemodynamic parameters during various stages of FRC and TLC breath-holds.

| | | FRC breath-hold | | TLC breath-hold | | | |
|---|---|---|---|---|---|---|---|
| | | Baseline | During | Baseline | BP nadir | First 30 s | Last 30 s |
| MAP (mm Hg) | Controls | 82 (78–86) | 90 (81–99)† | 83 (78–88) | 70 (62–78)† | 84 (80–88) | 108 (100–116)†,§ |
| | Divers | 81 (76–86) | 93 (86–100)† | 84 (78–90) | 54 (46–62)*,† | 72 (65–79)*,† | 115 (106–124)†,§ |
| SBP (mm Hg) | Controls | 135 (126–142) | 144 (131–159)† | 131 (125–137) | 114 (99–129)† | 138 (126–150) | 162 (148–176)†,§ |
| | Divers | 133 (125–141) | 147 (136–158)† | 134 (124–144) | 84 (71–97)*,† | 112 (98–126)*,† | 175 (159–191)†,§ |
| DBP (mm Hg) | Controls | 64 (61–67) | 69 (64–74)† | 66 (63–69) | 56 (50–62)† | 68 (64–72) | 84 (84–90)†,§ |
| | Divers | 63 (58–68) | 72 (66–78)† | 66 (60–72) | 44 (36–52)† | 59 (52–66)† | 90 (84–96)†,§ |
| HR (bpm) | Controls | 57 (51–63) | 63 (56–70) | 60 (53–67) | 76 (64–88) | 68 (59–77) | 60 (55–65)§ |
| | Divers | 64 (58–70) | 68 (62–74)† | 65 (59–71) | 85 (72–98)† | 83 (71–95)† | 64 (58–70)§ |
| SV (mL) | Controls | 128 (117–139) | 131 (115–147) | 122 (114–130) | 100 (80–120)† | 107 (86–128)† | 104 (85–123)†,§ |
| | Divers | 115 (101–129) | 111 (98–124) | 114 (97–131) | 66 (39–93)† | 77 (52–102)† | 98 (82–114)† |
| CO (L/min) | Controls | 7.4 (6.2–8.6) | 8.3 (6.6–10.0) | 7.3 (6.1–8.5) | 7.5 (5.9–9.1) | 7.3 (5.6–9.0) | 6.2 (4.8–7.6) |
| | Divers | 7.2 (6.6–7.8) | 7.5 (7.2–7.8) | 7.3 (6.7–7.9) | 5.1 (3.7–6.5)† | 5.9 (4.9–6.9)† | 6.2 (5.3–7.1)† |
| TPR (a.u.) | Controls | 11 (9–13) | 11 (9–13) | 12 (10–14) | 10 (8–12)† | 12 (8–16) | 18 (15–21)†,§ |
| | Divers | 11 (10–12) | 12 (11–13)† | 12 (11–13) | 12 (9–15) | 13 (11–15) | 19 (17–21)†,§ |
| $SaO_2$ (%) | Controls | 99 (99–99) | 99 (98–100) | 99 (99–99) | 99 (99–99) | 99 (99–99) | 97 (94–100)§ |
| | Divers | 99 (99–99) | 95 (92–98)† | 99 (99–99) | 99 (97–101) | 99 (98–100) | 89 (81–96)†,§ |

Values are means with 95% confidence intervals in the parentheses. FRC, functional residual capacity; TLC, total lung capacity.
† $P<0.05$ compared to baseline.
§ $P<0.05$ compared to first 30 s of breath-hold.
* $P<0.05$ between groups.

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more to an increase in AP/burst reflecting enhanced recruitment combined with possible repeated firing than to an increase in burst frequency (~5–25% increase in burst frequency *vs.* ~100% increase in AP content within burst).

The initial 30 s of the TLC breath-hold, characterized by low blood pressure, SV, and CO, represents a baroreceptor unloading stimulus. Although being a potent provocation for the increase in sympathetic neural activity, different patterns of activation were observed in this early phase, compared to the progressive chemoreflex stimulus of the prolonged breath-hold. In particular, within the first 30 s, there was a marked increase in burst frequency (~200%) accompanied by a small increase in the content of APs within single burst (~30%). The cause for such a different pattern of sympathetic activation is unclear. Perhaps, there might be a relatively higher threshold for the onset of the recruitment of new sympathetic neurons for baroreflex unloading than during increased chemoreflex stress. The possible rationale for this theory may lie in fact that we have observed some evidence of recruitment in divers group. Specifically, we observed an increasing trend in number of APs within single burst and number of active clusters in the divers, but not in controls. Due to deeper inhalation, divers reduced their MAP (~15% *vs.* ~35%, respectively) and SV (~20% *vs.* ~40%, respectively) significantly more than controls. Therefore they might have been able to reach threshold levels of baroreflex stimulation needed for onset of the recruitment of sympathetic neurons. Furthermore, Salmanpour et al. (in press) have recently utilized the same technique for AP detection during lower-body negative pressure (up to −60 mm Hg) to provoke baroreceptor unloading. Their study showed that the increase in sympathetic neural activity caused by baroreflex activation was attained by changes in the burst frequency and burst incidence alone without change in AP content or apparent recruitment of additional axons.

As expected, the FRC breath-hold lasted longer in BHD compared to controls. However, control subjects in this study held their breath after TLC breath-hold for approximately 3 min *vs.* 4 min in the BHD group. In our previous studies, control subjects usually achieved shorter breath-holds lasting approximately 1–1.5 min (Bakovic et al., 2003; Palada et al., 2007; Heusser et al., 2009). The reason for discrepancy in longer duration of the breath-holds in this group of controls is unclear. Our intention was to recruit a group of controls subjects who would be adequately matched with breath-hold divers, with the exception that they would not be intensively involved in breath-hold training. Therefore, most of the controls were kinesiology students that were regularly exposed to different sports, including water sports like swimming. It is possible that the occurrence of habituation to breath-holding in these individuals, combined with a positive psychological attitude towards achieving the best possible score, as seen in other sports including competitive apnea disciplines, may improve breath-hold time (Barwood et al., 2007), and may be the possible reason for such unusually long breath-holds in this group of controls. Meanwhile, the duration of the breath-hold in BHD of around 4 min was similar to our previous studies (Bakovic et al., 2003; Palada et al., 2007; Heusser et al., 2009).

### 4.3. Limitations

The major limitation of our study was the lack of measurement of arterial blood gasses. By measuring the arterial gas tensions the chemoreflex stimulus could be quantified with greater accuracy and perhaps ensure that the chemoreceptor stimulus was matched between conditions in both groups. Similar breath-hold times *per se* do not guarantee that comparable levels of $PaO_2$ and $PaCO_2$ are achieved at the end of a breath-hold in different subjects. For example, the maximal attained level of arterial gas tension may be influenced by different starting points caused by excessive hyperventilation before the breath-hold. Additionally, deeper inhalation and larger lung volumes provide an enlarged reservoir of $O_2$ as well as a larger dilution space which would reduce the slope of increase in $CO_2$. Moreover, different levels of metabolic rate among subjects will result in different speed of $O_2$ consumption and $CO_2$ production. Finally, it appears that the breakpoint for the breath-hold is not strictly related to certain arterial levels of $O_2$ and $CO_2$ (Kelman and Wann, 1971; Parkes, 2006). Consequently, the maximal change in $O_2$ and $CO_2$ levels caused by breath-holding highly depend on subject motivation to endure the increasing urge to breathe. However, our previous studies have shown that breath-hold divers have unchanged central (Dujic et al., 2008) or peripheral (Breskovic et al., 2010a; Breskovic et al., 2010b) chemosensitivity, cardiovagal and sympathetic baroreflex gains, or respiratory modulation of MSNA (Steinback et al., 2010a). In this study, at the end of the breath-holds, the divers and controls achieved similar levels of increase in MSNA (quantified using the integrated and raw signals); therefore, it might be concluded that the levels of chemoreflex stress were comparable between the groups.

## 5. Conclusion

In summary, our study has shown that prolonged breath-holds result in a considerable increase in MSNA which is reached both by an increase in burst incidence, (*i.e.* increased firing frequency of sympathetic neurons), by the recruitment of previously silent, larger (faster conducting) neurons, and possibly by repeated firing within the same burst. However, different patterns of activation of postganglionic sympathetic neurons were observed depending on whether the provocation was a TLC or FRC breath-hold. This latter observation points to the fact that a breath-hold (both FRC and TLC) can be used as method to investigate how the sympathetic nervous system grades its output in healthy subjects but also in different clinical pathologies. Furthermore, when exposed to the same levels of chemoreflex stimulus, control subjects have similar levels of MSNA obtained with the analogous strategy of sympathetic neuron recruitment and firing. Finally, regular and frequent exposure to the level of sympathoexcitation observed during breath-hold (especially TLC breath-hold) may pose a mechanism by which the pattern of basal sympathetic outflow can be altered by reduction in AP content within burst and by an increase in basal burst frequency. Additional studies are necessary to better investigate this finding.

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