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ORIGINAL PAPER

Sensitivity to hypercapnia and elimination of CO_2 following diving in Steller sea lions (*Eumetopias jubatus*)

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Abstract The diving ability of marine mammals is a function of how they use and store oxygen and the physiological control of ventilation, which is in turn dependent on the accumulation of CO_2. To assess the influence of CO_2 on physiological control of dive behaviour, we tested how increasing levels of inspired CO_2 (hypercarbia) and decreasing inspired O_2 (hypoxia) affected the diving metabolic rate, submergence times, and dive recovery times (time to replenish O_2 stores and eliminate CO_2) of freely diving Steller sea lions. We also measured changes in breathing frequency of diving and non-diving individuals. Our findings show that hypercarbia increased breathing frequency (as low as 2 % CO_2), but did not affect metabolic rate, or the duration of dives or surface intervals (up to 3 % CO_2). Changes in breathing rates indicated respiratory drive was altered by hypercarbia at rest, but blood CO_2 levels remained below the threshold that would alter normal dive behaviour. It took the sea lions longer to remove accumulated CO_2 than it did for them to replenish their O_2 stores following dives (whether breathing ambient air, hypercarbia, or hypoxia). This difference between O_2 and CO_2 recovery times grew with increasing dive durations, increasing hypercarbia, and was greater for bout dives, suggesting there could be a build-up of CO_2 load with repeated dives. Although we saw no evidence of CO_2 limiting dive behaviour, the longer time required to remove CO_2 may eventually exhibit control over the overall time they can spend in apnoea and overall foraging duration.

Keywords Dive behaviour · Gas exchange · Carbon dioxide · Steller sea lion

Abbreviations

F_iCO_2	Fractional content of inspired carbon dioxide
F_iO_2	Fractional content of inspired oxygen
P_{CO_2}	Partial pressure of carbon dioxide (in blood)
P_{O_2}	Partial pressure of oxygen (in blood)
bpm	Breaths per minute
PA_{CO_2}	Alveolar partial pressure of carbon dioxide
$\dot{V}_{O_2}$	Rate of oxygen consumption
$\dot{V}_{CO_2}$	Rate of carbon dioxide production

Introduction

Exhaustion of onboard oxygen stores is generally assumed to limit the time that diving vertebrates can remain submerged and has been the basis for calculating commonly used metrics—such as the aerobic dive limit—to compare the diving abilities of different species (reviewed in Kooyman and Ponganis 1998). However, physiological control of dive behaviour is inherently related to the control of ventilation. The observation that many marine mammals routinely end their dives long before they have depleted their O_2 stores but are also capable of diving well beyond their aerobic limits suggests that other factors must be involved in terminating a dive, such as a build-up of CO_2 (Butler 1982).

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CO_2 plays a central role in controlling ventilation in terrestrial mammals (Phillipson et al. 1981). However, its contribution to the control of diving in marine mammals is unclear (for review see Butler 1982). CO_2 is produced as O_2 stores are depleted during breath holding and results in an increase in the partial pressure of CO_2 (P_{CO_2}) in tissues and blood (Kooyman et al. 1980; Qvist et al. 1986). Small increases in blood P_{CO_2} in mammals stimulates respiratory drive, preventing the further rise of CO_2 by increasing ventilation rate and heart rate. However, this response would be disadvantageous for aquatic mammals, as it would severely limit submergence times. Marine mammals must therefore have adaptations to compensate for build-up of the CO_2 they produce while diving.

Early studies found marine mammals were less responsive to hypercarbia (high inspired CO_2) than terrestrial mammals, leading to suggestions that their relative insensitivity to CO_2 allowed them to dive longer than their terrestrial counterparts (Irving et al. 1935). Specifically, ventilation rates of restrained seals were observed to increase only slightly when the inspired CO_2 concentration was 5 %, while breathing frequency doubled when inspired CO_2 was 10 % (Irving 1938). Subsequent studies revealed that unrestrained marine mammals were nearly as sensitive to CO_2 as terrestrial mammals (i.e. increases in F_iCO_2 resulted in the same relative increase in ventilation), but that initiation of the ventilatory response may be blunted, i.e. occur at higher P_{CO_2} levels than for terrestrial mammals (Robin et al. 1963; Bentley et al. 1967; Påsche 1976a; Craig and Pasche 1980; Gallivan 1980; Butler 1982; Milsom et al. 1996; Kohin et al. 1999). Stimulation of the dive response (via trigeminal nerve input from facial receptors during submergence) may inhibit the response of the carotid bodies to rising P_{CO_2} levels while diving, contributing to the apparent insensitivity of marine mammals to CO_2 (de Burgh Daly et al. 1977; Elsner et al. 1977).

In addition to tolerating higher levels of CO_2, marine mammals also possess adaptations that increase the CO_2 carrying capacity of their blood. Elevated haemoglobin levels, which increase O_2 stores, can also carry more CO_2 as carboxyhaemoglobin. Marine mammal blood may also have a higher buffering capacity to protect against acidosis resulting from CO_2 build-up (Lenfant et al. 1969, 1970; Castellini and Somero 1981; Boutilier et al. 1993), which may contribute to the higher threshold of CO_2 required to elicit a ventilatory response in marine mammals as compared to terrestrial mammals.

A number of studies conducted in aquarium tanks suggest that breath-hold duration of marine mammals is affected by high blood P_{CO_2} levels (hypercapnia), indicating a potential effect of CO_2 on dive behaviour (Påsche 1976a, b; Craig and Pasche 1980; Gallivan 1980). CO_2 may also affect and determine the duration of inter-dive surface intervals, when diving mammals are thought to be primarily replenishing their O_2 stores (Boutilier et al. 2001). O_2 stores are generally restored faster than CO_2 is eliminated because of the longer time it takes to mobilize and remove CO_2 stores that have dissolved in tissues or have been buffered out of the blood (Boutilier et al. 2001). Hence, removal of CO_2 might regulate when the surface interval can end and the next dive can begin, thereby affecting overall foraging efficiency (percent of total time available for foraging).

Most studies on the effect of CO_2 have been undertaken with restrained animals or in restricted environments (i.e. on land or in small tanks). No studies have examined the effect of hypercarbia on ventilation rates or the behaviour of a voluntarily diving otariid. We therefore used a unique experimental set-up to examine the effect of hypercarbia in Steller sea lions (*Eumetopias jubatus*) that were actively foraging at depths typical of wild animals. We sought to examine the effects of hypercarbia and hypoxia on ventilation (through changes in breathing frequency) in non-diving animals and determine whether hypercarbia (and resultant hypercapnia) directly affected the behaviour (dive duration, inter-dive surface interval) or metabolic rates of animals diving naturally in an open ocean environment. Finally, we considered the role that CO_2 plays in controlling the dive behaviour of Steller sea lions by examining the effects of hypercarbia or hypoxia on the temporal relationship between CO_2 elimination and O_2 uptake following a dive (recovery). We hypothesized that hypercarbia would significantly increase breathing rates both before and after diving and result in either a decrease in dive durations or an increase the inter-dive surface durations.

Methods

Data collection

We used eight adult, female Steller sea lions that were raised at the Vancouver Aquarium (British Columbia, Canada). All animals were previously trained to use experimental equipment and performed all trials voluntarily under trainer control. Four of the sea lions (between 12 and 15 years old, mass range 159–230 kg) were housed at the Open Water Research Station (Port Moody, BC) and had been actively diving in the open ocean for research purposes since 2003 (F00SI, F00BO), 2005 (F00HA), or 2008 (F00YA). The other four sea lions, aged 9 years, were housed at the Vancouver Aquarium (mass range 157–182 kg). Animals were fed a diet of herring (*Clupea pallassi*) and market squid (*Doryteuthis opalescens*) supplemented with vitamins. All experiments were conducted under UBC Animal Care Permits #A07-0413 and #A11-0397.

Ventilation in resting animals

The effect of hypoxia and hypercarbia on ventilation rate (measured as breaths per minute) was initially examined on non-diving animals at the Vancouver Aquarium ($n = 4$). This was done to determine levels of hypoxia and hypercarbia to be tested on diving animals and to understand baseline changes in a resting, non-diving animal. Animals were fasted overnight prior to any trials, and the water temperature was within their assumed thermoneutral zone. Trials were carried out in a small covered pool using flow through respirometry, where breathing was restricted to a Plexiglas dome with air drawn through at a known rate of 200–350 L/min (depending on inspired gas) using a Sable Systems 500H mass flow generator and controller (Sable Systems Inc., Las Vegas, NV, USA). Animals were breathing either ambient air (control) or an altered inspired gas mixture. We created hypoxia (17, 18, 19 or 20 % O_2) or hypercarbia (1, 2, 3, 4 or 5 % CO_2) by adding either nitrogen or carbon dioxide gas, respectively, at known rates (monitored with a mass flowmeter; Omega, FMA-2322) to the incurrent air being drawn through the metabolic dome. Required flow rates for each level of inspired gas were experimentally determined prior to trials. Excurrent air was sub-sampled and scrubbed of water vapour; then, fractional concentrations of oxygen and carbon dioxide were measured using Sable system FC-1B and CA-1B analysers and recorded every 0.5 s (Sable Data Acquisition system, Sable Systems Inc.). Barometric pressure, relative humidity, and air temperature were also recorded. Metabolic data were analysed using Lab Analyst X (Warthog systems, Mark A. Chappell, University of California). Data were corrected for electronic drift by baselining gas concentrations to ambient air, or to the added gas for hypercarbia and hypoxia, at the beginning and end of the trial. Rates of oxygen consumption ($\dot{V}_{O_2}$) and carbon dioxide production ($\dot{V}_{CO_2}$) were calculated using equations 11.7 and 11.8 in Lighton (2008).

During the first set of trials (short-duration increase in F_iCO_2), animals breathed ambient air (20.94 % O_2 and 0.04 % CO_2) for the first 10 min followed by a stepwise increase in F_iCO_2 or decrease in F_iO_2. Breathing frequency (in breaths per minute; bpm) was calculated using the last ~6 min (when bpm was constant) of a 10-min period for each concentration exposure following a 2-min equilibration period for the inspired concentration in the metabolic dome to stabilize. In a subsequent set of trials (long duration increase in F_iCO_2), we repeated the hypercarbia trials (2 and 3 % CO_2) where the animals breathed a single concentration for 40 min (which was a comparable time frame for the dive trials—see following). Each animal ($n = 4$) completed one trial per inspired gas level, with treatments (ambient, 2 or 3 % CO_2) in a random order. For these trials (and dive trials), gas was added prior to the animal entering the chamber; hence, metabolic rate could be more accurately calculated from these added gas baselines (vs. Kohin et al. 1999). Estimates of metabolic and ventilation rates were calculated as the lowest 20-min average of a 40–45-min trial period (when $\dot{V}_{O_2}$ and bpm had reached a steady state).

Diving trials

Dive trials were conducted over a 3-month period (May–July 2011) with each animal typically doing 2 trials per week (maximum one per day). Using the same protocol as above, the sea lions breathed either ambient air or an altered inspired gas mixture of 2 % CO_2, 3 % CO_2, 19 % O_2, or 20 % O_2 both before and after their dive (for the number of trials completed by each animal see Table 1). Animals would not reliably enter the metabolic dome when CO_2 concentrations were >3 %, and only 3 of the 4 animals completed the 40-m bout dive at 3 % CO_2. Dive trials were also not undertaken under conditions of <19 % O_2 due to the logistics of the respirometry set-up (high flow rates required for dive trials precluded lower O_2 concentration beyond 19 %) as well as animal safety, given the open ocean conditions of the trials. Two sets of trials were conducted under each set of inspired gas conditions with animals diving either to 10 or 40 m depth (see below). The experimental set-up consisted of a floating platform with a square opening in the middle containing a floating transparent Plexiglas respirometry dome (100 L). Metabolic rate

Table 1 Number of successful diving trials (single long dives, and bouts of 4 consecutive dives) completed by each of the four Steller sea lions under each level of inspired gas

Dive type	Animal	Ambient air	2 % CO_2	3 % CO_2	20 % O_2	19 % O_2
Single	F00BO	3	3 (1)	2 (1)	2	2
	F97HA	3	3	2	2 (1)	4 (3)
	F00YA	3	5 (4)	2	2	2
	F97SI	5	3 (2)	2	2	3
Bout	F00BO	3	3 (2)	2 (1)	2	2
	F97HA	3	3	2	2	2
	F00YA	3	3 (1)	1 (0)	1	2
	F97SI	4	3	2	2	3 (2)

The number in brackets represents the number of trials in which CO_2 recovery time was determined

was measured in the dome using flow through respirometry as described above.

Animals were fasted overnight prior to trials and weighed each morning. They were fed <0.5 kg during transport to the dive site to minimize any effect of heat increment in feeding on metabolic rate. During trials, the animals wore a harness with a VHF transmitter and time depth recorder. Pre-dive metabolism was measured at the start of each trial while animals rested inside the metabolic dome for 5–10 min until $\dot{V}_{O_2}$ was constant (~3-min period). Animals then dove voluntarily to a predetermined depth (10 or 40 m), to the bottom of a pair of feeding tubes that delivered ~0.2 gram pieces of herring every 5 s for the duration of the dive. Fish were delivered alternately between two tubes ~6 m apart to create a "prey patch" at depth that encouraged the animals to remain active during the dive.

For each trial, the animals were directed to do a single long dive followed by a separate four-dive bout cycle, in which the animals chose both their dive and inter-dive surface interval duration. Following both the single dive and the dive bout, the sea lions were kept at the surface in the respirometry dome for a post-dive 'recovery' measurement (defined as the time it took for the $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ to return to within 5 % of baseline values). Diving metabolic rate (DMR) was calculated as the average $\dot{V}_{O_2}$ over the dive and following recovery period. For bout dives, DMR was the average $\dot{V}_{O_2}$ for the entire cycle of dives (including inter-dive surface intervals) and recovery period.

Statistical analysis

All data were analysed using R (R Development Core Team 2011). Data from each animal were treated as repeated measures by including animal ID as a random effect, using linear mixed-effects models (LME) from the nlme package (Pinheiro et al. 2011). Models were initially run using the maximum likelihood method as required for comparison of fixed factors on multiple models. For post-dive breathing rate, O_2 recovery time, CO_2 recovery time, and the difference in recovery time (CO_2–O_2), an ANCOVA (also with animal ID as a random effect) was performed to determine whether inspired gas (as a categorical variable) co-varied with dive duration (as a continuous variable). Type of dive (single or bout) and depth (10 or 40 m) were also tested as fixed factors. Finding significant multiple fixed factors resulted in comparing the nested models (with or without a fixed effect) using a log likelihood ratio test (LRT) to determine the best overall model to fit the data (Pinheiro and Bates 2000). Significance testing for the final model selected was undertaken using the restricted maximum likelihood method (F statistics and p values are reported from the final model). For significant categorical factors, post hoc tests (using the Bonferroni method) were performed to compare the means between multiple groups. R^2 statistics were calculated using the lmmfit package in R (Maj 2011). Values are reported as means (±SD), and significance was set at $\alpha = 0.05$.

Results

Hypercarbia and hypoxia on ventilation rate

Hypercarbia significantly affected breathing frequencies in all trials (Fig. 1). For the non-diving animals, short stepwise increases in F_iCO_2 (every 10 min) resulted in a significant increase in breathing frequency (LRT = 37.4, $F_{5,19} = 14.1$, $p < 0.001$) when concentrations reached 4 % CO_2 (9.5 ± 1.6 breaths per minute; bpm) and 5 % CO_2 (11.8 ± 1.4 bpm) compared to ambient air (0.04 % CO_2; 6.8 ± 1.5 bpm). There was a significant increase in breathing frequency during the longer 40-min exposure trials at lower concentrations of 2 % (6.4 ± 1.4 bpm) and 3 % CO_2 (9.0 ± 0.8 bpm) compared to when the sea lions were breathing ambient air (4.9 ± 1.2 bpm) (LRT = 19.0, $F_{2,6} = 25.7$, $p = 0.001$).

Similar levels of hypercarbia affected the breathing frequency of the diving animals, where pre-dive breathing frequency at 2 and 3 % CO_2 was significantly higher than when the animals were breathing ambient air (Fig. 2a, 5.41 ± 1.5, 7.28 ± 1.7, and 8.03 ± 1.7 bpm for ambient air, 2 and 3 % CO_2, respectively, LRT = 34.7, $F_{4,46} = 11.4$, $p < 0.001$) as was post-dive breathing frequency (9.00 ± 1.8, 12.1 ± 2.6, and 13.6 ± 2.1 bpm for ambient air, 2 and 3 % CO_2, respectively, LRT = 65.8, $F_{4,45} = 30.4$, $p < 0.001$). Post-dive breathing frequency (following a single dive) also significantly depended on dive duration ($F_{1,45} = 22.3$, $p < 0.001$). Hyprcarbia (3 % CO_2) also resulted in a significant increase in breathing frequency during the inter-dive surface intervals of a bout dive, ranging from 22.3 (±4.2) bpm in ambient air to 28.9 (±4.1) bpm in 3 % CO_2 (Fig. 2b, LRT = 11.1, $F_{4,40} = 2.87$, $p = 0.035$).

Hypoxia (to 17 % O_2) had no effect on the breathing frequency of either diving or non-diving sea lions (Fig. 1b). Unfortunately, we were unable to measure tidal volume, which may have been adjusted to facilitate changes in the overall ventilation rate.

Hypercarbia and hypoxia on dive behaviour and metabolism

There was no effect of hypercarbia or hypoxia on diving metabolic rate (measured as either $\dot{V}_{O_2}$ or $\dot{V}_{CO_2}$). There was also no measurable effect of hypercarbia or hypoxia on dive behaviour (dive or surface interval duration) at levels

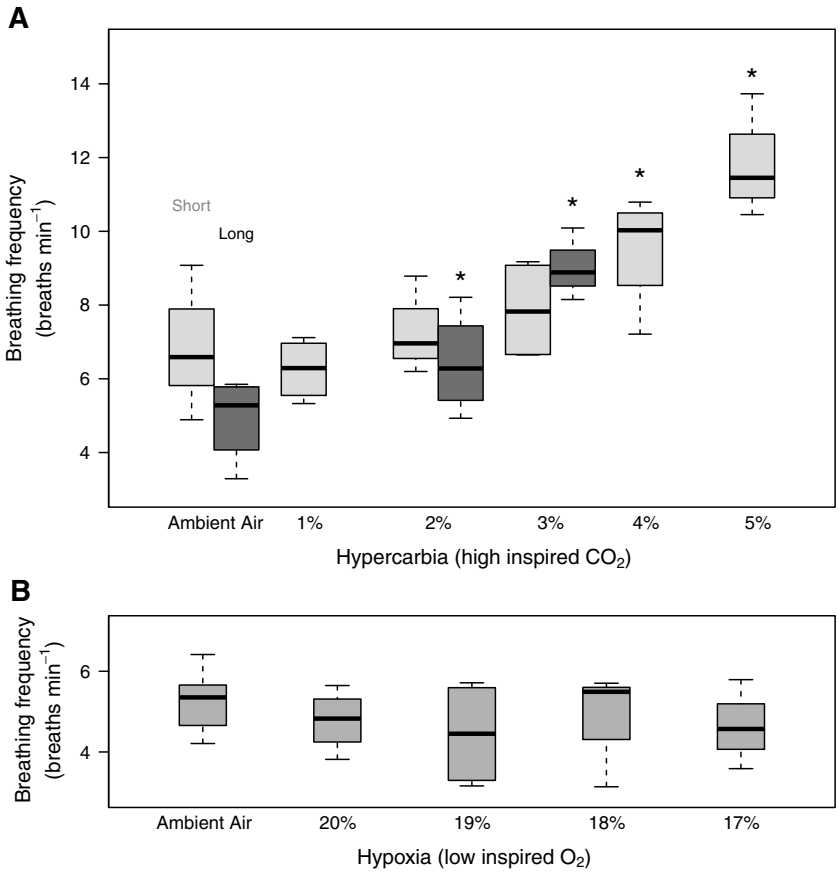

Fig. 1 Breathing frequency in non-diving, resting Steller sea lions ($n = 4$) as a function of inspired gas composition, including ambient air (20.9 % O_2 and 0.04 % CO_2). **a** Hypercarbia significantly increased breathing frequency when sea lions were subjected to either short-duration stepwise increases in F_iCO_2 (*lt. grey*, $p < 0.001$) or long duration (40 min; *dk. grey*). *Asterisk* indicates a significant difference from ambient conditions based on a linear mixed-effects model accounting for repeated measures between animals. **b** Hypoxia (short stepwise decrease in F_iO_2) had no effect on breathing frequency within the range used in this study

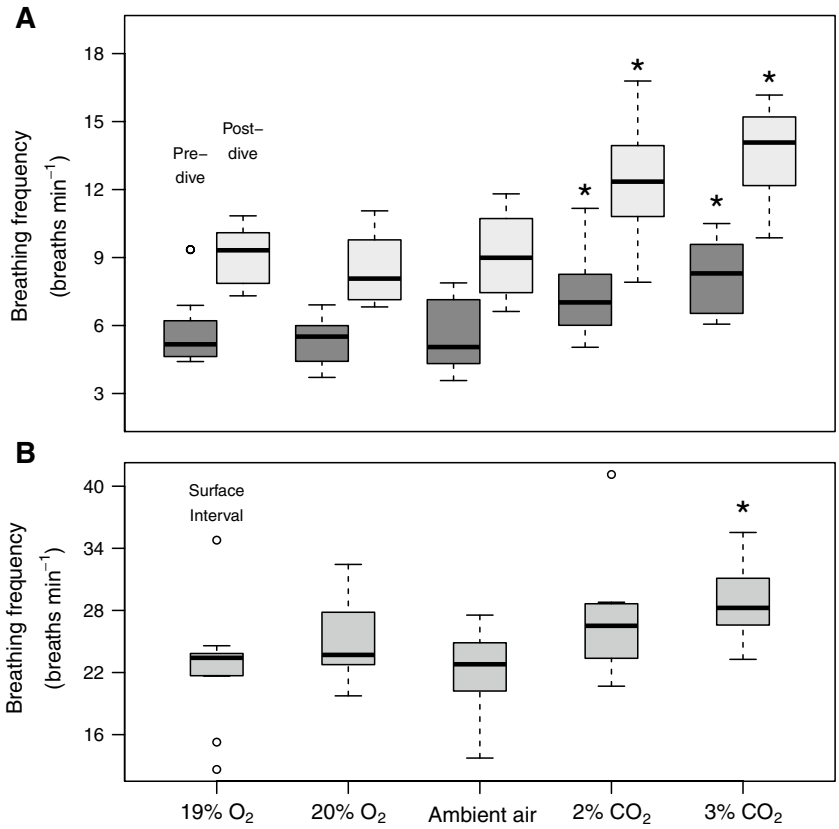

Fig. 2 Breathing frequency in diving Steller sea lions ($n = 4$) as a function of inspired gas composition. **a** Comparing pre-dive (3 min average, $p < 0.001$) and post-dive (over duration of recovery period, $p < 0.001$). **b** Breathing frequency during surface intervals between bout dives ($p = 0.007$). *Asterisk* indicates a significant difference from ambient conditions based on a linear mixed-effects model that accounted for repeated measures between animals and the significant effect of dive duration ($p < 0.001$) on post-dive values

up to 3 % CO_2 or down to 19 % O_2, despite there being a significant effect of hypercarbia on breathing rate and an observed initial behavioural response (adverse to staying in the metabolic dome) to increases in F_iCO_2 even as low as 1 %. There was a trend of decreasing dive duration relative to surface duration at the end of dive bouts with increasing levels of hypercarbia, but this was not significant.

Hypercarbia and hypoxia on post-dive recovery of CO_2 production and O_2 consumption

Following all bout dives, and all but two (96 %) of the single dives, it took longer for the $\dot{V}_{CO_2}$ of the sea lions to return to baseline levels (recover) than the $\dot{V}_{O_2}$ (Fig. 3). $\dot{V}_{O_2}$ recovery time following single dives was dependent on dive duration (longer dives resulted in longer recovery times; LRT = 9.83, $F_{1,50} = 10.4$, $p = 0.002$) but was unaffected by hypoxia or hypercarbia. However, $\dot{V}_{O_2}$ recovery time following dive bouts was not related to the duration of dive bouts or by hypercarbia or hypoxia. In contrast, $\dot{V}_{CO_2}$ recovery time following single dives was dependent on dive duration ($F_{4,39} = 33.5$, $p < 0.001$) and inspired gas (LRT = 12.07, $F_{4,39} = 3.00$, $p = 0.029$), as well as following dive bouts (duration; $F_{4,33} = 9.40$, $p = 0.004$, inspired gas; LRT = 10.5, $F_{4,33} = 2.68$, $p = 0.048$). Specifically, $\dot{V}_{CO_2}$ recovery times increased with increasing dive duration (and depth, which was interrelated to duration) and increasing hypercarbia.

The differences between O_2 and CO_2 recovery time increased with dive duration (single; $F_{1,39} = 10.0$, $p = 0.003$, bout; $F_{1,33} = 7.20$, $p = 0.011$) and significantly depended on inspired gas (single; LRT = 21.2, $F_{4,39} = 5.83$, $p < 0.001$, bout; LRT = 20.8, $F_{4,33} = 5.77$, $p = 0.001$). Hypercarbia (2 and 3 % inspired CO_2) resulted in a greater difference between recovery times (generally due to an increase in CO_2 recovery time but no change in O_2 recovery); hypoxia (to 19 % O_2) had no effect on $\dot{V}_{O_2}$ recovery, $\dot{V}_{CO_2}$ recovery, or the difference between the two (Fig. 4). It is important to note that we were unable to determine recovery time in 7 out of 55 single dives and 6 out of 48 bout dive trials (mostly hypercarbia; see Table 1), as $\dot{V}_{CO_2}$ did not reach pre-dive levels or a low enough slope to be considered stable and recovered. Hence, the average time for full $\dot{V}_{CO_2}$ recovery of Steller sea lions calculated in our study likely represents a slight underestimate.

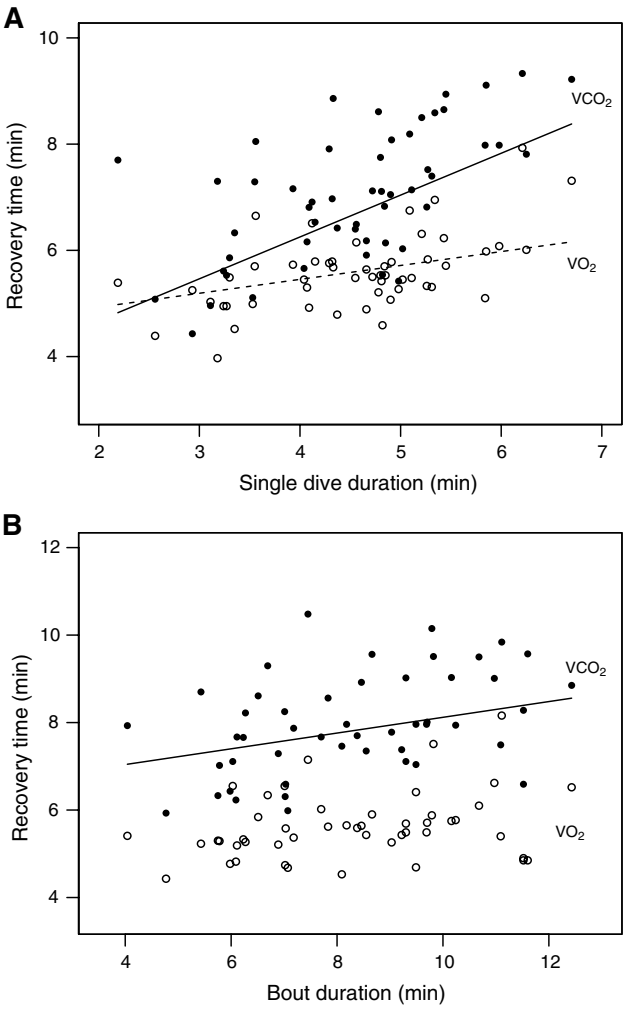

Fig. 3 Time for $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ to return to resting levels (recovery time) in Steller sea lions as a function of **a** single dive duration and **b** duration of a bout of four consecutive dives. CO_2 recovery was longer than O_2 recovery in all but two dives (96 %). Both O_2 ($R^2 = 0.23$, $p = 0.002$) and CO_2 ($R^2 = 0.40$, $p < 0.001$) recovery times increased with the duration of single dives. Following multi-dive bouts, only CO_2 recovery increased with duration ($R^2 = 0.28$, $p = 0.004$) and O_2 recovery was independent of duration ($R^2 = 0.07$, $p = 0.125$)

Discussion

Ventilation and dive behaviour

Past studies of how gas exchange affects dive behaviour have usually focused on the role that oxygen storage and utilization rates play in limiting dive duration, as well as on how the inter-dive surface durations are defined by the time required to refill those oxygen stores. However, control of dive behaviour must also depend to some extent on respiratory drive and therefore on P_{CO_2} levels (Butler and Jones 1997; Stephenson 2005).

We had expected hypercarbia or hypoxia to affect blood P_{CO_2} or P_{O_2} levels by altering the rate of diffusion of these gases between the blood and lungs in accordance with Fick's Law. This should have increased the required surface intervals relative to the duration of dives made by our sea lions based on the hypothesis that disfacilitation of respiratory drive is needed to initiate diving (i.e. P_{CO_2} levels

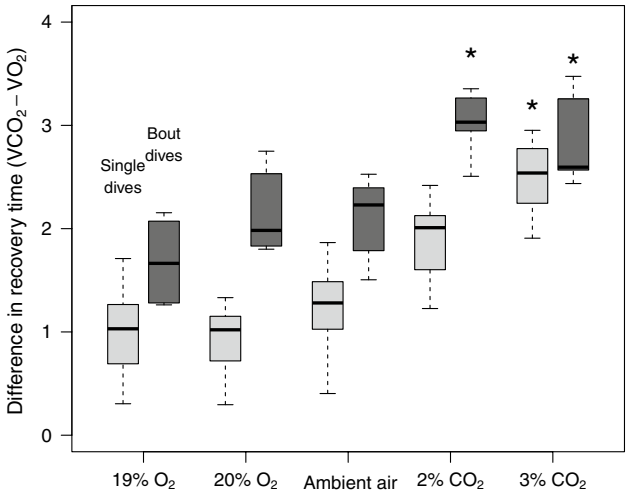

Fig. 4 Difference in recovery (duration required for CO_2 and O_2 levels to return to pre-dive levels) after completing a single dive (*lt. grey*) or a bout of 4 consecutive dives (*dk. grey*). All differences ($\dot{V}_{CO_2}$–$\dot{V}_{O_2}$) indicate that it took the sea lions longer to remove CO_2 than it did to replenish O_2 stores. *Asterisk* indicates a significant difference from ambient air conditions. Each *box plot* contains 6–8 data points that were derived from a linear mixed-effects model that accounted for the effect of dive duration and repeated measures between animals

must be reduced below some "threshold" value; Stephenson 2005). Such additional surface time would enable a sea lion to eliminate sufficient CO_2 to allow P_{CO_2} levels in their blood to return to pre-dive levels or the "threshold" value needed before being able to continue diving. However, we found that hypercarbia did not affect the duration of the surface intervals of our study animals. Failing to change the surface interval during hypercarbia suggests our sea lions were diving before P_{CO_2} had returned to resting levels—which should have limited their subsequent dive durations. Surprisingly, we observed no change in dive durations and could find no effect of hypercarbia on either the surface intervals or the dive durations chosen by our study animals.

Although there were no changes in duration of dives and surface times, we did observe significant changes in breathing frequency during hypercarbia before and after diving (as well as in resting animals)—indicating that hypercarbia affected respiratory drive. Breathing frequency increased at lower F_iCO_2 pre- and post-dive (diving animals) and during longer-duration exposures (resting animals) than during the short-duration exposures. This was likely due to the baselines being artificially elevated by the general decrease in breathing frequency that occurred over the duration of each trial (i.e. the breathing frequency for these trials was initially measured when breathing ambient air with hypercarbia following). Alternatively, the short (~10 min) initial exposure to hypercarbia in these trials may not have been long enough for blood P_{CO_2} levels to be affected in a resting animal. It is unclear exactly how our inspired CO_2 levels translated to arterial P_{CO_2} values, but they must have been elevated given the increase we observed in respiratory drive. Data from Påsche (1976a) indicate that an inspired CO_2 of 3 % resulted in a PA_{CO_2} of around 55-70 mmHg in harp and hooded seals. Hence, our inspired CO_2 levels likely altered the PA_{CO_2} of our sea lions.

We saw no effect of hypoxia on breathing frequency with inspired O_2 levels down to 17 %. This level of hypoxia may not have been low enough to elicit a ventilatory response, or the response may have been manifested as changes in tidal volume, as opposed to breathing frequency (Påsche 1976b). Previous studies have rarely found any effect of hypoxia on ventilation above ~13 % O_2 in phocid seals and manatees (Påsche 1976b; Gallivan 1980; Parkos and Wahrenbrock 1987; Milsom et al. 1996; Kohin et al. 1999).

Increased breathing frequency has been observed in many phocid seals at varying levels of hypercarbia, although generally at higher levels of inspired CO_2 than in our study (>5 % CO_2; Robin et al. 1963; Bainton et al. 1973; Påsche 1976a; Craig and Pasche 1980; Gallivan 1980; Parkos and Wahrenbrock 1987; Kohin et al. 1999). The slope of this response (PA_{CO_2} vs. ventilation rate) indicates phocids have a level of sensitivity to CO_2 that is similar to most terrestrial mammals (except possibly humans)—although the response of phocids may occur at a higher threshold PA_{CO_2} level (Bentley et al. 1967; Parkos and Wahrenbrock 1987; Butler and Jones 1997). Given that we observed increases in breathing frequency at 2 % CO_2 (lower than in previous studies), the response of Steller sea lions to hypercarbia is probably more similar to terrestrial mammals than phocids.

In general, seeing a response to hypercarbia at lower F_iCO_2 is not surprising given that previous studies have been on non-diving animals. Stresses associated with hypercarbia and the added exercise of diving to depth are likely much greater than in a resting animal due to the build-up of CO_2 in the body during apnoea. Hence, we believe the hyperventilation we observed in our diving animals was due to both the imposed hypercapnia and the hyperventilation that normally occurs prior to initiating a dive in order to lower P_{CO_2} (Stephenson 2005). However, drawing simple conclusions is complicated by the fact that other comparative studies have all been undertaken on phocid seals, which are generally considered better divers with more developed adaptations to diving than otariids (Kooyman 1989). A further complication to making simple comparisons is that overall changes in ventilation can be independently achieved through changes in breathing frequency, tidal volume (Påsche 1976a, b), or decreases in time spent apnoeic when at rest (as in Gallivan 1980). Hence, there may also have been a change in the tidal volumes of the sea lions that affected their overall ventilatory response to hypercarbia.

We saw significant ventilatory responses among the sea lions to hypercarbia at rest, but no change in dive behaviour (dive and inter-dive duration), suggesting there were other mechanisms involved in control of ventilatory drive during diving. This is consistent with earlier studies employing experimental forced "dives" in harbour seals that showed the dive response may inhibit the response of carotid bodies to elevated P_{CO_2} (de Burgh Daly et al. 1977; Elsner et al. 1977). Carotid bodies normally respond to increased blood P_{CO_2} with an increase in respiratory drive (stimulation to breathe). However, trigeminal nerve input while diving (stimulated by immersion of the facial receptors in water) has an inhibitory influence on chemoreceptor activity (de Burgh Daly et al. 1977; Elsner et al. 1977; Butler and Woakes 1982). Hence, an inspired CO_2 of 2 and 3 % likely resulted in an increase in arterial P_{CO_2} in our study, but was not great enough to override the inhibitory influence of the dive response on carotid body chemoreceptors. A similar inhibition of carotid bodies to hypercapnia (up to a critical point) was previously demonstrated during REM sleep in northern elephant seals (Milsom et al. 1996).

In contrast to our results, other studies of marine mammals have shown that breath-hold duration decreases at CO_2 levels as low as 3 % (Påsche 1976a; Gallivan 1980). This difference in results may be due to the artificial nature of the "dives" in the other studies that affected both motivation to extend dive durations and the extent of the dive response. The only other study that examined hypercapnia on actively diving animals concluded that "increasing alveolar CO_2 always completely inhibited diving behaviour" (Weddell seals; Parkos and Wahrenbrock 1987), but was not specific as to what level of PA_{CO_2} or F_iCO_2 this occurred. This suggests that higher levels of hypercarbia than what we tested would likely affect the dive behaviour of a freely diving sea lion.

It might be suggested that our sea lions did not dive close enough to their physiological limits for hypercarbia to limit their dive behaviour. However, the average dive duration of our study animals for single dives was 4.4 min, which is significantly longer than their calculated aerobic dive limit of 3 min (Gerlinsky et al. 2013) and much longer than the typical dive durations of Steller sea lions in the wild (2.0–2.4 min; Merrick et al. 1994; Merrick and Loughlin 1997; Loughlin et al. 1998).

Previous studies suggest that we would likely have seen an effect on dive duration at higher F_iCO_2 levels (Påsche 1976a, b; Craig and Pasche 1980; Gallivan 1980; Parkos and Wahrenbrock 1987). It is thus interesting to note that the threshold F_iCO_2 that might affect diving appeared to be the same concentration at which our sea lions would no longer voluntarily enter the breathing dome under hypercarbic conditions (~3 %). This suggests that our animals chose not to breathe high levels of CO_2 rather than increase their surface intervals or decrease their dive durations—despite the fact that this brought their feeding opportunities to an end. In contrast, sea lions motivated to dive more than normal (when nutritionally stressed) increased their dive duration while breathing ambient air (Gerlinsky 2013). However, they were unable to increase their dive durations when CO_2 was 2 %—suggesting that CO_2 limits dive duration under strenuous conditions.

Our data indicate that control of dive duration may depend on P_{O_2} in voluntarily diving animals. More surprising was our finding that inter-dive surface durations were not affected by hypercarbia, suggesting surface intervals may also be more limited by P_{O_2} or by a combination of P_{O_2} and P_{CO_2}. Increased alveolar ventilation would have also increased alveolar P_{O_2} resulting in faster uploading of O_2 stores, which could have confounded any effect of hypercarbia on surface interval duration.

Recovery from diving

Although there was no direct effect of CO_2 on dive behaviour, we saw evidence that CO_2 could indirectly affect diving by altering the surface intervals and effective recovery time following a dive. It takes longer for accumulated CO_2 stores to be removed from the body as CO_2 is sequestered in the blood and tissues as bicarbonate and needs to be converted back to CO_2 gas and bound to haemoglobin to be removed from the body (Boutilier et al. 2001; Falke et al. 2008). In nearly all dives, we observed that the $\dot{V}_{CO_2}$ of the sea lions took longer to return to baseline levels than their $\dot{V}_{O_2}$ regardless of whether it followed a single long dive or a bout of 4 consecutive dives with short surface intervals. This increased CO_2 recovery time as compared to O_2 suggests CO_2 could limit dive behaviour by imposing minimum surface interval durations. Similar results indicating that CO_2 limits dive behaviour and that P_{CO_2} is a potential signal to end the surface interval rather than P_{O_2} have been found in harbour porpoises (Boutilier et al. 2001) and grey seals (Reed et al. 1994).

Although we did not observe changes in dive behaviour under our experimental conditions, we predict that an increase in the number of consecutive dives in a bout should decrease dive durations as CO_2 further accumulates in the body. Indeed, there was some indication that the last dives in the bout were limited (i.e. surface intervals became slightly longer relative to dive durations), but this was not statistically significant and was likely confounded by variation in individual dive behaviour. Another potential reason we did not see the expected longer surface intervals (relative to dive duration) may be that this physiological response was counter to the animals' adverse behavioural response to hypercarbia (to decrease breathing duration in the metabolic dome, which would result in decreased surface time relative to dive durations).

Dive duration significantly affected $\dot{V}_{O_2}$ recovery following single dives only, unlike $\dot{V}_{CO_2}$ recovery, which was affected by the duration of both single dives and dive bouts (of 4 consecutive dives with short surface intervals). This suggests that O_2 stores during bouts were restored to a similar level after each surface interval, such that the animals always ended the bout at a similar level of O_2 depletion independent of duration. However, CO_2 probably continued to build up in their bodies over the course of the dive series as it could not be cleared at the same rate of O_2 store replenishment, resulting in a longer recovery at the end. This is consistent with the known higher buffering capacity of the muscle and blood for CO_2 (Lenfant et al. 1969; Castellini and Somero 1981) and suggests the animals were accumulating more of a "CO_2 load" over several repetitive dives. Under normal diving, CO_2 likely does not determine dive duration per se, but has more of an effect on the overall foraging efficiency by limiting the time an animal can spend in breath hold over the course of a foraging bout. While it is likely that O_2 stores limited individual dives, "CO_2 load" may eventually limit overall foraging for an animal performing several repetitive dives, such as in Steller sea lions.

The difference between recovery time for $\dot{V}_{CO_2}$ and $\dot{V}_{O_2}$ was amplified with increasing inspired CO_2 concentrations and decreased slightly with decreasing inspired O_2. The increase in $\dot{V}_{CO_2}$ recovery time further supports our conclusion that offloading of CO_2 was compromised during the recovery period by hypercarbia. This difference in recovery time was also significantly greater following the longer dives when the sea lions were nutritionally stressed (and had greater metabolic rates; Gerlinsky et al. 2014) with hypoxia resulting in a much smaller difference and hypercarbia in a much greater difference.

Conclusions

The limit to which we could expose the Steller sea lions to hypercarbia and still have them voluntarily participate in the experimental trials is likely similar to the physiological conditions under which CO_2 would affect diving. This may mean that sea lions in the wild are unlikely to stress themselves beyond a "CO_2 dive limit" (unless there is unusually high motivation to extend dive durations). Steller sea lions (and probably most marine mammals) appear to be similarly sensitive to CO_2 as terrestrial mammals. However, their increased capacity to store CO_2 and inhibition of carotid bodies during diving could contribute to the apparent "blunted" ventilatory response to CO_2 during forced dives and could to some extent uncouple the control of ventilation by CO_2 while diving.

Termination of a dive is undoubtedly a combination of factors that include behavioural and physiological limitations such as low O_2, high CO_2, and even rising pH levels from anaerobic metabolism (Noren et al. 2012). Our data support Stephenson's (2005) model that disfacilitation of respiratory drive initiates diving. Our data further suggest that P_{O_2} levels terminate a typical voluntary dive (based on the lack of behavioural responses to hypercarbia). When exposed to hypercarbia, the sea lions in our study were able to compensate with hyperventilation instead of increasing their surface intervals or decreasing their dive durations. Overall, our data suggest that O_2 storage is likely the limiting factor determining the duration of single dives, but that the accumulation of CO_2 over several dives ultimately limits the time an animal spends in apnoea during a bout of diving—and thus limits the overall time a sea lion can spend foraging.

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