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Measuring $\dot{V}O_2$ in hypoxic and hyperoxic conditions using dynamic gas mixing with a flow-through indirect calorimeter

Eric K. BIRKS[1], Hajime OHMURA[2]* and James H. JONES[3]

[1]Equine Sports Medicine Consultants, DE 19711, U.S.A.
[2]Sports Science Division, Equine Research Institute, Japan Racing Association, Tochigi 329-0412, Japan
[3]Department of Surgical and Radiological Sciences, School of Veterinary Medicine, University of California, Davis, CA 95616, U.S.A.

Measurements of gas exchange while breathing gases of different O_2 concentrations are useful in respiratory and exercise physiology. High bias flows required in flow-through indirect calorimetry systems for large animals like exercising horses necessitate the use of inconveniently large reservoirs of mixed gases for making such measurements and can limit the amount of equilibration time that is adequate for steady-state measurements. We obviated the need to use a pre-mixed reservoir of gas in a semi-open flow-through indirect calorimeter by dynamically mixing gases and verified the theoretical accuracy and utility of making such measurements using the mass-balance N_2-dilution method. We evaluated the accuracy of the technique at different inspired oxygen fractions by measuring exercising oxygen consumption ($\dot{V}O_2$) at two fully aerobic submaximal exercise intensities in Thoroughbred horses. Horses exercised at 24% and 50% maximum oxygen consumption ($\dot{V}O_2$ max) of each horse while breathing different O_2 concentrations (19.5%, 21% and 25% O_2). The N_2-dilution technique was used to calculate $\dot{V}O_2$. Repeated-measures ANOVA was used to tested for differences in $\dot{V}O_2$ between different inspired O_2 concentrations. The specific $\dot{V}O_2$ of the horses trotting at 24%$\dot{V}O_2$max and cantering at 50%$\dot{V}O_2$max were not significantly different among the three different inspired oxygen fractions. These findings demonstrate that reliable measurements of $\dot{V}O_2$ can be obtained at various inspired oxygen fractions using dynamic gas mixing and the N_2-dilution technique to calibrate semi-open-circuit gas flow systems.
Key words: *horse, N_2 dilution, oxygen consumption, training*

Investigations of oxygen transport to metabolizing tissues may require measurement of oxygen consumption ($\dot{V}O_2$) under conditions of varying inspired oxygen fraction (F_1O_2). Techniques in which inspiratory and expiratory volumes and O_2 concentrations are measured have proven satisfactory for determining $\dot{V}O_2$ in studies utilizing experimental subjects up to the size of humans, with certain limitations [2, 26]. However, when these methods are used for larger animals (*e.g.*, horses) during exercise [19, 20, 24], problems arise due to difficulties in accurately calibrating and measuring large ventilatory volumes and flows. There are also logistical problems with providing very large volumes of gases so that high bias flows can be maintained for a sufficient duration such that the animals equilibrate with the gas mixture and reach a steady-state during the exercise bout.

The N_2-dilution technique provides a simplified method for determining $\dot{V}O_2$ during exercise for animals of any size using an open-circuit system with a bias flow past the animal's face [7]. In this method, $\dot{V}O_2$ is determined by comparing changes in O_2 concentration downstream of where an animal consumes O_2 from a mask to changes observed when a calibrated flow of N_2 enters the mask to dilute the O_2 concentration downstream in the bias flow. Accuracy of this method requires only 1) that the bias flows are equal during calibration and measurement (but need not be accurately measured); 2) that an O_2 analyzer with

linear response is used (but need not be calibrated when the measurement is made breathing air); and 3) that a flow of N_2 equal to approx 5-times the $\dot{V}O_2$ of the animal is accurately measured during calibration. The rate of CO_2 production ($\dot{V}CO_2$) can be measured using an analogous CO_2 bleed-in technique [1]. The N_2-dilution technique has been used for numerous studies with mammals breathing air as well as some in which F_IO_2 was changed [1, 6, 9, 10–12, 14, 16, 17, 23].

Although the equations presented by Fedak *et al.* are theoretically sufficiently general to be applied to any combination of inspired O_2 and N_2, to our knowledge the use of this technique to determine $\dot{V}O_2$ under conditions of altered F_IO_2 has never been rigorously evaluated to validate its accuracy [7]. Here we tested the accuracy of the N_2-dilution technique for measuring $\dot{V}O_2$ under conditions of controlled hypoxia and hyperoxia in semi-open flow systems designed for equine athletes. We compared $\dot{V}O_2$ measured with the N_2-dilution technique in hypoxia, normoxia, and hyperoxia at identical submaximal exercise intensities for which net metabolic power is completely aerobic, insuring that "true" $\dot{V}O_2$ should be identical. In this paper we describe nuances of this technique and details of the analytical methods that are required for it to be accurate when used with altered F_IO_2.

Materials and Methods

The protocol for the study was approved by the University of California-Davis Animal Use and Care Committee.

Experimental subjects

Six Thoroughbred horses (3 geldings, 2 males, and 1 female; 3 year-olds, body weight 490 ± 31 (SD)) were studied. These horses had been trained on a treadmill (Mustang 2200, Graber AG, Fahrwangen, Switzerland) for 17 weeks at speeds up to 16 m/sec (specific $\dot{V}O_2max=2.59 \pm 0.10$ ml/(kg × sec)).

Open-circuit system design and gas analysis

The flow-through mask system utilized for these experiments has been described previously [1, 13, 15, 16, 18, 21, 23]. The basic elements of the system are a turbine, a downstream tube through which expired and bias flow gases are collected, a gas-tight mask, and an upstream tube in which O_2 and N_2 mix with the bias flow to produce the desired F_IO_2. The system used in this study incorporated a 25-hp (18.7-kW) turbine to draw air past subjects at bias flow rates of approximately 7,500 *l*/min (ATP) for horses. For the equine subjects, 25-cm dia PVC tubing carried bias flow gases to and from the mask. Flexible wire-reinforced polyvinyl tubing connected the mask to rigid conducting

tubes in the flow system.

A dual-channel O_2 analyzer (S-3/A II, Ametek Inc., Paoli, PA, U.S.A.) simultaneously monitored both upstream (inspired bias flow) and downstream (expired gas mixed with bias flow) O_2 concentrations. Gas was sampled from the upstream bias flow tubing 10 cm upstream of where the muzzlepiece of the mask attached. Prior to O_2 analysis, H_2O (for both samples) and CO_2 (for the downstream sample only) were removed from the gas stream with $CaSO_4$ (Drierite) and NaOH (Ascarite), respectively. Data were recorded on a PC (DI-720-USB and WinDaq Pro+, DATAQ Instruments Inc., Akron, OH, U.S.A.).

Experimental protocol

The normoxic $\dot{V}O_2max$ and the speed required to elicit it were determined for each of the horses prior to studies with altered F_IO_2. The $\dot{V}O_2max$ of each horse was identified using standard criteria, including no change in $\dot{V}O_2$ with increasing speed, respiratory exchange ratio (*RER*) >1.0, and plasma lactate accumulation rate exceeding 8 mM/min [1, 16, 21]. The temperature and relative humidity in the treadmill room were 20–25°C and 20–60%, respectively.

The horses warmed up by walking on the treadmill at 1.5 m/sec for 3 min, trotting at 4 m/sec for 3 min, and walking for 3 min at 1.5 m/sec. The horses equilibrated with the experimental gas mixture by breathing it during the entire warm-up period (9 min). The $\dot{V}O_2$ was determined following the warm-up period while the horses exercised at a trot (4 m/sec, 24% $\dot{V}O_2max$) and canter (8 m/sec; 50% $\dot{V}O_2max$) and breathed gas mixtures with F_IO_2 of 19.52 ± 0.03 (mean $\pm$ SD) % O_2, $20.95 \pm 0.00\%$ O_2, and $25.11 \pm 0.07\%$ O_2. The horses only ran once at any given F_IO_2.

Calibration

For calculation of the subject's $\dot{V}O_2$, N_2 was metered into the bias gas flow at the mask during calibration to obtain a decrease in O_2 concentration in the downstream sample (expired gas mixed with bias flow) similar to that measured while the horse exercised. At different F_IO_2, the unit flow of N_2 during calibration displaces different quantities of O_2. For example, at F_IO_2 of 0.16, every 1 *l* [STPD] min^{-1} of N_2 added into the system during calibration displaces 160 m*l* [STPD] O_2 min^{-1}, while at F_IO_2 of 0.26, 260 m*l* [STPD] O_2 min^{-1} are displaced.

Because F_IO_2 was altered in these experiments, it was necessary to enter it as a variable when calculating $\dot{V}O_2$. For these experiments, $\dot{V}O_2$ was calculated as

$$\dot{V}O_2 = \frac{F_IO_2 \bullet (\dot{V}N_2*/\Delta N_2*) \bullet \Delta O_2}{1 - F_{\bar{E}}O_2} \quad \text{(Eq. 1)}$$

where $\dot{V}N_2*$ is the flow rate of N_2 measured during calibra-

tion, ΔN_2^* is the O_2 analyzer deflection during calibration, ΔO_2 is the O_2 analyzer deflection measured with the subject connected to the system, and $F_{\bar{E}}O_2$ is the fractional O_2 concentration in the downstream tube (animal's expired gas mixed with bias flow). The quantity $\dot{V}N_2^*/\Delta N_2^*$ is a calibration factor representing the flow of calibration gas required to achieve a unit change in gas concentration. Calibration factors ($l\ N_2\ min^{-1}$) required to generate a 1% change in O_2 concentration during calibration were calculated. Calibrations were also performed at 15% and 30% O_2 concentrations to measure calibration factors even though the horses did not run at these concentrations.

Statistics

Repeated measures ANOVA was used to determined statistical differences, with *P*-value of 0.05 considered significant. Data are presented as mean ± SD. For comparisons that were not significantly different, we calculated the magnitudes of changes that could have been detected with 90% probability at the 0.05 α-level.

Results

Establishing F_IO_2

Adequacy of gas mixing at the mask and downstream was verified by detecting no change in bias-flow gas concentrations with diluent gas flowing when samples were drawn from the sample ports of the bias flow tubings at 5-mm intervals across their diameters. Nevertheless, in

conjunction with the bends in the tubing, gas mixing at the mask and downstream resulted in sufficient turbulence to mix the gases completely before the subject inspired the gas mixture or the downstream gas was sampled.

Figure 1 shows typical recordings of O_2 changes during experiments at the three F_IO_2. Changes observed in baseline (upstream) F_IO_2 during exercise were either added to (F_IO_2 > normoxic) or subtracted from (F_IO_2 < normoxic) deflections in $F_{\bar{E}}O_2$ recorded during exercise to calculate the total deflection for a given flow of calibration gas (Fig. 1).

Figure 2 shows the ratio of $\dot{V}N_2^*$ to O_2 analyzer deflection (calibration factor) obtained at identical bias flow rates at different F_IO_2 for equine systems.

F_IO_2 and oxygen consumption

Table 1 shows the specific $\dot{V}O_2$ of the horses at 24%$\dot{V}O_2$max and 50%$\dot{V}O_2$max while breathing 19.5%, 21% or 25% O_2 concentrations. The specific $\dot{V}O_2$ of the horses trotting at 24%$\dot{V}O_2$max and cantering at 50%$\dot{V}O_2$max were not significantly different among the three different F_IO_2, respectively.
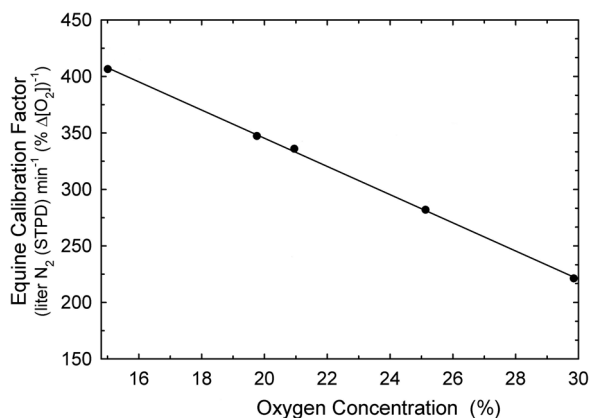
Discussion

In an open-flow system, the subject breathes into and out of a bias flow of gas drawn through a mask. Changes in O_2 and CO_2 concentrations in the gas flow downstream from the mask represent contributions, either consumed (O_2) or produced (CO_2), by the subject. The elegance of the
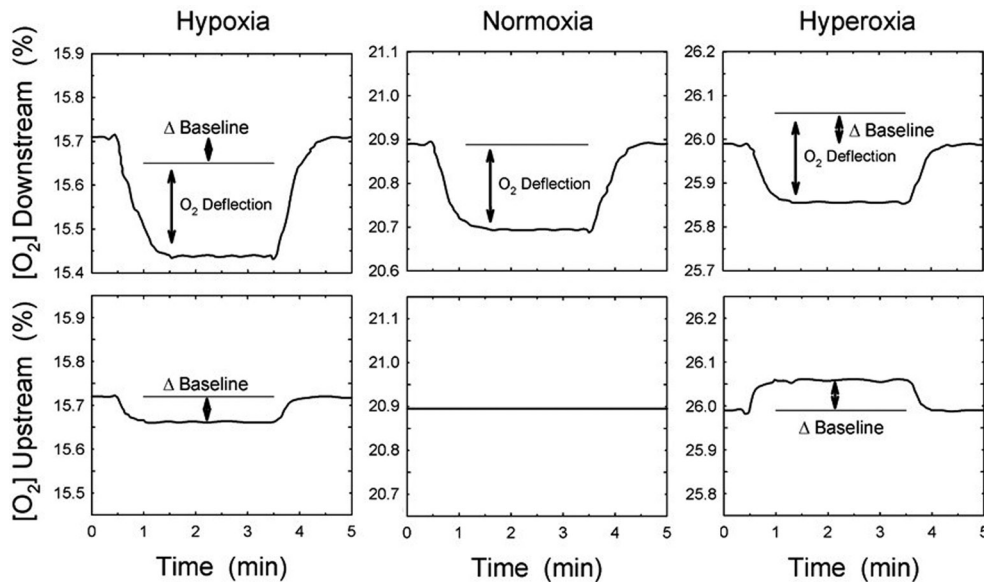
Fig. 1. Recordings of upstream (lower panels) and downstream (upper panels) O_2 concentrations while breathing through a semi-open flow-through indirect calorimeter with dynamic gas mixing in hypoxia (15.77% O_2, left panels), normoxia (20.94% O_2, center panels), or hyperoxia (26.04% O_2, right panels).

N_2-dilution technique lies in the fact that it is unnecessary to use calibrated flow meters for the bias flow and in the fact that for studies conducted in normoxia, it is unnecessary to use calibrated O_2 or CO_2 analyzers, thus avoiding compounded errors associated with them, simplifying and reducing errors in the entire measurement procedure [7].

With the N_2-dilution technique, it is only necessary to monitor the relative magnitudes of the changes in gas concentration with a linearly responding O_2 analyzer while the subject is being measured and when the system is calibrated. Calibration is achieved by accurately measuring the flow of calibration gas necessary to produce an identical (or proportional) change in gas concentration when the subject is not connected to the system as occurred during exercise. To calculate $\dot{V}O_2$, the calibration gas is used to displace bias flow gas at a rate proportional to the amount of O_2 consumed by the subject during exercise. The flow of N_2 required for calibration is only a fraction of the bias flow (approx 5-times the subject's $\dot{V}O_2$) and can be measured more accurately than measuring total bias flow, particularly for large animals, e.g., horses (bias flows ~6,000–10,000 l min^{-1}, N_2 calibration flow ~300–500 l (STPD) min^{-1}). It is

also possible to use electronic mass flowmeters or controllers to measure calibration gas flows, because they are calibrated with dry gases of known composition, whereas they are inaccurate when used to measure bias flow because the gas composition varies as does the water vapor concentration of the bias gas when the animal expires into it. Both of these factors affect the calibration of a mass flowmeter.

The equation used to calculate $\dot{V}O_2$ in these experiments (Eq. 1) is a modified form of equation 11b from Fedak et al. [7]; it assumes a respiratory exchange ratio (RER) of 1.0 and ignores the correction for H_2O vapor production ($\dot{V}H_2O$). If $F_IO_2-F_{\bar{E}}O_2=0.01$, this assumption results in a 0.1% error for each 0.1 unit that RER differs from 1.0, and there is a maximum 8% error from not correcting for $\dot{V}H_2O$. For the submaximal exercise intensities and bias flows utilized in our study, the value of $F_IO_2-F_{\bar{E}}O_2$ typically ranged from 0.002 to 0.006; thus the error associated with uncorrected RER is generally much less than 0.1%. The maximum error due to $\dot{V}H_2O$ would result only if the inspired air contained no H_2O vapor and the downstream flow was completely saturated at 40°C with H_2O vapor originating from the animal. Because the bias flow was adjusted to approx 10-times the subjects' minute ventilation, the maximum value of this error, as used in our system, is <1%. The workloads utilized during these measurements were of sufficiently low intensity (24% and 50% of $\dot{V}O_2$ max), and the magnitude of hypoxia was sufficiently modest, so O_2 delivery was assumed to play no role in limiting $\dot{V}O_2$.

The present study demonstrates that the simple N_2-dilution technique for calibrating open-flow systems yields unbiased values for $\dot{V}O_2$ with varying F_IO_2. No detectable nor systematic changes in $\dot{V}O_2$ were measured with F_IO_2 of very different composition. If the calculations for $\dot{V}O_2$ were biased, they would be expected to systematically alter calculated $\dot{V}O_2$ in opposite directions for hyperoxia and hypoxia, yet we detected no systematic bias in any of the measurements with markedly different gas compositions.

The N_2-dilution technique provides several distinct advantages over other commonly used $\dot{V}O_2$-measuring techniques with the high bias flows necessary for large exercising animals. In general, most other techniques require accurate measurement not only of F_IO_2 and $F_{\bar{E}}O_2$ but also water vapor fraction, either (or both) inspiratory or expiratory flows ($\dot{V}_I$ or $\dot{V}_E$), and/or bias flow [2, 4, 22, 25,

Fig. 2. Calibration factors (l N_2 (STPD) min^{-1} required to generate a 1% change in O_2 concentration during calibration for horses semi-open flow-through indirect calorimeters. Data include 15% and 30% O_2 concentration calibrations even though the horses did not run at these concentrations, so they were not used for $\dot{V}O_2$ comparisons.

Table 1. Specific $\dot{V}O_2$ of horses exercising at different intensities while breathing different concentrations of inspired O_2

Exercise intensity	24%$\dot{V}O_2$max			50%$\dot{V}O_2$max		
Inspired O_2	19.5%	21%	25%	19.5%	21%	25%
Specific $\dot{V}O_2$ (ml/(kg × sec))	0.59 ± 0.28	0.57 ± 0.08	0.68 ± 0.21	1.42 ± 0.35	1.43 ± 0.24	1.44 ± 0.19

mean ± SD.

26]. Accurate measurements of ventilation volumes require large calibrated flow meters and thus are often difficult to obtain, especially for exercising horses with tidal volumes >15 l, ventilation rates exceeding 2.2 Hz, and peak flows approaching 100 P s^{-1} during maximal exercise [6, 8]. Open-flow techniques for measuring $\dot{V}O_2$ do not require the use of valves to separate inspiratory and expiratory flows. Valving can increase impedance, thereby increasing the energy cost of ventilation, and possibly contribute to limiting $\dot{V}O_2$. In the flow-through system used in this study, bias gas flowed with minimal resistance through the mask with no valves to impede ventilation. For the equine systems used in the present study, the pressure drop at the mask was minimal (<4 cm H$_2$O) at the flow rates used in the experiments.

A major criticism of techniques for determining $\dot{V}O_2$ that require measurement of ventilation volumes is that the necessary assumption of no net N$_2$ exchange may not be correct [3, 5], even under steady-state conditions. This could be a major source of error, especially for those methods in which either, but not both, $\dot{V}_I$ or $\dot{V}_E$ is measured. This potential difficulty is best overcome by measuring both $\dot{V}_I$ and $\dot{V}_E$ using a technique that is independent of measuring either ventilatory volume as in the present study.

We observed that F_IO_2 changed slightly as exercise intensity increased (Fig. 1, top) with F_IO_2 set by a constant flow of diluent gas ($\dot{V}_{dil}$) when the subjects' total ventilation ($\dot{V}_{sub}$) increased. These changes are consistent with $\dot{V}_E$ being greater than $\dot{V}_I$. Total bias flow downstream of the animal can be expressed by the following equation:

$$\dot{V}_{total} = \dot{V}_{amb} + \dot{V}_{dil} + \dot{V}_E - \dot{V}_I \quad (Eq.\ 2)$$

where $\dot{V}_{total}$ is the total air flow set by the demand of the turbine; $\dot{V}_{dil}$ is the diluent gas flow, either O$_2$ or N$_2$, added to alter F_IO_2; $\dot{V}_E$ and $\dot{V}_I$ are the expiratory and inspiratory volumes, respectively; and $\dot{V}_{amb}$ is the ambient air necessary to meet the remainder of the turbine's demand. When $\dot{V}_E = \dot{V}_I$, the F_IO_2 established by altering $\dot{V}_{dil}$ remains constant. However, if $\dot{V}_E$ exceeds $\dot{V}_I$ with constant $\dot{V}_{dil}$, F_IO_2 changes in proportion to the product of $\dot{V}_E/\dot{V}_I$ and $\dot{V}_{sub}$. If $\dot{V}_{dil}$ were O$_2$ (hyperoxia), an increase in $[(\dot{V}_E/\dot{V}_I) \times \dot{V}_{sub}]$ would cause $\dot{V}_{amb}$ to decrease to maintain the equality in *Eq. 2* and F_IO_2 must increase. Conversely, if $\dot{V}_{dil}$ were N$_2$ (hypoxia), increased $[(\dot{V}_E/\dot{V}_I) \times \dot{V}_{sub}]$ would decrease F_IO_2. This change in baseline (F_IO_2) during a run must be measured and added or subtracted when calculating the change in [O$_2$] due to the animal's metabolism (Fig. 1). During calibration, when N$_2$ added at the mask reduces the upstream flow and hence alters F_IO_2 (if $\dot{V}_{dil}$ is not adjusted), the deflection in downstream [O$_2$] must be calculated from the altered baseline, as the downstream deflection is generated by the additional $\dot{V}N_2*$ as well as $\dot{V}_{dil}$ in the system.

In most methods used for determining $\dot{V}O_2$ with varying F_IO_2, the experimental subject breathes from a stored, pre-mixed source. While this approach eliminates slight differences in F_IO_2 from a pre-determined value due to $\dot{V}_E/\dot{V}_I$ inequalities, leaks in the inspired-gas line would introduce unmeasured errors. Any such leaks in the open-flow system are either dynamically corrected if upstream, as F_IO_2 is measured at the mask, or if downstream, affect measurements equally for the exercising subject and during calibration. The only location in the open-flow system at which it is critical not to have leaks is at the mouthpiece or mask, which is also essential for the closed system. Leaks at this location would produce unmeasured changes in F_IO_2 that would consequently affect the $\dot{V}O_2$ computations by either method. Flooding the subjects' faces with He when they were connected to the system resulted in an undetectable He concentration downstream. Therefore, we presume that equally undetectable volumes of air could have leaked inward to bias the measurements. This assumption appears valid, as leakage would have systematically biased $\dot{V}O_2$ measurements in hyperoxia high and in hypoxia low (*i.e.*, decreasing or increasing $F_{\bar{E}}O_2$ relative to F_IO_2, respectively), which did not occur. When the system is used in normoxia, leakage around the animal's face is part of $\dot{V}_{amb}$ and does not affect the calculated $\dot{V}O_2$ at all.

The present study demonstrates that reliable measurements of $\dot{V}O_2$ can be obtained using the N$_2$-dilution technique at various F_IO_2, ranging from 0.19 to 0.25, using an open-circuit gas system during submaximal exercise. We measured identical $\dot{V}O_2$ at different F_IO_2 at work rates in which identical aerobic power was generated in horses.

Acknowledgments

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